Title

PNPLA3 polymorphism influences the association between high-normal TSH level and NASH in euthyroid adults with biopsy-proven NAFLD

Short title: NASH and high-normal TSH levels

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Abbreviations

NAFLD, nonalcoholic fatty liver disease; *PNPLA3*, patatin-like phospholipase domain-containing protein 3; NASH, nonalcoholic steatohepatitis; CI, confidence interval; HCC, hepatocellular carcinoma; TSH, thyroid stimulating hormone; FT4, free thyroxine; FT3, free triiodothyronine; NAS, NAFLD activity score; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; WBC, white blood cells; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low-density lipoprotein cholesterol; OR, odds ratio; SD, standard derivation; GGT, gamma-glutamyl transferase; HSL, hormone sensitive lipase; ATLG, adipose triglyceride lipase; SREBP1c, sterol regulatory element-binding protein1c; SRE, sterol-response element.

Abstract

Aim: We aimed to evaluate the association between serum thyroid stimulating hormone (TSH) levels, *within the reference range*, and the histological severity of nonalcoholic fatty liver disease (NAFLD), and whether this association was modulated by the patatin-like phospholipase domain-containing 3 (*PNPLA3*) rs738409 polymorphism.

Materials and Methods: We enrolled 327 euthyroid individuals with biopsy-proven NAFLD, who were subdivided into two groups, i.e., a 'strict-normal' TSH group (TSH level 0.4 to 2.5 mIU/l; n=283) and a 'high-normal' TSH group (TSH level 2.5 to 5.3 mIU/l with normal thyroid hormones; n=44). Logistic regression analyses were performed to assess the association between TSH status and presence of nonalcoholic steatohepatitis (NASH) after stratifying subjects by *PNPLA3* genotypes.

Results: Compared to strict-normal TSH group, patients with high-normal TSH levels were younger and had a greater prevalence of NASH and higher histologic NAFLD activity score. After stratifying by *PNPLA3* genotypes, the significant association between high-normal TSH levels and presence of NASH was restricted only to carriers of the *PNPLA3* G risk allele and remained significant even after adjustment for potential confounding factors (adjusted-odds ratio 3.279; 95%CI 1.298-8.284; p=0.012).

Conclusion: In euthyroid individuals with biopsy-proven NAFLD, we found a significant association between high-normal TSH levels and NASH. After stratifying

by *PNPLA3* rs738409 genotypes, this association was observed only among carriers of the *PNPLA3* G risk allele.

Keywords: PNPLA3, NASH, TSH, euthyroidism

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a highly prevalent disease affecting up to 25%-30% of the world's adult population.[1, 2] NAFLD is regarded as a complex disease trait such that interactions between the environment and a susceptible genetic background induce disease phenotypic expression and influence disease outcome. Multiple genome-wide association studies have clearly identified the I148M patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) variant as the most common genetic determinant of the presence and severity of NAFLD.[3-6]

It is known that the development and progression of NAFLD usually occur in the presence of profound derangements of glucose and lipid metabolism, and dysregulation of energy homeostasis. Thyroid hormones are critical regulators of energy homeostasis and exert prominent direct effects on lipid and glucose metabolism.[7] Currently, observational studies have reported that overt or subclinical hypothyroidism is significantly associated with the presence and severity of NAFLD.[8-10] There is also some experimental evidence suggesting that increasing levels of thyroid-stimulating hormone (TSH) *per se* may induce liver fat accumulation and the underlying mechanism is thought to involve TSH-induced liver fat accumulation principally mediated by the hepatic TSH receptor increasing hepatic

sterol regulatory element-binding protein1c (SREBP-1c) activity via activation of the cAMP/PKA/PPARα pathway.[11]

An updated meta-analysis of fifteen observational studies involving ~44,000 subjects has recently confirmed the existence of a significant association between primary hypothyroidism (defined either by the history of levothyroxine replacement therapy or by the presence of subclinical and overt hypothyroidism) and the risk of having NAFLD. This risk tended to increase across the different definitions used for diagnosing hypothyroidism (use of levothyroxine replacement therapy >overt hypothyroidism >subclinical hypothyroidism), and seemed to further increase with increased severity of NAFLD.[12] Furthermore, some epidemiological evidence is also emerging to suggest that increasing levels of TSH, within the normal reference range, are also associated with an increased prevalence of NASH and advanced fibrosis in euthyroid individuals with biopsy-proven NAFLD.[13, 14]

