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Doctoral Dissertation

瘦性非酒精性脂肪肝的血清標記物與單核苷酸多型性之探討

Biomarkers and Single Nucleotide Polymorphisms of Lean Non-alcoholic Fatty Liver Disease

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本論文係盧佳文（學號 D06421006）在國立臺灣大學醫學院臨床醫學所完成之博士學位論文，於民國 112 年 6 月 7 日承下列考試委員審查通過及口試及格，特此證明。

The undersigned, appointed by the Graduate Institute of Clinical Medicine, National Taiwan University, on 7th Jun. 2023 have examined a PhD dissertation entitled above presented by Chia-wen Lu (student ID D06421006) candidate and hereby certify that it is worthy of acceptance.

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中文摘要

背景

瘦型脂肪肝患者在脂肪肝中的比例越來越高。既是肝細胞激素又是脂肪激素的胎球蛋白-A 還有脂聯素/瘦素比率和瘦性脂肪肝之間的關聯從未被研究過，此外，亞洲人群中瘦型脂肪肝的遺傳特徵也尚不清楚。

主旨

本研究的目的是要探討調整中樞型肥胖和胰島素抵抗後，瘦型脂肪肝和非瘦型脂肪肝與血清胎球蛋白-A 及脂聯素/瘦素比率濃度之間的關聯。此外，和瘦型對照組相比，我們旨在檢視瘦型脂肪肝的有或沒有與 *PNPLA3* 和 *SAMM50* 單核苷酸多型性的相關風險。

方法

此論文是從兩個不同的人群和數據庫中整理的三項研究。在生物標誌物和瘦型脂肪肝的研究中，納入對象是台灣北部新竹市的社區成年人。根據身體質量指數和腹部超音波的判定，將受試者分別分為瘦型對照組、瘦型脂肪肝組、單純超重/肥胖（非瘦型）組和超重/肥胖脂肪肝組。實驗室中使用酶聯免疫吸附測定法測量血清胎球蛋白-A、脂聯素和瘦素。之後，以多變量邏輯回歸分析估計在調整可能的干擾因子後，胎球蛋白-A 和脂聯素/瘦素比率在不同血清濃度中患有瘦型脂肪肝的差異。我們使用接收者操作特徵曲線(以下稱 ROC 曲線)分析評估脂聯素/瘦素比率對瘦型脂肪肝的診斷準確度。

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至於單核苷酸多型性和瘦型脂肪肝相關的研究，是一項於 2022 年在台灣哈佛健診進行的病例對照研究。納入身體質量指數低於 24 kg/m^2 的成年人，並藉由腹部超音波分類是否有脂肪肝。基於 NHGRI-EBI 網站庫料庫並使用 Global Screening Array-24 v1.0 BeadChip 於單核苷酸多態性的選擇，我們去除重複和不顯著的變異後，選擇了 *PNPLA3* 基因中的 rs12483959 和 *SAMM50* 基因中的 rs3761472。統計方法則使用了多重邏輯回歸模型和 ROC 曲線分析評估。

結果

胎球蛋白-A 最高三分位數與最低三分位數血清濃度的脂肪肝勝算比為 2.62（95% CI：1.72-3.98；趨勢 P<0.001）。在以身體質量指數做分層分析，並調整可能的干擾因子後，胎球蛋白-A 高三分位數與最低三分位數的瘦型脂肪肝勝算比為 2.09（95% CI：1.09-3.98；趨勢 P 為 0.026）；與瘦型對照組相比，在調整年齡、性別、吸煙習慣、運動習慣、胰島素阻抗、和肝功能後，脂聯素/瘦素比率在瘦型脂肪肝勝算比為 0.28(95%CI: 0.12-0.69)。脂聯素/瘦素比率用以診斷脂肪肝的 ROC 曲線為 0.85（95% CI：0.82-0.88）(P<0.001)。在基因研究中，共有 1,652 名的瘦型對照組和 602 名瘦型脂肪肝患者被納入哈佛數據庫。*PNPLA3* rs12483959 (OR: 3.06; 95% CI: 2.15-4.37)和 *SAMM50* rs3761472 (OR: 2.90; 95% CI: 2.04-4.14)的 GG 基因型在調整年齡、性別、身體質量指數後，得到瘦型脂肪肝風險較高。*PNPLA3* rs738409 和 *SAMM50* rs3761472 檢測瘦型脂肪肝的 ROC 曲線下面積分別為 0.859（95%CI：0.841，0.877）和 0.860（95%CI：

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0.843，0.877）。

結論

胎球蛋白-A 和脂聯素/瘦素比率可能是早期區分瘦型脂肪肝和瘦型對照組的良好生物標誌物。針對基因變異，*PNPLA3* rs738409 和 *SAMM50* rs3761472 基因多態性與亞洲人群中的瘦性脂肪肝的高風險獨立相關。這些都有待進一步研究

關鍵字

瘦性非酒精性脂肪肝、代謝相關性脂肪肝、胎球蛋白-A、脂聯素、瘦素、單核苷酸多型性

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Abstract

Background: The prevalence of lean non-alcoholic fatty liver disease (NAFLD) is on the rise, contributing to a growing proportion of liver diseases. However, the phenotypic and genetic characteristics of lean NAFLD in Asian populations have yet to be fully understood.

Aims: Our study aims to investigate the correlation between serum levels of fetuin-A and the AL ratio in lean and non-lean individuals, considering their NAFLD status and adjusting for central obesity and insulin resistance. Furthermore, we intend to assess the varying risks of lean NAFLD in the presence or absence of *PNPLA3* and *SAMM50* variants, comparing them to lean individuals without NAFLD.

Methods: Three studies were conducted using data from two distinct populations and databases. The first set of studies included community-based adults residing in Hsinchu City, Northern Taiwan. The participants were categorized into different groups based on their BMI and ultrasonographic indicators of fatty liver, including lean controls, lean NAFLD, non-lean individuals with simple overweight/obesity, and overweight/obese individuals with NAFLD. Enzyme-linked immunosorbent assay was employed to measure serum levels of fetuin-A, adiponectin, and leptin. For the study related to SNPs and lean NAFLD, it was a cohort study conducted in the HAVO Health Exam Clinic from 2022 in Taiwan. Adults with a body mass index less than 24

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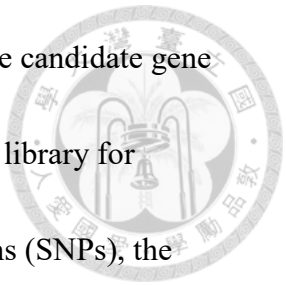

kg/m^2 were enrolled. Fatty liver was defined by ultrasonography. The candidate gene approach employed in the study relied on the NHGRI-EBI website's library for selecting relevant genes. To analyze single nucleotide polymorphisms (SNPs), the Global Screening Array-24 v1.0 Bead Chip was utilized for the selection process. After eliminating duplicates and insignificant variants, rs12483959 in the PNPLA3 gene and rs3761472 in the SAMM50 gene were chosen for analysis. Multiple logistic regression models and ROC curves were employed in these studies.

Results: The odds ratio (OR) for having NAFLD in the highest tertile compared to the lowest tertile of fetuin-A was 2.62 (95% CI: 1.72-3.98; P for trend < 0.001). When stratified by BMI and adjusted for confounding factors, the OR for having lean NAFLD in the highest versus the lowest tertile of fetuin-A was 2.09 (95% CI: 1.09-3.98; P for trend 0.026). Compared with the lean controls, the odds of having lean NAFLD for the highest versus the lowest tertile of AL ratio was 0.28(95%CI: 0.12-0.69) after adjustment. Regarding the diagnostic performance of NAFLD, incorporating the AL ratio, BMI, triglyceride levels, and AST/ALT ratio, the ROC analysis yielded an area under the curve (AUC) of 0.85 (95% CI: 0.82-0.88) for all NAFLD (P < 0.001). A total of 1,652 lean controls and 602 lean NAFLD patients were enrolled in HAVO database. After adjustment, individuals with GG genotypes of *PNPLA3* rs12483959 had a higher risk of fatty liver with an odds ratio (OR) of 3.06

doi:10.6342/NTU202301314

(95% CI: 2.15-4.37). Similarly, those with GG genotypes of *SAMM50* rs3761472 also had an increased risk of fatty liver with an OR of 2.90 (95% CI: 2.04-4.14). The ROC analysis demonstrated good discriminatory ability for *PNPLA3* rs738409 and *SAMM50* rs3761472 in identifying lean NAFLD. The areas under the ROC curves were 0.859 (95% CI: 0.841, 0.877) for *PNPLA3* rs738409 and 0.860 (95% CI: 0.843, 0.877) for *SAMM50* rs3761472.

Conclusions: The findings suggest that Fetuin-A and the AL ratio have the potential to serve as promising biomarkers for early differentiation between lean NAFLD patients and lean controls, irrespective of insulin resistance. Additionally, the gene variants *PNPLA3* rs738409 and *SAMM50* rs3761472 are independently linked to an increased risk of fatty liver in lean individuals of Asian descent. These results indicate the need for further investigation and research in this area to better understand the implications and potential clinical applications of these biomarkers and gene variants in lean NAFLD.

Key words: lean NAFLD, MAFLD, fetuin-A, adiponectin, leptin, single nucleotide polymorphism

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CONTENT

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doi:10.6342/NTU202301314

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I、INTRODCTION

Background

Patients with lean NAFLD make up an increasing subset of liver diseases. The association between lean NAFLD and fetuin-A, adiponectin/leptin (AL) ratio which serve as both a hepatokine and an adipokine, has never been examined. Besides, the genetic features of lean NAFLD in Asian populations remain unclear, too.

Aims

The aim of our study is to explore the association of serum gradients of fetuin-A and AL ratio among lean and non-lean patients, and those with NAFLD versus non-NAFLD after adjusting for central obesity and insulin resistance. Besides, we aimed to examine the different risks for lean NAFLD with and without *PNPLA3* and *SAMM50* variants in lean NAFLD patients compared with lean controls.

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II、LITERATURE REVIEWS

2.1 The definition and prevalence of lean NAFLD

Nonalcoholic fatty liver disease (NAFLD) is a significant health concern characterized by its increasing incidence and prevalence, along with its association with various comorbidities. The incidence of NAFLD ranges from 28 to 52 cases per 1,000 person-years, and the overall prevalence is approximately 25% (1). NAFLD is commonly linked to obesity, type 2 diabetes, dyslipidemia, and metabolic syndrome (MetS) (2). Consequently, there is a growing recognition of the need for a unified terminology, with metabolic-associated fatty liver disease (MAFLD) emerging as a synonymous term encompassing a spectrum of diseases from NAFLD (3). However, there is a subset of individuals who present with lean NAFLD, characterized by the presence of NAFLD despite having a normal body mass index (BMI) (4). Lean NAFLD patients differ from non-lean NAFLD patients in several aspects. They tend to be younger and exhibit higher hemoglobin levels (5), an elevated ratio of alanine aminotransferase (ALT) to aspartate aminotransferase (AST) (6), and lower levels of insulin resistance and MetS (7). Compared to healthy subjects, lean NAFLD patients are more likely to have dyslipidemia (8) and are predisposed to central obesity and insulin resistance (9). Nonetheless, it is important to note that both non-lean and lean NAFLD patients share common metabolic features such as insulin resistance and

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dyslipidemia (7). From a histological perspective, lean NAFLD tends to exhibit less severe steatosis, with fewer than 5% of hepatocytes displaying abnormal fat accumulation (10). The prevalence of non-alcoholic steatohepatitis (NASH), characterized by ballooning degeneration, lobular or portal inflammation, and fibrosis, appears to be similar between lean and non-lean NAFLD patients (10, 11). Overall, the limited data available and the conflicting results obtained thus far, coupled with the increasing population of lean NAFLD patients, have raised significant concerns and highlighted the need for further research in this area.

For the pathophysiology of NAFLD, the mainstream mechanisms are insulin resistance and increased adiposity that lead to metabolic dysregulation with significant liver involvement (12). In detail, the metabolic phenotype of NAFLD is characterized mainly by insulin resistance due to the hepatic oversupply with sugar, lipid and etc, while the genetic component is characterized by the impaired hepatic mitochondrial function, leading to chronic inflammation (13) The pathogenesis of NAFLD represents the metabolic dysfunction clinically of a complex interplay between lifestyle, environmental and genetic factors along with a key role for epigenetic changes. (1, 14). NAFLD is predominantly characterized by metabolic dysfunctions, with approximately 50% of individuals having coexisting type 2 diabetes (T2D), around 70% experiencing cardiovascular diseases, and more than

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90% being severely obese (2, 15). Recognizing the close association of NAFLD with metabolic dysregulation, a group of over 1,000 specialists across 134 countries has advocated for the endorsement of metabolic-associated fatty liver disease (MAFLD) as a unifying term for NAFLD, emphasizing its connection to metabolic dysfunction (16, 17).

As mentioned, there has been paradoxically a growing subset of patients who are inflicted with NAFLD, but their BMI is classified as lean (defined as BMI <25 in the Western region and BMI <23 in the Asian region) (4). Lean NAFLD varies in prevalence among different ethnic groups or with diagnostic approaches, accounting for 5% to 8% in Caucasian subjects and 16% to 18% in the Asia-Pacific region (18). Without obesity as a prerequisite for NAFLD, lean NAFLD shares similar severities of advanced diseases and mortality similar to its obese counterpart (19). Therefore, the new definition of MAFLD, i.e. NAFLD required an evidence of hepatic steatosis, detected either by imaging techniques, blood biomarkers/scores or by liver histology and involves one of the three following phenotypes, 1) overweight/obesity, 2) the presence of type 2 diabetes mellitus, or 3) lean subjects with evidence of metabolic dysregulation (20). Since liver biopsy is not feasible for widespread screening, ultrasound remains the most practical imaging modality for NAFLD detection (21). However, there is a pressing need for a reliable biomarker or scoring system for early

doi:10.6342/NTU202301314

detection and diagnosis of NAFLD, particularly for the easily overlooked population i.e. lean NAFLD. Currently, the fatty liver index (FLI), which incorporates BMI, waist circumference (WC), gamma-glutamyl transferase and triglyceride levels, is one of the most established scoring systems for NAFLD (22). Plasma cytokeratin 18 (CK18) fragment level has been the most extensively evaluated biomarker of steatohepatitis and is a marker of hepatocyte apoptosis (23). However, none of the aforementioned biomarkers or scores are specifically tailored for the early detection of lean NAFLD.

2.2 The biomarkers for lean NAFLD

Fetuin-A, also known as Alpha$_2$-Heremans-Schmid glycoprotein (AHSG), is a multifunctional glycoprotein that is predominantly synthesized and secreted by the liver, but is also produced by adipose tissue and other organs. (24). It plays a vital role in various biological processes, although its precise function is not fully elucidated. Research has implicated fetuin-A in the regulation of insulin resistance, inflammation, and cell adhesion (25). One of the most documented functions of fetuin-A is to act as an endogenous inhibitor of insulin receptor tyrosine kinase, which triggers insulin resistance (26). Therefore, fetuin-A has been highly correlated with diabetes, obesity, and MetS in previous studies (27, 28). Fetuin-A has been shown to promote lipid-induced insulin resistance by activating TLR4 signaling in liver and muscle cells,

doi:10.6342/NTU202301314

leading to increased expression of genes involved in lipid metabolism and inflammation. Fetuin-A was assumed to act as an endogenous ligand of Toll-like receptor 4 to stimulate chronic adipose inflammation (29). Fetuin-A stimulates the secretion of inflammatory cytokines such as TNF-alpha and interleukin-6 in adipose tissue (30). In addition to its role in insulin resistance, fetuin-A has also been implicated in inflammation and cell adhesion. For example, fetuin-A has been shown to inhibit the adhesion of leukocytes to endothelial cells, suggesting a role in the regulation of immune responses. Moreover, fetuin-A has been shown to modulate the activity of various proteases, such as matrix metalloproteinases and tissue inhibitor of metalloproteinases, which are involved in tissue remodeling and repair. With roles in both insulin resistance and chronic inflammation, circulating fetuin-A levels have been found to be significantly correlated with NAFLD patients (31). Several human studies have reported an association between elevated serum fetuin-A levels and the development and progression of NAFLD (32). However, the exact mechanisms by which fetuin-A contributes to the pathogenesis of NAFLD are not fully understood and further studies are needed to clarify this relationship. Similar, the association between lean NAFLD and fetuin-A has never been studied.

Other than Fetuin-A, adiponectin and leptin are promising biomarkers which were discovered in the 1990s. Adiponectin is a hormone secreted by adipose tissue

doi:10.6342/NTU202301314

that was first identified in 1995 by Scherer et al. Since then adipose tissue has gradually transformed from a simple energy reservoir to a highly active endocrine organ (33). It is one of the most abundant adipokines produced by adipose tissue and plays a key role in regulating metabolism and inflammation. Adiponectin exists in several isoforms, including a low molecular weight isoform, a medium molecular weight isoform, and a high molecular weight isoform, with the last isoform being the most biologically active (34). Adiponectin has a variety of physiological functions, including regulation of insulin sensitivity, glucose and lipid metabolism, inflammation, and cardiovascular function. It enhances insulin sensitivity by stimulating glucose uptake and fatty acid oxidation in skeletal muscle and liver cells, thereby promoting glucose utilization and reducing blood glucose levels. Adiponectin also has a beneficial effect on lipid metabolism by increasing fatty acid oxidation and decreasing lipogenesis in adipose tissue and liver cells. In addition to its metabolic effects, adiponectin has anti-inflammatory properties. It inhibits the production of pro-inflammatory cytokines such as TNF-α and IL-6 and promotes the production of anti-inflammatory cytokines such as IL-10. This anti-inflammatory effect is thought to contribute to the cardioprotective effects of adiponectin, as chronic inflammation is a risk factor for cardiovascular disease (35). Adiponectin levels are negatively correlated with obesity, insulin resistance, and type 2 diabetes. Low levels of

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adiponectin have been shown to be an independent risk factor for the development of these metabolic disorders (36). Conversely, increased levels of adiponectin have been associated with a reduced risk of metabolic disorders and cardiovascular disease (34).

Leptin is positively correlated with obesity and insulin resistance (37), while adiponectin shows a good ability to enhance insulin sensitivity and counteract the development of diabetes (38, 39). Additionally, leptin dually exerts antisteatotic proinflammatory and profibrogenic actions for NAFLD. The net effect however remains unclear (40). In contrast, adiponectin consistently promotes anti-inflammatory and antifibrotic activity (41). Consequently, adiponectin to leptin ratio was assumed to correlate negatively with low-grade chronic inflammation (42), atherosclerosis risk (43) and cardiovascular disorders (35, 44). A few human studies have elaborated the association between the adiponectin, leptin or AL ratio and NAFLD (45, 46) while few were related to lean NAFLD. No matter obese or not, adiponectin is a biomarker for NAFLD subjects indicating the progression to steatohepatitis in a biopsy proven study (47) and the development of NAFLD in a Korea cohort (48). And, lean subjects with evidence of NAFLD have lower adiponectin concentrations than lean controls in Caucasian populations. In the other hand, leptin levels reflect total body fat and insulin resistance (49) that correlate positively with hepatic steatosis in diabetes subjects (50). Taking together, AL ratio

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were associated with the severity of steatosis in a Japanese study (45) and was a predictor of NAFLD in obese adults that correlated with liver function and insulin resistance better than each single adipokine (46). However, it is important to note that there is currently no investigation specifically exploring the relationship between lean NAFLD and the AL ratio. Further research is needed to elucidate the role of the AL ratio in lean NAFLD and its potential as a diagnostic or prognostic marker in this specific population.

Theoretically, the AL ratio could serve as a potential marker to distinguish individuals with lean NAFLD from those without NAFLD, particularly in the early stages of the disease and independent of insulin sensitivity. It is true that in theory, lean individuals without NAFLD tend to have higher adiponectin levels and lower leptin levels compared to those with NAFLD. However, NAFLD itself is a chronic inflammatory condition that can affect adipokine levels and disrupt the expected balance. The AL ratio, which take into accounts both adiponectin and leptin levels, may provide a more comprehensive assessment of the metabolic status and inflammatory state associated with NAFLD. By examining the AL ratio, it may be possible to identify subtle alterations in adipokine profiles that differentiate lean individuals with NAFLD from those without NAFLD, even in the early stages of the disease. To test the hypothesis, further research is needed to investigate the AL ratio in

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lean individuals with and without NAFLD, specifically focusing on early stages of the disease and considering potential confounding factors such as insulin sensitivity. Longitudinal studies and larger sample sizes would help establish the utility of the AL ratio as a diagnostic or prognostic marker for lean NAFLD.

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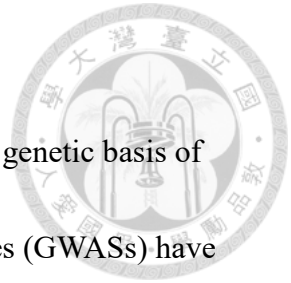

2.3 SNPs for lean NAFLD

Numerous gene studies have been conducted to investigate the genetic basis of lean NAFLD. Among these studies, genome-wide association studies (GWASs) have consistently identified single nucleotide polymorphisms (SNPs) in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene as the most extensively studied SNPs associated with lean NAFLD (51, 52). The *PNPLA3* gene encodes a protein called adiponutrin, which is expressed in both adipocytes and hepatocytes and plays a crucial role in lipid metabolism (53). Briefly, the *PNPLA3* variant results in the production of a protein that is less efficient in lipid metabolism, leading to the accumulation of fat in the liver. A common genetic variant of the *PNPLA3* gene, rs738409, is strongly associated with the development and progression of NAFLD (54). The *PNPLA3* rs738409 variant seemed to be more common in Japanese, Hong Kong and Sri Lankan patients with lean NAFLD than in those with obese NAFLD, but there was no difference in Western countries. (55-57) The exact mechanism by which the *PNPLA3* gene contributes to the development of NAFLD is not fully understood. However, it is thought that the G allele may cause the *PNPLA3* protein to be less effective at breaking down triglycerides, which could lead to the accumulation of fat in the liver. In addition, the presence of the *PNPLA3* rs738409 G allele has been associated with an earlier presentation of NAFLD in Taiwanese children. (58) It is

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noteworthy that the association between *PNPLA3* variants and lean NAFLD appears particularly strong. This suggests that even individuals without traditional risk factors for NAFLD, such as obesity, may still be at risk of developing the condition if they carry the *PNPLA3* rs738409 G allele. Based on these findings, we hypothesize that *PNPLA3* variants independently contribute to the risk of NAFLD in lean adults within the Taiwanese population. Further research is needed to explore this relationship and understand the underlying mechanisms in detail. Sorting and assembly machinery component 50 homolog (*SAMM50*) and the well-known *PNPLA3* are both located on chromosome 22q13. *SAMM50* is a mitochondrial outer membrane protein involved in the reduction of oxidative stress. It was first identified as a component of the SAM complex, which is involved in the assembly of beta-barrel proteins in the outer mitochondrial membrane. (59) In Asian studies, *SAMM50* polymorphisms contributed to the occurrence and severity of fatty liver in the Chinese Han, Korean and Japanese populations. (60, 61) While *SAMM50* deficiency has been linked to mitochondrial function and morphology, there is currently no evidence to suggest that *SAMM50* polymorphisms are directly associated with fatty liver disease or its severity. Notably, there have been no studies to date examining the association of *SAMM50* specifically in lean subjects with and without NAFLD. Further research is needed to explore the potential involvement of *SAMM50* in lean NAFLD and elucidate its specific role in

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the pathogenesis and progression of the disease. Understanding the genetic factors, such as SAMM50 polymorphisms, can contribute to a comprehensive understanding of the underlying mechanisms and aid in the development of targeted interventions for lean NAFLD patients.

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III、MATERIALS AND METHODS

3.1 Fetuin-A and lean NAFLD

3. 1.1 Study Subjects

This study was conducted in the community of Hsinchu City, Northern Taiwan. All the participants completed standardized questionnaires through individual interviews. The exclusion criteria were excessive alcohol use, which was defined as drinking more than 20 g of alcohol daily for women and 30 g for men, and chronic liver diseases, which included chronic hepatitis, autoimmune, drug-induced, vascular, and inherited hemochromatosis, and Wilson disease. In total, 606 adults older than 20 years were enrolled. Prior to participation, informed consent forms were signed, and information regarding age, sex, cigarette smoking, exercise habits, and previous diseases was collected. participants were classified as current smokers if they had been smoking for more than 6 months prior to the study. Noncurrent smokers were defined as individuals who had quit smoking for more than 12 months before the study or had never smoked. The presence of a regular exercise habit was assessed through a simple yes or no question: “Do you have a regular exercise habit?” Weight and height were measured by a standard electronic scale and stadiometer. Blood pressure (BP) was measured by a sphygmomanometer. Waist circumference (WC) was measured by the same trained operator. This study was approved by the

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Institutional Review Board of National Taiwan University Hospital (IRB NO. 201210012RIC).

3.1.2 Ultrasonography assessment

Abdominal ultrasonography was performed after at least eight hours of fasting by a well-trained examiner with a 3.5–5 MHz transducer and a high-resolution B-mode scanner (Hitachi Aloka ProSound α 6). The ultrasound measurements were performed by three experienced research physicians. Before the study, all three physicians reached a consensus regarding the standard procedure for ultrasound scanning, including the scoring of ultrasonographic fatty liver indicator (US-FLI) and the sequence of acquiring liver images. The severity of NAFLD was calculated using the US-FLI score, which ranges from 0 to 8(21). The US-FLI is composed of five indicators: (1) the presence of liver-kidney contrast graded as mild/moderate (score 2) and severe (score 3); and (2) the presence (score 1) or absence (score 0) of posterior attenuation of the ultrasound beam, vessel blurring, difficult visualization of the gallbladder wall, difficult visualization of the diaphragm, and areas of focal sparing (score of 1 each). The subjects were then divided into four groups: (1) lean non-NAFLD group: US-FLI score <2, BMI<24 kg/m^2; (2) lean NAFLD group: US-FLI score ≥ 2, BMI< 24 kg/m^2; (3) non-lean, non-NAFLD group: US-FLI score <2, BMI ≥24 kg/m^2; (4) non-lean NAFLD group: US-FLI score ≥ 2, BMI ≥24 kg/m^2.

doi:10.6342/NTU202301314

3.1.3 Blood Analysis

Venous blood samples were collected after at least eight hours of fasting. Serum glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides were measured by an automatic spectrophotometric assay (HITACHI 7250, Japan). Fasting insulin levels were assessed by a micro-particle enzyme immunoassay using an AxSYM system (Abbott Laboratories, Dainabot Co, Tokyo, Japan). To estimate the degree of insulin resistance, the homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using the formula: HOMA-IR = (fasting insulin × fasting plasma glucose) / 22.5. Glucose was measured in mmol/L, and insulin was measured in mU/L (62). Serum fetuin-A was measured using a quantitative sandwich enzyme immunoassay technique. Prior to the analysis, the serum samples were diluted 4,000-fold. This immunoassay was calibrated against highly purified NS0-expressing recombinant human fetuin-A (R&D Inc. Minneapolis, USA).

