Title

The *HSD17B13* rs72613567 variant is associated with lower levels of albuminuria in patients with biopsy-proven nonalcoholic fatty liver disease

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List of Abbreviations

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; CKD, chronic kidney disease; *HSD17B13*, 17-beta hydroxysteroid dehydrogenase 13; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; HOMA-IR, homeostasis model assessment of

insulin resistance; eGFR, estimated glomerular filtration rate; ACR, albumin to creatinine ratio; NGAL, neutrophil gelatinase-associated lipocalin; BUN, blood urea nitrogen; SCr, serum creatinine; UA, uric acid; ALB, albumin; ALP, alkaline phosphatase.

Abstract

Background and Aims: Several susceptibility gene variants predisposing to nonalcoholic fatty liver disease (NAFLD) have been identified in chronic kidney disease (CKD). Evidence supports that 17-beta hydroxysteroid dehydrogenase 13 (*HSD17B13*) rs72613567 plays a role in NAFLD development by affecting lipid homeostasis. Since lipid droplets may accumulate in the kidneys and contribute to renal injury, we investigated the association between the *HSD17B13* rs72613567 variant and markers of renal function/injury in NAFLD.

Methods and Results: We measured estimated glomerular filtration rate (eGFR), urinary/serum neutrophil gelatinase-associated lipocalin (NGAL), and urinary albumin-to-creatinine ratio (u-ACR) in individuals with biopsy-proven NAFLD. Multivariable regression analyses were undertaken to examine the associations between the *HSD17B13* rs72613567 variant and markers of renal function/injury. Individuals were stratified by *HSD17B13* rs72613567 genotypes into -/-, A/- and A/A groups. *HSD17B13* rs72613567 genotypes were not significantly associated with eGFR and urinary/serum NGAL levels. Conversely, the prevalence of abnormal albuminuria in the A/- + A/A group was lower than in the -/- group (4.92% vs. 19.35%, p=0.001). Additionally, the mean u-ACR levels were lower among carriers of the A/- or A/A genotypes with coexisting hypertension or diabetes, than among those with the -/- genotype. The risk of abnormal albuminuria (adjusted-odds ratio 0.16, p=0.001) remained significantly lower in the A/- + A/A group after adjustment for established renal risk factors and histologic

severity of NAFLD.

Conclusion: *HSD17B13* rs72613567: A allele is associated with a lower risk of having abnormal albuminuria, but not with lower eGFR or urinary/serum NGAL levels, in patients with biopsy-proven NAFLD.

Keywords: 17-beta hydroxysteroid dehydrogenase 13; nonalcoholic fatty liver disease; albuminuria; chronic kidney disease; liver biopsy

1. INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver lipid-associated conditions ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis [1]. NAFLD is strongly associated with insulin resistance, dyslipidemia, chronic inflammation and increased oxidative stress [2], which are also shared risk factors for the development and progression of chronic kidney disease (CKD) [3]. Several observational studies have consistently shown that NAFLD (as assessed by either imaging techniques or biopsy) is significantly associated with an increased prevalence of abnormal albuminuria and CKD [4].

Growing evidence indicates that some susceptible genetic variants may confer a greater susceptibility to NASH and these genetic variants have been identified across different populations, i.e. the I148M polymorphism in the *patatin like phospholipase containing domain 3* (*PNPLA3*) and the E167K allele in the *transmembrane 6 superfamily member 2 gene* (*TM6SF2*) [5-8]. Our previous study has also reported that individuals with biopsy-confirmed NAFLD carrying the *PNPLA3 I148M* allele are at higher risk of having early renal glomerular and tubular injury [9].