It is currently not known as to whether any association between increasing TSH levels (*within the reference range*) and NASH is affected by the *PNPLA3* rs738409 polymorphism, which is the key genetic variant associated with increased susceptibility to NASH.[3-5]

Therefore, in a large sample of euthyroid subjects with biopsy-confirmed NAFLD, the major aims of our cross-sectional study were: a) to assess the association between serum TSH levels within the euthyroid range and the histological severity of NASH; and b) to examine whether this association was modulated by the patatin-like phospholipase domain-containing 3 (*PNPLA3*) rs738409 polymorphism.

Materials and methods

Study population

In this study, all participants were recruited at the First Affiliated Hospital of Wenzhou Medical University from a well-characterized cohort of adult individuals (namely the Prospective Epidemic Research Specifically of NASH [PERSONS] cohort), who attended our Liver clinic for suspected NAFLD from December 2016 to July 2019.[15] From an initial sample of 966 adult individuals with suspected NAFLD (based on imaging techniques and/or elevated serum liver enzyme levels), we subsequently excluded 639 subjects for the following reasons: (1) positivity for serum markers of viral hepatitis A (n=2), hepatitis B (n=184), hepatitis C (n=8) or hepatitis E (n=2); (2) alcohol consumption in excess of 140 g per week for men and 70 g per week for women (n=108); (3) drug-induced hepatitis (n=3), autoimmune hepatitis (n=17) or liver cancers (n=8); (4) thyroid cancers (n=18) or history of other active extra-hepatic cancers (n=8); (5) abnormal thyroid function tests (overt or subclinical hypothyroidism or hyperthyroidism) or use of levothyroxine replacement therapy (n=17); (6) hepatic steatosis <5% on liver histology (n=39); (7) refuse liver biopsy (n=26); and (8) missing biochemical data, including *PNPLA3* genotyping (n=206).

As a consequence of the above-mentioned exclusion criteria (as shown in **Figure 1**), 327 (257 men and 70 women) euthyroid individuals with biopsy-proven NAFLD were included in the final statistical analysis for the present study.

The research protocol was approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical University. The protocol conformed to the ethical

guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from each participant included in the study.

Liver histology

The detailed methodology for liver biopsies has been described previously.[15] A single experienced liver pathologist examined all liver biopsies. Briefly, NAFLD was defined as steatosis grade >5% and definite NASH was diagnosed with score ≥1 for each of the three histological components of steatosis, inflammation and hepatocellular ballooning while NAFLD activity score (NAS) ≥ 5 was diagnosed according to the NASH-Clinical Research Network classification.[16] Significant fibrosis was defined as liver fibrosis ≥ F2 on histology, while advanced fibrosis was defined by liver fibrosis ≥ F3 according to the Brunt's histologic criteria.[17]

Analysis of the PNPLA3 rs738409 polymorphism

The methods used for analysis in blood samples of the *PNPLA3* rs738409 polymorphism have been described elsewhere.[18] Locus-specific PCR and detection primers were designed using Assay Design Suite v3.1. MALDI-TOF mass spectrometry was used to detect allele type following DNA amplification by multiplex PCR.

Clinical, laboratory data and thyroid function testing

In brief, data collection for anthropometric measurements, BMI, blood pressure (BP) were conducted by standardized methods.[18] Hypertension was defined as BP ≥ 140/90 mmHg or drug treatment. Diabetes was defined by a fasting glucose concentration ≥ 126 mg/dl (≥ 7 mmol/l) or hemoglobin A1c (HbA1c) ≥ 6.5% (≥ 48

mmol/mol) or use of anti-hyperglycemic drugs. Hyperlipidemia was defined as total cholesterol ≥ 6.2 mmol/l, LDL-cholesterol ≥ 4.1 mmol/l or triglycerides ≥ 2.2 mmol/l or use of any lipid-lowering agents.

