Title Page

Low skeletal muscle mass is associated with more severe histological features of non-alcoholic fatty liver disease in men

Short Title: Low skeletal muscle mass in NAFLD

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The authors declare that they do not have anything to disclose regarding any funding or conflict of interest with respect to this manuscript.

List of Abbreviations

NAFLD, non-alcoholic fatty liver disease; BMI, body mass index; ASM%, weight-adjusted appendicular skeletal muscle; HOMA-IR, homeostasis model assessment-insulin resistance; AST, aspartate aminotransferase; ALT, alanine aminotransferase;

GGT, γ-glutamyltranspeptidase; ALP, alkaline phosphatase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CK-18, cytokeratin-18; *PNPLA3*, patatin-like phospholipase domain-containing 3; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; BIA, bioelectrical impedance analyzer; CI, confidence interval; NASH-CRN, NASH-Clinical Research Network; OR, odds ratio.

Ethical approval: 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 1964 Helsinki declaration and its later amendments.

Informed consent: Written informed consent was obtained from all participants included in the study.

Abstract

Background/Purpose of the study: Although low skeletal muscle mass is associated with non-alcoholic fatty liver disease (NAFLD), it is currently uncertain whether there are associations between weight-adjusted appendicular skeletal muscle (ASM%), severity of histological features of NAFLD, and the patatin-like phospholipase domain-containing 3 (*PNPLA3*) rs738409 polymorphism. Our aim was to test for a possible influence of the *PNPLA3* rs738409 variant on the association between ASM% and severity of NAFLD histological features.

Methods: We enrolled 401 Chinese men with biopsy-proven NAFLD. Using a bioelectrical-impedance body composition analyzer (BIA, Inbody 720, Japan Inc., Tokyo), we calculated the ASM% as the percentage of total appendicular skeletal muscle mass (ASM, kg)/total body mass (kg) ×100.

Results: Compared to those with high ASM%, patients with low ASM% (≤30.6, i.e., the median value of distribution of the whole sample) had a greater severity of individual histological features of NAFLD. These patients also had a higher risk of severe steatosis and non-alcoholic steatohepatitis (NASH) (adjusted-odds ratio [OR] 2.34, 95%CI 1.39-3.93, and adjusted-OR 2.22, 95%CI 1.30-3.77) even after adjusting for age, body mass index, diabetes, and serum creatinine levels. Carriage of the G allele of *PNPLA3* rs738409 *plus* low ASM% was associated with a higher risk of severe steatosis and presence of liver fibrosis (OR 3.02, 95%CI 1.46-6.26, P=0.003 and OR 2.18, 95%CI 1.03-4.60, P=0.041 respectively), and there was a non-significant but borderline increased risk of NASH (OR 2.00, 95%CI 0.98-4.06,

P=0.056).

Conclusions: Low ASM% and the presence of a G allele within *PNPLA3* rs738409 is associated with more severe histological features of NAFLD.

Key words: Skeletal muscle mass; Non-alcoholic fatty liver disease; Sarcopenia; Patatin-like phospholipase domain-containing 3.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become an important cause of chronic liver disease worldwide. The prevalence of NAFLD has doubled over the past two decades, and the burden of ill health associated with NAFLD will continue to grow with the continuing epidemic of obesity and a sedentary lifestyle. NAFLD occurs in about one-quarter of adults in the general population, of which approximately a third can progress to non-alcoholic steatohepatitis (NASH) [1]. In subjects with NASH the incidence of cirrhosis is high, with as many as 25% developing cirrhosis within 10 years [2, 3]. In subjects developing the more advanced stages of NAFLD, there is also a higher risk of all-cause mortality, severe liver-related complications (mostly liver failure or hepatocellular carcinoma), and extra-hepatic diseases (mostly cardiovascular and renal diseases) [4-6].

Sarcopenia is a syndrome caused by involuntary loss of skeletal muscle mass and strength [7], which is closely related to aging, diabetes, obesity and cardiovascular disease. Recent studies have shown that low skeletal muscle mass is associated with greater systemic insulin resistance, low-grade inflammation, lower plasma adiponectin levels, vitamin D deficiency and reduced physical activity [8]. This suggests that there is an interplay between the liver and skeletal muscle tissue. In fact, previous studies have reported that low skeletal muscle mass may influence the physiopathology of NAFLD independent of obesity and insulin resistance, as well as contributing to a poor prognosis in patients with end-stage liver disease [9-13].