Recently, 17-beta hydroxysteroid dehydrogenase 13 (*HSD17B13*) has been identified as a liver-specific, lipid droplet-associated protein that is attracting ever-growing attention in the field of NAFLD research [10, 11]. *HSD17B13* was first cloned by Liu and

coworkers from a human liver cDNA library in 2007 [12]. *HSD17B13* was found to be located on the surface of hepatic lipid droplets, thereby potentially playing an important role in the pathogenesis of NAFLD [13, 14]. In addition, a common loss-of-function variant in *HSD17B13* rs72613567 might also represent a potential therapeutic target for reducing the progression of NAFLD to NASH and cirrhosis [15]. Recently, in a sample of 684 Italian obese children, Sessa et al. found that carriers of the *HSD17B13* rare A allele had higher levels of estimated glomerular filtration rate (eGFR) levels than homozygous children, both among subjects with and without NAFLD, and independently of *PNPLA3 I148M* and *TM6SF6 E167K* polymorphisms [16]. To date, it is uncertain whether the function of the *HSD17B13* rs72613567 variant may also influence kidney function in adult individuals. Albuminuria is a urinary biomarker of early renal damage and often considered as an early marker of subsequent changes in kidney structure, reflecting disease progression [17]. Since it is known that lipid droplets can also accumulate in the kidneys and altered lipid metabolism can contribute to the development of abnormal albuminuria and renal injury [18-21], we hypothesized and tested that an association may exist between the *HSD17B13* rs72613567 variant and measures of kidney function/injury, in a cohort of Chinese adults with biopsy-proven NAFLD.

2. MATERIALS AND METHODS

2.1 Patients

In this cross-sectional study, we enrolled 215 middle-aged individuals with NAFLD without other competing causes of liver and kidney diseases, who consecutively attended the First Affiliated Hospital of Wenzhou Medical University between December 2016 and February 2019. The diagnosis of NAFLD was confirmed by liver biopsy in all participants. Detailed criteria for inclusion and exclusion have been reported previously [9]. The study was approved by the local ethics committee at our hospital. Written informed consent was obtained from participants and personal information and records were omitted and de-identified prior to analysis.

2.2 Liver histology

An ultrasound-guided liver biopsy was performed using a 16-gauge Hepafix needle (Gallini, Modena, Italy). Liver biopsy specimens were excluded if the biopsy length was less than 10 mm, contained fewer than six portal spaces, and had more than two fragmented tissue cores. Liver biopsy specimens were placed in formalin solution for fixation, embedded in paraffin blocks and stained with hematoxylin-eosin and Masson's trichrome. All biopsy specimens were analyzed by an experienced liver pathologist, who was blinded to participants' clinical data. The histological features of NAFLD were defined according to the NASH Clinical Research Network classification [22] and the Brunt's histological criteria [23].

2.3 Genetic analysis

Blood samples were collected from all participants and approximately 20 ng of genomic DNA from each blood sample was extracted for genetic analysis. DNA was extracted using the DNA Blood Mini Kit (QIAGEN) and stored at -20 °C for the analysis. *HSD17B13* rs72613567 genotyping was performed using the TaqMan assay platform according to the manufacturer's protocol. The forward primer sequence was CAGATTATGGCCTGTATTGGAGAC; the reverse primer sequence was GCTCTATTGGTGTTTTAGTATTTGGGT; the probe reporter 1 sequence was CTACAGAAGTAAGTACAGCAC; and the probe reporter 2 sequence was ACTACAGAAGTTAAGTACAGCA.

2.4 Clinical and laboratory parameters

Demographic data, anthropometric/clinical parameters and comorbidities were recorded prior to liver biopsy in all participants. Body mass index (BMI) was calculated as body weight in kilograms divided by height in meters squared. Presence of overweight/obesity was defined as BMI ≥25 kg/m^2. Venous blood samples were collected in the morning after an overnight fast. Complete blood count, serum liver enzymes (aspartate aminotransferase [AST], alanine aminotransferase [ALT], gamma-glutamyltransferase [GGT]), creatinine, urea nitrogen, glucose, uric acid and plasma lipid profile were measured by standard laboratory procedures at the Central Laboratory

of our hospital, using the relative reference techniques on an automated analyzer (Abbott AxSYM). Homeostasis model assessment of insulin resistance (HOMA-IR) score was calculated for estimating insulin resistance.