Blood routine parameters, including hemoglobin, platelets, white blood cells (WBC), and biomarkers such as serum albumin, HbA1c, fasting glucose, fasting C-peptide, fasting insulin, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglyceride, liver enzymes [i.e. gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST)] and uric acid levels were centrally analyzed by an automated analyzer (Abbott AxSYM, Park, IL). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: HOMA-IR score = [fasting insulin (μIU/ml) ×fasting glucose concentration (mmol/l)]/22.5. Serum levels of thyroid-stimulating hormone (TSH, reference range in our laboratory: 0.4-5.3 mIU/l), free triiodothyronine (FT3, reference range: 3.3-6.5 pmol/l) and free thyroxine (FT4, reference range: 7.6-16.0 pmol/l) were analyzed in our central Laboratory on a fully automated UniCel Dxl 800 Access 2 analyzer (Beckman Coulter, California, USA) using microparticle chemiluminescent assays.

Statistical analysis

Statistical data analysis was performed by using the SPSS Version 25.0. Data for continuous variables were expressed as means ± SD or as percentages for categorical variables. Among the euthyroid patients with histologically-proven NAFLD, 283 patients with TSH between 0.4 to 2.5 mIU/l were assigned to the strict-normal TSH group, while the remaining 44 euthyroid patients with TSH between 2.5 to 5.3 mIU/l

were assigned to the high-normal TSH group. We adopted this lower limit of normality for TSH level, i.e. 2.5 mIU/l, according to the 2003 National Academy of Clinical Biochemistry guidelines.[19, 20]The Student's *t*-test, the Welsh's *t*-test (or unequal variances *t*-test) and the Fisher's exact test (for categorical variables) were used to compare statistical differences in clinical, biochemical and liver histology characteristics of NAFLD patients grouped according to either serum TSH levels ('strict-normal' group *vs*. 'high-normal' group) or *PNPLA3* rs738409 genotypes (CC *vs*. GC/GG genotypes combined). Univariable and multivariable logistic regression analyses were performed to test the association between TSH status and risk of having definite NASH after stratifying subjects by *PNPLA3* rs738409 genotypes. Statistical significance was considered at $p<0.05$ (two-tailed).

Results

Baseline characteristics of patients stratified by NASH status

We studied 327 euthyroid adults with histologically proven NAFLD, in whom the mean age was 41.8 years and the mean BMI was 26.8 kg⁄m^2. By study design, patients with overt or subclinical hypothyroidism or hyperthyroidism and those treated with levothyroxine replacement therapy were excluded from the study. After stratifying these euthyroid patients by the presence of NASH on histology (**supplementary Table 1)**, patients with NASH (n=117) had higher serum TSH levels (1.9±0.8 vs. 1.6±0.7 mIU/l, $p=0.003$) than those belonging to the non-NASH group (n=210). In addition, patients with NASH were more likely to be younger, more centrally obese, and to have dyslipidemia, greater insulin resistance and higher values of serum uric acid and liver enzymes.

Baseline characteristics of patients stratified by TSH status

As shown in **Table 1**, compared to the strict-normal TSH group, patients with high-normal TSH levels were more likely to be younger, had a higher prevalence of NASH, had more severe steatosis and a higher total NAS score. They also had higher serum albumin and uric acid levels. No significant differences were found in liver fibrosis stage. In addition, the two patient groups were comparable for all other clinical and biochemical variables, including serum liver enzymes, FT3 and FT4 levels as well as *PNPLA3* rs738409 genotypes.

Baseline characteristics of patients stratified by TSH status and PNPLA3 rs738409 genotypes

As shown in **Table 2**, when patients were simultaneously stratified by TSH status and *PNPLA3* rs738409 genotypes, we found that the significant association between high-normal TSH levels and the presence of NASH was restricted only to carriers of the *PNPLA3* G risk allele. Indeed, patients carrying the *PNPLA3* G risk allele with high-normal TSH levels had a higher total NAS score and an almost two-fold higher prevalence of NASH (65.5% *vs.* 33.7%) compared with their counterparts with strict-normal TSH levels. Conversely, no significant association was found between high-normal TSH levels and presence of NASH or total NAS score among carriers of the *PNPLA3* CC genotype.