The rs738409 C>G p.I148M variant in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene is a key genetic factor that is strongly associated with a greater susceptibility to NAFLD development and progression [14]. The *PNPLA3* gene variant is associated with more severe liver damage and a lower risk of cardiovascular disease [15, 16]. However, a previous study showed that the *PNPLA3* GG gene variant did not increase the risk of NAFLD in individuals with low appendicular skeletal muscle (regardless of their obesity status); thus suggesting that carriers of the *PNPLA3* GG genotype might have uncoupling of the association between low skeletal muscle mass and NAFLD [17].

To better understand the potential crosstalk between low skeletal muscle mass and NAFLD, we examined the association between low weight-adjusted appendicular skeletal muscle (ASM%) and the severity of individual histological features of NAFLD, and also explored the association between the *PNPLA3* rs738409 polymorphism and ASM% in men with biopsy-confirmed NAFLD.

Patients and methods

Participants

All study participants were from the First Affiliated Hospital of Wenzhou Medical University (China). Our study is a part of observational cohort study of prospective epidemic research specifically on NASH (PERSONS). Participants were enrolled

153 from December 2016 to September 2020, and the diagnostic criteria for NAFLD have
154 been described previously [18]. The following exclusion criteria were applied: 1)
155 presence of viral hepatitis such as hepatitis B or C; 2) significant alcohol consumption
156 (≥ 140 g/week); 3) autoimmune hepatitis; 4) drug-induced liver injury; 5) other
157 known causes of chronic liver diseases; 6) incomplete clinical/biochemical data; and
158 7) patients of female sex. Ultimately, 401 men with biopsy-proven NAFLD were
159 included in the final analysis.
160
161 The study was approved by the Research Ethics Committees of the First Affiliated
162 Hospital of Wenzhou Medical University. Each participant provided written informed
163 consent for participation in the study.
164
165 **Anthropometric and biochemical measurements**
166 A questionnaire regarding the past history of diabetes, hypertension, alcohol
167 consumption, use of medications and face-to-face interview for each participant was
168 completed by a well-trained investigator. Standing height and weight were measured
169 without shoes and outer clothing. Body mass index (BMI) was calculated using the
170 formula weight (kilograms) divided by height squared in meters. Hypertension was
171 diagnosed as blood pressure ≥130/85 mmHg or use of any anti-hypertensive drugs.
172 Diabetes was diagnosed according to widely used biochemical criteria, or self-
173 reported history of diabetes, or treatment with any hypoglycemic drugs. Venous blood
174 samples were collected after at least 12 hours fasting overnight. Fasting blood glucose

(FBG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltranspeptidase (GGT), alkaline phosphatase (ALP), bilirubin, creatinine, uric acid, triglycerides, high-density lipoprotein (HDL) cholesterol and low density lipoprotein (LDL) cholesterol were centrally analyzed by using an automated analyzer (Abbott AxSYM). Platelet count was measured with an automated blood cell counter (Mindray BC-6800Plus). HemoglobinA1c (HbA1c) was measured using HbA1c Analytical Column 1000 (Trinity Biotech, USA). Fasting insulin was quantified using an electro-chemiluminescence immunoassay (Beckman Coulter). Homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated by multiplying the FBG (mmol/L) and fasting insulin (mU/L) and dividing by 22.5 [19]. All anthropometric and laboratory data were obtained from participants within 24 hours of liver biopsy examination.

Serum cytokeratin-18 measurement

Serum CK-18 M30 and M65 concentrations were measured by using commercially available ELISA kits (Herui Biomed Company Limited, Suzhou, China). Both intra- and inter-assay coefficients of variation were <15%, as described previously [18].

Liver histology

All liver biopsy specimens were assessed and reviewed by a single experienced liver pathologist (X.D. Wang), who was blinded to all participants' clinical characteristics. NAFLD was defined by observation of steatosis grade >5%. The three histological

features of NASH, including steatosis, lobular inflammation, hepatocellular ballooning as well as the histological NAFLD activity score (NAS), were defined and graded according to the NASH-Clinical Research Network (CRN) Scoring System [20]. Liver fibrosis was graded according to the Brunt's histological criteria [21]. Significant fibrosis was defined by a fibrosis stage of 2 or greater.

PNPLA3 genotype

In all participants the *PNPLA3* rs738409 was genotyped on human peripheral blood using the MassARRAY platform (Agena Bioscience, San Diego, CA, USA). Assay Design Suite v3.1 was used to design the Locus-specific PCR and detection primers. After the DNA samples were amplified via multiplex PCR, allele detection was performed through MALDI-TOF mass spectrometry [18].