3. 1.4 Statistical analysis

The participants were categorized into tertiles based on their serum fetuin-A levels. Continuous variables are presented as mean ± standard deviation (SD), while categorical variables are presented as numbers and percentages. Multivariate logistic regression analyses were conducted to determine the odds ratios of having NAFLD

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across the fetuin-A tertiles. The analysis was adjusted for factors such as age, sex, current smoking, exercise habit, WC, and HOMA-IR. Stratified analyses were performed based on BMI status. To assess the relationship between serum fetuin-A concentrations and NAFLD groups, adjusted least square means were calculated using general linear models. The adjustments included age, sex, current smoking, exercise habit, waist circumference, and HOMA-IR. Statistical analyses were performed using SPSS statistical software (V.17, SPSS, Chicago, Illinois, USA). A p value of <0.05 was considered to be statistically significant.

3.2 AL ratio and lean NAFLD

3.2.1 Study Subjects

This study was derived from a previous investigation on the association between Fetuin-A and lean NAFLD (63). However, in this study, individuals with diabetes were excluded to align with the emerging consensus that considers diabetes as a distinct category separate from lean NAFLD due to differences in genotype and phenotype. The exclusion criteria please referred to the previous published study (63). In total, 575 adults older than 20 years with diabetes were enrolled. Information about age, sex, cigarette smoking, exercise habits, and previous diseases was obtained after informed consent forms were signed. BP and WC were measured by the same trained operator. Body fat percentage was measured through bioelectrical impedance analysis

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by a portable body composition analyzer (TANITA BC-418, Japan). This study was approved by the Institutional Review Board of National Taiwan University Hospital (IRB NO. 201210012RIC).

3.2.2 Ultrasonography assessment

Abdominal ultrasonography was performed by three experienced physicians using a 3.5–5 MHz transducer and a high-resolution B-mode scanner (Hitachi Aloka ProSound Alpha 6, Japan). For the detail of the scoring protocol please refer to our previous study (63).

3.2.3 Definition of Lean and NAFLD groups

The cut-off points for BMI categories in Taiwan are defined as follows: <18.5 kg/m^2: underweight, 18.5–23.9 kg/m^2: normal weight, 24–26.9 kg/m^2: overweight, ≥27 kg/m^2: obesity (33). In our study, the subjects were then divided into the following groups: (1) lean controls: US-FLI score <2, BMI<24 kg/m^2; (2) lean NAFLD group: US-FLI score ≥ 2, BMI< 24 kg/m^2; (3) simple overweight/obesity group: US-FLI score <2, BMI ≥24 kg/m^2; and (4) overweight/obesity NAFLD group: US-FLI score ≥ 2, BMI ≥24 kg/m^2.

3.2.4 Blood Analysis

After a minimum fasting period of eight hours, venous blood samples were collected from the participants. The collected samples were used to measure various

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parameters including serum glucose, HbA1c, total cholesterol, HDL-C, LDL-C, triglycerides, and HOMA-IR. (63) Serum adiponectin (As One International INC, Santa Clara, CA, USA) was diluted to 10x during pre-treatment, incubated at 100ºC for 5 minutes and then diluted to 5100x finally. Serum leptin (R&D Inc. Minneapolis, USA) was diluted to 30x using dilution buffer. The limit of detection (LOD) was 23.4 pg/mL and 7.8 pg/mL for adiponectin and leptin, respectively. The intra-assay and inter-assay coefficients of variation (CVs) were all less than 5%. Both adiponectin and leptin were then measured by enzyme-linked immunosorbent assay following manufacturer's protocol as previously described (35).

3.2.5 Statistical Analysis

Continuous variables are presented as mean±SD, while categorical variables are presented as number (percentage). The differences between the four groups were assessed using the chi-squared test for categorical variables and one-way analysis of variance (ANOVA) for continuous variables. Subsequently, Tukey's post hoc analysis was conducted to compare the healthy control, lean NAFLD, overweight controls, and overweight NAFLD groups in terms of basic demographic characteristics, leptin, adiponectin, and the AL ratio. To explore the relationship between the AL ratio and metabolic factors, multivariate linear regression analyses were performed. The dependent variables included lean controls, lean NAFLD, simple overweight/obesity,

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and overweight/obesity NAFLD groups, while the independent variable was the tertiles of the AL ratio. Furthermore, multivariate logistic regression models were employed to investigate the odds of having NAFLD in relation to the tertiles of the AL ratio after adjusting for age, sex, current smoking, exercise habits, HOMA-IR, and AST/ALT ratio. ROC analysis, with the calculation of the area under the ROC curve (AUC), was conducted to evaluate the diagnostic performance of the AL ratio for NAFLD. All statistical analyses were performed using the SPSS statistical software package. (V.17, SPSS, Chicago, Illinois, USA). A p value of <0.05 was considered to be statistically significant.

3.3. SNP and lead ALFD study population

3.3.1 Study Subjects

This was a cohort study conducted in the HAVO Health Exam Clinic. The HAVO database included individuals aged more than 20 years who received a self-paid health check-up and SNP genotyping from Jan 2020 to the end of 2021. All subjects completed standardized questionnaires through individual interviews. The health survey questionnaire asked participants questions regarding socio-demographics, lifestyles and medical history. The health check-up included physical exams, blood analyses, and abdominal ultrasonography. Informed consent forms were obtained from all participants. The study was approved by the Institutional Review Board of

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For lean NAFLD, the exclusion criteria were 1) body mass index ≥ 24 kg/m^2; 2) inability to undergo an abdominal ultrasound examination; and 3) incomplete SNP genotyping. The content of the physical examination included weight and height, which were measured by a standard electronic scale and stadiometer, and blood pressure (BP), which was measured by an electronic sphygmomanometer. Waist circumference was measured horizontally through the middle point between the upper border of the iliac bones and the lower border of the ribs. The content of the blood test included fasting glucose, triglycerides and high-density lipoprotein cholesterol (HDL-C). Abdominal ultrasonography was performed by trained physicians. Fatty liver was binarily defined by the presence or absence of liver-kidney contrast. We used the BMI cutoff of 24 kg/m^2 to define lean and overweight-obese subjects.(64) Participants were considered to have metabolic syndrome if they met ≥3 of the following criteria: waist circumference ≥90 cm in men or ≥80 cm in women; serum triglycerides ≥ 150 mg/dL; HDL-C < 40 mg/dL in men or <50 mg/dL in women; systolic BP ≥ 130 and/or diastolic BP ≥ 85 mm Hg; and fasting glucose ≥100 mg/dL.

3.3.2 Selection of single nucleotide polymorphisms

We searched candidate genes and NAFLD-related SNPs on the website of the most up-to-date SNPs reported in the publications of the National Human Genome

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Research Institute (NHGRI) and the European Bioinformatics Institute (EMBL-EBI) (Supplement A and B). The website of NHGRI-EBI Catalog of Published Genome-Wide Association Studies (GWAS) (65) collects a total of 67 documented SNPs in *PNPLA3* and 12 SNPs in *SAMM50* which were found to be related to NAFLD. From the HAVO database, a total of 1,652 lean controls and 602 lean NAFLD patients were extracted by criteria. Global Screening Array (GSA)-24 v1.0 BeadChip (Infinium, California, USA) was then used for genotyping. The basic microarray technical data of the Asian Screening Array (ASA) were downloaded from the Illumina official website (66). The main sources of ASA chips were from East Asian and Southeast Asian populations, such as China, Japan, South Korea, Mongolia, and Singapore. A total of more than 9,000 subjects were enrolled, and whole-gene sequencing data were obtained. A total of approximately 642,824 SNPs were screened. Genotype calls were highly accurate with 99.5% call rates; otherwise, they were considered too far from the cluster centroid to have reliable genotype calls, reproducibility or Mendelian consistency. After matching 67 SNPs in *PNPLA3* and 12 SNPs in *SAMM50* to the ASA chip, we excluded 60 SNPs in the *PNPLA3* gene and 8 SNPs in the *SAMM50* gene. Then, we excluded one SNP for duplication and statistical non-significance and five SNPs in *PNPLA3* and 2 SNPs in *SAMM50* that had high collinearity (Pearson correlation coefficient > 0.95). As a result, rs12483959 in *PNPLA3* and rs3761472 in

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SAMM50 entered further statistical analyses. The flow chart of SNP selection for lean NAFLD is shown in Figure1.

3.3.3 Statistical analysis

Data are presented as the mean±SD for continuous variables and number (percentage) for categorical variables. Differences between the groups were examined using the chi-squared test for categorical variables and Student's *t* test or one-way analysis of variance (ANOVA) for continuous variables. Multivariate logistic regression analyses were performed to estimate the relationship between the odds of having fatty liver in relation to *PNPLA3* rs738409 and *SAMM50* rs3761472 after adjustment for age, sex, smoking, drinking, BMI and metabolic factors (waist circumference, fasting glucose, systolic BP, diastolic BP, triglycerides and HDL-C). We performed ROC analysis to determine the diagnostic performance of *PNPLA3* rs738409 and *SAMM50* rs3761472 for lean NAFLD. All analyses were performed using SPSS statistical software (V.17, SPSS, Chicago, Illinois, USA) and R software (R-4.2.2). A p value of <0.05 was considered to be statistically significant.

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IV、RESULTS

4.1. Fetuin-A and lean NAFLD

4.1.1. General Characteristics

Table 1 presents the key characteristics of the participants. The average age of the participants was 42.6±11.5 years, and 61.7% of them were female. The mean serum concentrations of fetuin-A were as follows: 689.4±672.4 mg/L, 882.6±731.3 mg/L, 829.3±429.3 mg/L, and 855.9±467.0 mg/L for the four groups (Figure 2). Notably, the lean NAFLD group exhibited the highest level of fetuin-A. A post hoc analysis (Table 2) revealed that the lean NAFLD group shared similar metabolic factors with the non-lean, non-NAFLD group. Both lean and non-lean NAFLD had high levels of fetuin A, while non-lean NAFLD apparently had more metabolic factors and high BMI, waist circumference, and body fat percentage.

4.1.2. Association of fetuin-A and NAFLD

To investigate the relationship between fetuin-A concentration gradients and NAFLD, multiple logistic regression analyses were conducted, and the odds ratios (ORs) of having NAFLD were examined based on tertiles of serum fetuin-A levels (Table 3). Adjusting for age, gender, current smoking, and exercise habit, the OR of having NAFLD was 2.62 (95% CI: 1.72-3.98; P for trend<0.001) for the highest tertile compared to the lowest tertile of fetuin-A. After further adjustment for age, sex,

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current smoking, exercise, and waist circumference (WC), the OR decreased to 1.80 (95% CI: 1.10-2.94, P for trend 0.02). However, after additional adjustment for HOMA-IR, the ORs became statistically insignificant (1.5; 95% CI: 0.92-2.67; P for trend 0.099).

Table 4 presents the ORs of having NAFLD based on multiple logistic regression analyses stratified by BMI and tertiles of serum fetuin-A levels. When BMI was less than 24 kg/m^2, the crude OR of having NAFLD for the highest tertile versus the lowest tertile of fetuin-A was 1.95 (95% CI: 1.14-3.34; P for trend<0.018). After adjusting for age, sex, current smoking, exercise, WC, and HOMA-IR, the OR remained elevated at 2.09 (95% CI: 1.09-3.98; P for trend 0.026). In contrast, for BMI greater than 24 kg/m^2, both the crude and adjusted ORs of having NAFLD for the highest tertile compared to the lowest tertile of fetuin-A were not statistically significant, with values of 1.35 (95% CI: 0.57-3.21; P for trend<0.603) and 0.69 (95% CI: 0.24-1.95; P for trend 0.422), respectively.

The least square means (±SDs) of the serum fetuin-A concentrations in relation to the four groups were 732.4 (617.0-847.9) mg/L, 920.3 (790.5-1050.1) mg/L, 860.0 (678.5-1041.6) mg/L, and 833.3 (723.7-942.9) mg/L after adjusting for age, sex, current smoking, current drinking, exercise habit, WC, and HOMA-IR (Figure 3).

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4.2. AL ratio and lean NAFLD

4.2.1. General Characteristics

Table 5 presents the basic characteristics of the participants. The average age of the participants was 42.8±11.5 years, with 61.3% of them being female. Out of the 575 subjects included in the study, 200 subjects (34.8%) were diagnosed with overweight/obesity NAFLD, and 105 subjects (18.3%) had lean NAFLD. As diabetes was excluded, our study group consisted of a substantial proportion of metabolically healthy individuals (MetS factors: 1.08±1.11). Tukey's post hoc analysis was conducted to examine the differences between the groups (Table 6). Notably, the AL ratio emerged as a specific indicator distinguishing the lean control group from both the lean and overweight/obesity NAFLD groups, surpassing the differentiating capabilities of adiponectin or leptin alone (Figure 3-9). Comparing the lean NAFLD group with the simple overweight/obesity group, significant differences were observed in BMI, fat percentage, and waist circumference. However, no significant differences were found in other metabolic parameters, including blood pressure, lipid profile, glucose, insulin resistance, or inflammatory biomarkers such as AST, ALT, and CRP.

4.2.2. Association of AL ratio and NAFLD

In order to examine the relationship between each factor of metabolic syndrome

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and the AL ratio, multivariate linear regression models were utilized, adjusting for age and sex (Table 7). The analysis revealed significant associations between the AL ratio and various metabolic factors. The AL ratio exhibited negative associations with body fat percentage, BMI, waist circumference (WC), systolic blood pressure (SBP), diastolic blood pressure (DBP), triglycerides (TG), glucose, and homeostatic model assessment of insulin resistance (HOMA-IR) (all $p < 0.001$). On the other hand, a positive association was observed between the AL ratio and high-density lipoprotein (HDL) cholesterol ($p < 0.001$). These findings highlight the AL ratio as a consistent and robust biomarker for detecting metabolic dysfunction.

To investigate the significance of the AL ratio as a parameter for metabolic syndrome, multivariate logistic regression models were employed to assess the odds of having NAFLD based on different cut-off values (Table 8-10) and tertiles of serum AL ratio (Table 11). The analysis revealed that the highest tertile of the AL ratio was associated with reduced odds of having NAFLD compared to the lowest tertile, with an odds ratio (OR) of 0.34 (95% CI: 0.17-0.71; P for trend<0.001). Upon further adjustment for the AST/ALT ratio, the OR of having NAFLD for the highest tertile of the AL ratio remained significant at 0.37 (95% CI: 0.18-0.77, P for trend 0.008). These findings emphasize the potential of the AL ratio as a valuable parameter for assessing NAFLD risk in individuals with metabolic syndrome.

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Table 12 presents the odds ratios (ORs) of having non-alcoholic fatty liver disease (NAFLD) based on tertiles of serum AL ratio, stratified by BMI. Among individuals with a BMI < 24 kg/m^2, the adjusted OR of having NAFLD for the highest versus the lowest tertile of AL ratio was 0.28 (95% CI: 0.12-0.69; P for trend 0.005). Similarly, among individuals with a BMI > 24 kg/m^2, the adjusted OR of having NAFLD for the highest versus the lowest tertile of AL ratio was 0.30 (95% CI: 0.09-0.96; P for trend 0.043).

In addition, the AL ratio, BMI, triglyceride levels, and AST/ALT ratio were selected for the diagnosis performance of NAFLD using a ROC analysis. The area under the ROC curve (AUROC) for all subjects was 0.85 (95% CI: 0.82-0.88). Stratified by gender, the AUROC was 0.83 (95% CI: 0.78-0.87) for females and 0.86 (95% CI: 0.81-0.91) for males (all p < 0.001). These results highlight the diagnostic performance of the AL ratio in identifying NAFLD, with promising accuracy across different BMI categories and gender groups (Figure 10).

4.3. SNPs for lean NAFLD

4.3.1 Baseline characteristics

A total of 1,652 lean controls and 602 lean NAFLD patients were enrolled. The average age was 43.8±11.5 years, and 1,130 (50.1%) patients were male (Table 13). In the lean NAFLD group, the subjects were older, and the percentage of metabolic syndrome was higher than that in the lean control group (case vs. control: 10.5% vs. 1.5%). Waist circumference, systolic BP, diastolic BP, fasting glucose and triglycerides were significantly higher and HDL-C was significantly lower in lean

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NAFLD patients than in lean controls.

4.3.2 Distribution of genotypes in lean subjects

The gene location, cytogenic region, most severe consequence of the SNPs related *PNPLA3* and *SAMM50* were shown in Table 14-17. The distribution of *PNPLA3* rs738409 (CC/CG/GG) and *SAMM50* rs3761472 (AA/AG/GG) in lean NAFLD patients differed significantly from that in lean controls. In subjects with the *PNPLA3* rs738409 CC/CG/GG genotypes, 193 (22.6%), 216 (26.1%) and 123 (36.1%) had lean NAFLD compared with 660 (77.4%), 611 (73.9%) and 218 (63.9%) lean controls, respectively [OR=1.11 (0.90-1.36) for the CG genotype, OR=1.77 (1.36-2.29) for the GG genotype (reference: CC genotype)]. In subjects with the *SAMM50* rs3761472 AA/AG/GG genotypes, 197 (22.9%), 287 (27.2%) and 118 (35.0%) had lean NAFLD compared with 664 (77.1%), 769 (72.8%) and 219 (65.0%) lean controls, respectively [OR=1.26 (1.02-1.55) for the AG genotype, OR=1.82 (1.38-2.39) for the GG genotype (reference: AA genotype)] (table 18).

4.3.3 Clinical characteristics according to genotype

We sorted the subjects into categories by genotype at locus rs738409 (representative of the *PNPLA3* gene) or locus rs3761472 (representative of the *SAMM50* gene). The rs738409 SNP posed a low, moderate and high risk of fatty liver in the CC, CG, and GG genotypes, respectively, and the rs3761472 SNP posed a low, moderate and high risk of fatty liver in the AA, AG, and GG genotypes, respectively.

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Among the different gene risks of fatty liver in the lean population, there were no significant differences in body mass index, age, sex, smoking or drinking. Additionally, none of the metabolic factors, including waist circumference, systolic BP, diastolic BP, fasting glucose, triglycerides and HDL-C, were significantly different among the different genotypes. The only difference among the CC, CG, and GG genotypes of *PNPLA3* rs738409 or the AA, AG, and GG genotypes of *SAMM50* rs3761472 was the presence or absence of fatty liver (Table 18).

4.3.4 Independent risk factors for lean NAFLD

Table 19 presents the odds ratios (ORs) of developing fatty liver in lean individuals based on gene variants of PNPLA3 rs738409 and SAMM50 rs3761472, as determined by logistic regression models with adjustments. Regarding the PNPLA3 rs738409 SNP, after adjusting for age, sex, smoking, and drinking, the OR of developing lean NAFLD for the GG genotype compared to the CC genotype was 2.17 (95% CI: 1.62-2.92; P for trend <0.001). Further adjustment for BMI and metabolic factors, including waist circumference, fasting glucose, systolic BP, diastolic BP, triglycerides, and HDL-C, revealed an increased OR of 3.06 (95% CI: 2.15-4.37; P for trend <0.001) for the GG genotype compared to the CC genotype. Similarly, for the SAMM50 rs3761472 SNP, after adjustment for age, sex, smoking, and drinking, the OR of developing lean NAFLD for the GG genotype compared to the AA genotype

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was 2.03 (95% CI: 1.51-2.73; P for trend <0.001). Upon further adjustment for BMI and metabolic factors, the OR of developing lean NAFLD for the GG genotype compared to the AA genotype was 2.90 (95% CI: 2.04-4.14; P for trend <0.001). These findings indicate that both the PNPLA3 rs738409 and SAMM50 rs3761472 gene variants are significantly associated with an increased risk of developing fatty liver in lean individuals, even after accounting for potential confounding factors.

To further evaluate the diagnostic performance of these gene variants for lean NAFLD in lean subjects, we utilized age, sex, waist circumference, fasting glucose, systolic BP, diastolic BP, triglycerides, and HDL-C as predictors. The area under the ROC was calculated for each variant. The AUC for rs738409 was 0.859 (95% CI: 0.841, 0.877), while the AUC for rs3761472 was 0.860 (95% CI: 0.843, 0.877) (Figure 11). These results demonstrate that both gene variants have good abilities in identifying lean individuals at risk of NAFLD.

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V、DISCUSSIONS

5.1. Fetuin-A and lean NAFLD

This study provides the first evidence of a positive association between the serum fetuin-A gradient and the risk of lean NAFLD. The findings are as follows: First, the highest tertile of serum fetuin-A was associated with a 2.09-fold increased risk of lean NAFLD compared to the lowest tertile, while no significant association was observed in non-lean NAFLD. Second, a dose-response relationship was identified between the serum fetuin-A gradient and non-lean NAFLD, even after adjusting for age, sex, current smoking, exercise habit, WC, and HOMA-IR (P for trend <0.05). Third, both lean and non-lean NAFLD patients exhibited elevated levels of fetuin-A. However, non-lean NAFLD patients displayed additional metabolic factors, higher BMI, waist circumference, and body fat percentage. Interestingly, the direct association between fetuin-A and the risk of lean NAFLD persisted even after adjusting for WC and HOMA-IR. This suggests that there may be unidentified factors influencing this association, beyond central obesity and insulin resistance, which were only accounted for in the lean subjects.

The name "fetuin" suggests that its highest concentration is found in fetal blood. While fetuin levels decrease significantly in adults, it serves various important functions. Specifically, fetuin-A is predominantly expressed in the liver (over 95%)

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and is secreted by hepatocytes and adipocytes (67). It is well known that fetuin-A is involved in the development of insulin resistance in both animal and human studies (68, 69), and thus contributes to the development of NAFLD. Animal studies have demonstrated that fetuin-A promotes lipid-induced inflammation by binding free fatty acids to Toll-like receptor 4 (33, 70), potentially exacerbating the progression of NAFLD. It is not surprising that previous studies (37) have found significantly elevated levels of fetuin-A in NAFLD. Furthermore, biopsy-proven human studies (71) have consistently shown higher circulating levels of fetuin-A and greater hepatic expression of fetuin-A in individuals with NAFLD compared to healthy controls, regardless of the histological state and BMI class. These findings suggest that the conventional BMI-based concept for NAFLD or MAFLD may need to be reconsidered, as fetuin-A levels remain elevated even in lean individuals with NAFLD. To date, there is a lack of data regarding the relationship and underlying mechanisms between lean NAFLD and the serum gradient of fetuin-A. However, we propose a hypothesis that lean NAFLD, despite being associated with fewer metabolic dysfunctions compared to non-lean NAFLD, may exhibit heightened inflammation and oxidative stress, leading to disease progression. Experimental studies have demonstrated that fetuin-A plays a role in promoting the expression of proinflammatory cytokines at both the mRNA and protein levels (20, 72). Moreover,

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fetuin-A has been shown to chronically respond to inflammatory stimuli (73), contributing to the transition from steatohepatitis to non-alcoholic steatohepatitis (NASH) (74, 75). In a study involving 1,339 Caucasian individuals with biopsy-proven NAFLD, it was observed that both lean and non-lean NAFLD patients could progress to advanced liver disease, metabolic comorbidities, cardiovascular disease, and liver-related mortality, irrespective of obesity progression (19).

Previous observations have revealed that lean NAFLD patients tend to be younger and exhibit fewer metabolic clinical features, yet they experience similar histological severity, comorbidities, and mortality rates compared to non-lean NAFLD patients (76). It is noteworthy that lean NAFLD develops prior to the onset of obesity and metabolic dysfunction, making it challenging to rely on conventional metabolic factors for early detection. Given that liver fat accumulation and chronic inflammation serve as sensitive and early indicators in these subsets, fetuin-A, functioning as both a hepatokine and an adipokine, holds promise as a surrogate biomarker that is independent of central obesity and insulin resistance. The strength of our study lies in being the first to establish a link between serum fetuin-A levels and lean NAFLD, providing evidence for a dose-dependent relationship between fetuin-A and the risk of lean NAFLD.

There are some limitations in our study. It is important to acknowledge certain

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limitations of our study. Firstly, being a cross-sectional study, we cannot establish a causal relationship between lean NAFLD and the serum gradient of fetuin-A. Despite adjusting for potential confounding factors, there may still be unmeasured and unidentified variables that could contribute to residual effects. For instance, the duration of NAFLD could potentially impact serum fetuin-A levels over time, but we did not collect longitudinal data from lean or non-lean NAFLD individuals. Further investigations using well-designed animal models and prospective cohorts are needed to explore the pathophysiology of lean NAFLD and its relationship with fetuin-A. Secondly, we did not perform liver biopsy, which is considered the gold standard for diagnosing NAFLD. Although ultrasonography has limitations in determining the severity of NAFLD, it is widely recognized as a screening tool for NAFLD [2]. In our study, we utilized US-FLI, a commonly used ultrasonographic scoring system, as a surrogate modality for diagnosing NAFLD [19, 33]. Future studies should focus on combining ultrasonographic assessment with surrogate biomarkers to enhance the accuracy and precision of noninvasive approaches for diagnosing NAFLD.