2.5 Kidney function parameters, CKD and other comorbidities

Glomerular filtration rate (eGFR) was estimated using the CKD-Epidemiology Collaboration equation [24]. Albuminuria was measured with an immuno-nephelometric assay on a morning spot urine sample and expressed as the albumin-to-creatinine ratio (ACR); abnormal albuminuria was defined as urinary ACR ≥30 mg/g creatinine. Urinary and serum levels of NGAL (neutrophil gelatinase-associated lipocalin) were measured using an up-converting phosphor technology (Hotgen, Beijing) according to the manufacturer's instructions. Reference urinary and serum NGAL values were obtained from a consecutive sample of Chinese healthy volunteers (*n*=25) without known kidney diseases; their mean u-NGAL and serum NGAL levels were 31.2±12.8 ng/mL and 31.5±11.3 ng/mL, respectively. CKD was defined as the presence of eGFR <60 ml/min/1.73 m^2 and/or u-ACR ≥30 mg/g creatinine. Hypertension was defined as blood pressure ≥130/85 mmHg or the use of any anti-hypertensive drugs. Diabetes was defined by a fasting glucose level ≥7.0 mmol/L or HbA1c ≥6.5% (≥ 48 mmol/mol) or prescribed hypoglycemic medications. Hyperuricemia was defined as a serum uric acid level >420 μmol/L for men and >360 μmol/L for women, respectively, or use of allopurinol. Dyslipidemia was defined as any

of the following criteria: total cholesterol >5.17 mmol/L; triglycerides >1.70 mmol/L; high-density lipoprotein (HDL)-cholesterol <1.0 mmol/L for women and <1.3 mmol/L for men and low-density lipoprotein (LDL)-cholesterol ≥3.4 mmol/L, or use of any lipid-lowering agents.

2.6 Statistical analysis

Data are expressed as numbers (percentages) for categorical variables, as means ± standard deviations for normally distributed continuous variables or as medians (inter-quartile ranges) for non normally distributed variables. The *HSD17B13* rs72613567 A/- and A/A genotypes were included either separately or combined into a single category (using the -/- genotype as the reference category) in statistical analyses. Baseline characteristics of the study population, stratified by *HSD17B13* rs72613567 genotypes, were compared using the one-way analysis of variance (ANOVA) for normally distributed continuous variables or the Kruskal-Wallis test for non normally distributed continuous variables, as well as the Pearson χ^2 or the Fisher's exact test for categorical variables. Univariable and multivariable logistic or linear regression analyses were used to examine the association between *HSD17B13* rs72613567 genotypes and markers of kidney dysfunction. We also converted u-ACR and u-NGAL levels to natural logarithms to normalize their distributions before statistical analyses. In multivariable logistic or linear regression models, model 1 was adjusted for age, sex, BMI, HOMA-IR score, hyperuricemia, hypertension, and pre-existing type 2 diabetes; and model 2 was further

adjusted for eGFR levels, presence of NASH (defined as NAFLD Activity Score ≥4) and the histological severity of liver fibrosis. The covariates included in these multivariable regression models were selected on the basis of their biological plausibility. All statistical tests were 2-sided and a *p*-value of <0.05 (two-tailed) was considered statistically significant. All statistical analyses were conducted using the SPSS version 22.0 (SPSS, Chicago, IL).

3. RESULTS

3.1 Baseline characteristics of NAFLD patients stratified by *HSD17B13* rs72613567 genotypes

Among the 215 adult individuals with biopsy-confirmed NAFLD included in the study (mean age 41±13 years, BMI 26.8±5 kg/m^2), 43.2% were -/- homozygotes, 12.1% were A/A homozygotes and 44.7% were A/- heterozygotes, corresponding to an A-allele frequency for the *HSD17B13* rs72613567 variant of 34.4%. The distribution of this genetic variant was in Hardy-Weinberg equilibrium (p=0.98). Because of the relatively low number of individuals who were homozygous for the A/A allele, we merged the groups of individuals with at least one A allele for further statistical analysis and compared these subjects with those who did not have an A allele (i.e. the -/- genotype group). Comparisons of the patients' characteristics between the three *HSD17B13* genotypes are summarized in **Table 1**. Individuals carrying the -/- genotype had a significantly higher prevalence of abnormal albuminuria (defined as u-ACR ⩾30 mg/g) than those with A/- genotype (19.3% *vs*. 6.3%) or those with A/- + A/A genotype (19.3% *vs*. 4.9%), respectively. Conversely, compared to those carrying the *HSD17B13* rs72613567: A variant, the carriers of the -/- genotype did not differ in terms of age, sex, BMI, serum liver enzymes, lipid profile, serum uric acid, HOMA-IR score, comorbid conditions (including hypertension and type 2 diabetes), as well as serum or urinary NGAL levels, eGFR and blood urea nitrogen values. Interestingly, as shown in **Figure 1**, individuals carrying the *HSD17B13* rs72613567 A/- or A/A genotypes were more