BIA measurement and definition of low ASM%

In all participants the BIA was measured using a bioelectrical-impedance body composition analyzer (BIA, Inbody 720, Japan Inc., Tokyo, Japan). The whole-body BIA was measured between the bilateral upper and lower limbs with the subject in a supine position. The ASM% was calculated as the percentage of total appendicular skeletal muscle mass (ASM, kg)/total body mass (kg) ×100. In this study, taking the median value of distribution in the whole sample of participants as the cut-off point, we defined ASM% ≤30.6 as "low ASM%" (indicative of sarcopenia) and ASM% >30.6 as "high ASM%".

Statistical analysis

Statistical analyses were performed using R software (version 3.5.2, R Foundation for Statistical Computing, Vienna, Austria). Participants were divided into two groups according to median values of ASM%. Continuous variables were expressed as mean ± standard deviation (SD) and comparisons between the two groups were made by using the Student's *t*-test. Categorical variables were expressed as frequency (%) and comparisons between the two groups were made by using the chi-square test. Logistic regression analyses were used to investigate the association between low ASM% and severity of individual histological features of NAFLD, which was our primary study outcome. The relationship between ASM%, *PNPLA3* rs738409 G variant and severity of NAFLD histology were also analysed by logistic regression analyses, as the secondary outcome of the study. Statistical analyses were two-sided and statistical significance was set at $p<0.05$.

Results

Baseline characteristics of participants

A total of 401 men with biopsy-proven NAFLD were included in the study. Baseline characteristics of all the participants stratified by low ASM% (i.e., below the median) and high ASM% (above the median) values are summarized in **Table 1**. Participants with low ASM% had significantly higher values of BMI, HOMA-IR score, serum aminotransferases, uric acid, total cholesterol, LDL-cholesterol, CK-18 M-30, M-65,

as well as a lower age and lower serum creatinine levels, compared to those with a high ASM%. In addition, those with a low ASM% also had a greater severity of individual histological features of NAFLD, including steatosis, lobular inflammation, fibrosis and NAS score (all $P< 0.01$) (**Table 1**). No significant differences in the frequency of the *PNPLA3* rs738409 genotypes CC, CG and GG were found between the two groups of patients.

NAFLD severity and low ASM%

We performed a stratified analysis according to the severity of NAFLD pathological status. As shown in **Figure 1**, we found that participants with more severe liver histology had a higher prevalence of low ASM%. In particular, the presence of a low ASM% was higher in participants with worse NAFLD outcomes, such as severe steatosis (62.7% vs. 35.3%), severe inflammation (66.7% vs. 45.9%), presence of fibrosis (54.1% vs. 39.4%) and definite NASH (68.1% vs. 38.2%), compared to those with less severe NAFLD histological features. (**Figure 1**).

Low ASM% is related to more severe histological features of NAFLD

We also analyzed the association between ASM% and the pathological severity of NAFLD. In an unadjusted regression model, patients with low ASM% had a higher risk of severe steatosis (OR 3.07, 95% CI 2.04-4.63, $P<0.001$), severe inflammation (OR 2.35, 95%CI 1.41-3.93, $P=0.001$), presence of fibrosis (OR 1.81, 95% CI 1.16 - 2.83, $P=0.009$) and NASH (OR 3.46, 95% CI 2.27-5.28, $P<0.001$), compared to those

with a high ASM% (**Figure 2A**). After adjustment for age, BMI, diabetes and serum creatinine levels, only the associations between low ASM% and both severe steatosis and NASH remained statistically significant (adjusted-OR 2.34, 95%CI 1.39-3.93, and adjusted-OR 2.22, 95%CI 1.30-3.77) (**Figure 2B**).