5.2. AL ratio and lean NAFLD

A logical inference between lean NAFLD and AL ratio was well demonstrated in the study. First, we demonstrated that in this population that was younger and

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healthier, the AL ratio was indeed a strong and good parameter in relation to each metabolic factor and HOMA-IR. Then, the association between the serum AL ratio and the risk of NAFLD was examined. In the section of crude OR, both the lean NAFLD and overweight/obesity NAFLD groups showed a decreased risk from the lowest tertile of AL ratio to the highest tertile of AL ratios compared with that of the lean controls and simple overweight/obesity groups, respectively. (P for a trend <0.001) Then, we removed the effects of HOMA-IR and AST/ALT ratio to determine the amount of residual effect differences that were left between the case and controls (lean controls vs. lean NAFLD; simple overweight/obesity vs. overweight/obesity NAFLD). The analysis revealed a consistent and significant reduction in the risk of NAFLD across increasing tertiles of the AL ratio, indicating a lower risk in individuals with higher AL ratios (p for trend <0.05). Furthermore, the AUROC curve, which assesses the discriminatory power of the AL ratio, demonstrated strong performance with an AUROC value ranging from 0.83 to 0.86. These results confirm the potential of the AL ratio as a reliable indicator for predicting and evaluating the risk of NAFLD.

Adipose tissue, functioning as a complex endocrine organ, plays a vital role in the development of NAFLD through its communication with the liver via the secretion of adipokines (36, 77). Among these adipokines, adiponectin and leptin

doi:10.6342/NTU202301314

exhibit contrasting roles in relation to BMI. As individuals transition from being lean to overweight and eventually obese, accompanied by an increase in adiposity, there is a parallel decrease in serum adiponectin levels and an increase in serum leptin levels (44, 78). Apart from their impact on insulin sensitivity and adipocyte lipid storage, adiponectin and leptin also contribute to inflammatory or anti-inflammatory processes (39, 79).Some observational studies have demonstrated that the linkage between anti-inflammation and adiponectin is at least partially independent of obesity (80), and this result is consistent with our study. Consequently, the AL ratio has been suggested to be a marker of low-grade chronic inflammation in populations with impaired insulin functions and obesity (44, 45, 81). Some studies propose that the AL ratio is positively associated with arteriosclerosis, intima media thickness of the common artery and CVD. (35, 82). A Japanese health survey delineated cross-sectionally that the AL ratio was associated with the severity of steatosis by ultrasonography (45). Another study elucidated that the AL ratio could be a noninvasive predictor of NAFLD in obese children, which better correlates with weight and HOMA-IR than each single adipokine (46). Compared to MALFD, leptin is more robust in the effect of obesity, while adiponectin could interfere with the presentation of NAFLD regardless of HOMA-IR and adiposity. Therefore, the AL ratio could be independently used to distinguish the lean NAFLD individuals from the lean control individuals.

doi:10.6342/NTU202301314

Since 2020, the term MAFLD has been adopted as the preferred terminology for the condition previously known as NAFLD (3, 16). It has been observed that lean NAFLD patients, despite being younger and having fewer metabolic clinical features, exhibit similar histological severity, comorbidities, and mortality compared to NAFLD patients (2). Given that lean NAFLD develops before the onset of overweight or increased adiposity, alternative methods such as imaging modalities or biomarkers can be employed for early detection rather than relying solely on BMI. In our study, we specifically excluded individuals with diabetes, as its pathophysiology may involve distinct pathways and progression trajectories (16, 83). Our study focused on early-stage NAFLD patients with fewer metabolic syndromes. Considering that liver fat accumulation and chronic inflammation are highly sensitive and early indicators in these particular subgroups, the AL ratio emerged as a promising biomarker for the early identification of lean NAFLD.

The prevalence of lean NAFLD appears to be higher in the Asian population, indicating the influence of ethnic differences and genetic variants (8). A recent meta-analysis reported that the prevalence of lean NAFLD among non-obese individuals in Asia can reach up to 40.75% (84). In line with these findings, our study observed that 105 out of 322 lean subjects (33%) in our population had lean NAFLD. Furthermore, the prevalence of lean NAFLD among NAFLD patients in Asia has been reported to

doi:10.6342/NTU202301314

range from 12% to 47%, which is consistent with our own finding of 105 out of 305 NAFLD subjects (34.4%) (85).

Our study has several limitations that should be acknowledged. Firstly, we did not conduct liver biopsies, which is considered the gold standard for diagnosing NAFLD. However, due to the high prevalence and diverse presentation of NAFLD, performing liver biopsies on a large scale is impractical. Instead, we used a validated and widely applied ultrasonographic scoring system, US-FLI, to assess the presence of NAFLD. Although ultrasonography cannot provide information about the severity of NAFLD, the use of US-FLI as a reliable screening tool for NAFLD has been well-established in previous studies (78). Furthermore, US-FLI has been extensively used in real-world settings as a substitute modality for diagnosing NAFLD (4). Another limitation of our study is its cross-sectional design, which only allows us to establish associations rather than infer causality between the AL ratio and NAFLD. Additionally, we did not collect information on the duration of NAFLD, which could potentially impact the serum AL ratio. Although we adjusted for insulin resistance as a confounding factor, it is important to note that we used an indirect measurement of insulin resistance through equations based on fasting glucose and insulin levels, rather than the gold standard glucose clamp technique. While this indirect measurement is commonly used in clinical practice, it may not capture all aspects of insulin resistance

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accurately. While our study showed a significant association between the AL ratio and lean NAFLD, it is important to note that as a cross-sectional study, it cannot provide information on early detection. To develop a more robust detection model or validate our findings, a longitudinal cohort study would be necessary. Longitudinal studies allow for the assessment of temporal relationships and can provide valuable insights into the predictive value of the AL ratio for the development and progression of lean NAFLD over time. Such studies would help establish the potential utility of the AL ratio as an early detection tool for lean NAFLD.

5.3. SNPs for lean NAFLD

In our study, we investigated the association between gene variants and the risk of lean NAFLD. Specifically, we focused on the G alleles in *PNPLA3* rs738409 and *SAMM50* rs3761472. Previous studies have reported conflicting results regarding the association between *PNPLA3* rs738409 and lean NAFLD, often due to differences in ethnicities. Additionally, the relationship between *SAMM50* and NAFLD has not been explored in previous studies. Using a large sample size in a cohort study, we observed that the G alleles of *PNPLA3* rs738409 and *SAMM50* rs3761472 were more prevalent in lean NAFLD individuals compared to lean controls. Even after adjusting for various factors including sex, age, smoking, drinking, BMI, fasting glucose, systolic BP, diastolic BP, triglycerides, and HDL-C, these two gene variants showed

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significantly higher odds ratios in lean NAFLD patients compared to lean controls. These findings suggest that the *PNPLA3* and *SAMM50* gene variants independently contribute to the development of lean NAFLD, beyond the influence of BMI and metabolic syndrome. These results further support the unique pathophysiology of lean NAFLD in Asian populations. The variant *PNPLA3* rs738409 was noted to be associated with NAFLD in a meta-analysis (53), among Asian populations (61) and in pediatric patients in Taiwan (58). However, its association with lean subjects remains under debate in Western countries (86). In Japanese studies, *PNPLA3* rs738409 was strongly associated with the development and progression of nonobese NAFLD rather than obese NAFLD (87), which did not differ in metabolic morbidities and sex (57). These findings suggest that the *PNPLA3* rs738409 variant plays a crucial role in the pathogenesis and progression of NAFLD in non-obese individuals, irrespective of metabolic factors and gender. In Hong Kong, it was observed that among individuals with NAFLD, lean subjects had a higher likelihood of carrying the *PNPLA3* rs738409 GG genotype compared to overweight and obese subjects (88). Additionally, patients with NAFLD who carry the *PNPLA3* G allele but are not obese have an increased risk of developing steatohepatitis or advanced fibrosis (55). Similarly, in a 7-year prospective community cohort study conducted in Sri Lanka, *PNPLA3* variants were found to be strongly associated with lean NAFLD (56). These findings further support

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the notion that *PNPLA3* variants play a significant role in the development of NAFLD, particularly in lean individuals. Our study not only supported the higher association between the *PNPLA3* rs738409 GG genotype and lean NAFLD in Taiwan but also demonstrated that the association was independent of BMI and metabolic dysfunction.

The role of *SAMM50* in NAFLD is thought to be related to fatty acid oxidation and intracellular lipid accumulation (59). Few studies have demonstrated the association between *SAMM50* and NAFLD. In a Chinese Han population, the *SAMM50* rs3761472 G allele created susceptibility to NAFLD (89). *SAMM50* may interact with *PNPLA3* to increase susceptibility to NAFLD in Chinese (90) and Mexican populations (91). *SAMM50* and *PNPLA3* may affect the severity of NAFLD in Korean children (92) and adults (93) and the progression of NAFLD (60). This was the first study to demonstrate that there was an independently higher risk of lean NAFLD among subjects with the *SAMM50* rs3761472 G allele variant after removing the effects of BMI and metabolic factors.

Recently, an international expert panel introduced the term metabolic dysfunction-associated fatty liver disease (MAFLD) as a new definition for fatty liver disease (20). This updated terminology has improved the clinical management of liver diseases, particularly in the Asia-Pacific region, where the prevalence of lean

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individuals with fatty liver is higher compared to Western countries (94). It has become evident in recent years that MAFLD is primarily a metabolic disorder, irrespective of body weight status (12). However, identifying fatty liver disease in patients with normal body weight remains challenging in clinical practice. There is currently no reliable biomarker, and liver biopsy is not commonly used for detecting lean NAFLD (95). In our study, we found that lean individuals carrying the *PNPLA3* rs12483959 or *SAMM50* rs3761472 GG alleles had approximately a 3-fold higher risk of fatty liver disease, even after adjusting for BMI and metabolic factors. Additionally, the area under the ROC curve (0.86) demonstrated a good performance for *PNPLA3* rs738409 and *SAMM50* rs3761472 in the detection of lean NAFLD.

There are several limitations to consider in our study. Firstly, it was a case-control study, where the exposure variable was the presence of *PNPLA3* rs12483959 or *SAMM50* rs3761472 GG alleles, and the outcome variable was the presence of fatty liver disease. It's important to note that the genetic makeup of individuals remains constant after birth, which provides a characteristic feature of the study cohort. Secondly, although we were able to establish a link between the *PNPLA3* rs12483959 and *SAMM50* rs3761472 GG alleles and lean NAFLD, we were unable to assess the severity and progression of fatty liver disease among lean individuals with and without these genetic variants.

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In conclusion, the *PNPLA3* rs738409 and *SAMM50* rs3761472 gene polymorphisms are associated with a higher risk of fatty liver in lean individuals independent of BMI and metabolic syndrome in Asian populations. Further investigation is warranted.

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VI、CONCLUSIONS

In conclusion, our study suggests that both Fetuin-A and the AL ratio could serve as potential biomarkers for early detection of lean NAFLD, independent of insulin resistance. Additionally, we found a significant association between the *PNPLA3* rs738409 and *SAMM50* rs3761472 gene polymorphisms and an increased risk of fatty liver in lean individuals, even after adjusting for BMI and metabolic syndrome.

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Tables

Table 1 Baseline characteristics among the lean, non-lean, NAFLD, and non-NAFLD groups

	Lean		Non-lean		P value
	Non-NAFLD N=227	NAFLD N=108	Non-NAFLD N=54	NAFLD N=217	
Age (years)	41.1±11.0	42.6±11.6	44.5±11.3	43.7±11.8	0.061
Male (%)	47(20.7%)	37(34.3%)	25(46.3%)	123(56.7%)	<0.001
BMI (kg/m^2)	20.6±1.8	21.9±1.5	26.0±1.7	28.1±4.0	<0.001
WC (cm)	73.1±6.1	77.6±6.5	85.4±6.2	91.1±8.3	<0.001
Body fat (%)	25.6±6.2	26.6±6.0	30.0±7.9	32.4±8.4	<0.001
Systolic BP	115.7±15.7	121.6±15.3	122.6±17.0	130.4±15.3	<0.001
Diastolic BP	72.9±11.2	77.2±9.5	77.9±13.8	82.2±12.2	<0.001
TCHO (mmol/L)	190.0±33.8	196.9±39.8	194.6±29.3	201.7±35.5	0.007
TG (mmol/L)	74.2±37.2	109.2±78.9	95.0±43.5	160.2±113.8	<0.001
HDL-C(mmol/L)	66.7±15.0	57.3±13.2	59.5±13.5	49.7±12.6	<0.001
LDL-C(mmol/L)	114.5±31.2	125.4±37.1	123.0±29.2	131.7±32.5	<0.001
Glucose(mmol/L)	83.7±13.0	85.3±8.7	87.0±10.4	94.2±22.8	<0.001
Insulin(U/mL)	5.29±4.24	6.77±5.21	7.1±3.9	11.5±8.9	<0.001
HOMA-IR	0.68±0.55	0.86±0.65	0.91±0.49	1.49±1.10	<0.001
Smoke (%)	16(7.0)	11(10.2)	5(9.3)	35(16.1)	0.022
Exercise (%)	100(44.1)	46(42.6)	27(50.0)	92(42.4)	0.782
GOT	20.3±6.8	21.7±7.0	21.5±5.9	25.8±10.2	<0.001
GPT	17.2±9.4	23.8±16.5	21.4±10.6	36.7±27.8	<0.001
CRP (mg/dL)	0.11±0.31	0.10±0.13	0.17±0.28	0.22±0.25	<0.001
Metabolic factors	0.39±0.62	0.91±0.89	1.15±0.90	2.14±1.18	<0.001
MetS (%)	2(2.5)	6(7.5)	4(5.0)	68(85)	<0.001
Fetuin-A (mg/L)	689.4±672.4	882.6±731.3	829.3±429.3	855.9±467.0	0.009

ANOVA was applied to test the difference among groups.

Abbreviations: NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; WC, waist circumference; BP, blood pressure; TCHO, total cholesterol; TG, triglycerides, HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; CRP, C-reactive protein; MetS: metabolic syndrome. Significant level: P<0.05

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Table 2. Comparison of lean, non-lean, NAFLD, and non-NAFLD groups in metabolic variables using Tukey's post hoc analysis.

lean/NAFLD:	+/-vs+/+	+/-vs-/-	+/-vs-/+	+/+vs-/-	+/+vs-/+	-/-vs-/+
Age (years)	0.663	0.199	0.079	0.754	0.856	0.966
Male (%)	0.059	0.002	<0.001	0.400	<0.001	0.451
BMI (kg/m^2)	0.001.	<0.001	<0.001	<0.001	<0.001	<0.001
WC (cm)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Body fat (%)	0.610	<0.001	<0.001	0.024	<0.001	0.122
Systolic BP	0.007	0.019	<0.001	0.977	<0.001	0.006
Diastolic BP	0.007	0.022	<0.001	0.986	0.002	0.075
TCHO (mmol/L)	0.339	0.822	0.003	0.980	0.658	0.553
TG (mmol/L)	0.001	0.321	<0.001	0.711	<0.001	<0.001
HDL-C(mmol/L)	<0.001	0.003	<0.001	0.745	<0.001	<0.001
LDL-C(mmol/L)	0.023	0.315	<0.001	0.971	0.359	0.296
Glucose(mmol/L)	0.833	0.5448	<0.001	0.928	<0.001	0.024
Insulin(U/mL)	0.303	0.352	<0.001	0.994	<0.001	<0.001
HOMA-IR	0.303	0.324	<0.001	0.990	<0.001	<0.001
GOT	0.499	0.767	<0.001	1.000	<0.001	0.003
GPT	0.017	0.463	<0.001	0.876	<0.001	<0.001
CRP (mg/dL)	0.961	0.439	<0.001	0.321	<0.001	0.672
Fetuin-A (mg/L)	0.030	0.413	0.019	0.951	0.981	0.991

Four groups: lean (+) NAFLD (-), lean (+) NAFLD (+), lean (-) NAFLD (-), and lean (-) NAFLD (+)

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Table 3 Odds ratios of having NAFLD derived from multiple logistic regression analyses in tertiles of serum fetuin-A levels

	Q1(N=202) (≤ 821 mg/L)	Q2 (N=201) (822-1012 mg/L)	Q3(N=203) (1013-1224 mg/L)	P for trend
Model 1	1.00	2.49(1.64-3.77) **	2.62(1.72-3.98) **	<0.001
Model 2	1.00	1.55(0.94-2.56)	1.80(1.10-2.94) *	0.020
Model 3	1.00	1.49(0.87-2.57)	1.57(0.92-2.67)	0.099

Model 1: adjusted for age, gender, current smoking, and exercise habit.

Model 2 adjusted for variables in model 1, plus WC as a confounding factor.

Model 3 adjusted for variables in model 2, plus HOMA-IR as a confounding factor.

HOMA-IR, homeostasis model assessment of insulin resistance. *For $p<0.05$; **For $p<0.001$.

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Table 4 Odds ratios of having NAFLD derived from multiple logistic regression analyses in tertiles of serum fetuin-A levels, stratification by BMI

Lean NAFLD				
	Q1(N=158)	Q2 (N=75)	Q3(N=102)	P for trend
Model 1	1.00	1.01(0.53-1.90)	1.95(1.14-3.34) *	0.018
Model 2	1.00	1.26(0.63-2.50)	2.26(1.26-4.07) *	0.007
Model 3	1.00	1.33(0.63-2.82)	2.09(1.09-3.98) *	0.026
Overweight-obese NAFLD				
	Q1(N=44)	Q2 (N=126)	Q3(N=101)	P for trend
Model 1	1.00	1.48(0.65-3.38)	1.35(0.57-3.21)	0.603
Model 2	1.00	1.20(0.47-3.02)	0.89(0.34-2.33)	0.688
Model 3	1.00	0.95(0.35-2.56)	0.69(0.24-1.95)	0.422

Model 1: adjusted for age, gender, current smoking, and exercise habit.

Model 2 adjusted for variables in model 1, plus WC as a confounding factor.

Model 3 adjusted for variables in model 2, plus HOMA-IR as a confounding factor.

HOMA-IR, homeostasis model assessment of insulin resistance. *For $p<0.05$; **For $p<0.001$

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Table 5 Baseline characteristics among the lean controls, lean NAFLD, simple overweight/obesity and overweight/obesity NAFLD groups

	BMI<24		BMI≥24	
	Lean controls	Lean NAFLD	Simple overweight/obesity	Overweight/obesity NAFLD
	N=217	N=105	N=53	N=200
Age (years)	41.20±10.94	42.96±11.59	44.38±11.34	43.33±11.80
Male (%)	45(20.7) [c, d]	36(34.3) [d]	25(47.2) [a]	113(56.5) [a, b]
Smoke (%)	14(6.5) [d]	11(10.5)	5(9.4)	33(16.5) [a,]
Exercise (%)	95(43.8)	45(42.9)	26(49.1)	86(43.0)
BMI (kg/m^2)	20.66±1.80 [b, c, d]	21.83±1.54 [a, c, d]	25.95±1.75 [a, b, d]	27.98±3.91 [a, b, c]
fat percentage (%)	27.54±8.08 [b, c, d]	30.76±7.33 [a, d]	33.89±7.12 [a]	36.15±7.89 [a, b]
WC (cm)	73.13±6.13 [b, c, d]	77.56±6.58 [a, c, d]	85.72±5.92 [a, b, d]	90.40±7.88 [a, b, c]
Systolic BP	115.80±15.52 [b, c, d]	122.12±15.15 [a, d]	122.63±17.16 [a, d]	129.99±15.12 [a, b, c]
Diastolic BP	73.00±11.18 [b, c, d]	77.50±9.46 [a, d]	78.06±13.91 [a]	81.75±11.93 [a, b]
TCHO (mg/dL)	189.78±33.99 [d]	197.60±40.05	194.00±29.23	202.64±35.34 [a]
TG (mg/dL)	74.00±35.43 [b, d]	109.36±79.36 [a, d]	96.02±43.21 [d]	157.51±113.57 [a, b, c]
HDL-C (mg/dL)	66.78±14.96 [b, c, d]	57.13±13.35 [a, d]	59.09±13.23 [a, d]	49.93±12.61 [a, b, c]
LDL-C (mg/dL)	114.12±31.13 [b, d]	126.20±37.28 [a]	122.49±29.21	132.86±32.16 [a]
Glucose (mg/dL)	82.65±8.66 [d]	85.50±8.65 [d]	86.51±9.83 [d]	89.39±9.43 [a, b, c]
Insulin (μIU/mL)	5.13±3.28 [d]	6.71±5.24 [d]	7.15±3.87 [d]	11.30±9.03 [a, b, c]
HOMA-IR	0.66±0.42 [d]	0.86±0.65 [d]	0.92±0.49 [d]	1.44±1.11 [a, b, c]
GOT (U/L)	20.46±6.86 [d]	21.75±7.03 [d]	21.57±6.00 [d]	25.40±10.07 [a, b, c]
GPT (U/L)	17.22±9.48 [b, d]	23.96±16.61 [a, d]	12.48±10.74 [d]	35.69±27.84 [a, b, c]

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CRP (mg/dL)	0.114±0.320 [d]	0.091±0.123 [d]	0.173±0.277	0.207±0.250 [a, b]
Metabolic factors (n)	0.39±0.62 [b, c, d]	0.92±0.90 [a, d] -	1.15±0.91 [a, d]	2.01±1.24 [a, b, c]
MetS (%)	1(0.5) [d]	6(6.2) [d]	4(8.5) [d]	56(28.0) [a, b, c]
Adiponectin(μg/mL)	18.13±8.55 [b, d]	13.60±8.00 [a, d]	15.01±8.82 [d]	9.82±5.71 [a, b, c]
Leptin (ng/mL)	8.16±6.30 [c, d]	9.42±7.21 [d]	12.48±10.74 [a]	15.20±11.35 [a, b]
AL ratio ($x10^3$)	6.43±18.36 [b. d]	2.26±1.93 [a]	2.23±2.32	1.13±1.14 [a]

Abbreviations: NAFLD, non-alcoholic fatty liver disease; BMI, body mass index; WC, waist circumference; BP, blood pressure; TCHO, total cholesterol; TG, triglycerides, HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; GOT: glutamic oxaloacetic transaminase; GPT: glutamic pyruvic transaminase; CRP: C-reactive protein; MetS: metabolic syndrome; AL ratio: adiponectin-leptin ratio

Data are presented as the mean±SD for continuous variables and number (percentage) for categorical variables. Differences between the four groups were examined using the chi-squared test for categorical variables and one-way analysis of variance (ANOVA) for continuous variables. Tukey's post hoc analysis was applied to examine the differences among the healthy control, lean NAFLD, overweight controls and overweight NAFLD groups in terms of basic demographic characteristics, leptin, adiponectin, and AL ratio."

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The four groups were represented with [a]: lean controls; [b]: lean NAFLD; [c]: simple overweight/obesity; [d]: overweight/obesity NAFLD. If a significant level $p<0.05$ was achieved between any two of the four groups, a superscript was added to the corresponded columns.

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Table 6 Comparison of lean controls, lean NAFLD, simple overweight/obesity and overweight/obesity NAFLD groups in metabolic variables using Tukey's post hoc analysis.

Lean/NAFLD:	Health/lean MAFLD	Health/Overweight	Health/ MAFLD	Lean MAFLD/ Overweight	Lean MAFLD/ MAFLD	Overweight/MAFLD
Age (years)	0.642	0.314	0.130	0.884	0.936	0.985
Male (%)	0.088	0.002	<0.001	0.352	<0.001	0.552
BMI (kg/m^2)	0.002	<0.001	<0.001	<0.001	<0.001	<0.001
fat percentage (%)	0.003	<0.001	<0.001	0.084	<0.001	0.205
WC (cm)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Systolic BP	0.005	0.031	<0.001	0.997	<0.001	0.008
Diastolic BP	0.006	0.024	<0.001	0.992	0.006	0.122
TCHO (mg/dL)	0.237	0.860	0.002	0.929	0.736	0.460
TG (mg/dL)	0.002	0.310	<0.001	0.761	<0.001	<0.001
HDL-C(mg/dL)	<0.001	0.002	<0.001	0.832	<0.001	<0.001
LDL-C(mg/dL)	0.010	0.336	<0.001	0.907	0.457	0.237
Glucose(mg/dL)	0.793	0.682	<0.001	0.984	<0.001	0.014
Insulin	0.390	0.353	<0.001	0.983	<0.001	<0.001
HOMA-IR	0.390	0.331	<0.001	0.977	<0.001	<0.001
GOT	0.566	0.828	<0.001	0.999	<0.001	0.005
GPT	0.021	0.497	<0.001	0.872	<0.001	<0.001
CRP	0.891	0.448	0.001	0.250	0.001	0.799
Adiponectin	<0.001	0.055	<0.001	0.703	<0.001	<0.001
Leptin	0.596	0.008	<0.001	0.185	<0.001	0.185
A/L ratio	0.010	0.072	<0.001	1.000	0.822	0.914

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Table 7. Relation between the serum adiponectin-leptin ratio and metabolic factors in multivariate linear regression models after adjusting for age and sex

Variables	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6	Model 7	Model 8	Model 9
	ß (SE)	ß (SE)	ß (SE)	ß (SE)	ß (SE)	ß (SE)	ß (SE)	ß (SE)	ß (SE)
	P value	P value	P value	P value	P value	P value	P value	P value	P value
BMI	-0.252(0.108)								
(kg/m^2)	<0.001								
Fat (%)		-0.365(0.060)							
		<0.001							
WC			-0.296(0.050)						
(cm)			<0.001						
SBP				-0.141(0.030)					
(mmHg)				0.001					
DBP					-0.133(0.040)				
(mmHg)					0.002				
HDL-C						0.158(0.033)			
(mg/dl)						<0.001			
TG							-0.165(0.005)		
(mg/dl)							<0.001		
Glucose								-0.102(0.028)	
(mg/dl)								0.015	

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HOMA-IR	-0.203(0.260) <0.001

Abbreviations: NAFLD, non-alcoholic fatty liver disease; BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides, HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance

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Table 8. Odds ratios of having MAFLD related groups derived from multinomial regression analyses in relation to adiponectin leptin ratio

1. Compared with healthy group (healthy group: OR 1.00 as reference group)						
	Lean MAFLD		Overweight		MAFLD	
	OR	P value	OR	P value	OR	P value
Model 1	0.72	<0.001	0.65	<0.001	0.25	<0.001
Model 2	0.76	<0.001	0.63	0.96	0.37	0.92
Model 3	0.80	0.002	1.42	0.98	0.86	1.00
2. Compared with lean MAFLD group (Reference: MAFLD group)						
	Healthy		Overweight		MAFLD	
	OR	P value	OR	P value	OR	P value
Model 1	-	-	0.91	0.33	0.35	<0.001
Model 2	-	-	0.84	0.99	0.49	0.94
Model 3	-	-	1.78	0.97	1.08	1.00
3. Compared with lean MAFLD group (Reference: overweight group)						
	Healthy		Lean MAFLD		MAFLD	
	OR	P value	OR	P value	OR	P value
Model 1	-	-	-	-	2.61	<0.001
Model 2	-	-	-	-	0.58	<0.001
Model 3	-	-	-	-	0.61	0.001

Model 1: adjust for age and gender

Model 2: adjust for age, gender, and BMI

Model 3: adjust for age, gender, BMI and WC

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Table 9 Odds ratios of having MAFLD derived from multiple logistic regression analyses in serum gradient of adiponectin leptin level, stratification by BMI

Lean MAFLD				
	Low(N=59) Ratio <1	Moderate (N=97) 1≤Ratio<2	High(N=166) Ratio ≥2	P for trend
Model 1	1.00	0.48(0.24-0.95) *	0.17(0.08-0.35) **	<0.001
Model 2	1.00	0.56(0.25-1.14)	0.32(0.14-0.72) *	0.006
Model 3	1.00	0.57(0.27-1.21)	0.38(0.16-0.95) *	0.037
MAFLD				
	Low(N=144) Ratio <1	Moderate (N=56) 1≤Ratio<2	High(N=53) Ratio ≥2	P for trend
Model 1	1.00	0.38(0.16-0.92) *	0.12(0.05-0.31) **	<0.001
Model 2	1.00	0.52(0.21-1.33)	0.23(0.08-0.68) *	0.008
Model 3	1.00	0.78(0.29-2.09)	0.44(0.13-1.44)	0.178

Model 1: adjusted for age, gender, current smoking, and exercise habit.