likely to have a lower risk of abnormal albuminuria than those carrying the -/- genotype, but comparable values of abnormal u-NGAL. Additionally, no significant differences were observed in the prevalence of NASH (defined as NAS ≥4) and the individual histologic features of NAFLD across the three *HSD17B13* rs72613567 genotypes (**Table 1**).

3.2 *HSD17B13* rs72613567 genotypes related to risk of albuminuria

As shown in **Figure 2**, we found that the carriers of *HSD17B13* rs72613567: A-allele had significantly lower mean levels of logarithmically transformed urinary ACR) than those carrying the -/- genotype, mostly among those with coexisting type 2 diabetes or hypertension. Therefore, to further understand the association between albuminuria and the *HSD17B13* rs72613567 genotypes, logistic and linear regression analyses were performed. **Table 2** shows that there was a significant association between the *HSD17B13* rs72613567 A/- or A/A genotypes and lower risk of abnormal albuminuria. This association remained statistically significant even after adjustment for age, sex, BMI, HOMA-IR score, hypertension, diabetes and hyperuricemia (adjusted model 1), as well as after additional adjustment for eGFR levels, presence of NASH and the histological severity of liver fibrosis. Furthermore, as shown in **Table 3,** a linear regression model adjusted for the aforementioned covariates confirmed that individuals carrying the A/- or A/A genotypes had significantly lower urinary ACR levels (included as a continuous variable) than those carrying the -/- genotype . Conversely, no

significant differences were observed in u-NGAL levels across the different *HSD17B13* genotypes.

3.3 *HSD17B13* rs72613567 genotypes related to albuminuria stratified by sex, age and BMI

Since age, sex and obesity are common risk factors for CKD, we examined the association between the *HSD17B13* rs72613567 variant and the risk of abnormal albuminuria stratified by sex, age (above or below 50 years) or presence of overweight/obesity **(Table 4)**. In the fully adjusted model (model 2), the association between the *HSD17B13* rs72613567 variant and the risk of abnormal albuminuria remained statistically significant with an adjusted odds ratio of 0.16 (95% CI 0.04-0.69) among overweight/obese individuals (BMI ≥25 kg/m^2) carrying the A/- genotype, and with an adjusted odds ratio of 0.12 (95% CI 0.03-0.49) among those carrying the A/- + A/A genotypes, respectively. Men with the *HSD17B13* rs72613567 A/- or A/A genotypes also had a lower risk of abnormal albuminuria than those carrying the -/- genotype (model 2, adjusted odds ratio=0.08, 95% CI 0.01-0.40). Similar results were also found in younger (age <50 years) individuals with the *HSD17B13* rs72613567 A/- or A/A genotypes. No significant differences were observed between the rs72613567 A/- or A/A genotypes and the risk of abnormal albuminuria among patients with or without hypertension, and among those with or without pre-existing type 2 diabetes (data not shown).

4. DISCUSSION

The main and novel findings of this study are that in our cohort of Chinese adult individuals with biopsy-proven NAFLD the frequency of the *HSD17B13* rs72613567: A allele is 34.5%, and individuals who are carrying the *HSD17B13 rs72613567* A/- or A/A genotypes, are at significantly lower risk of having abnormal albuminuria (especially in men, in individuals who are overweight or obese, and in those with age <50 years). To the best of our knowledge, this is the first study to investigate the association between the *HSD17B13* rs72613567 variant and markers of kidney function/injury in adults with biopsy-proven NAFLD.