High-normal TSH levels and increased risk of having NASH in carriers of the PNPLA3 G risk allele

As shown in **Table 3**, in univariable logistic regression analysis, there was no significant association between high-normal TSH levels and presence of NASH among carriers of the *PNPLA3* CC genotype, whereas this association was statistically significant among patients carrying the *PNPLA3* G risk allele (unadjusted-OR, 3.745; 95%CI, 1.652–8.492; p=0.002). Notably, this association remained statistically significant even after adjustment for age, sex, BMI, hypertension, diabetes, dyslipidemia, serum albumin and HOMA-IR score (adjusted-OR 3.279; 95% CI 1.298-8.284; p=0.012). The results remained essentially unchanged even when we adjusted for waist circumference instead of BMI (adjusted-OR 2.966; 95% CI 1.180-7.456; p=0.021).

Discussion

The novel findings of our study are that in euthyroid adults with biopsy-proven NAFLD there is a strong association between high-normal TSH levels and presence of NASH. However, after stratifying by *PNPLA3* rs738409 genotypes, this association remained significant only amongst carriers of the *PNPLA3* G risk allele even after adjustment for age, sex, obesity, diabetes, HOMA-IR score, dyslipidemia and other potential confounders.

It is well known that thyroid hormones plays an important role in regulating body weight, lipid metabolism and insulin sensitivity, and emerging evidence also suggests that thyroid hormone regulation (even in the euthyroid range) may be involved in the development of NAFLD.[21] Gou *et al*. reported that increased serum TSH levels, irrespective of serum FT3 and FT4 levels, were an important

risk factor for the development and progression of NAFLD.[22] Furthermore, Carulli *et al*. suggested that high-though-normal TSH values were strongly associated with the presence of NASH[23] in a small series of euthyroid patients, although in another study this result was not confirmed.[24] More recently, in a sample of 425 South Korean adults with histologically-proven NAFLD, Kim *et al*. reported that NAFLD subjects with subclinical hypothyroidism or low-normal thyroid function had higher proportions of NASH and advanced liver fibrosis compared to those with strict-normal thyroid function. These authors also reported that risks of NASH and advanced fibrosis increased progressively with serum levels of TSH.[13]

To the best of our knowledge, this is the first study to show that the association between high-normal TSH levels and NASH (and total NAS score) markedly varied according to the *PNPLA3* rs738409 polymorphism. Although a significant association between TSH levels and liver fibrosis stage was not observed in our study, we found that high-normal TSH levels were strongly and independently associated with NASH only among *PNPLA3* GG and CG carriers, but not among those carrying the *PNPLA3* CC genotype.

To date, the biological mechanisms underlying the association between high-normal TSH levels and NASH and the possible influence of *PNPLA3* polymorphism on this association are poorly understood and remain largely speculative. It is known that triacylglycerol lipase, encoded by the *PNPLA3* protein, is a mediator for triacylglycerol hydrolysis in adipocytes.[25] This process of triacylglycerol hydrolysis is mainly controlled by the sterol regulatory element-binding protein1c

(SREBP1c).[26] Interestingly, one of the functions of the thyroid is to enhance SREBP1c gene expression favoring the cleavage of this enzyme, and then facilitating the binding to the sterol response elements (SREs) in the promoters of target genes.[27], Yan *et al*. also found that TSH level *per se* may increase hepatic fat content in a mice model, mainly through the upregulation of SREBP-1c activity.[11] The mechanism underlying TSH-induced hepatic fat accumulation is mainly explained by its role in the activation of the cAMP/PKA/PPARα pathway. The TSH receptor (TSHR) is a trigger for hepatic SREBP-1c activity that ultimately results in increased expression of genes that are implicated in hepatic lipogenesis through decreased AMPK in the cAMP/PKA/PPARα pathway.[11] As mentioned above, the *PNPLA3* G allele encoding the l148M variant reduces hydrolase activity of *PNPLA3*, and increases hepatic fat accumulation; consequently, the *PNPLA3* G allele is strongly associated with increased susceptibility to development and progression of NAFLD and hepatocellular carcinoma.[28-30]