Model 2 adjusted for variables in model 1, plus fat percentage as a confounding factor.

Model 3 adjusted for variables in model 2, plus HOMA-IR as a confounding factor.

HOMA-IR, homeostasis model assessment of insulin resistance

*For $p<0.05$; **For $p<0.001$

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Table 10. Odds ratios of having MAFLD derived from multiple logistic regression analyses in serum gradient of leptin adiponectin level, stratification by BMI

Lean MAFLD				
	Low(N=166) Ratio <0.5	Moderate (N=97) 0.5≤Ratio<1	High(N=59) Ratio ≥1	P for trend
Model 1	1.00	2.86(1.51-5.41) *	5.94(2.89-12.21) **	<0.001
Model 2	1.00	2.22(1.06-4.65) *	4.51(1.98-10.24) **	<0.001
Model 3	1.00	1.48(067-3.31)	2.61(1.05-6.46) *	0.037
MAFLD				
	Low(N=53) Ratio <0.5	Moderate (N=56) 0.5≤Ratio<1	High(N=144) Ratio ≥1	P for trend
Model 1	1.00	3.16(1.29-7.73) *	8.36(3.26-21.41) **	<0.001
Model 2	1.00	2.32(0.88-6.12)	3.47(1.16-10.42) *	0.027
Model 3	1.00	1.78(0.64-4.95)	2.30(0.70-7.62)	0.178

Model 1: adjusted for age, gender, current smoking, and exercise habit.

Model 2: adjusted for variables in model 1, plus HOMA-IR as a confounding factor.

Model 3: adjusted for variables in model 2, plus fat percentage as a confounding factor.

HOMA-IR, homeostasis model assessment of insulin resistance

*For p<0.05; **For p<0.001

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Table 11. Odds ratios of having NAFLD in relation to the serum tertile of adiponectin-leptin (AL x10^3) ratio using multiple logistic regression analyses

	AL x10^3 ratio <0.91 N=190	0.91≤ AL x10^3 ratio <2.36 N=193	AL x10^3 ratio ≥2.36 N=192	P for trend
Model 1	1.00	0.28(0.17-0.44) **	0.07(0.04-0.12) **	<0.001
Model 2	1.00	0.58(0.34-1.00) *	0.22(0.12-0.43) **	<0.001
Model 3	1.00	0.66(0.37-1.12)	0.34(0.17-0.71) **	<0.001
Model 4	1.00	0.67(0.38-1.21)	0.37(0.18-0.77) *	0.008

Model 1: adjusted for age, sex, current smoking, and exercise habits.

Model 2: adjusted for variables in Model 1, plus BMI

Model 3: adjusted for variables in Model 2, plus HOMA_IR

Model 4: adjusted for variables in Model 2, plus GOT/GPT ratio

*For p<0.05; **For p<0.001

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Table 12. Odds ratios of having NAFLD in relation to serum tertile of adiponectin leptin level using multiple logistic regression analyses, stratification by BMI

Lean NAFLD				
	AL <0.91 N=53	0.91≤AL <2.36 N=120	AL ≥2.36 N=149	P for trend
Model 1	1.00	0.58(0.29-1.14)	0.16(0.08-0.36) **	<0.001
Model 2	1.00	0.59(0.29-1.21)	0.26(0.11-0.61) *	0.002
Model 3	1.00	0.62(0.30-1.29)	0.28(0.12-0.69) *	0.005
Overweight NAFLD				
	AL <0.91 N=137	0.91≤AL <2.36 N=73	AL ≥2.36 N=43	P for trend
Model 1	1.00	0.34(0.14-0.80) *	0.11(0.04-0.30) **	<0.001
Model 2	1.00	0.59(0.23-1.51)	0.28(0.09-0.89) *	0.031
Model 3	1.00	0.61(0.23-1.58)	0.30(0.09-0.96) *	0.043

Model 1: adjusted for age, sex, current smoking, and exercise habits.

Model 2: adjusted for variables in Model 2, plus HOMA_IR

Model 3: adjusted for variables in Model 2, plus GOT/GPT ratio

*For p<0.05; **For p<0.001

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Table 13. Basic characteristics and biochemical profiles of the HAVO study population

	Lean Control (n=1652)	Lean NAFLD (n=602)	P value
Male (%)	733 (44.4)	397 (65.9)	<0.001
Age (years)	42.0±11.4	48.6±10.3	<0.001
Smoking (%)	332 (20.4)	202 (33.9)	<0.001
Drinking (%)	799 (49.3)	314 (52.8)	0.146
BMI (kg/m^2)	21.0±1.83	22.6±1.1	<0.001
WC (cm)	74.8±6.3	81.4±5.0	<0.001
SBP (mmHg)	114.3±12.1	119.5±12.9	<0.001
DBP (mmHg)	70.0±9.1	74.3±9.4	<0.001
Glu AC (mg/dL)	87.5±13.8	95.6±24.1	<0.001
TCHO (mg/dL)	190.6±35.9	201.4±34.7	<0.001
LDL (mg/dL)	120.8±34.0	134.7±33.6	<0.001
TG (mg/dL)	83.7±40.9	136.5±88.2	<0.001
HDL (mg/dL)	59.0±13.8	49.6±11.7	<0.001
MetS (%)	25 (1.5)	63 (10.5)	<0.001

Abbreviations: BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP: diastolic blood pressure; TCHO, total cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol.

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Table 14 A. Gene location, cytogenetic region and most severe consequence of the SNPs related to *PNPLA3*

Number	SNP	Location	Cytogenetic region	Most severe consequence
1	rs738409	22:43928847	22q13.31	Missense variant
2	rs2896019	22:43937814	22q13.31	Intron variant
3	rs12483959	22:43930116	22q13.31	Intron variant
4	rs2281135	22:43936690	22q13.31	Intron variant
5	rs4823173	22:43932850	22q13.31	Intron variant
6	rs3747207	22:43928975	22q13.31	Intron variant
7	rs738408	22:43928850	22q13.31	Synonymous variant
8	rs2076211	22:43933198	22q13.31	Intron variant
9	rs2294915	22:43945024	22q13.31	Intron variant
10	rs2294433	22:43933395	22q13.31	Intron variant
11	rs1977080	22:43934151	22q13.31	Intron variant
12	rs1977081	22:43934248	22q13.31	Intron variant
13	rs2281293	22:43938962	22q13.31	Intron variant
14	rs12485100	22:43929636	22q13.31	Intron variant
15	rs4823178	22:43938649	22q13.31	Intron variant
16	rs12484809	22:43929751	22q13.31	Intron variant
17	rs1883349	22:43936063	22q13.31	Intron variant
18	rs4823179	22:43945313	22q13.31	Intron variant
19	rs12484795	22:43947746	22q13.31	Intron variant
20	rs2073081	22:43939864	22q13.31	Intron variant
21	rs12484466	22:43934333	22q13.31	Intron variant
22	rs12484700	22:43931393	22q13.31	Intron variant
23	rs78569621	22:43933700	22q13.31	Intron variant
24	rs9625963	22:43931339	22q13.31	Intron variant
25	rs2072907	22:43936773	22q13.31	Intron variant
26	rs1997693	22:43935633	22q13.31	Intron variant
27	rs1010023	22:43940218	22q13.31	Intron variant
28	rs12484801	22:43929685	22q13.31	Intron variant
29	rs13055874	22:43945792	22q13.31	Intron variant
30	rs13055900	22:43945786	22q13.31	Intron variant
31	rs13056638	22:43935898	22q13.31	Intron variant
32	rs16991158	22:43931299	22q13.31	Intron variant
33	rs1810508	22:43947271	22q13.31	3'-UTR variant

doi:10.6342/NTU202301314

34	rs1883348	22:43935935	22q13.31	Intron variant
35	rs1010022	22:43940430	22q13.31	Intron variant
36	rs11090617	22:43930820	22q13.31	Intron variant
37	rs13056555	22:43943646	22q13.31	Intron variant
38	rs16991175	22:43939451	22q13.31	Intron variant
39	rs2072905	22:43937599	22q13.31	Intron variant
40	rs2281137	22:43936613	22q13.31	Intron variant
41	rs2401512	22:43938065	22q13.31	Intron variant
42	rs34879941	22:43936998	22q13.31	Intron variant
43	rs36069781	22:43944206	22q13.31	Intron variant
44	rs4823181	22:43945726	22q13.31	Intron variant
45	rs73176497	22:43941077	22q13.31	Intron variant
46	rs8142145	22:43940616	22q13.31	Intron variant
47	rs926633	22:43941653	22q13.31	Intron variant
48	rs9625962	22:43930392	22q13.31	Intron variant
49	rs2294919	22:43946445	22q13.31	3'-UTR variant
50	rs2008451	22:43947089	22q13.31	3'-UTR variant
51	rs2072906	22:43937292	22q13.31	Intron variant
52	rs2076207	22:43937490	22q13.31	Intron variant
53	rs2281138	22:43936597	22q13.31	Intron variant
54	rs2294916	22:43945042	22q13.31	Intron variant
55	rs2896020	22:43938088	22q13.31	Intron variant
56	rs34352134	22:43939536	22q13.31	Intron variant
57	rs34376930	22:43939573	22q13.31	Intron variant
58	rs35621602	22:43939526	22q13.31	Intron variant
59	rs36055245	22:43931312	22q13.31	Intron variant
60	rs4823176	22:43938596	22q13.31	Intron variant
61	rs4823177	22:43938606	22q13.31	Intron variant
62	rs4823180	22:43945418	22q13.31	Intron variant
63	rs149157446	22:43909694	22q13.31	Intergenic variant
64	rs738491	22:43958231	22q13.31	Intron variant
65	rs76015644	22:43959813	22q13.31	Intron variant
66	rs117826724	22:43964121	22q13.31	Intron variant
67	rs80634	22:43897188	22q13.31	Intergenic variant

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Table 14 B. Gene location, cytogenetic region and most severe consequence of the SNPs related to *SAMM50*

	SNP	Location	Cytogenetic region	Most severe consequence
1	rs2073080	22:43998522	22q13.31	Intron variant
2	rs2143571	22:43995806	22q13.31	Intron variant
3	rs2235776	22:43982119	22q13.31	Intron variant
4	rs3761472	22:43972242	22q13.31	Missense variant
5	rs4823182	22:43981562	22q13.31	Intron variant
6	rs7587	22:43990401	22q13.31	Synonymous variant
7	rs6006469	22:43987737	22q13.31	Noncoding transcript exon variant
8	rs2294922	22:43983685	22q13.31	Intron variant
9	rs5764430	22:43965617	22q13.31	Intron variant
10	rs738491	22:43958231	22q13.31	Intron variant
11	rs76015644	22:43959813	22q13.31	Intron variant
12	rs117826724	22:43964121	22q13.31	Intron variant

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Table 15. The odds of having fatty liver in lean subjects in relation to *PNPLA3* gene after adjustment by logistic regression models

	Low Risk	Moderate Risk	High Risk	P for trend
rs12483959	196(22.9)	282(36.7)	124(36.6)	
Model 1	1.00	1.29(1.03-1.61)	2.17(1.62-2.92)	<0.001
Model 2	1.00	1.32(1.04-1.69)	2.49(1.80-3.44)	<0.001
Model 3	1.00	1.42(1.09-1.85)	3.06(2.15-4.37)	<0.001
rs2281135	190(22.7)	286(27.0)	125(35.2)	
Model 1	1.00	1.31(1.04-1.64)	2.01(1.50-2.70)	<0.001
Model 2	1.00	1.37(1.07-1.75)	2.38(1.73-3.29)	<0.001
Model 3	1.00	1.43(1.10-1.86)	2.86(2.01-4.07)	<0.001
rs2896019	193(22.6)	216(26.1)	123(36.1)	
rs738409	193(22.6)	216(26.1)	123(36.1)	
Model 1	1.00	1.16(0.93-1.44)	1.95(1.47-2.59)	<0.001
Model 2	1.00	1.14(0.89-1.44)	2.17(1.59-2.96)	<0.001
Model 3	1.00	1.19(0.92-1.55)	2.59(1.85-3.63)	<0.001

Model 1: adjusted for age, sex, smoking and drinking

Model 2: adjusted for variables in Model 1, plus BMI

Model 3: adjusted for variables in Model 2, plus metabolic factors: WC, fasting glucose, systolic blood pressure, diastolic blood pressure, TG and HDL

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Table 16. The odds of having fatty liver in lean subjects in relation to *SAMM50* gene after adjustment by logistic regression models

	Low Risk	Moderate Risk	High Risk	P for trend
rs2143571	197 (22.9)	287 (27.2)	118 (35.0)	
Model 1	1.00	1.30 (1.04-1.62)	2.03 (1.51-2.73)	<0.001
Model 2	1.00	1.36 (1.0-1.73)	2.37 (1.71-3.29)	<0.001
Model 3	1.00	1.40 (1.08-1.83)	2.90 (2.04-4.14)	<0.001
rs3761472	199 (23.2)	286 (27.0)	117 (34.7)	
Model 1	1.00	1.30 (1.04-1.62)	2.03 (1.51-2.73)	<0.001
Model 2	1.00	1.36 (1.07-1.73)	2.37 (1.71-3.29)	<0.001
Model 3	1.00	1.40 (1.08-1.83)	2.90 (2.04-4.14)	<0.001
rs2073080	199(23.3)	286(26.9)	117(34.7)	
Model 1	1.00	1.25(1.00-1.56)	1.94(1.44-2.61)	<0.001
Model 2	1.00	1.34(1.05-1.70)	2.38(1.72-3.30)	<0.001
Model 3	1.00	1.37(1.06-1.79)	2.83(1.98-4.04)	<0.001

Model 1: adjusted for age, sex, smoking and drinking

Model 2: adjusted for variables in Model 1, plus BMI

Model 3: adjusted for variables in Model 2, plus metabolic factors: WC, fasting glucose, systolic blood pressure, diastolic blood pressure, TG and HDL

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Table 17A. SNPs of *PNPLA3* gene which are associated with lean NAFLD (BMI<24kg/m^2) in a Taiwanese population, N=2254

SNP	Chromosome	Position	Genotypes	Lean Control N (%)	Lean NAFLD N (%)	OR (95%CI)	P for trend
rs738491	22q13.31	43958231	CC	438(76.7)	133(23.3)	1.00	0.022
			TC	806(73.3)	294(26.7)	1.20(0.95-1.52)	
			TT	356(68.9)	161(31.1)	1.49(1.14-1.95)	
rs12483959	22q13.31	43930116	GG	661(77.1)	196(22.9)	1.00	<0.0001
			AG	775(73.3)	282(36.7)	1.22(1.00-1.52)	
			AA	215(63.4)	124(36.6)	1.95(1.48-2.56)	
rs2076211	22q13.31	43933198	GG	634(77.2)	187(22.8)	1.00	<0.0001
			AG	759(73.3)	277(26.7)	1.23(1.00-1.53)	
			AA	209(62.8)	124(37.2)	2.01(1.53-2.65)	
rs2281135	22q13.31	43936690	GG	646(77.3)	190(22.7)	1.00	<0.0001
			AG	775(73.0)	286(27.0)	1.25(1.01-1.54)	
			AA	230(64.8)	125(35.2)	1.84(1.40-2.41)	
rs2896019	22q13.31	43937814	TT	621(77.1)	184(22.9)	1.00	<0.0001
			TG	757(73.1)	279(26.9)	1.24(1.00-1.54)	
			GG	224(64.2)	125(35.8)	1.88(1.43-2.48)	
rs738409	22q13.31	43928847	CC	660(77.4)	193(22.6)	1.00	<0.0001
			CG	611(73.9)	216(26.1)	1.11(0.90-1.36)	
			GG	218(63.9)	123(36.1)	1.77(1.36-2.29)	
rs926633	22q13.31	43941653	GG	622(77.2)	184(22.8)	1.00	<0.0001
			AG	749(73.2)	274(26.8)	1.24(1.00-1.53)	
			AA	224(64.0)	126(36.0)	1.90(1.45-2.50)	

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Table 17B. SNPs of *SAMM50* gene which are associated with lean NAFLD (BMI<24kg/m^2) in a Taiwanese population, N=2254

SNP	Chromosome	Position	Genotypes	Lean Control N (%)	Lean NAFLD N (%)	OR (95%CI)	P for trend
rs738491	22q13.31	43958231	CC	438(76.7)	133(23.3)	1.00	0.022
			TC	806(73.3)	294(26.7)	1.20(0.95-1.52)	
			TT	356(68.9)	161(31.1)	1.49(1.14-1.95)	
rs2143571	22q13.31	43995806	GG	657(76.8)	199(23.2)	1.00	<0.0001
			AG	775(73.0)	286(27.0)	1.26(1.02-1.55)	
			AA	220(65.3)	117(34.7)	1.82(1.38-2.39)	
rs3761472	22q13.31	43998522	AA	664(77.1)	197(22.9)	1.00	<0.0001
			AG	769(72.8)	287(27.2)	1.26(1.02-1.55)	
			GG	219(65.0)	118(35.0)	1.82(1.38-2.39)	
rs2073080	22q13.31	43972242	CC	655(76.7)	199(23.3)	1.00	<0.0001
			CT	777(73.1)	286(26.9)	1.21(0.98-1.49)	
			TT	220(65.3)	117(34.7)	1.75(1.33-2.30)	

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Table 18A. Correlation between genotype at locus rs738409 (representative of *PNPLA3* gene)

	CC (N=853)	CG (N=827)	GG (N=341)	P value
Male (%)	421 (49.4)	426 (51.5)	160 (46.9)	0.407
Age (years)	43.9±11.6	43.3±11.2	44.2±11.5	0.362
Smoking (%)	201 (24.0)	204 (24.8)	75 (22.5)	0.868
Drinking (%)	403 (48.2)	410 (50.0)	179 (53.8)	0.273
BMI (kg/m^2)	21.4±1.8	21.5±1.8	21.4±1.8	0.746
WC (cm)	76.5±6.8	76.8±6.7	76.4±6.5	0.612
SBP (mmHg)	115.6±12.6	115.8±12.3	115.0±12.4	0.771
DBP (mmHg)	71.2±9.3	71.4±9.5	70.8±8.9	0.671
Glu AC (mg/dL)	89.3±16.1	90.0±17.7	89.8±20.2	0.809
TCHO (mg/dL)	192.4±34.1	194.1±36.1	191.3±39.0	0.544
LDL (mg/dL)	123.4±32.4	126.1±34.9	122.0±36.5	0.116
TG (mg/dL)	99.4±71.7	95.0±55.5	96.5±55.8	0.231
HDL (mg/dL)	56.5±14.2	56.4±13.5	56.5±13.4	0.821
MetS (%)	37 (4.4)	34 (4.1)	11 (3.3)	0.563
Fatty liver (%)	193 (22.6)	216 (26.1)	123 (36.1)	<0.001

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Table 18B. Correlation between genotype at locus rs3761472 (representative of *SAMM50* gene)

	AA (861)	AG (N=1056)	GG (N=337)	P value
Male (%)	425 (49.4)	545 (51.6)	160 (47.5)	0.354
Age (years)	43.8±11.6	43.6±11.3	44.2±11.7	0.670
Smoking (%)	195 (23.0)	266 (25.5)	73 (22.2)	0.330
Drinking (%)	403 (47.7)	529 (50.8)	181 (54.8)	0.081
BMI (kg/m^2)	21.4±1.8	21.5±1.8	21.4±1.7	0.896
WC (cm)	76.4±6.7	76.7±6.7	76.5±6.5	0.554
SBP (mmHg)	115.6±12.5	116.0±12.6	115.0±12.4	0.421
DBP (mmHg)	71.2±9.3	71.3±9.5	70.6±8.9	0.509
Glu AC (mg/dL)	89.1±16.1	90.2±17.9	89.6±20.0	0.443
TCHO (mg/dL)	192.4±33.8	194.8±36.4	191.9±39.2	0.243
LDL (mg/dL)	123.3±32.3	126.1±35.3	122.8±36.8	0.124
TG (mg/dL)	99.8±72.2	97.3±54.8	94.2±54.8	0.353
HDL (mg/dL)	56.5±14.2	56.4±13.7	56.7±13.5	0.935
MetS (%)	39 (4.6)	41 (3.9)	8 (2.4)	0.225
Fatty liver (%)	197 (22.9)	287 (27.2)	118 (35.0)	<0.001

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Table 19. The odds of having fatty liver in lean subjects in relation to *PNPLA3* (rs738409) and *SAMM50* (rs3761472) gene variants after adjustment by logistic regression models

	Low Risk	Moderate Risk	High Risk	P for trend
rs738409	196(22.9)	282(36.7)	124(36.6)	
Model 1	1.00	1.29(1.03-1.61)	2.17(1.62-2.92)	<0.001
Model 2	1.00	1.32(1.04-1.69)	2.49 (1.80-3.44)	<0.001
Model 3	1.00	1.42(1.09-1.85)	3.06 (2.15-4.37)	<0.001
rs3761472	199 (23.2)	286 (27.0)	117 (34.7)	
Model 1	1.00	1.30 (1.04-1.62)	2.03 (1.51-2.73)	<0.001
Model 2	1.00	1.36 (1.07-1.73)	2.37 (1.71-3.29)	<0.001
Model 3	1.00	1.40 (1.08-1.83)	2.90 (2.04-4.14)	<0.001

Model 1: adjusted for age, sex, smoking and drinking

Model 2: adjusted for variables in Model 1 plus BMI

Model 3: adjusted for variables in Model 2 plus metabolic factors: WC, fasting glucose, systolic blood pressure, diastolic blood pressure, TG and HDL

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Figures

Figure 1.