To date, emerging evidence suggests that there is a link between NAFLD and increased risk of CKD[1]. Among the genetic factors, a non-synonymous single nucleotide polymorphism in the *PNPLA3* gene (i.e. a major common genetic variant strongly associated with greater susceptibility to NASH and advanced fibrosis) has been associated with lower eGFR values in patients with NAFLD from different ethnicities. The mechanisms underlying this association appear to be related to lipid droplet accumulation in the kidneys, and the release of multiple proinflammatory and profibrogenic factors [25-27]. Evidence also supports the notion that HSD17B13 is another liver-specific lipid droplet-associated protein that has retinol dehydrogenase activity, and plays a role in insulin resistance, adipogenesis, hepatic lipid metabolism

and development of NAFLD [14, 28]. It has been reported that *HSD17B13* expression was up-regulated in livers of individuals with NAFLD, and over-expression of HSD17B13 in C57BL/6 mice significantly increased hepatic *de novo* lipogenesis, mainly through the increase in the regulatory element-binding protein-1c transcription activity [13].

In a large cohort study of 111,612 individuals from the Danish general population, genotyping of *HSD17B13* rs72613567, the investigators identified 47.8% -/- homozygotes, 42.6% -/A heterozygotes and 9.6% A/A homozygotes, respectively. These investigators also showed that the A/A genotype of *HSD17B13* was significantly associated with lower rates of liver-related mortality, and with lower serum alanine aminotransferase levels, especially in people with obesity, in heavy drinkers and in individuals carrying three or four steatogenic alleles in the *PNPLA3* and *TM6SF2* genes [15]. In our study, we did not find any significant association between the *HSD17B13* rs72613567 variant and the presence of NASH or the individual histological components of NASH, which is also consistent with the results recently reported by Koo et al. in a cohort of 453 South Korean individuals with biopsy-proven NAFLD [29]. Currently, a significant association between the *HSD17B13* rs72612567 variant and NAFLD has been reported only in Caucasian individuals [13, 30, 31]. However, further large studies are needed to better examine this issue in different ethnic groups.

In the present study, we have shown that the risk of having abnormal albuminuria was markedly lower among carriers of the rs72613567: A allele than among those carrying the rs72613567 -/- genotype . Since *HSD17B13* is also expressed in the kidneys and its enzyme activity may play a role in cholesterol and fatty acid metabolism, it is reasonable to assume that an aberrantly high expression and activity of this enzyme may promote ectopic lipid accumulation, which has a role in the development of lipid-mediated kidney disease [12]. The concept of lipid nephrotoxicity was firstly proposed in 1982, and increasing evidence now supports the notion that dyslipidemia may directly contribute to the development and progression of glomerulosclerosis [21]. Although there is no strong association between the *HSD17B13* rs72613567 variant and plasma lipid profile, a large body of clinical and experimental evidence suggests that accumulation of renal lipids may contribute to the development and progression of kidney disease [19, 32]. Renal lipids may increase the rate of reactive oxygen species to further elevate plasma oxidized low-density lipoprotein (LDL) levels that can accumulate in renal mesangial cells [33, 34]. Mesangial cells can bind oxidized-LDL particles to increase cell proliferation via multiple downstream effects, and renal lipids may also act as mediators of inflammation and induce mesangial cells to secrete proinflammatory cytokines [32, 35]. Experimentally, it has been reported that glomerular macrophages from hypercholesterolemic rats overexpress transforming growth factor-β1 messenger RNA, causing glomerular matrix expansion [36, 37]. Intracellular accumulation of saturated fatty acids and cholesterol may also result in

endoplasmic reticulum (ER) stress, which in turn causes macrophage apoptosis that activates oxidative stress pathways [35, 38]. Collectively, dyslipidemia may induce low-grade inflammation, increase oxidative and ER stress, which are all involved in the development and progression of CKD [37]. Therefore, it is plausible to speculate that the *HSD17B13* rs72613567 -/- genotype is a risk factor for abnormal albuminuria in people with NAFLD, and the loss-of-function variant in *HSD17B13* (rs72613567:A allele) may exert a "protective" effect on the development of early kidney dysfunction.