Interaction between the *PNPLA3* rs738409 variant and ASM%

Mutations in gene coding *PNPLA3* rs738409 are associated with NAFLD, so it is important to study the interaction between *PNPLA3* rs738409 polymorphism and low skeletal muscle mass. To test for the influence of the *PNPLA3* rs738409 variant on the associations between ASM% and severity of NAFLD histological features, we stratified patients into four groups based on both their *PNPLA3* rs738409 genotype and median value of ASM%. As shown in **Figure 3**, the prevalence of severe steatosis, severe inflammation, presence of fibrosis and NASH changed according to the effects of *PNPLA3* rs738409 G allele and ASM% (all $P< 0.05$) (**Figure 3, A-E**). In further statistical analyses, using patients with *PNPLA3* rs738409 CC and low ASM% as the reference category, we found that carriage of the G allele of *PNPLA3* rs738409 *plus* low ASM% had a higher risk of having severe steatosis (adjusted-OR 3.02, 95% CI 1.46-6.26, P=0.003), presence of fibrosis (OR 2.18, 95% CI 1.03-4.60, P=0.041) and a borderline trend for higher risk of having NASH (OR 2.00, 95% CI 0.98-4.06, P=0.056) after adjusting for age, BMI, diabetes and serum creatinine (**Table 2**). Thus, these data suggest that the carriage of G allele of *PNPLA3* rs738409 may exacerbate the effect of low ASM% on the risk of having more severe

285 histological features of NAFLD.

286

287 **Discussion**

288 The results of our cross-sectional study show that in Chinese men with biopsy-proven
289 NAFLD, the presence of low ASM% (indicative of possible sarcopenia) was
290 associated with more severe histological features of NAFLD, and the carriage of G
291 allele of *PNPLA3* rs738409 was associated with increasing histological severity of
292 NAFLD, especially in the presence of a low ASM%.

293

294 Accumulating evidence has shown that loss of skeletal muscle mass and strength may
295 be a novel risk factor for progression of NAFLD [9, 12, 13, 22-24]. Our previous
296 meta-analysis of six studies showed that loss of skeletal muscle mass was a risk factor
297 for development and progression of NAFLD, but in that meta-analysis the effect of
298 the *PNPLA3* rs738409 genotype was not studied [25].

299

300 It is well known that the *PNPLA3* rs738409 variant is a genetic factor that is strongly
301 associated with a greater susceptibility to NAFLD and NASH. To date, there are very
302 few studies focussing on the effect of *PNPLA3* rs738409 polymorphism on the
303 association between low skeletal muscle mass and NAFLD. It has been reported that
304 the presence of the *PNPLA3* rs738409 G allele and an increase in skeletal muscle
305 mass to body fat mass, were predictive factors of a serum ALT reduction >30% from
306 baseline, that was achieved by decreasing caloric intake by 500–1000 kcal/day and

307 undertaking moderate-intensity exercise [26]. Patients carrying the *PNPLA3* rs738409
308 GG genotype may also be more sensitive to the beneficial effects of lifestyle
309 modification [27]. This finding suggests that the *PNPLA3* rs738409 G allele may play
310 a role in loss of skeletal muscle mass and NAFLD, although it is unclear whether loss
311 of skeletal muscle mass is the cause or the consequence of NAFLD. In contrast, it has
312 been shown that the *PNPLA3* GG gene variant did not increase the risk of NAFLD in
313 individuals with low ASM%, regardless of obesity status [17]. Thus, understanding
314 the crosstalk between skeletal muscle and the liver may provide a new perspective to
315 better decipher the complex and intertwined mechanisms of NAFLD development.
316
317 The major finding of our cross-sectional study was that participants with severe liver
318 injury had a higher prevalence of low skeletal muscle mass. Indeed, severe steatosis,
319 severe inflammation, presence of fibrosis and a greater NAS were significantly
320 associated with a low ASM%. There was a relatively high frequency of liver fibrosis
321 in our study though our participants were relatively young (especially in the low
322 ASM% group at 36.4 years), which might be partly related to a sedentary lifestyle and
323 physical inactivity. Furthermore, the *PNPLA3* G-allele carriers with low ASM% had
324 an increased likelihood of having severe liver disease compared to those carrying
325 *PNPLA3* CC genotype or high ASM%, thus suggesting that low skeletal muscle mass
326 might aggravate the pathological state of *PNPLA3* I148M-driven NAFLD. Although a
327 previous study does not support our findings, that study did not assess liver severity
328 with histology [17]. That said, further studies are needed to better understand the