It is widely accepted that hormone sensitive lipase and adipose triglyceride lipase (ATLG) are two important rate-limiting enzymes in the lipolysis process.[31] As early as 1969, Birnbaumer *et al.* confirmed that the receptor TSHR is also expressed in the plasma membrane of rat fat cells,[32] and TSH may promote the differentiation of pre-adipocytes into mature adipocytes.[33] TSH may also directly inhibit the expression of ATGL in mouse mature adipocytes by activating the cAMP/PKA pathway, thereby inhibiting basal lipolysis of triglycerides.[34]

Recent phase 2 randomized placebo-controlled trials also showed that resmetirom (MGL-3196) and VK2809, i.e., new liver-directed, orally active, selective thyroid

hormone receptor β (THR-β) agonists, significantly reduced liver fat content, after 12 weeks and 36 weeks of treatment, in euthyroid patients with biopsy-confirmed NASH.[35, 36]

Taken together, we consider that the findings from experimental and intervention studies provide further support for the results of our study. We have shown that high-normal TSH levels were significantly associated with higher total NAS score, presence of NASH and higher serum ALT levels, but that the proportion of patients with NASH and the histologic grade of steatosis were increased among those patients with high-normal TSH levels who carried the *PNPLA3* G risk allele. We suggest that if the promising results of the two recently phase 2 randomized controlled trials using the new selective THR-β agonists (discussed above) are confirmed in future larger trials, our findings may highlight a subgroup of patients with NASH who would benefit most from treatment with selective THR-β agonists (i.e. patients who have high-normal TSH levels and the *PNPLA3* G risk allele). That said, we recognize that further mechanistic studies are certainly needed to better understand the complex interconnections among NASH, *PNPLA3* polymorphism and high-normal TSH levels.

Our study has some important limitations that should be mentioned. Firstly, the cross-sectional design of the study does not allow establishing causal or temporal relationships between high-normal TSH levels, NASH and the *PNPLA3* rs738409 polymorphism, although this limitation is, at least in part, mitigated by the fact that the genetic variant under study is inherited and, therefore, reverse causation does not apply. Secondly, our study includes only a single ethnic group of Chinese individuals. Multiple studies demonstrated that the *PNPLA3* rs738409 G allele is more common in

Asian individuals without metabolic syndrome [1, 37] and, therefore, additional studies are required in other ethnic populations to confirm our findings. Finally, the relatively small number of patients with "high-normal" TSH, especially when patients were stratified by the *PNPLA3* genetic variant, suggests that larger cohorts of patients with biopsy-proven NAFLD are needed to further confirm our findings.

In conclusion, the results of our study show for the first time that in euthyroid patients with biopsy-confirmed NAFLD there is a significant association between high-normal TSH levels and the presence of NASH. After stratifying by *PNPLA3* genotype, this association was observed only among carriers of the *PNPLA3* G risk allele and remained significant even after adjusting for age, sex, obesity, diabetes, HOMA-IR score, dyslipidemia and other potential confounding factors.

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

Declaration of conflicting interests

All authors: nothing to declare.

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Legend to the Tables

Table 1. Baseline characteristics of euthyroid individuals with biopsy-proven NAFLD stratified by serum TSH status. The Student's *t*-test, the Welsh's *t*-test (for continuous variables) and the Fisher exact test (for categorical variables) were used to compare statistical differences in clinical, biochemical and liver histology characteristics between the groups.

Table 2. Baseline characteristics of euthyroid individuals with NAFLD stratified by both *PNPLA3* rs738409 genotypes and serum TSH status. The Student's *t*-test, the Welsh's *t*-test and the Fisher exact test were used to compare statistical differences in clinical, biochemical and liver histology characteristics between the groups.

Table 3. Risk of NASH based on TSH status (strict-normal *vs*. low-normal TSH group), stratified by *PNPLA3* rs738409 genotypes.

Supplementary Table 1. Baseline characteristics of euthyroid individuals with biopsy-proven NAFLD stratified by presence of NASH.

Figure Legends

Figure 1. Flowchart of the study.