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*Article*

# Independently Dose-responsive Association 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 diseases. 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 nonlean patients. The study comprised 606 adults from the community, stratified into lean or nonlean (BMI </≥ 24 kg/m2) 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 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 nonlean 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

## 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 1,000 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 nonlean NAFLD, patients with lean NAFLD are younger and have higher hemoglobin levels [5], an elevated ALT/AST ratio [6], and less insulin resistance

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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 nonlean NAFLD share metabolic features of insulin resistance 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.

Feutin-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 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, and Wilson disease. In total, 606 adults older than 20 years were enrolled. Information about age, sex, cigarette smoking, 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. Blood pressure (BP) was measured by a sphygmomanometer. Waist circumference (WC) was measured by the same trained operator. This study was approved by the Institutional Review Board of National Taiwan University Hospital (IRB NO. 201210012RIC).

### *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 $\alpha$ 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[19]. 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)

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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/m2; (2) lean NAFLD group: US-FLI score ≥ 2, BMI< 24 kg/m2; (3) nonlean, non-NAFLD group: US-FLI score <2, BMI ≥24 kg/m2; (4) nonlean NAFLD group: US-FLI score ≥ 2, BMI ≥24 kg/m2.

*2.3. 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 microparticle enzyme immunoassay using an AxSYM system (Abbott Laboratories, Dainabot Co, Tokyo, Japan). The homeostatic model assessment of insulin resistance (HOMA-IR) was applied to calculate the estimated degree of insulin resistance (HOMA-IR=fasting insulin × fasting plasma glucose/22.5, with glucose presented in mmol/L and insulin presented in mU/L) [20]. Serum fetuin-A was measured using a quantitative sandwich enzyme immunoassay technique after a dilution of 4,000-fold. This immunoassay was calibrated against highly purified NS0-expressing recombinant human fetuin-A (R&D Inc. Minneapolis, USA).

2.4. Statistical analysis

Participants were divided into tertiles according to the serum concentrations of fetuin-A. Data are presented as the mean±SD for continuous variables and number (percentage) for categorical variables. Multivariate logistic regression analyses were performed to estimate the odds ratio of having NAFLD among the tertiles of fetuin-A after adjustment for age, sex, current smoking, exercise habit, WC, and 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, sex, current smoking, exercise habit, weight circumference, and homeostasis model assessment of insulin resistance. 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. 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, and 61.7% of the participants were female. 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. The highest level of fetuin-A was found in the lean NAFLD group. In a post hoc analysis (Supplementary A), the lean NAFLD group shared similar metabolic factors with the nonlean, non-NAFLD group. However, patients in the former group had presentation of NAFLD and patients in the latter had a significantly higher BMI, waist circumference, and body fat percentage. Both lean and nonlean NAFLD had high levels of fetuin A, while nonlean 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.

*3.2. Association of fetuin-A and NAFLD*

To further clarify the association between 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 2. 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, current smoking, and exercise habit. After adjustment for age, sex, current smoking, exercise, 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

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for trend 0.02). However, after further adjusting for HOMA-IR, the ORs became insignificant (1.5; 95% CI: 0.92-2.67; P for trend 0.099).

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

| | Lean | | Nonlean | | P value |
|---|---|---|---|---|---|
| | Non-NAFLD | NAFLD | Non-NAFLD | NAFLD | |
| | N=227 | N=108 | N=54 | 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

Stratified by BMI, the ORs of having NAFLD derived from multiple logistic regression analyses in tertiles of serum fetuin-A are shown in Table 3. When BMI <24 kg/m2, 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, sex, current smoking, exercise, WC, and 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/m2, 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.

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 1).

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Table 2 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 inslin resistance. *For p<0.05; **For p<0.001.

Table 3 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

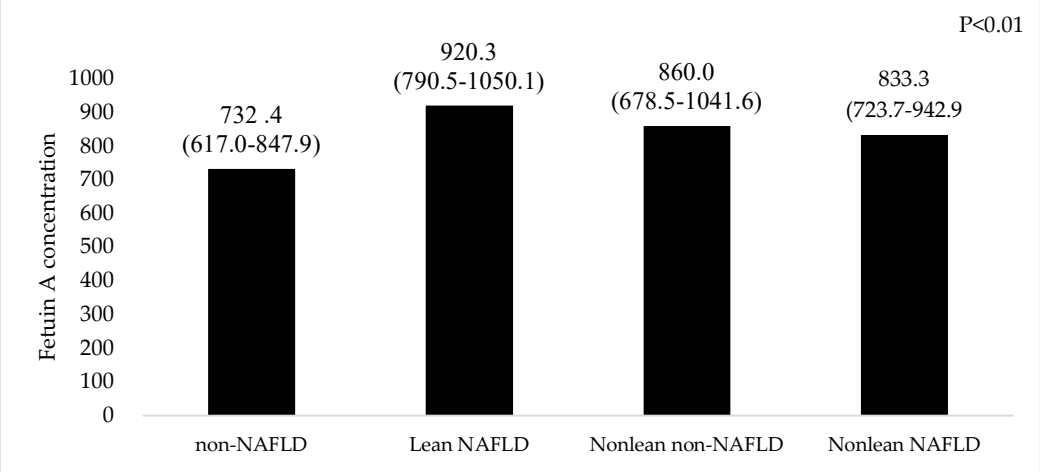


**Figure 1.** Comparison of serum concentrations of Fetuin A in relation to the group of NAFLD after adjusting age, gender, current smoking, exercise habit, weight circumference and homeostasis model assessment of insulin resistance by least square means method.

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## 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 nonlean NAFLD. Second, we also found that there was a dose-response relationship between the serum fetuin-A gradient and nonlean NAFLD after adjusting for age, sex, current smoking, exercise habit, WC, and HOMA-IR (P for trend <0.05). Third, both lean and nonlean NAFLD had high levels of fetuin-A, while nonlean NAFLD apparently had more metabolic factors and high 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 HOMA-IR implied that as yet unidentified factors affected this association beyond 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 [21], and serves pleiotropic functions. In adults, fetuin-A is secreted by hepatocytes and adipocytes and predominantly (>95%) expressed in the liver [22]. It is well known that fetuin-A is involved in the development of insulin resistance in both animal and human studies [23, 24], 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, 25], most likely contributing even further to the progression of NAFLD. It is not surprising that fetuin-A levels were significantly elevated in NAFLD 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 than in healthy controls regardless of the histological state and BMI class [26], 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 nonlean 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, 27] and chronically responds to inflammatory stimuli [28], leading to the progression from steatohepatitis to NASH [29, 30]. In a study cohort comprising 1,339 Caucasian biopsy-proven NAFLD patients, it was found that both lean and nonlean NAFLD may progress to advanced liver disease, metabolic comorbidities, cardiovascular disease, and liver-related mortality, independent of the progression to obesity [31].

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 counterparts [32]. 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 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 demonstrated 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 nonlean 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

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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 [19, 33]. 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/xxx/s1,Table S1: Comparison of lean, nonlean, NAFLD, and non-NAFLD groups in metabolic variables using Tukey's post hoc analysis.

**Author Contributions:** KC Huang conceptualized and designed the study, secured funding for the study. CW Lu performed the formal analyses and prepared the original draft. YC Lee, CH Chiang, HH Chang, WS Yang and KC Huang contributed medical expertise and reviewed the manuscript. All authors read and approved the final version of the manuscript.

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**Informed Consent Statement:** Informed consent forms were signed by every participants after comprehensive explanation

**Data Availability Statement:** The rights to the data belong to our research group. Selected variables could be provided after application.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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