329 complex relationship between *PNPLA3* gene polymorphism and loss of skeletal
330 muscle mass in NAFLD.
331
332 To date, skeletal muscles are thought also to be an endocrine organ that secretes a
333 variety of myokines, including cytokines and proteins with function in regulating
334 muscle metabolism, as well as participating in the inter-tissue cross-talk between
335 skeletal muscle and other tissues, such as the liver, adipose tissue and brain [22]. Loss
336 of skeletal muscle mass has been reported to be an important predictor of NAFLD in a
337 retrospective study [28]. While the increase in skeletal muscle mass index (SMI) may
338 help prevent NAFLD progression [29], skeletal muscle also plays a key role in the
339 body's glucose metabolism and insulin signal transmission [30]. Loss of skeletal
340 muscle mass may cause dysglycaemia and greater insulin resistance, which ultimately
341 contribute to the rising risk of NAFLD [22]. In our study, participants with low
342 ASM% also had higher HOMA-IR score compared to those with high ASM%, as well
343 as a greater risk of having more severe histological features of NAFLD. These results
344 further highlight the role of IR as a key connector between low skeletal muscle mass
345 and NAFLD.
346
347 A recent study has shown that sex also plays a role in the association between low
348 skeletal muscle mass and NAFLD [31]. In this study, the authors found that in a
349 cohort of 4210 participants with type 2 diabetes, low skeletal muscle mass was
350 independently associated with NAFLD only in men, whereas this association was

attenuated in women after adjusting for clinical risk factors. Similarly, another smaller study also showed that the skeletal muscle mass index was not related to the severity of liver steatosis (as detected by controlled attenuation parameter) in women [32]. Multiple physiological-related factors such as estrogens, muscle mass, muscle strength, skeletal muscle metabolism, body fat distribution and even different lifestyles may explain the reported sex-specific effects on the interplay between low skeletal muscle mass and NAFLD [31-34].

Limitations

Some important limitations of our study must be acknowledged. First, we included only men in our cohort as we did not have sufficient women with these measurements to test the associations in women. Second, we used BIA for assessing skeletal muscle mass index. However, previous studies have validated the use of BIA *vs*. dual-energy X-ray absorptiometry (DXA) or magnetic resonance imaging (MRI) [35]. The results we obtained for BIA estimating ASM% were appropriate and support the use of an easy-available and cheap instrument for assessing bioimpedance in people with NAFLD [29]. Another limitation is the lack of any data on gait speed or grip strength that could help better characterize muscle function. Third, the cross-sectional design of the study limits our ability to establish temporal or causal associations between ASM% and the severity of NAFLD histology. Fourth, liver biopsy is the gold standard procedure for diagnosing and evaluating the severity of NAFLD and NASH. However, concordance rates between pathologists for the evaluation of NAFLD are

poor [36, 37]. Ideally, at least two pathologists and a centralized pathological diagnosis are required for verification and achieving consensus on liver histology. Our study was a single center study that employed only one expert pathologist. Thus, it is possible that the histological diagnosis might be inaccurate. That said, with only one pathologist any misclassification bias should be consistent across all specimens in our study. Finally, our participants were relatively young and of Chinese ethnicity. Consequently, our findings need to be further validated in older patients and other ethnic groups.

Conclusions

Our results show that in Chinese men with biopsy-confirmed NAFLD, the presence of low ASM% was strongly associated with more severe histological features of NAFLD, and the carriage of G allele of *PNPLA3* rs738409 was associated with more severe histological features of NAFLD, especially in the presence of a low ASM%.

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TABLE TITLES

Table 1. Baseline characteristics of men with biopsy-proven NAFLD, stratified by median weight-adjusted appendicular skeletal muscle mass (ASM%).

Table 2. Interactions of the *PNPLA3* rs738409 genetic variant and low or high weight-adjusted appendicular skeletal muscle mass on the severity of liver histology features in men with biopsy-proven NAFLD.

FIGURE LEGENDS

Figure 1. Prevalence of low ASM% in men with biopsy-proven NAFLD, stratified by the severity of histological features of NAFLD.

The prevalence of low ASM% was significantly higher in participants with worse histological features of NAFLD, including severe steatosis, inflammation, presence of fibrosis or definite NASH compared to those with mild NAFLD. Prevalence of low ASM% did not differ significantly between participants with severe ballooning and those with mild ballooning.

Figure 2. Associations between low ASM% and the severity of histological features of NAFLD.

A. Unadjusted logistic regression analyses between low ASM% and severity of NAFLD histology features**. B.** Adjusted logistic regression analyses between low ASM% and severity of NAFLD histology features. Data have been adjusted for age, BMI, serum creatinine level and presence of diabetes.

Figure 3. Prevalence of severe steatosis (A), severe ballooning (B), severe inflammation (C), presence of liver fibrosis (D) or definite NASH (E) in men with biopsy-proven NAFLD, who were stratified by *PNPLA3* genotypes and low or high ASM%.