Recently, Sessa et al. have also reported a significant, inverse association between the *HSD17B13* rs72613567 variant and eGFR levels in a cohort of Italian obese children and adolescents, irrespective of the presence of NAFLD defined by ultrasound [16]. In line with this, the results of our study showed that in adults with biopsy-proven NAFLD those carrying the A/- or A/A genotypes had a lower risk of having abnormal albuminuria than those carrying the -/- genotype, especially in men, in those with overweight or obesity, or in those with younger age. It is well known that men progress to end-stage kidney disease more rapidly than women [39]; similarly, obesity is also an established risk factor for CKD [40]. In our study, we also found that the carriers of *HSD17B13* rs72613567: A allele had lower levels of albuminuria than those carrying the -/- genotype mostly in subjects with coexisting type 2 diabetes or hypertension. As diabetes and hypertension are well established risk factors for CKD, it is perhaps not surprising that the "protective" effect of the *HSD17B13* rs72613567 variant on kidney

function may be stronger in individuals with coexisting type 2 diabetes or hypertension.

Our study has some important limitations that should be discussed. First, the design of our study was cross-sectional and does not allow for establishing temporality or causality of the observed associations, although this limitation is mitigated by the fact that the genetic variant under study is inherited and, therefore, reverse causation does not apply. Second, detailed information about specific renal pathology associated with NAFLD was not available in our study. Third, in this single-center study we enrolled individuals with biopsy-proven NAFLD belonging to a single Asian ethnicity and further large cohort studies are needed to externally validate our findings in other ethnic groups. Fourth, there are no specific functional studies that have explored the association between the *HSD17B13* rs72613567 variant and risk of CKD to date. However, we consider that this genetic variant may exert its "protective" effect on renal function through the modulation of multiple inflammatory and fibrogenic pathways, which are the same mechanisms that have been also postulated for the protective effect of the *HSD17B13* rs72613567 variant on the development and progression of NAFLD.

In conclusion, the findings of our study show that *HSD17B13* rs72613567 A/- or A/A genotypes are significantly associated with a lower likelihood of having abnormal albuminuria, but not with lower u-NGAL or eGFR levels, in patients with biopsy-proven NAFLD. This association appears to be independent of established renal risk

factors, presence of NASH and histological severity of liver fibrosis. Our findings suggest novel insights into the pathophysiology of the *HSD17B13* rs72613567 variant in the development of early kidney dysfunction. Further mechanistic studies are now needed to better understand the underlying mechanisms related to the *HSD13B17*'s effect on risk of albuminuria.

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All authors do not have nothing to declare.

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LEGENDS TO THE TABLES

Table 1. Baseline characteristics of patients with biopsy-proven NAFLD stratified by *HSD17B13* rs72613567 genotypes.

Table 2. Association between *HSD17B13* rs72613567 genotypes and abnormal albuminuria in patients with biopsy-proven NAFLD.

Table 3. Association between *HSD17B13* rs72613567 genotypes and natural logarithmic conversion of urinary albumin-to-creatinine ratio [ln (u-ACR), mg/g], and urinary neutrophil gelatinase-associated lipocalin [ln (u-NGAL), ng/mL] in patients with biopsy-proven NAFLD.

Table 4. Association of *HSD17B13* rs72613567 genotypes with abnormal albuminuria in patients with biopsy-proven NAFLD stratified by overweight/obesity, sex or age, respectively.

LEGENDS TO THE FIGURES

Figure 1. Proportions of subjects with different u-ACR categories, u-NGAL categories (A) according to the *HSD17B13* rs72613567 genotypes. * $p<0.05$

Figure 2. Logarithmically transformed albuminuria (ln u-ACR levels) in NAFLD individuals with either diabetes (A) or hypertension (B), stratified by *HSD17B13* rs72613567 genotypes. Data are presented as mean with 95% confidence intervals (CI). * $p<0.05$