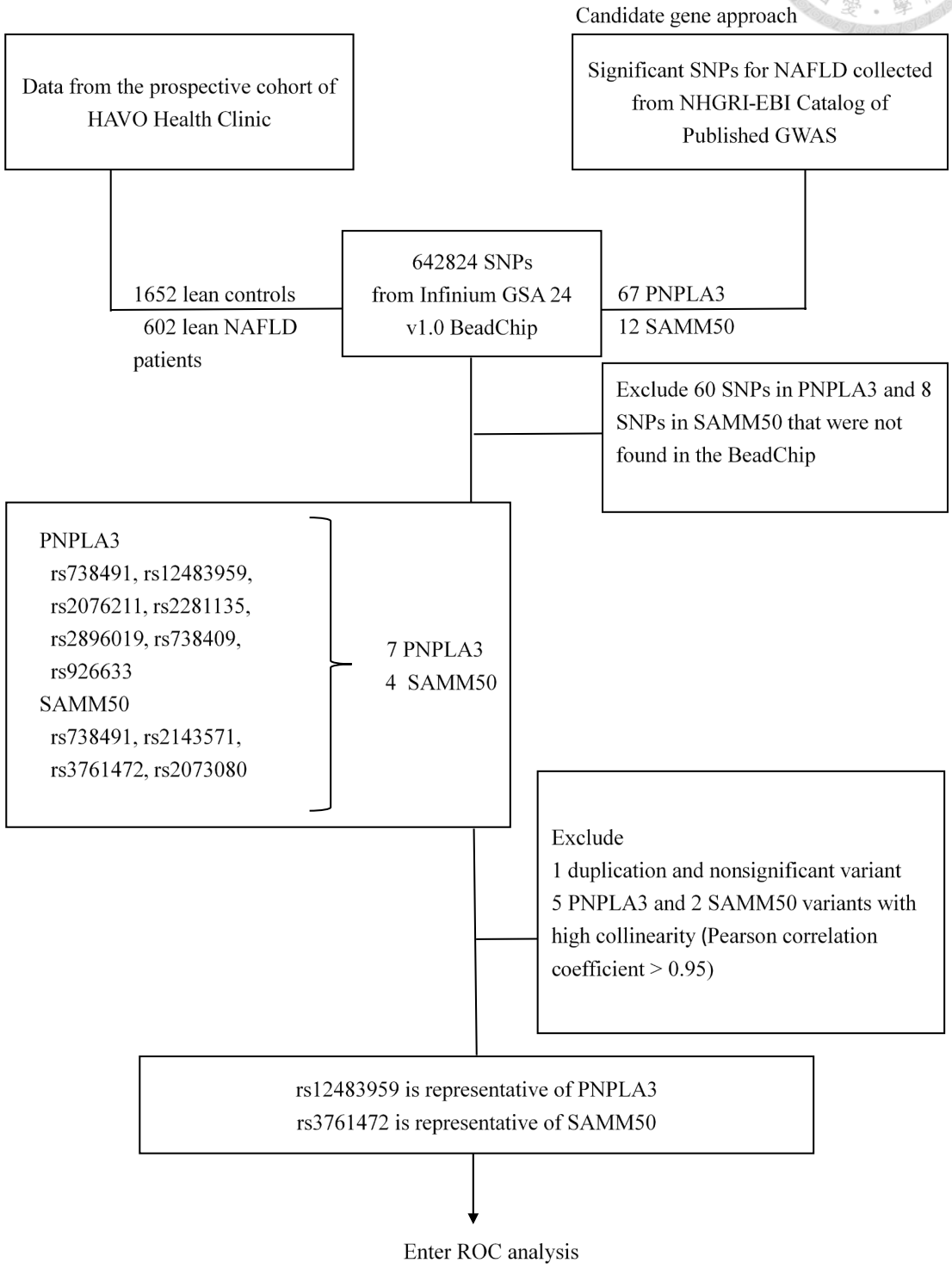

Fig. 1. Flow chart of SNP selection for lean NAFLD

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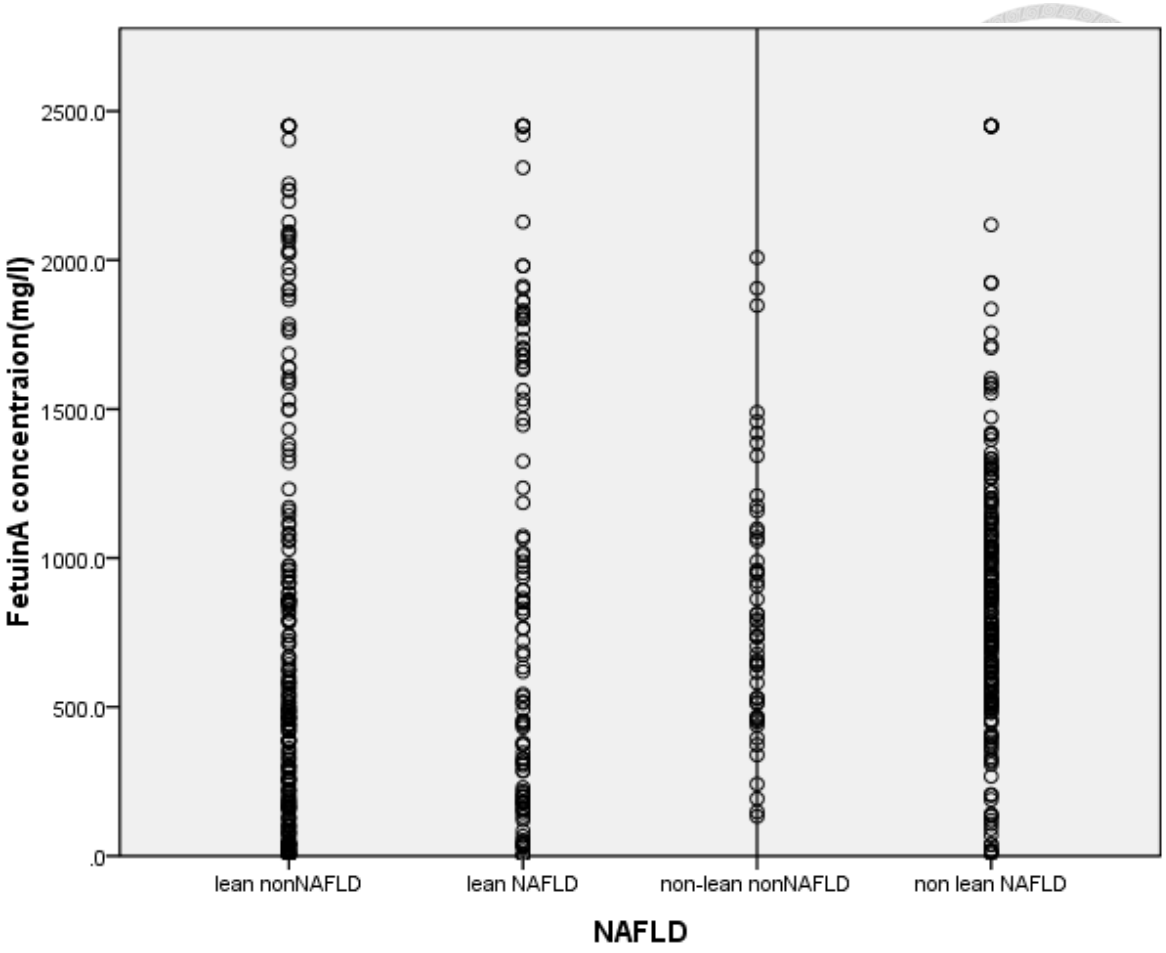

Figure 2. The distribution of Fetuin A concentration among lean non NAFLD, lean NAFLD, non-lean non-NAFLD and non lean NAFLD groups

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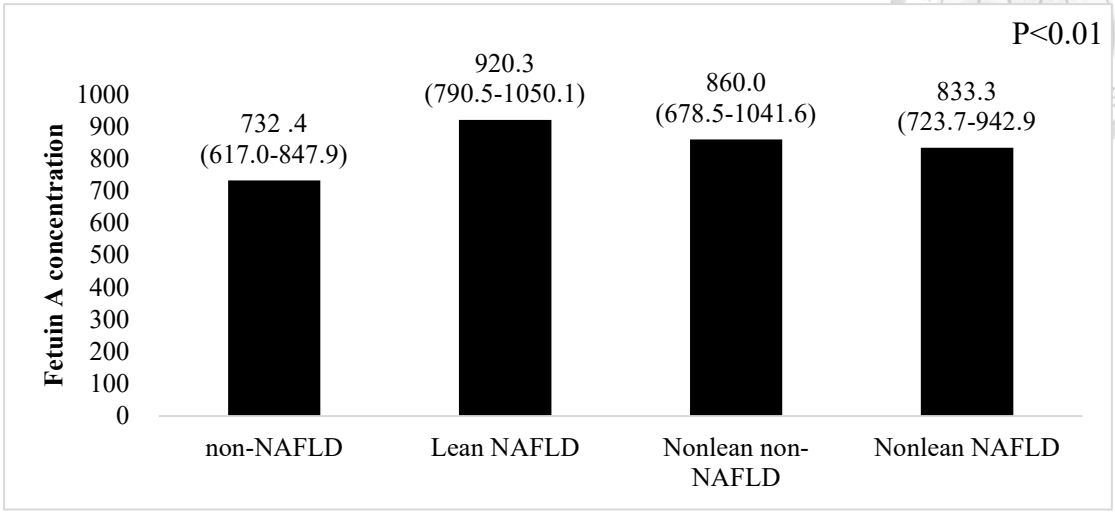

Fig 3. Comparison of serum concentrations of Fetuin A in relation to the group of NAFLD. Comparison of serum concentrations of Fetuin A in relation to the group of NAFLD after adjusting age, gender, current smoking, exercise habit, weight circumference and homeostasis model assessment of insulin resistance by least square means method.

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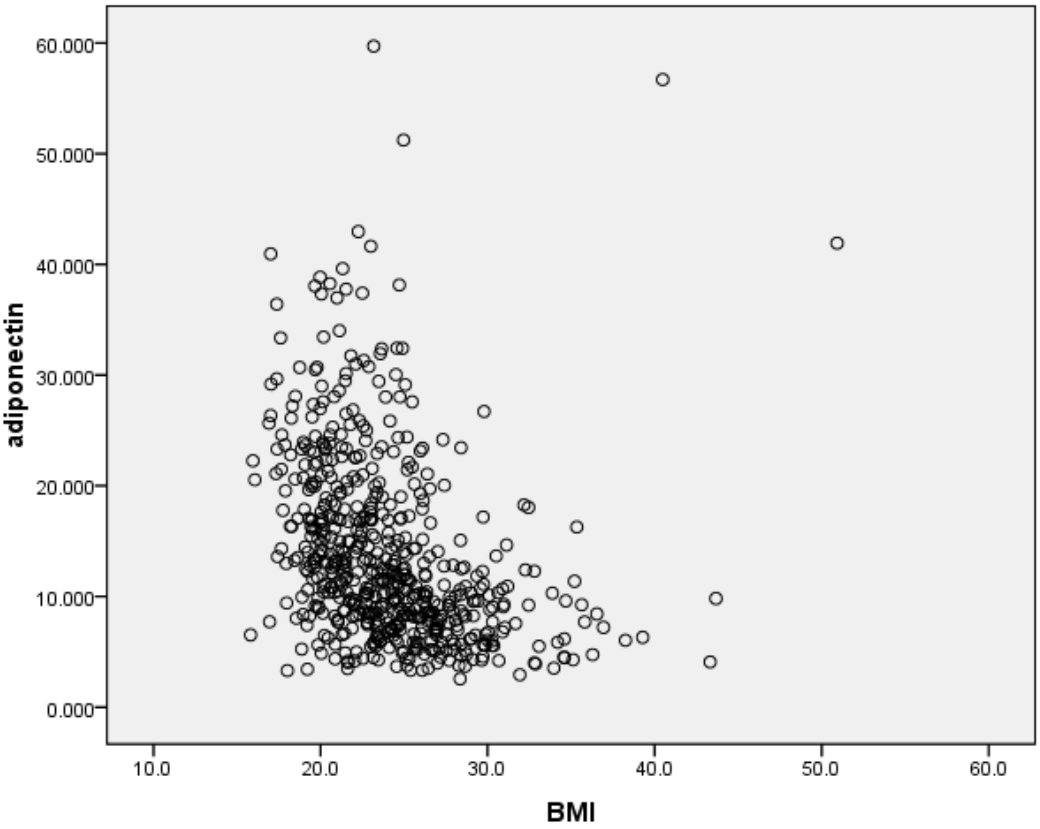

Figure 4.The serum concentration of adiponectin showed a negative relation with BMI

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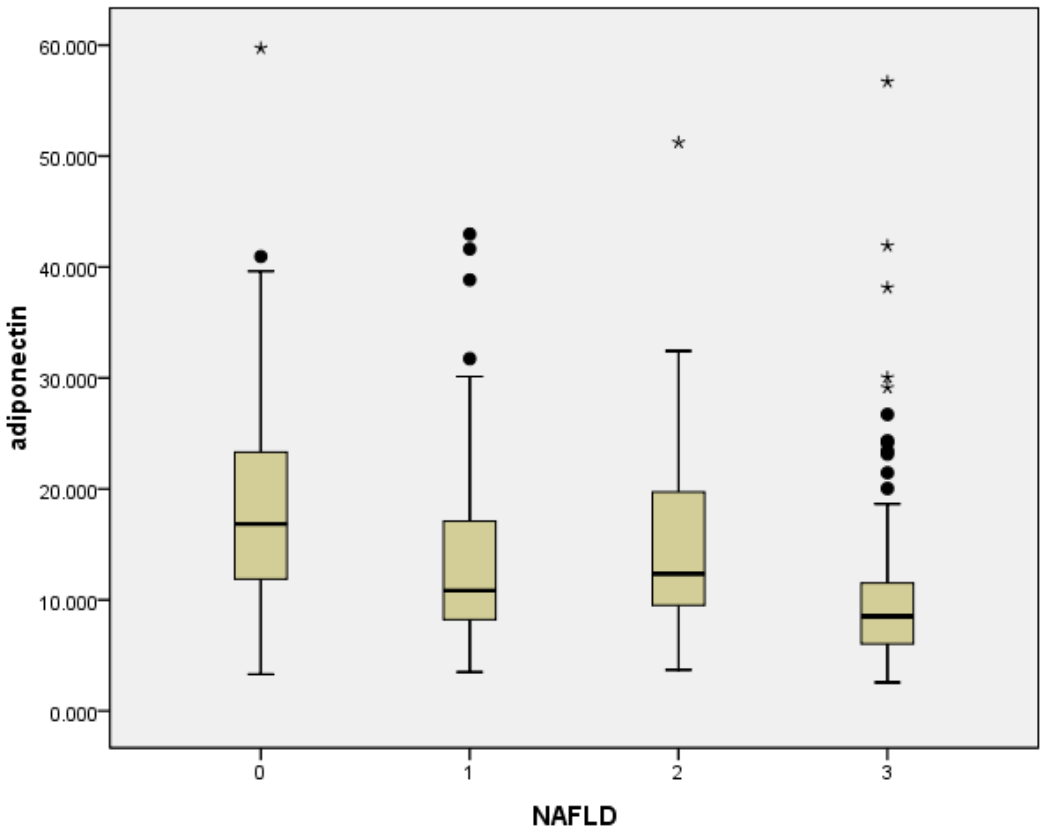

Figure 5. The distribution of adiponectin concentration among groups

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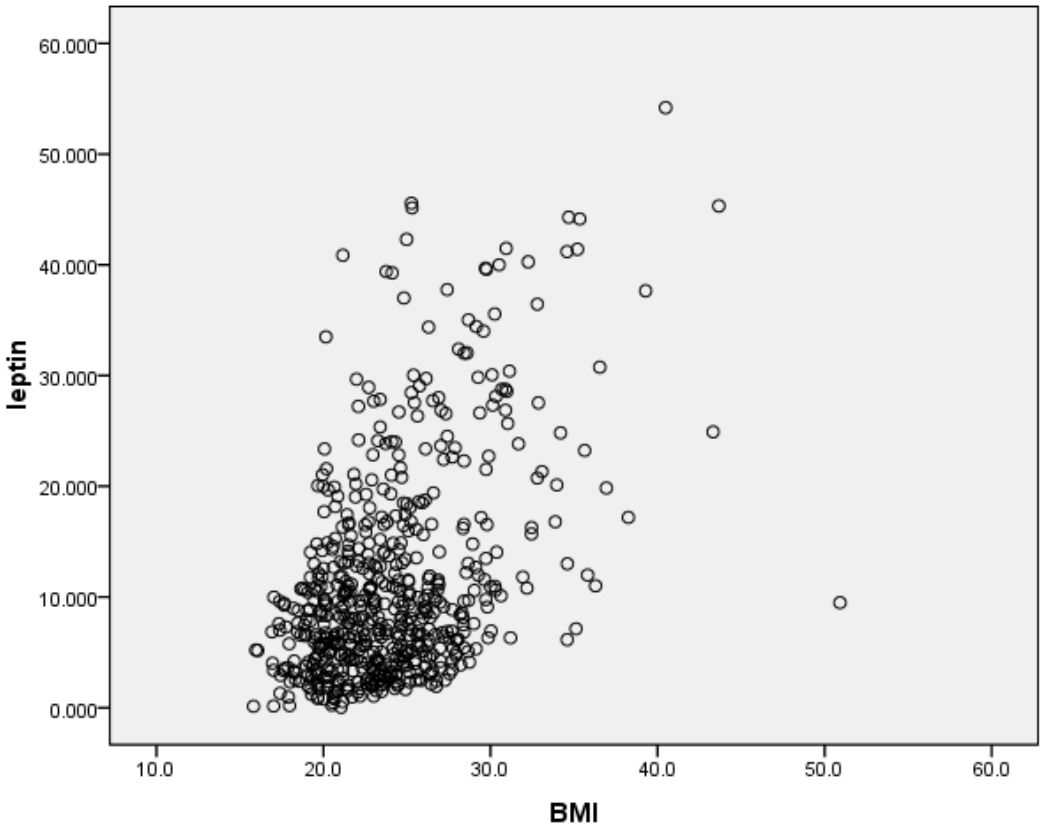

Figure 6. The serum concentration of leptin showed a positive relation with BMI

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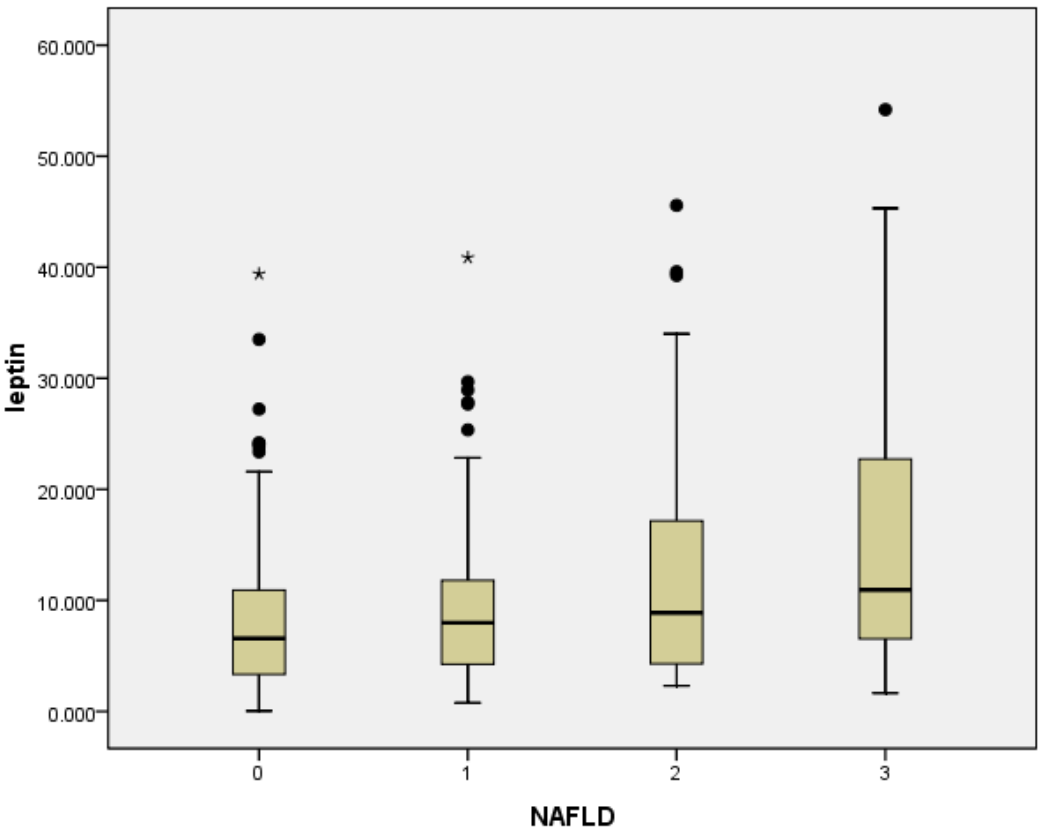

Fig 7. The distribution of leptin concentration among groups

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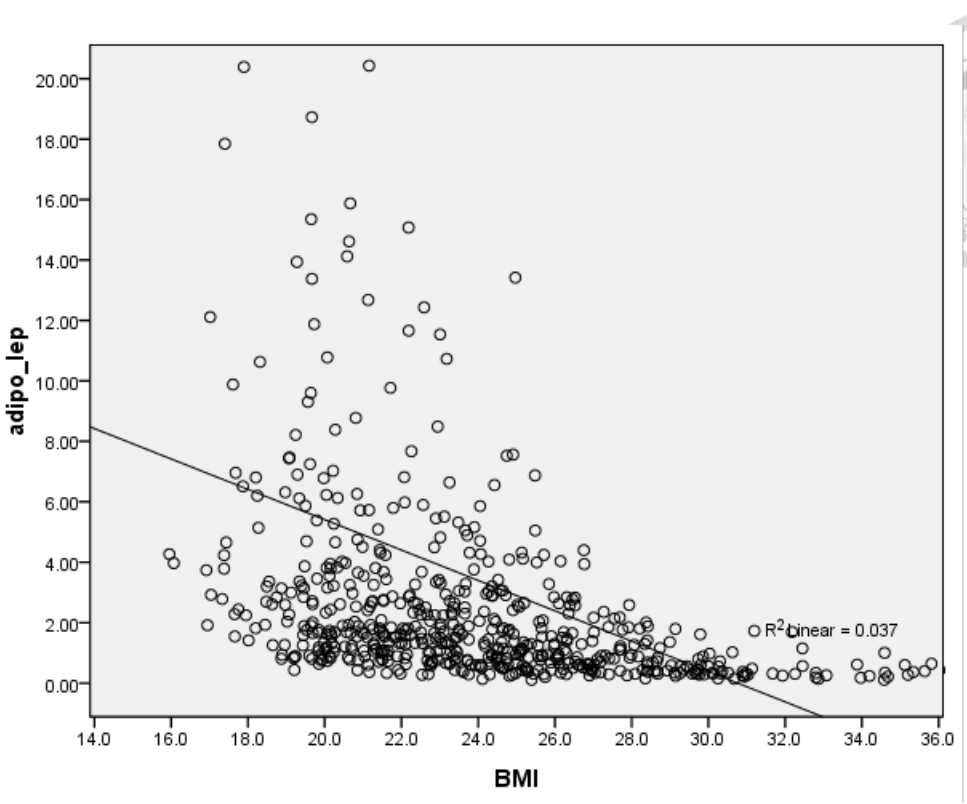

Fig 8. The serum concentration of adiponectin-leptin ratio showed a negative relation with BMI

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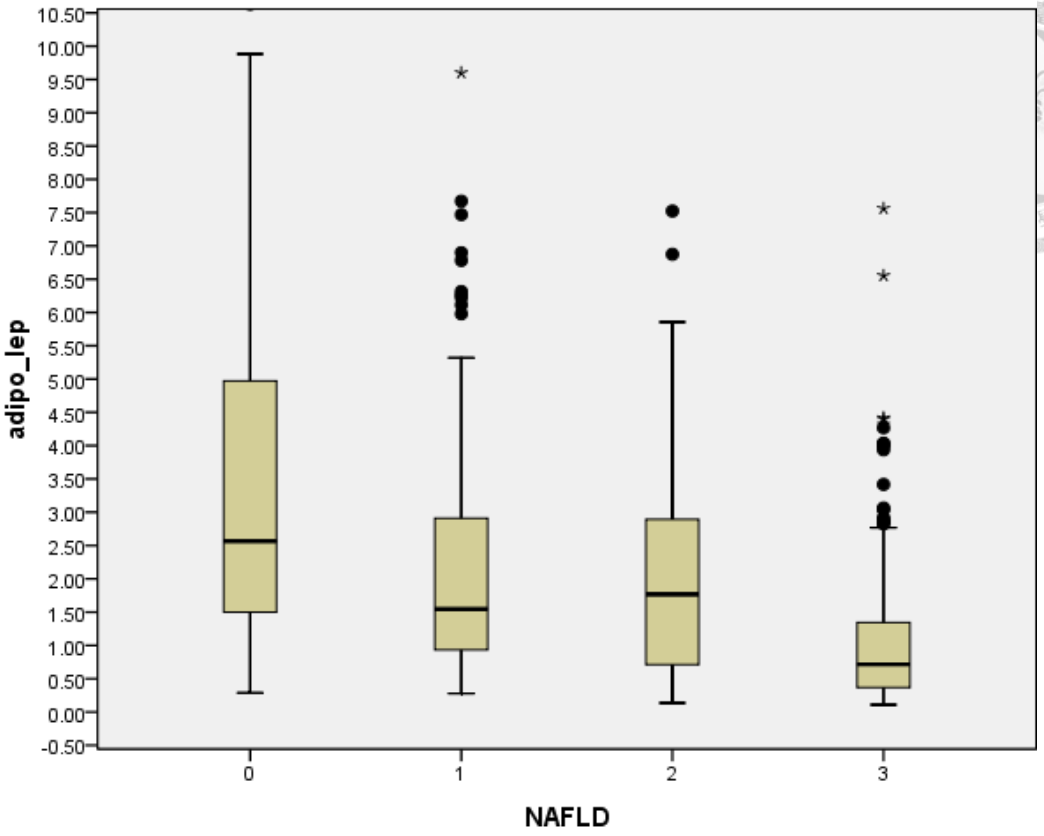

Fig 9. The distribution of adiponectin-leptin ratio among groups

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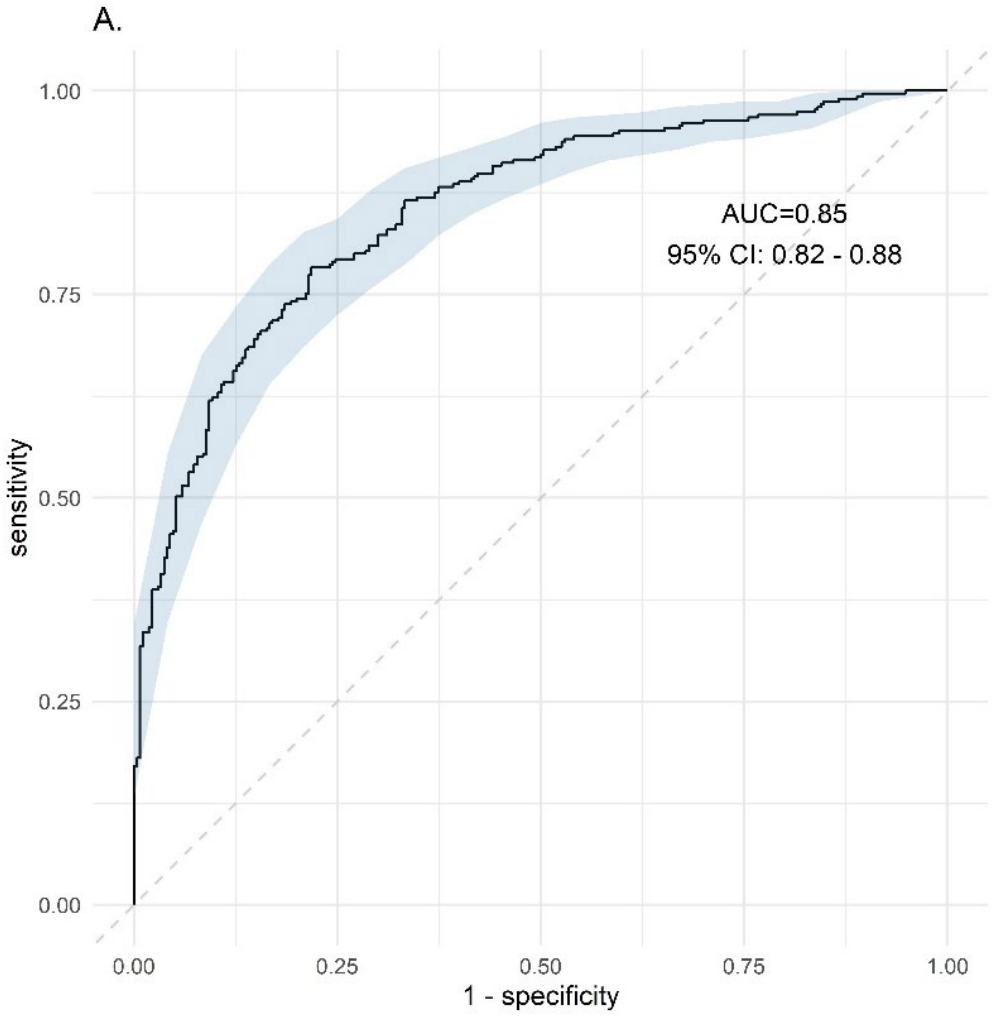

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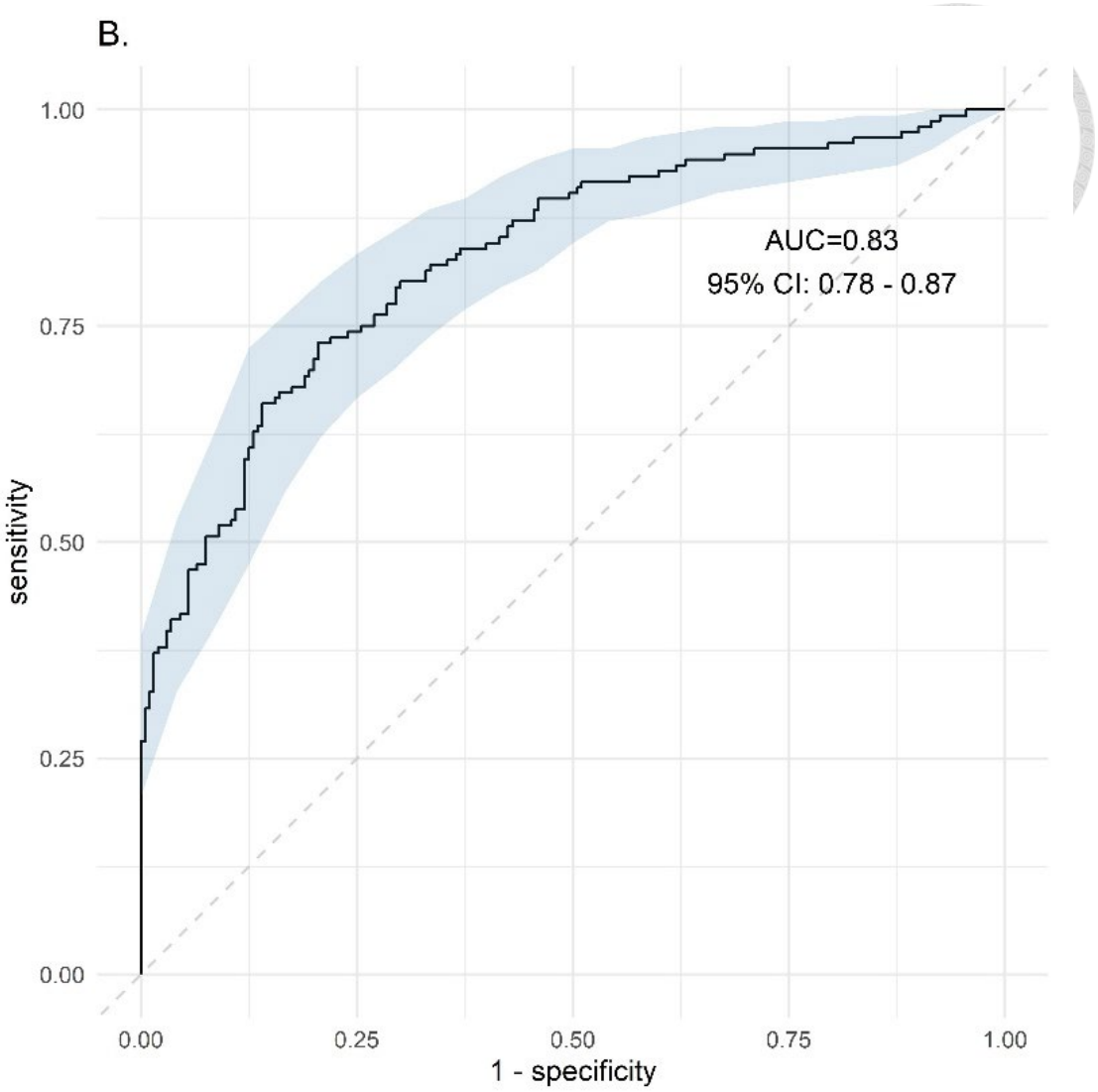

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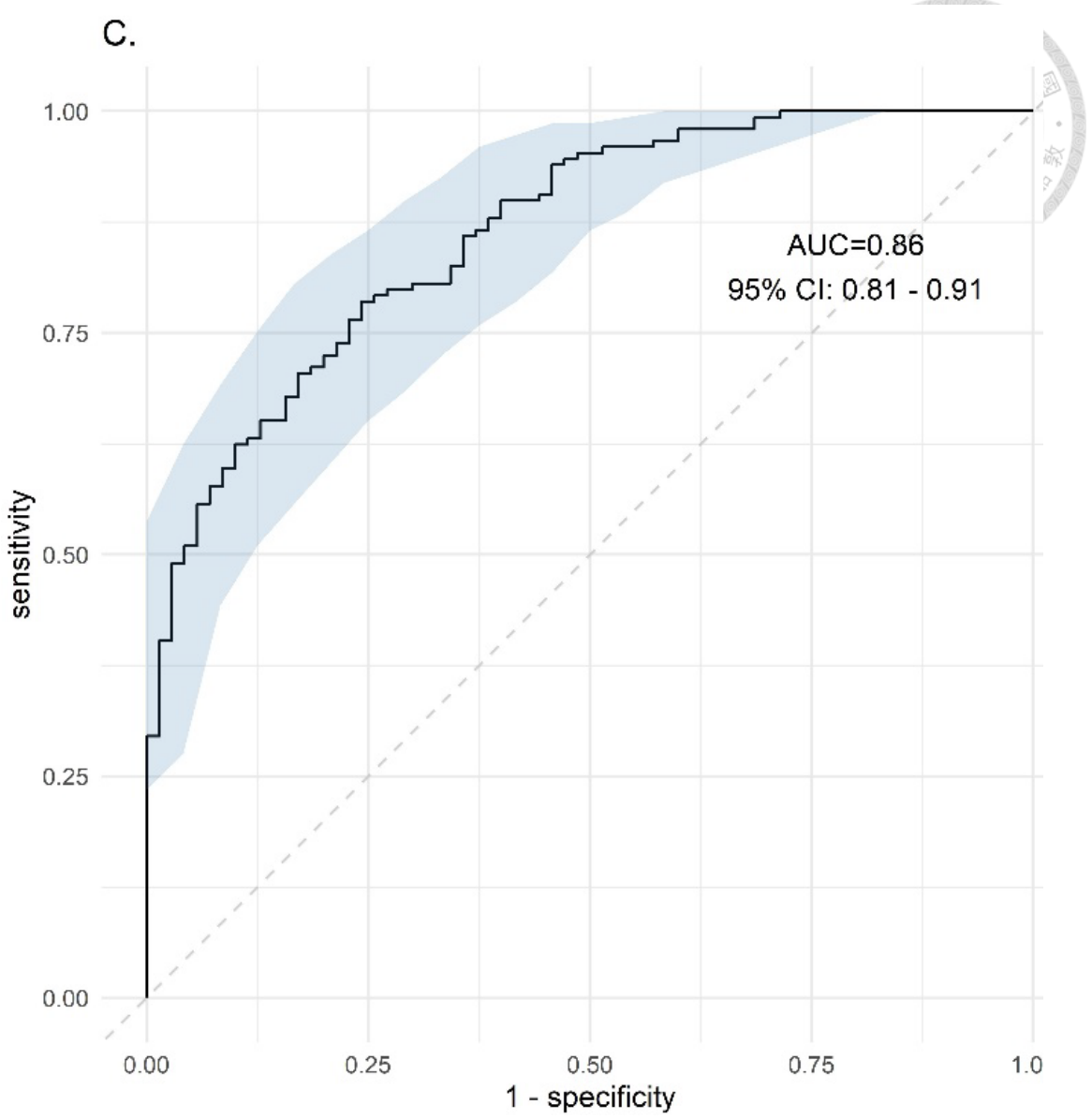

Figure 10. Receiver operating characteristic (ROC) for the diagnosis of NAFLD

Receiver operating characteristic (ROC) for the diagnosis of NAFLD. Except adiponectin-leptin ratio, BMI, triglyceride and AST/ALT ratio were selected. A. all subjects, AUROC was 0.85 (95% CI: 0.82-0.88), B. female subjects, AUROC was 0.83 (0.78-0.87), and C. male subjects, AUROC was 0.86 (081-0.91). All $p<0.001$.

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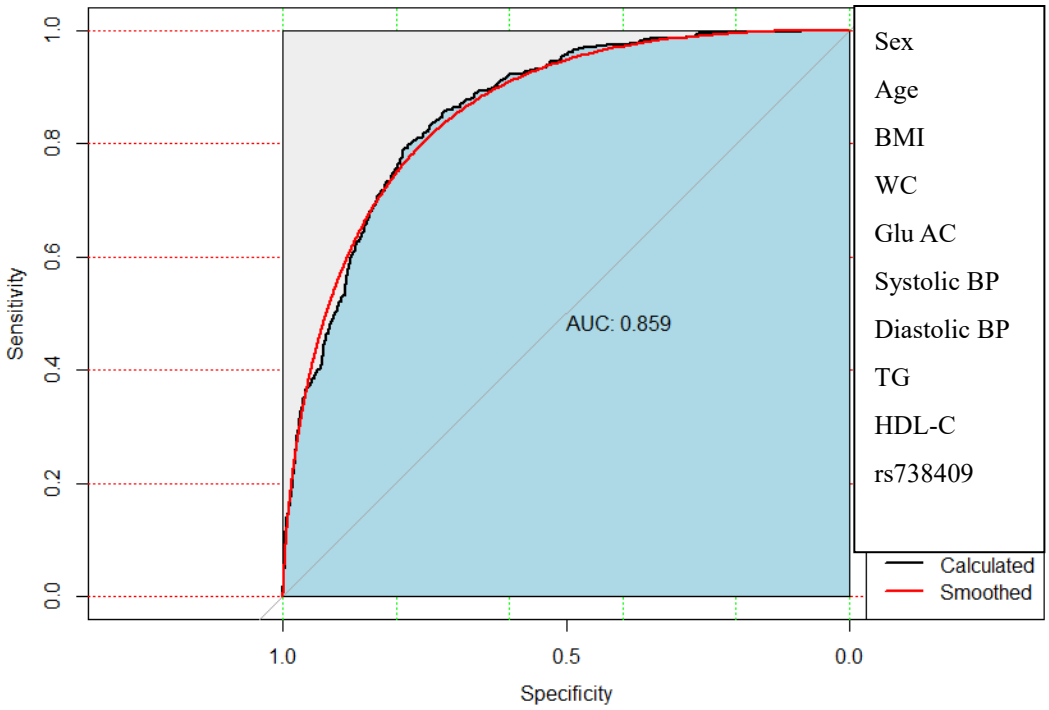
A



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B.

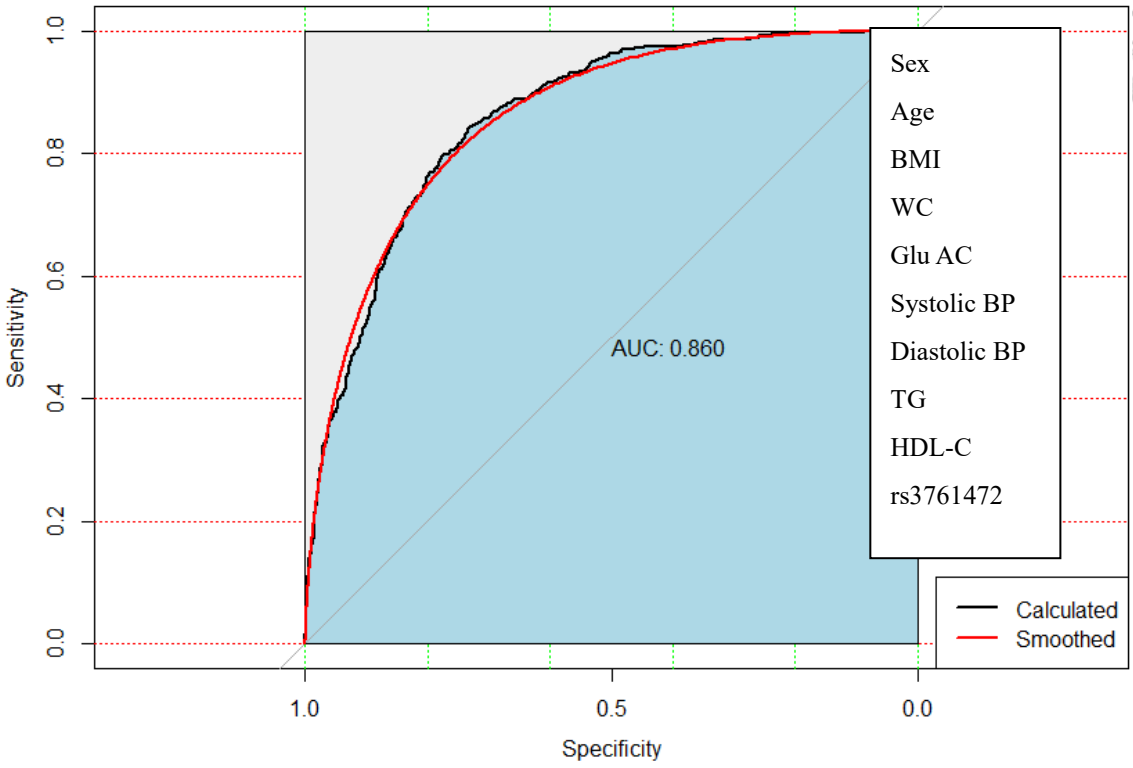

Figure 11. The area under the ROC curve for gene variants in the detection of lean NAFLD. The area under the ROC curve for gene variants in the detection of lean NAFLD. Other than SNPs, sex, age, body mass index, waist circumference, systolic BP, diastolic BP, HDL and triglycerides were included in the analysis. The areas under the ROC curve for *PNPLA3* rs738409 and *SAMM50* rs3761472 in the detection of lean NAFLD were 0.859 (95%CI: 0.841, 0.877) and 0.860 (95%CI: 0.843, 0.877), respectively. A. *PNPLA3* rs738409 and B. *SAMM50* rs3761472.

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nutrients

MDPI

Article

Independent Dose–Response Associations between Fetuin-A and Lean Nonalcoholic Fatty Liver Disease

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Abstract: Patients with lean NAFLD make up an increasing subset of liver disease patients. The association between lean NAFLD and feutin-A, which serves as a hepatokine and adipokine, has never been examined. Our study aimed to explore the association of serum fetuin-A among lean and non-lean patients. The study comprised 606 adults from the community, stratified into lean or non-lean (BMI </≥ 24 kg/m^2) and NAFLD or non-NAFLD (scoring of ultrasonographic fatty liver indicator, US-FLI ≥ 2/<2). Multivariate logistic regression analyses were performed to estimate the odds ratio of having NAFLD among the tertiles of fetuin-A after adjustment. The least square means were computed by general linear models to estimate marginal means of the serum fetuin-A concentrations in relation to the NAFLD groups. The odds ratio (OR) of having NAFLD for the highest versus the lowest tertile of fetuin-A was 2.62 (95% CI: 1.72–3.98; p for trend < 0.001). Stratifying by BMI, the OR of having lean NAFLD for the highest versus the lowest tertile of fetuin-A was 2.09 (95% CI: 1.09–3.98; p for trend 0.026), while non-lean NAFLD had no significant association with the fetuin-A gradient after adjustments. Fetuin-A was positively associated with lean NAFLD after adjusting for central obesity and insulin resistance.

Keywords: central obesity; fetuin-A; lean NAFLD; insulin resistance

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a growing health concern due to its increasing incidence and prevalence and its impact on associated comorbidities. The incidence of NAFLD is 28–52 per 1000 person-years, and the prevalence of NAFLD is approximately 25% [1]. It is well established that NAFLD is commonly associated with obesity, type 2 diabetes (T2DM), dyslipidemia, and metabolic syndrome (MetS) [2]. Therefore, a synonymous terminology is developing for diseases ranging from NAFLD to metabolic-associated fatty liver disease (MAFLD) [3]. However, there has been an increasing subset of patients with lean NAFLD, where they have NAFLD but also a normal body mass index [4]. Compared with non-lean NAFLD, patients with lean NAFLD are younger and have higher hemoglobin levels [5], an elevated ALT/AST ratio [6], and less insulin resistance and MetS [7]. Compared with healthy subjects, lean NAFLD patients have more dyslipidemia [8] and easier central obesity and insulin resistance [9]. Overall, in terms of phenotype, patients with non-lean NAFLD share metabolic features of insulin resistance

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and dyslipidemia with lean NAFLD patients [7]. From a histological perspective, lean NAFLD seems to have less severe steatosis [10], where >5% of hepatocytes are considered abnormal; lean NAFLD also has similar prevalence of NASH, where ballooning degeneration, lobular or portal inflammation, and fibrosis are present [10,11]. In general, the limited data, conflicting results, and increasing population of lean NAFLD patients have evoked remarkable concern.

Fetuin-A, also named Alpha2-Heremans-Schmid glycoprotein, is synthesized in hepatocytes and secreted into the bloodstream [12]. One of the most documented functions of fetuin-A is to act as an endogenous inhibitor of the insulin receptor tyrosine kinase, which triggers insulin resistance [13]. Therefore, fetuin-A has been highly correlated with T2DM, obesity, and MetS in previous studies [14,15]. Recently, fetuin-A was assumed to act as an endogenous ligand of Toll-like receptor 4 to stimulate chronic adipose inflammation [16]. Fetuin-A stimulates the secretion of inflammatory cytokines, such as TNF-alpha and interleukin-6, in adipose tissue [17]. With roles in both insulin resistance and chronic inflammation, circulating fetuin-A levels have been found to be significantly correlated with NAFLD patients [18]. However, the association between lean NAFLD and fetuin-A has never been studied. Therefore, we focused on a young adult population and conducted a community-based investigation to examine the clinical characteristics and metabolic factors of four groups: lean (+) NAFLD (−), lean (+) NAFLD (+), lean (−) NAFLD (−), and lean (−) NAFLD (+). The study also aimed to explore the association of serum gradients of fetuin-A among four groups (lean/NAFLD: +/−, +/+, −/−, −/+) after adjusting for insulin resistance and central obesity.

2. Materials and Methods

2.1. Study Subjects

This study was conducted in the community of Hsinchu City, Northern Taiwan. All the participants completed standardized questionnaires through individual interviews. The exclusion criteria were excessive alcohol use, which was defined as drinking more than 20 g of alcohol daily for women and 30 g for men, and chronic liver diseases, which included chronic hepatitis, autoimmune, drug-induced, vascular, and inherited hemochromatosis, as well as Wilson disease. According to the recommendation of World Health Organization, both men and women were suggested to drink less than two standard drinks per day, i.e., 20 g of pure ethanol per day [19]. The amount of alcohol in any drink is calculated by the following equation: pure alcohol mass equals volume (L) × alcohol percentage (%) × volumetric mass density (g/L) [20]. Subjects who drank more than the limited amount were excluded to confirm that we only enrolled NAFLD. In total, 606 adults aged 20 to 80 years old were enrolled. Information about age, gender, personal habits including cigarette smoking and exercise habits, and previous diseases was obtained after informed consent forms were signed. Current smokers were defined as those who had been smoking for more than 6 months prior to participating in this study. Noncurrent smokers were defined as those who had quit smoking for more than 12 months before the study or who had never been smokers. Exercise habit was defined by the following yes or no question: “Do you have a regular exercise habit?”. Weight and height were measured by a standard electronic scale and stadiometer. Waist circumference (WC) was measured at the level of the umbilicus by a by the same trained operator while the nearest millimeter was recorded. Blood pressure (BP) was measured by a sphygmomanometer. The first and fifth Korotkoff phases were used to determine systolic blood pressure (BP) and diastolic BP, respectively [21] This study was approved by the Institutional Review Board of National Taiwan University Hospital (IRB NO. 201210012RIC).

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2.2. Ultrasonography Assessment

Abdominal ultrasonography was performed after at least eight hours of fasting by a well-trained examiner with a 3.5–5 MHz transducer and a high-resolution B-mode scanner (Hitachi Aloka ProSound α 6). The ultrasound measurements were performed by three experienced research physicians. Before the study, all three physicians reached a consensus regarding the standard procedure for ultrasound scanning, including the scoring of ultrasonographic fatty liver indicator (US-FLI) and the sequence of acquiring liver images. The severity of NAFLD was calculated using the US-FLI score, which ranges from 0 to 8 [22]. The US-FLI is composed of five indicators: (1) the presence of liver-kidney contrast graded as mild/moderate (score 2) and severe (score 3); and (2) the presence (score 1) or absence (score 0) of posterior attenuation of the ultrasound beam, vessel blurring, difficult visualization of the gallbladder wall, difficult visualization of the diaphragm, and areas of focal sparing (score of 1 each). The subjects were then divided into four groups: (1) lean non-NAFLD group: US-FLI score < 2, BMI < 24 kg/m^2; (2) lean NAFLD group: US-FLI score ≥ 2, BMI< 24 kg/m^2; (3) non-lean, non-NAFLD group: US-FLI score < 2, BMI ≥ 24 kg/m^2; (4) non-lean NAFLD group: US-FLI score ≥ 2, BMI ≥ 24 kg/m^2.

2.3. Blood Analysis

Venous blood was sampled after ≥8 h of fasting. Serum glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides were measured by an automatic spectrophotometric assay (HITACHI 7250, Tokyo, Japan). Fasting insulin levels were examined by a microparticle enzyme immunoassay using an AxSYM system (Abbott Laboratories, Dainabot Co, Tokyo, Japan). We estimated the intensity of insulin resistance by an indirect assessment, the homeostatic model assessment of insulin resistance (HOMA-IR). The convert equation was HOMA-IR = fasting insulin × fasting plasma glucose/22.5, with glucose shown in mmol/L and insulin shown in mU/L [23]. Serum fetuin-A was measured using a quantitative sandwich enzyme immunoassay technique after a 4000-fold dilution. This immunoassay was calibrated against highly purified NS0-expressing recombinant human fetuin-A (R&D Inc. Minneapolis, MN, USA).

2.4. Statistical Analysis

Subjects were sorted into tertiles according to the serum levels of fetuin-A. Basic demographic characteristics are shown as the mean ± standard deviation for the continuous parameters and cases (%) for the categorical parameters. Multivariate logistic regression analyses were performed to calculate the odds ratio of having NAFLD among the tertiles of fetuin-A after adjustment for age, gender, personal habits, WC, and the HOMA-IR, stratified by BMI or not. The least square means were computed by general linear models to estimate marginal means of the serum fetuin-A concentrations in relation to the NAFLD groups after adjusting for age, gender, personal habits, weight circumference, and the homeostasis model assessment of insulin resistance. We conducted statistical analyses by applying SPSS statistical software (V.17, SPSS, Chicago, IL, USA). We assumed a statistical significance whenever the p value < 0.05.

3. Results

3.1. General Characteristics

The basic characteristics of the participants are shown in Table 1. The mean age of the participants was 42.6 ± 11.5 years old, the median was 41.0 years old (25th/75th: 34.0/50.0 years old), and 61.7% of the participants were female and 38.3% of the participants were male. The mean serum concentrations of fetuin-A were 689.4 ± 672.4 mg/L, 882.6 ± 731.3 mg/L, 829.3 ± 429.3 mg/L, and 855.9 ± 467.0 mg/L in the four groups, respectively. The scattered plots and box plot representing the distribution of subjects among four groups are shown in Supplementary Figure S1. The highest level of fetuin-A was

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found in the lean NAFLD group. In a post hoc analysis (Table 2), the lean NAFLD group shared similar metabolic factors with the non-lean, non-NAFLD group. However, patients in the former group had a presentation of NAFLD and patients in the latter had a significantly higher BMI, waist circumference, and body fat percentage. Both lean and non-lean NAFLD had high levels of fetuin-A, while non-lean NAFLD apparently had more metabolic factors and high BMI, waist circumference, and body fat percentage. This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

Table 1. Baseline characteristics among the lean, non-lean, NAFLD, and non-NAFLD groups.

	Lean		**Non-lean**		***p* Value**
	Non-NAFLD N = 227	**NAFLD N = 108**	**Non-NAFLD N = 54**	**NAFLD N = 217**	
Age (years)	41.1 ± 11.0	42.6 ± 11.6	44.5 ± 11.3	43.7 ± 11.8	0.061
Male (%)	47 (20.7%)	37 (34.3%)	25 (46.3%)	123 (56.7%)	<0.001
Female (%)	180 (79.3%)	71 (65.7%)	29 (53.7%)	94 (43.3%)	<0.001
BMI (kg/m^2)	20.6 ± 1.8	21.9 ± 1.5	26.0 ± 1.7	28.1 ± 4.0	<0.001
WC (cm)	73.1 ± 6.1	77.6 ± 6.5	85.4 ± 6.2	91.1 ± 8.3	<0.001
Body fat (%)	25.6 ± 6.2	26.6 ± 6.0	30.0 ± 7.9	32.4 ± 8.4	<0.001
Systolic BP	115.7 ± 15.7	121.6 ± 15.3	122.6 ± 17.0	130.4 ± 15.3	<0.001
Diastolic BP	72.9 ± 11.2	77.2 ± 9.5	77.9 ± 13.8	82.2 ± 12.2	<0.001
TCHO (mmol/L)	190.0 ± 33.8	196.9 ± 39.8	194.6 ± 29.3	201.7 ± 35.5	0.007
TG (mmol/L)	74.2 ± 37.2	109.2 ± 78.9	95.0 ± 43.5	160.2 ± 113.8	<0.001
HDL-C (mmol/L)	66.7 ± 15.0	57.3 ± 13.2	59.5 ± 13.5	49.7 ± 12.6	<0.001
LDL-C (mmol/L)	114.5 ± 31.2	125.4 ± 37.1	123.0 ± 29.2	131.7 ± 32.5	<0.001
Glucose (mmol/L)	83.7 ± 13.0	85.3 ± 8.7	87.0 ± 10.4	94.2 ± 22.8	<0.001
Insulin (U/mL)	5.29 ± 4.24	6.77 ± 5.21	7.1 ± 3.9	11.5 ± 8.9	<0.001
HOMA-IR	0.68 ± 0.55	0.86 ± 0.65	0.91 ± 0.49	1.49 ± 1.10	<0.001
Current smoker (%)	16 (7.0)	11 (10.2)	5 (9.3)	35 (16.1)	0.022
Exercise (%)	100 (44.1)	46 (42.6)	27 (50.0)	92 (42.4)	0.782
GOT	20.3 ± 6.8	21.7 ± 7.0	21.5 ± 5.9	25.8 ± 10.2	<0.001
GPT	17.2 ± 9.4	23.8 ± 16.5	21.4 ± 10.6	36.7 ± 27.8	<0.001
CRP (mg/dL)	0.11 ± 0.31	0.10 ± 0.13	0.17 ± 0.28	0.22 ± 0.25	<0.001
Metabolic factors	0.39 ± 0.62	0.91 ± 0.89	1.15 ± 0.90	2.14 ± 1.18	<0.001
MetS (%)	2 (2.5)	6 (7.5)	4 (5.0)	68 (85)	<0.001
Fetuin-A (mg/L)	689.4 ± 672.4	882.6 ± 731.3	829.3 ± 429.3	855.9 ± 467.0	0.009

ANOVA was applied to test the difference among groups. Abbreviations: NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; WC, waist circumference; BP, blood pressure; TCHO, total cholesterol; TG, triglycerides, HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; CRP, C-reactive protein; MetS: metabolic syndrome. Current smokers were defined as those who had been smoking for more than 6 months prior to participating in this study. Noncurrent smokers were defined as those who had quit smoking for more than 12 months before the study or who had never been smokers. Exercise habit was defined by the following yes or no question: "Do you have a regular exercise habit?". Significance level: $p < 0.05$.

Table 2. Comparison of lean, non-lean, NAFLD, and non-NAFLD groups in metabolic variables.

Lean/NAFLD:	**+/− vs. +/+**	**+/− vs. −/−**	**+/− vs. −/+**	**+/+ vs. −/−**	**+/+ vs. −/+**	**−/− vs. −/+**
Age (years)	0.663	0.199	0.079	0.754	0.856	0.966
Male (%)	0.059	0.002	<0.001	0.400	<0.001	0.451
BMI (kg/m^2)	0.001	<0.001	<0.001	<0.001	<0.001	<0.001

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WC (cm)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Body fat (%)	0.610	<0.001	<0.001	0.024	<0.001	0.122
Systolic BP	0.007	0.019	<0.001	0.977	<0.001	0.006
Diastolic BP	0.007	0.022	<0.001	0.986	0.002	0.075
TCHO (mmol/L)	0.339	0.822	0.003	0.980	0.658	0.553
TG (mmol/L)	0.001	0.321	<0.001	0.711	<0.001	<0.001
HDL-C (mmol/L)	<0.001	0.003	<0.001	0.745	<0.001	<0.001
LDL-C (mmol/L)	0.023	0.315	<0.001	0.971	0.359	0.296
Glucose (mmol/L)	0.833	0.5448	<0.001	0.928	<0.001	0.024
Insulin (U/mL)	0.303	0.352	<0.001	0.994	<0.001	<0.001
HOMA-IR	0.303	0.324	<0.001	0.990	<0.001	<0.001
GOT	0.499	0.767	<0.001	1.000	<0.001	0.003
GPT	0.017	0.463	<0.001	0.876	<0.001	<0.001
CRP (mg/dL)	0.961	0.439	<0.001	0.321	<0.001	0.672
Fetuin-A (mg/L)	0.030	0.413	0.019	0.951	0.981	0.991

Turkey post hoc analysis was performed to compare each two groups within the four groups to know which two groups were significantly different in the ANOVA analysis. Four groups: lean (+) NAFLD (−), lean (+) NAFLD (+), lean (−) NAFLD (−), and lean (−) NAFLD (+).

3.2. Association of Fetuin-A and NAFLD

To further clarify the association between the concentration gradients of fetuin-A and NAFLD, multiple logistic regression analyses were applied to examine the odds ratios (ORs) of having NAFLD derived from tertiles of serum fetuin-A levels in Table 3. The OR of having NAFLD for the highest versus the lowest tertile of fetuin-A was 2.62 (95% CI: 1.72–3.98; p for trend < 0.001) adjusting for age, gender, and personal habits. After adjustment for age, gender, personal habits, and WC, the OR of having NAFLD for the highest versus the lowest tertile of fetuin-A was 1.80 (95% CI: 1.10–2.94, p for trend 0.02). However, after further adjusting for the HOMA-IR, the ORs became insignificant (1.5; 95% CI: 0.92–2.67; p for trend 0.099).

Table 3. Odds ratios of having NAFLD derived from multiple logistic regression analyses in tertiles of serum fetuin-A levels.

	Q1 (N = 202) (≤821 mg/L)	**Q2 (N = 201) (822–1012 mg/L)**	**Q3 (N = 203) (1013–1224 mg/L)**	***p* for Trend**
Model 1	1.00	2.49 (1.64–3.77) **	2.62 (1.72–3.98) **	<0.001
Model 2	1.00	1.55 (0.94–2.56)	1.80 (1.10–2.94) *	0.020
Model 3	1.00	1.49 (0.87–2.57)	1.57 (0.92–2.67)	0.099

Model 1: adjustment of age, gender, and personal habits. Model 2: adjustment of age, gender, personal habits, and waist circumference. Model 3: adjustment of age, gender, personal habits, waist circumference, and the homeostasis model assessment of insulin resistance. * For $p < 0.05$; ** For $p < 0.001$.

Stratified by BMI, the ORs of having NAFLD derived from multiple logistic regression analyses in tertiles of serum fetuin-A are shown in Table 4. When BMI < 24 kg/m^2, the crude OR of having NAFLD for the highest versus the lowest tertile of fetuin-A was 1.95 (95% CI: 1.14–3.34; p for trend < 0.018). After adjusting for age, gender, personal habits, WC, and the HOMA-IR, the OR of having NAFLD for the highest versus the lowest tertile of fetuin-A was 2.09 (95% CI: 1.09–3.98; p for trend 0.026). When BMI > 24 kg/m^2, both the crude ORs and the adjusted ORs of having NAFLD for the highest versus the lowest tertile of fetuin-A were insignificant, being 1.35 (95% CI: 0.57–3.21; p for trend < 0.603) and 0.69 (95% CI: 0.24–1.95; p for trend 0.422), respectively.

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Table 4. Odds ratios of having NAFLD derived from multiple logistic regression analyses in tertiles of serum fetuin-A levels, with stratification by BMI.

	Lean NAFLD			
	Q1 (N = 158)	**Q2 (N = 75)**	**Q3 (N = 102)**	***p* for Trend**
Model 1	1.00	1.01 (0.53–1.90)	1.95 (1.14–3.34) *	0.018
Model 2	1.00	1.26 (0.63–2.50)	2.26 (1.26–4.07) *	0.007
Model 3	1.00	1.33 (0.63–2.82)	2.09 (1.09–3.98) *	0.026
	Overweight/Obese NAFLD			
	Q1 (N = 44)	**Q2 (N = 126)**	**Q3 (N = 101)**	***p* for Trend**
Model 1	1.00	1.48 (0.65–3.38)	1.35 (0.57–3.21)	0.603
Model 2	1.00	1.20 (0.47–3.02)	0.89 (0.34–2.33)	0.688
Model 3	1.00	0.95 (0.35–2.56)	0.69 (0.24–1.95)	0.422

Model 1: adjustment of age, gender, and personal habits. Model 2: adjustment of age, gender, personal habits, and waist circumference. Model 3: adjustment of age, gender, personal habit, waist circumference, and the homeostasis model assessment of insulin resistance. * For $p < 0.05$;

The least square means (±SDs) of the serum fetuin-A concentrations in relation to the four groups were 732.4 (617.0–847.9) mg/L, 920.3 (790.5–1050.1) mg/L, 860.0 (678.5–1041.6) mg/L, and 833.3 (723.7–942.9) mg/L, respectively, after adjusting for age, gender, personal habits, WC, and the HOMA-IR (Figure 1).

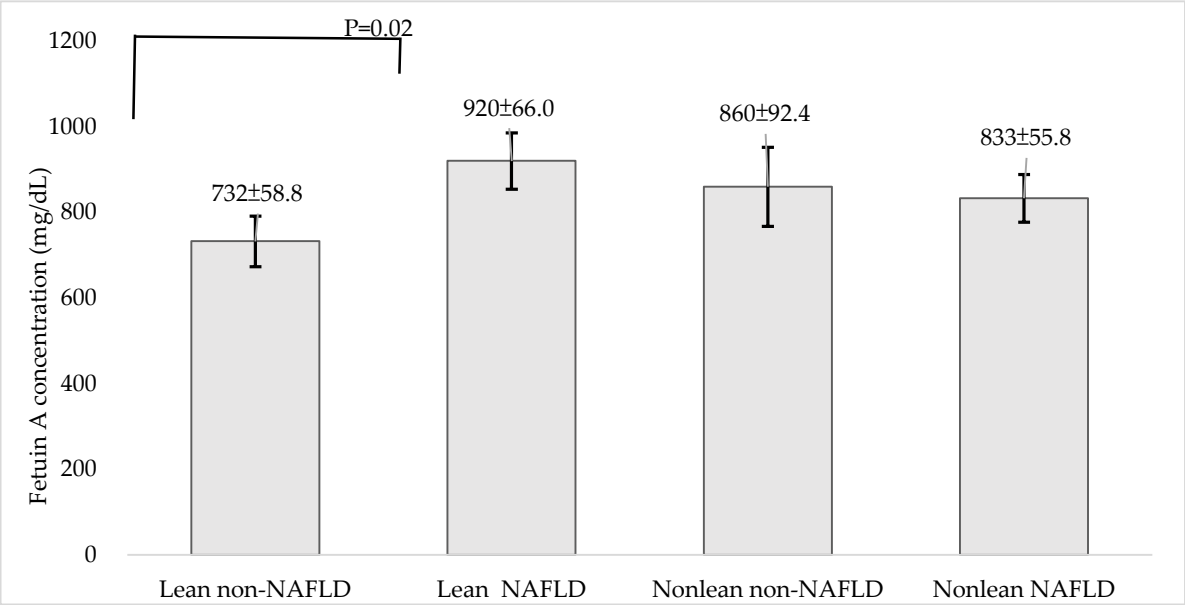

Figure 1. Comparison of serum concentrations of fetuin-A in relation to the groups of NAFLD after adjusting age, gender, personal habits, weight circumference, and the homeostasis model assessment of insulin resistance by least square means method. Data are shown as mean ± SD with error bars. Statistical significance was only found between lean non-NAFLD and lean NAFLD ($p < 0.05$) groups, but not found between the non-lean non-NAFLD and non-lean NAFLD ($p = 0.798$) groups.

4. Discussion

This is the first study to demonstrate that there is a positive association between the serum fetuin-A gradient and the risk of lean NAFLD. First, a 2.09-fold risk of lean NAFLD was found in the highest tertile compared with the lowest tertile of serum fetuin-A, while no significance was found in non-lean NAFLD. Second, we also found that there was a dose–response relationship between the serum fetuin-A gradient and non-lean NAFLD after adjusting for age, gender, personal habits, WC, and the HOMA-IR (p for trend < 0.05). Third, both lean and non-lean NAFLD had high levels of fetuin-A, while non-lean NAFLD

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apparently had more metabolic factors and higher BMI, waist circumference, and body fat percentage. The persistence of a direct relationship between fetuin-A and the risk of lean NAFLD after adjusting for WC and the HOMA-IR implied that still unidentified factors affected this association beyond the central obesity and insulin resistance that were only captured in the lean subjects.

The name fetuin implies that its amount is highest in fetal blood. Fetuin is found in significantly lower concentrations in adults [24] and serves pleiotropic functions. In adults, fetuin-A is secreted by hepatocytes and adipocytes and predominantly (>95%) expressed in the liver [25]. It is well known that fetuin-A is involved in the development of insulin resistance in both animal and human studies [26,27], and thus contributes to the development of NAFLD. Fetuin-A promotes lipid-induced inflammation by binding free fatty acids to Toll-like receptor 4 in animal studies [16,28], most likely contributing even further to the progression of NAFLD. It is not surprising that fetuin-A levels were significantly elevated in NAFLD patients in previous studies [18]. In biopsy-proven human studies, both circulating levels of fetuin-A and the hepatic expression of fetuin-A were higher in NAFLD patients than in healthy controls regardless of the histological state and BMI class [29], implying that the BMI-oriented concept for NAFLD or MAFLD might need to be reconsidered. To date, there have been no data on the relationship or the underlying mechanisms between lean NAFLD and the serum gradient of fetuin-A. We boldly hypothesize that, although lean NAFLD is associated with fewer metabolic dysfunctions than non-lean NAFLD, it might be prone to more progressive inflammation and oxidative stress. Experimental studies have shown that fetuin-A promotes the expression of proinflammatory cytokines at the mRNA and protein levels [12,30] and chronically responds to inflammatory stimuli [31], leading to the progression from steatohepatitis to NASH [32,33]. In a study cohort comprising 1339 Caucasian biopsy-proven NAFLD patients, it was found that both lean and non-lean NAFLD may progress to advanced liver disease, metabolic comorbidities, cardiovascular disease, and liver-related mortality, independent of the progression to obesity [34].

It is interesting but puzzling that fetuin-A is prone to be elevated in early NAFLD, and that it is more prominent in lean NAFLD. We boldly hypothesized that the amount of adipose composition reflects the capacity of lipid storage to some extent. Therefore, lacking adipose tissue in lean subjects is thought to be because of less fat storage capacity and is associated with lipid accumulation in ectopic sites [35,36]. After triglycerides are eventually saturated in adipocytes, the liver was recognized as the most sensitive and vulnerable ectopic site for fat deposition, leading to fatty liver disease [37,38]. Although lipodystrophy might be specific for acquired or congenital loss of adipose tissue, more and more evidence supported that within lean people in the general population, some features of lipodystrophy exist, i.e., insulin resistance and accumulation of lipids in the liver [39,40]. Furthermore, an animal model has demonstrated that a lean mouse phenotype with fatty liver was probably a consequence of adipocyte dysfunction [41]. Inspiringly, we found that lean NAFLD subjects shared similar risks of metabolic factors, including fasting glucose, insulin resistance, lipid profiles, and blood pressure, with non-lean, non-NAFLD subjects. In our data, lean NAFLD subjects had a normal BMI and fatty liver disease, while non-lean non-NAFLD subjects were overweight or obese with a significantly higher fat percentage and waist circumference. In line with our findings, lipodystrophy limited the lipid accumulation in lean NAFLD subjects, causing ectopic fat accumulation in the liver. We thus inferred that the role of fetuin-A, majorly as a hepatokine and minorly as an adipokine, was reasonable for the highest concentration in the lean NAFLD group.

It has been observed that lean NAFLD patients are younger and have fewer metabolic clinical features but share similar histological severity, comorbidities, and mortality with their non-lean counterparts [42]. Lean NAFLD subjects develop NAFLD prior to obesity and metabolic dysfunction, and conventional metabolic factors cannot be used for early detection. Since liver fat accumulation and chronic inflammation are very sensitive

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and early indicators in these subsets, fetuin-A, as a hepatokine and an adipokine, could be used as a surrogate biomarker independent of central obesity and insulin resistance. The strengths of our study therefore cannot be ignored. We were the first to link the serum level of fetuin-A with lean NAFLD and to demonstrate a dose escalation of fetuin-A for the risk of lean NAFLD.

There are some limitations in our study. First, this is a cross-sectional study, and we could not interfere with the causal relationship between lean NAFLD and the serum gradient of fetuin-A. Despite the collection and adjustment of probable confounders, there could be unmeasured and undefined factors indicating possible residual effects. For example, the duration of NAFLD may potentially influence serum fetuin-A levels over time, but we did not collect longitudinal data from lean or non-lean NAFLD individuals. The relationship between lean NAFLD and fetuin-A warrants more investigation through basic and clinical studies to clarify the pathophysiology of lean NAFLD and fetuin-A with well-designed animal models and prospective cohorts. Second, we did not perform liver biopsy, which is the gold standard for the diagnosis of NAFLD. Although the ultrasonographic approach could not distinguish the severity of NAFLD, it has been acknowledged as a screening tool for NAFLD [2]. In addition, we applied US-FLI, an extensively applied ultrasonographic scoring system, as a substitute modality for the diagnosis of NAFLD [22,43]. Although the bias of misclassification by ultrasound could exist and attenuate the association, we still demonstrated a statistical significance between the non-NAFLD and NAFLD group. Furthermore, we did not check inflammatory markers, such as TNF-alpha and IL-6 levels, as well as their association with fetuin-A, to clarify the inflammatory status probably related to the underlying mechanism. Further studies should focus on the combination of ultrasonographic assessment and surrogate biomarkers to improve the accuracy and precision of noninvasive approaches for NAFLD.

5. Conclusions

In conclusion, we found that serum fetuin-A has a dose–response association with lean NAFLD independent of insulin resistance and central obesity. In order to address the increasing subset of lean NAFLD patients and reappraise BMI-approached MAFLD, further investigations are needed to explore the mechanisms connecting fetuin-A to lean NAFLD as well as their clinical application.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/nu13092928/s1, Figure S1A. Scattered plots of fetuin-A concentration among four groups. The data showed as a collection of points, each having the value of fetuin-A concentration on the vertical axis and the category of group in the horizontal axis. Suppl. 1B. Box plot of fetuin-A concentration among four groups. The lines from bottom to top represented Q1, Q2, Q3 and Q4 values, respectively, The box showed the interquartile range, the distance between Q3 and Q1. The larger data than Q4 pointed any outliers.

Author Contributions: K.-C.H. guided and planned this investigation as well as recruited funds. C.-W.L. handled the statistical approaches and wrote the manuscript. Y.-C.L., C.-H.C., H.-H.C., W.-S.Y., and K.-C.H. contributed medical expertise and reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Adiponectin–leptin ratio for the early detection of lean non-alcoholic fatty liver disease independent of insulin resistance

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ORIGINAL ARTICLE

Adiponectin–leptin ratio for the early detection of lean non-alcoholic fatty liver disease independent of insulin resistance

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ABSTRACT

Background: Lean Non-alcoholic Fatty Liver Disease (NAFLD) shares a similar disease burden to those of their overweight counterparts and should be detected early. We hypothesized that the adiponectin–leptin ratio (AL ratio) could be a good marker for early detection of lean NAFLD independent of insulin resistance.

Materials and methods: A total of 575 adults without diabetes were enrolled in a community-based study. The subjects were stratified into the lean controls, lean NAFLD, simple overweight/obesity and overweight/obesity NAFLD groups according to body mass index (BMI) and ultrasonographic fatty liver indicators. Serum adiponectin and leptin levels were measured by enzyme-linked immunosorbent assay. Multivariate logistic regression analyses were performed to estimate the odds ratio of having NAFLD in relation to the tertiles of serum AL concentration after adjustment. Receiver operating characteristic (ROC) analyses were applied to evaluate the diagnostic performance of the AL ratio for NAFLD.

Results: The mean age of the participants was 42.8 ± 11.5 years. Comparing with the lean controls, the odds of having lean NAFLD for the highest versus the lowest tertile of AL ratio was 0.28(95%CI: 0.12–0.69) after adjustment. Putting AL ratio, BMI, triglyceride, AST/ALT ratio to the diagnosis performance of NAFLD, the ROC was 0.85 (95% CI: 0.82–0.88), 0.83 (95% CI 0.78–0.87) and 0.86 (95% CI 081–0.91) for all NAFLD, NAFLD in women and NAFLD in men, respectively. ($p < .001$).

Conclusions: The study revealed that the AL ratio could be a good biomarker to early distinguish lean NAFLD patients from lean controls independent of insulin resistance. [AQ3]

KEY MESSAGES

1. The prevalence of non-alcoholic fatty liver disease (NAFLD) increases globally and is related to liver diseases and metabolic dysfunctions. Lean subset of NAFLD shares a similar disease burden to those of their overweight counterparts and should be detected early.
2. Adiponectin–leptin ratio were associated with the severity of steatosis and was a predictor of obese NAFLD better than each single adipokine. To date, there is no investigation that explores specifically for the relationship between lean NAFLD and AL ratio.
3. Our study found that adiponectin–leptin ratio is a sole independent marker regardless of insulin resistance in lean NAFLD. Having lean NAFLD for the highest versus the lowest tertile of adiponectin–leptin ratio was 0.28(95%CI: 0.12–0.69) after adjustment of age, sex, current smoking, exercise habits, HOMA-IR and AST/ALT. ROC for the NAFLD performance is good for the early detection (0.85; 95% CI: 0.82–0.88). Further rigorous investigation is necessary and should be promptly performed.

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Lean NAFLD; leptin; adiponectin; adiponectin-leptin ratio; insulin resistance

Introduction

The prevalence of nonalcoholic fatty liver disease (NAFLD), recently termed metabolic dysfunction-associated fatty liver disease (MAFLD), is increasing globally [1]. The incidence of NAFLD is estimated to be 28–52 per 1,000 person/years, and the prevalence is approximately a quarter of adult population [2]. NAFLD is a formidable public health issue that is

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widely associated with hepatic and extrahepatic comorbidities or complications, such as cirrhosis, hepatocellular carcinoma, diabetes, metabolic syndrome and cardiovascular diseases [3]. Moreover, the core pathophysiology of insulin resistance and increased adiposity of NAFLD attribute its cause to metabolic dysregulation with significant liver involvement [4]. As we know, the metabolic phenotype of NAFLD is characterized mainly by insulin resistance due to the hepatic oversupply with sugar, lipid and etc, while the genetic component is characterized by the impaired hepatic mitochondrial function, leading to chronic inflammation [5]. The pathogenesis of NAFLD represents the metabolic dysfunction clinically of a complex interplay between lifestyle, environmental and genetic factors along with a key role for epigenetic changes [6]. As a fatty live disease mainly composed of metabolic dysfunctions, the prevalence of NAFLD is approximately one quarter in the world in which composed of 50% of T2D, approximately 70% among cardiovascular diseases and more than 90% of severely obese patients [7]. Therefore, an endorsement by more than 1000 specialists over 134 countries have emphasized that MAFLD is an overarching term associated with metabolic dysregulation [8].

Paradoxically, there has been a growing subset of patients who are inflicted with NAFLD, but their body mass index (BMI) is classified as lean (defined as BMI <25 in the Western region and BMI <23 in the Asian region) [9]. Lean NAFLD varies in prevalence among different ethnic groups or with diagnostic approaches, accounting for 5% to 8% in Caucasian subjects and 16% to 18% in the Asia-Pacific region [10]. Without obesity as a prerequisite for NAFLD, lean NAFLD shares similar severities of advanced diseases and mortality similar to its obese counterpart [11]. Therefore, the new definition of MAFLD, i.e. NAFLD required an evidence of hepatic steatosis, detected either by imaging techniques, blood biomarkers/scores or by liver histology and involves one of the three following phenotypes, (1) overweight/obesity, (2) the presence of type 2 diabetes mellitus or (3) lean subjects with evidence of metabolic dysregulation [12]. Since liver biopsy cannot be applied widely, ultrasound is the most practical imaging modality for screening NAFLD [13]. Nevertheless, a reliable biomarker or score is urgently needed for early detection and diagnosis of NAFLD, especially for easily ignored populations, i.e. lean NAFLD. Currently, the fatty liver index (FLI), which incorporates BMI, waist circumference (WC), gamma-glutamyl transferase and triglyceride levels, may be the most established index for scoring NAFLD [14]. The plasma cytokeratin 18 (CK18) fragment level is the most extensively evaluated biomarker of steatohepatitis and is a marker of hepatocyte apoptosis [15]. However, none of the above biomarkers/scores are specific for early detection of lean NAFLD.

Adiponectin and leptin were discovered in the 1990s. Since then adipose tissue has gradually transformed from a simple energy reservoir to a highly active endocrine organ [16,17]. Leptin is positively correlated with obesity and insulin resistance [18], while adiponectin shows a good ability to enhance insulin sensitivity and counteract the development of diabetes [19,20]. Additionally, leptin dually exerts anti-steatotic proinflammatory and profibrogenic actions for NAFLD. The net effect however remains unclear [21]. In contrast, adiponectin consistently promotes anti-inflammatory and antifibrotic activity [22]. Consequently, adiponectin to leptin ratio was assumed to correlate negatively with low-grade chronic inflammation [23], atherosclerosis risk [24] and cardiovascular disorders [25,26]. A few human studies have elaborated the association between the adiponectin, leptin or AL ratio and NAFLD [27,28] while few were related to lean NAFLD. No matter obese or not, adiponectin is a biomarker for NAFLD subjects indicating the progression to steatohepatitis in a biopsy proven study [29] and the development of NAFLD in a Korea cohort [30]. And, lean subjects with evidence of NAFLD have lower adiponectin concentrations than lean controls in Caucasian populations [6]. In the other hand, leptin levels reflect total body fat and insulin resistance [31] that correlate positively with hepatic steatosis in diabetes subjects [32]. Taking together, AL ratio were associated with the severity of steatosis in a Japanese study [27] and was a predictor of NAFLD in obese adults that correlated with liver function and insulin resistance better than each single adipokine [28]. To date, there is no investigation that explores specifically for the relationship between lean NAFLD and AL ratio.

Theoretically, lean subjects with normal BMIs and adiposity should have higher circulating adiponectin and lower circulating leptin. However, NAFLD itself is a chronic process of liver inflammation which may alter the level of circulating adiponectin and leptin. As the result, whether the AL ratio is the same for lean subjects with or without NAFLD remains unclear. We hypothesized that the AL ratio could distinguish patients with lean NAFLD from those without NAFLD in the very early stage independent of insulin sensitivity. Therefore, we conducted this community-based study to enrol young adults without diabetes and applied strict ultrasound scoring to investigate the

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relationship between AL ratio in the four groups: lean controls, lean NAFLD, simple overweight/obesity and overweight/obesity NAFLD groups. We also applied ROC analyses to find a most suitable diagnostic performance of NAFLD using AL ratio and available biomarkers in clinical setting.

Materials and methods

Study subjects

This study was conducted cross-sectionally in a community in Northern Taiwan. All the participants enrolled when they received a regular health check-up in National Taiwan University Hospital, Hsin-Chu branch. All the subjects completed standardized questionnaires through individual interview regarding socio-demographics, smoking, drinking, exercise and medical history. Subjects who had a history of diabetes, were taking antihyperglycemic agents or insulin or fasting serum glucose $\geq$126 mg/dl or haemoglobin A1c $\geq$6.5% were excluded. In total, 575 adults older than 20 years were enrolled. Weight, height and Blood pressure (BP) were measured by calibrated, electronic stadiometers and sphygmomanometers. WC was measured horizontally through the middle point between the upper border of iliac bones and the lower border of the ribs. Body fat percentage was measured through bioelectrical impedance analysis by a portable body composition analyser (TANITA BC-418, Japan). Abdominal ultrasonography was performed by three experienced physicians using a 3.5–5 MHz transducer and a high-resolution B-mode scanner (Hitachi Aloka ProSound Alpha 6, Japan). The severity of NAFLD was calculated using the US-FLI score [9]. The details about the including and excluding criteria, questionnaires, the scoring of fatty liver by abdominal ultrasonography and blood analyses please refer to our published study [33]. Informed consent forms were signed. This study was approved by the Institutional Review Board of National Taiwan University Hospital (IRB NO. 201210012RIC).

Definition of lean and NAFLD groups

The cut-off points for BMI categories in Taiwan are defined as follows: $<$18.5 kg/m^2: underweight, 18.5–23.9 kg/m^2: normal weight, 24–26.9 kg/m^2: overweight, $\geq$27 kg/m^2: obesity [34]. The subjects were then divided into the following groups [1]: lean controls: US-FLI score $<$2, BMI $<$ 24 kg/m^2 [2]; lean NAFLD group: US-FLI score $\geq$2, BMI$<$ 24 kg/m^2 [3]; simple overweight/obesity group: US-FLI score $<$2, BMI $\geq$24 kg/m^2; and [4] overweight/obesity NAFLD group: US-FLI score $\geq$2, BMI $\geq$24 kg/m^2 [33].

Blood analysis

Serum adiponectin (As One International INC, Santa Clara, CA, USA) was diluted to 10x during pre-treatment, incubated at 100 °C for 5 min and then diluted to 5100x finally. Serum leptin (R&D Inc. Minneapolis, USA) was diluted to 30x using dilution buffer. The limit of detection (LOD) was 23.4 pg/mL and 7.8 pg/mL for adiponectin and leptin, respectively. The intra-assay and inter-assay coefficients of variation (CVs) were all less than 5%. Both adiponectin and leptin were then measured by enzyme-linked immunosorbent assay following manufacturer's protocol as previously described [35].

Statistical analysis

Data are presented as the mean ± SD for continuous variables and number (percentage) for categorical variables. Differences between the four groups were examined using the chi-squared test for categorical variables and one-way analysis of variance (ANOVA) for continuous variables. Tukey's post hoc analysis was applied to examine the differences among the healthy control, lean NAFLD, overweight controls and overweight NAFLD groups in terms of basic demographic characteristics, leptin, adiponectin and AL ratio. Multivariate linear regression analyses were performed to estimate the relationship between the AL ratio and metabolic factors. We put lean controls, lean NAFLD, simple overweight/obesity and overweight/obesity NAFLD groups as dependent variables and the tertiles of AL ratio as an independent variable. Then, multivariate logistic regression models were applied to examine the odds of having NAFLD in relation to the tertiles of AL ratio after adjustments age, sex, current smoking, exercise habits, HOMA-IR and AST/ALT ratio. We performed receiver operating characteristic (ROC) analysis with the area under the ROC curve (AUC) to evaluate the diagnostic performance of the AL ratio for NAFLD. All analyses were performed using SPSS statistical software (V.17, SPSS, Chicago, Illinois, USA). A p value of $<$.05 was considered to be statistically significant.

Results

The basic characteristics of the participants are shown in Table 1. The mean age of the participants was

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Table 1. Baseline characteristics among the lean controls, lean NAFLD, simple overweight/obesity and overweight/obesity NAFLD groups.

	BMI < 24		BMI ≥ 24	
	Lean controls $N = 217$	Lean NAFLD $N = 105$	Simple overweight/obesity $N = 53$	Overweight/obesity NAFLD $N = 200$
Age (years)	41.20 ± 10.94	42.96 ± 11.59	44.38 ± 11.34	43.33 ± 11.80
Male (%)	45(20.7)[c,d]	36(34.3) [d]	25(47.2) [a]	113(56.5) [a, b]
Smoke (%)	14(6.5)[d]	11(10.5)	5(9.4)	33(16.5) [a]
Exercise (%)	95(43.8)	45(42.9)	26(49.1)	86(43.0)
BMI (kg/m^2)	20.66 ± 1.80 [b, c, d]	21.83 ± 1.54 [a, c, d]	25.95 ± 1.75 [a, b, d]	27.98 ± 3.91 [a, b, c]
Fat percentage (%)	27.54 ± 8.08 [b, c, d]	30.76 ± 7.33 [a, d]	33.89 ± 7.12 [a]	36.15 ± 7.89 [a, b]
WC (cm)	73.13 ± 6.13 [b, c, d]	77.56 ± 6.58 [a, c, d]	85.72 ± 5.92 [a, b, d]	90.40 ± 7.88 [a, b, c]
Systolic BP	115.80 ± 15.52 [b, c, d]	122.12 ± 15.15 [a, d]	122.63 ± 17.16 [a, d]	129.99 ± 15.12 [a, b, c]
Diastolic BP	73.00 ± 11.18 [b, c, d]	77.50 ± 9.46 [a, d]	78.06 ± 13.91 [a]	81.75 ± 11.93 [a, b]
TCHO (mg/dL)	189.78 ± 33.99 [d]	197.60 ± 40.05	194.00 ± 29.23	202.64 ± 35.34 [a]
TG (mg/dL)	74.00 ± 35.43 [b, d]	109.36 ± 79.36 [a, d]	96.02 ± 43.21 [d]	157.51 ± 113.57 [a, b, c]
HDL-C (mg/dL)	66.78 ± 14.96 [b, c, d]	57.13 ± 13.35 [a, d]	59.09 ± 13.23 [a, d]	49.93 ± 12.61 [a, b, c]
LDL-C (mg/dL)	114.12 ± 31.13[b, d]	126.20 ± 37.28 [a]	122.49 ± 29.21	132.86 ± 32.16 [a]
Glucose (mg/dL)	82.65 ± 8.66 [d]	85.50 ± 8.65 [d]	86.51 ± 9.83 [d]	89.39 ± 9.43 [a, b, c]
Insulin (μIU/mL)	5.13 ± 3.28 [d]	6.71 ± 5.24 [d]	7.15 ± 3.87 [d]	11.30 ± 9.03 [a, b, c]
HOMA-IR	0.66 ± 0.42 [d]	0.86 ± 0.65 [d]	0.92 ± 0.49 [d]	1.44 ± 1.11 [a, b, c]
GOT (U/L)	20.46 ± 6.86 [d]	21.75 ± 7.03 [d]	21.57 ± 6.00 [d]	25.40 ± 10.07 [a, b, c]
GPT (U/L)	17.22 ± 9.48 [b, d]	23.96 ± 16.61 [a, d]	12.48 ± 10.74 [d]	35.69 ± 27.84 [a, b, c]
CRP (mg/dL)	0.114 ± 0.320 [d]	0.091 ± 0.123 [d]	0.173 ± 0.277	0.207 ± 0.250 [a, b]
Metabolic factors (n)	0.39 ± 0.62 [b, c, d]	0.92 ± 0.90 [a, d] -	1.15 ± 0.91 [a, d]	2.01 ± 1.24 [a, b, c]
MetS (%)	1(0.5) [d]	6(6.2) [d]	4(8.5) [d]	56(28.0) [a, b, c]
Adiponectin(μg/mL)	18.13 ± 8.55 [b, d]	13.60 ± 8.00 [a, d]	15.01 ± 8.82 [d]	9.82 ± 5.71 [a, b, c]
Leptin (ng/mL)	8.16 ± 6.30 [c, d]	9.42 ± 7.21 [d]	12.48 ± 10.74 [a]	15.20 ± 11.35 [a, b]
AL ratio (x10^3)	6.43 ± 18.36 [b, d]	2.26 ± 1.93 [a]	2.23 ± 2.32	1.13 ± 1.14 [a]

Abbreviations: NAFLD: non-alcoholic fatty liver disease; BMI: body mass index; WC: waist circumference; BP: blood pressure; TCHO: total cholesterol; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; GOT: glutamic oxaloacetic transaminase; GPT: glutamic pyruvic transaminase; CRP: C-reactive protein; MetS: metabolic syndrome; AL ratio: adiponectin-leptin ratio.

Notes: Data are presented as the mean ± SD for continuous variables and number (percentage) for categorical variables. Differences between the four groups were examined using the chi-squared test for categorical variables and one-way analysis of variance (ANOVA) for continuous variables. Tukey's post hoc analysis was applied to examine the differences among the healthy control, lean NAFLD, overweight controls and overweight NAFLD groups in terms of basic demographic characteristics, leptin, adiponectin and AL ratio.

The four groups were represented with [a]: lean controls; [b]: lean NAFLD; [c]: simple overweight/obesity; [d]: overweight/obesity NAFLD. If a significant level $p < .05$ was achieved between any two of the four groups, a superscript was added to the corresponded columns.

42.8 ± 11.5 years, and 61.3% of the participants were female. Of the 575 subjects included, 200 subjects (34.8%) had overweight/obesity NAFLD, and 105 subjects (18.3%) had lean NAFLD. Since we excluded diabetes, our study group had a high proportion of metabolically healthy subjects (MetS factors: 1.08 ± 1.11). Tukey's post hoc analysis was performed to test the differences between groups. Importantly, the AL ratio can specifically tell the lean control from the lean or overweight/obesity NAFLD group rather than adiponectin or leptin alone. To compare the lean NAFLD group and simple overweight/obesity group, we found that their BMI, fat percentage and waist circumference were significant differences while there were no differences in any metabolic parameters, including blood pressure, lipid profile, glucose, insulin resistance or inflammatory biomarkers such as AST, ALT and CRP.

To further clarify the association between each factor of metabolic syndrome and the AL ratio, we applied multivariate linear regression models after adjusting for age and sex (Table 2). The AL ratio was negatively associated with body fat percentage, BMI, WC, SBP, DBP, TG, glucose and HOMA-IR (all $p < .001$) while positively associated with HDL ($p < .001$). These results impressed that AL ratio is a consistent and strong biomarker for detecting metabolic dysfunction.

Knowing that the AL ratio was a good parameter in relation to each factor of metabolic syndrome, multivariate logistic regression models were performed to explore the odds of having NAFLD in relation to the tertiles of serum AL ratio (Table 3). The OR of having NAFLD for the highest versus the lowest tertile of AL ratio was 0.34 (95% CI: 0.17–0.71; p for trend $< .001$). After further adjustment of AST/ALT ratio, the OR of having NAFLD for the highest versus the lowest tertile of AL ratio was 0.37 (95% CI: 0.18–0.77, p for trend .008).

Stratified by BMI, the ORs of having NAFLD derived from multiple logistic regression analyses in tertiles of serum AL ratio are shown in Table 4. When BMI <24 kg/m^2, the OR of having NAFLD for the highest versus the lowest tertile of AL ratio after adjustment was 0.28 (95% CI: 0.12–0.69; p for trend .005). When

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Table 2. Relation between the serum adiponectin-leptin ratio and metabolic factors in multivariate linear regression models after adjusting for age and sex.

Variables	Model 1 ß (SE) *p* Value	Model 2 ß (SE) *p* Value	Model 3 ß (SE) *p* Value	Model 4 ß (SE) *p* Value	Model 5 ß (SE) *p* Value	Model 6 ß (SE) *p* Value	Model 7 ß (SE) *p* Value	Model 8 ß (SE) *p* Value	Model 9 ß (SE) *p* Value
BMI (kg/m^2)	−0.252(0.108) <.001								
Fat (%)		−0.365(0.060) <.001							
WC (cm)			−0.296(0.050) <.001						
SBP (mmHg)				−0.141(0.030) .001					
DBP (mmHg)					−0.133(0.040) .002				
HDL-C (mg/dl)						0.158(0.033) <.001			
TG (mg/dl)							−0.165(0.005) <.001		
Glucose (mg/dl)								−0.102(0.028) .015	
HOMA-IR									−0.203(0.260) <.001

Abbreviations: NAFLD: non-alcoholic fatty liver disease; BMI: body mass index; WC: waist circumference; SBP: systolic blood pressure; DBP: diastolic blood pressure; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C:low-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance.

Table 3. Odds ratios of having NAFLD in relation to the serum tertile of adiponectin-leptin (AL × 10^3) ratio using multiple logistic regression analyses.

	AL × 10^3 ratio <0.91 *N* = 190	0.91≤ AL ×10^3 ratio <2.36 *N* = 193	AL × 10^3 ratio ≥2.36 *N* = 192	*p* for trend
Model 1	1.00	0.28(0.17–0.44) **	0.07(0.04–0.12) **	<.001
Model 2	1.00	0.58(0.34–1.00) *	0.22(0.12–0.43) **	<.001
Model 3	1.00	0.66(0.37–1.12)	0.34(0.17–0.71) **	<.001
Model 4	1.00	0.67(0.38–1.21)	0.37(0.18–0.77) *	.008

Notes: Model 1: adjusted for age, sex, current smoking and exercise habits; Model 2: adjusted for variables in Model 1, plus BMI; Model 3: adjusted for variables in Model 2, plus HOMA_IR; Model 4: adjusted for variables in Model 2, plus GOT/GPT ratio.
* $p < .05$; ** $p < .001$.

Table 4. Odds ratios of having NAFLD in relation to serum tertile of adiponectin leptin level using multiple logistic regression analyses, stratification by BMI.

Lean NAFLD	AL <0.91 *N* = 53	0.91 ≤ AL <2.36 *N* = 120	AL ≥2.36 *N* = 149	*p* for trend
Model 1	1.00	0.58(0.29–1.14)	0.16(0.08–0.36) **	<.001
Model 2	1.00	0.59(0.29–1.21)	0.26(0.11–0.61) *	.002
Model 3	1.00	0.62(0.30–1.29)	0.28(0.12–0.69) *	.005
Overweight NAFLD				
	AL <0.91 *N* = 137	0.91 ≤ AL <2.36 *N* = 73	AL ≥2.36 *N* = 43	*p* for trend
Model 1	1.00	0.34(0.14–0.80) *	0.11(0.04–0.30) **	<.001
Model 2	1.00	0.59(0.23–1.51)	0.28(0.09–0.89) *	.031
Model 3	1.00	0.61(0.23–1.58)	0.30(0.09–0.96) *	.043

Notes: Model 1: adjusted for age, sex, current smoking and exercise habits; Model 2: adjusted for variables in Model 2, plus HOMA_IR; Model 3: adjusted for variables in Model 2, plus GOT/GPT ratio.
*$p < .05$; **$p < .001$.

BMI ≥24 kg/m^2, the OR of having NAFLD for the highest versus the lowest tertile of AL ratio after adjustment was 0.30 (95% CI: 0.09–0.96; *p* for trend .043).

The AL ratio, BMI, triglyceride and AST/ALT ratio were selected for the diagnosis performance of NAFLD using ROC analysis curve. For all subjects, the AUROC was 0.85 (95% CI: 0.82–0.88). For female and male, AUROC was 0.83 (0.78–0.87) and 0.86 (081–0.91), respectively (all $p < .001$) (Figure 1).

Discussion

A logical inference between lean NAFLD and AL ratio was well demonstrated in the study. First, we demonstrated that in this population that was younger and healthier, the AL ratio was indeed a strong and good parameter in relation to each metabolic factor and HOMA-IR. Then, the association between the serum AL ratio and the risk of NAFLD was examined. In the section of crude OR, both the lean NAFLD and overweight/obesity NAFLD groups showed a decreased risk from the lowest tertile of AL ratio to the highest tertile of AL ratios compared with that of the lean controls and simple overweight/obesity groups, respectively (*p* for a trend <.001). Then, we removed the effects of HOMA-IR and AST/ALT ratio to determine the amount of residual effect differences that were left between the case and controls (lean controls

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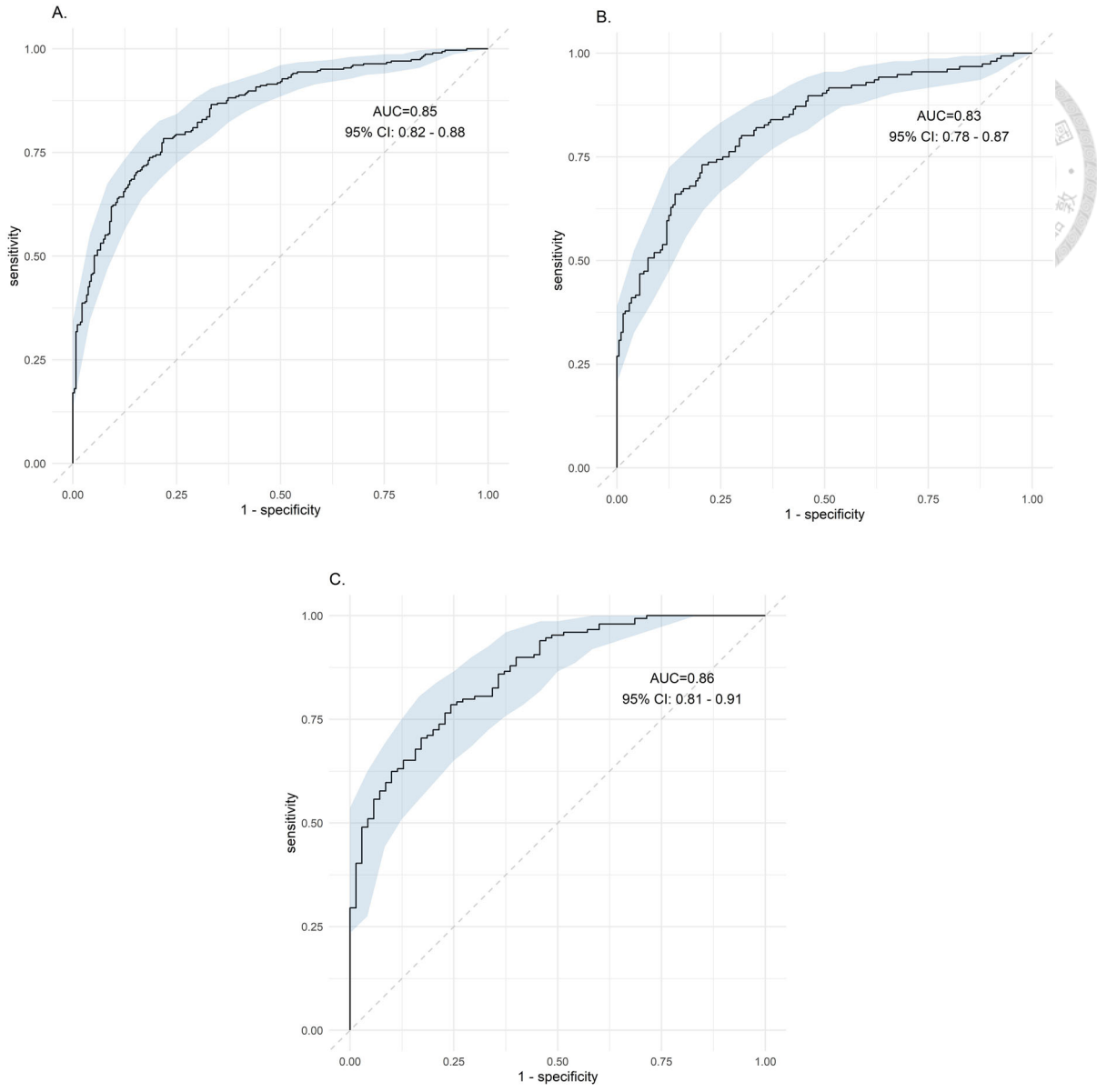

Figure 1. Receiver operating characteristic (ROC) for the diagnosis of NAFLD. Except adiponectin-leptin ratio, BMI, triglyceride and AST/ALT ratio were selected. (A) All subjects, AUROC was 0.85 (95% CI: 0.82–0.88), (B) female subjects, AUROC was 0.83 (0.78–0.87), and (C) male subjects, AUROC was 0.86 (081–0.91). All $p < .001$.

vs. lean NAFLD; simple overweight/obesity vs. overweight/obesity NAFLD). As a result, a persistent lower risk of NAFLD was found in the lowest tertile of AL ratio to the highest tertile of AL ratios (p for trend $<.05$). The AUROC curve also performed very well at the level of 0.83–0.86.

Adipose tissue, as a sophisticated endocrine organ, performs crosstalk with the liver by circulating adipokines for the development of NAFLD [36,37]. Among adipokines, adiponectin and leptin have contrary roles in relation to BMI. As the gradual transition from lean to overweight to obesity occurred, which is associated with the accumulation of adiposity, the serum adiponectin level decreased in parallel with the increase in serum leptin levels [25,38]. In addition to altering insulin sensitivity and the function of adipocyte lipid storage, adiponectin and leptin are related to inflammatory or anti-inflammatory functions [20,39]. Some observational studies have demonstrated that the linkage between anti-inflammation and adiponectin is at least partially independent of obesity [40], and this result is consistent with our study. Consequently, the AL ratio has been suggested to be a marker of low-grade chronic inflammation in populations with impaired insulin functions and obesity [25,27,41]. Some studies propose that the AL ratio is positively associated with arteriosclerosis, intima media thickness of the common artery and CVD [26,42].

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A Japanese health survey delineated cross-sectionally that the AL ratio was associated with the severity of steatosis by ultrasonography [27]. Another study elucidated that the AL ratio could be a noninvasive predictor of NAFLD in obese children, which better correlates with weight and HOMA-IR than each single adipokine [28]. Compared to MALFD, leptin is more robust in the effect of obesity, while adiponectin could interfere with the presentation of NAFLD regardless of HOMA-IR and adiposity. Therefore, the AL ratio could be independently used to distinguish the lean NAFLD individuals from the lean control individuals.

Since 2020, MAFLD has been used as the main terminology instead of NAFLD [8,43]. It has been indicated that although lean NAFLD patients are younger and have fewer metabolic clinical features, they share similar histological severity, comorbidities and mortality with NAFLD patients [44]. Because lean NAFLD subjects develop fatty liver disease prior to becoming overweight or having increased adiposity, we could utilize image modality or biomarker rather than BMI for early detection. We excluded diabetes because its pathophysiology could be another pathway and progression trajectory [8,45]. We enrolled early-stage NAFLD patients with less metabolic syndromes. Since liver fat accumulation and chronic inflammation are very sensitive and early indicators in these subsets, the AL ratio was suggested to be a good early classifier for lean NAFLD.

Lean NAFLD is more prevalent in Asia area that reflects ethnic differences and genetic variants [46]. In a recent meta-analysis, the prevalence of lean NAFLD among non-obese population was up to 40.75% in Asian [47]. In line with our study, we found that 105 of the 322 lean subjects (33%) had lean NAFLD in our population. And, the prevalence of lean NAFLD in the NAFLD subjects in Asia is varied, ranging from 12% to 47% which was also consistent with our finding (105 of 305, 34.4%) [48].

NAFLD composed of 50% of T2D [7] and encountered a changing of terminology to MAFLD after 2021(8). MAFLD separated diabetes as a unique category from the other two categories, obese or lean with metabolic dysfunction, for its different pathophysiology [12]. Compared with our previous published article extracted from the same population [33], we excluded diabetes in this study for better understanding and detecting the lean NAFLD. General speaking, the metabolic phenotype of NAFLD is characterized mainly by insulin resistance while the genetic component is characterized by the impaired hepatic mitochondrial function [5]. That's why we chose adiponectin and leptin, both as adipokines and hepatokines, to detect lean NAFLD. Furthermore, we performed an AUROC analysis to consolidate the hypothesis that adiponectin/leptin ratio is good performance in NAFLD detection.

There are some limitations in our study. First, we did not perform liver biopsy. Although liver biopsy is the gold standard for NAFLD, the high prevalence and variable presentation of NAFLD make performing biopsies less practical. Nevertheless, we applied a strict echo score, the US-FLI, which has been well validated and applied in previous studies. Although the ultrasonographic approach cannot determine the severity of NAFLD, it has been validated by US-FLI as a reliable dichotomous screening tool for NAFLD [38]. In addition, US-FLI has been applied extensively as a substitute modality for the diagnosis of NAFLD in the real world [9]. Second, this was a cross-sectional study, and we could only determine the association rather than a causal relationship between the AL ratio and NAFLD. We tried to enrol early NAFLD patients, but we did not record the duration of NAFLD that may potentially influence the serum AL ratio. We adjusted insulin resistance as a pivotal step to demonstrate that persistent low-grade inflammation of lean NAFLD plays a key role and could independently be related to the AL ratio; however, we applied an indirect measurement by the equation that was transformed by fasting glucose and insulin instead of a standard glucose clamp technique. Although we demonstrated a significant association between AL ratio and NAFLD, focussing on lean NAFLD, the cross-sectional study could not infer an early detection. For a better detection model or further validation model, a longitudinal cohort is warranted.

Conclusions

In conclusion, this was the first investigation to link the negative association between serum AL ratio and lean NAFLD. Our study found that the AL ratio is a sole independent marker regardless of insulin resistance in lean NAFLD. Combination of AL ratio, BMI as well as triglyceride and AST/ALT ratio, ROC for the NAFLD performance is good for the early detection. Further rigorous investigation is necessary and should be promptly performed.

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Ethical approval

This study was approved by the Institutional Review Board of National Taiwan University Hospital (IRB NO. 201210012RIC).

Author contributions

All authors were involved in the conception and design of the work. WS Yang and KC Huang conceptualized and designed the study, secured funding for the study. CW Lu performed the formal analyses and prepared the original draft. KC Yang, YC Chi, TY Wu, CH Chiang and HH Chang contributed medical expertise and reviewed the manuscript. All authors read and approved the final version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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