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Case Control Study

Targeted metabolomics study of fatty acid metabolism in lean metabolic-associated fatty liver disease patients

Sun PQ *et al*. Fatty acid metabolism in lean liver

Abstract

BACKGROUND

The annual incidence of metabolic-associated fatty liver disease (MAFLD) in China has been increasing and is often overlooked owing to its insidious characteristics. Approximately 50% of the patients had normal weight or were not obese, referred to as "lean MAFLD". Available studies on this group of patients are limited. Because MAFLD is related to abnormal lipid metabolism, lipid-targeted metabolomics was used in this study to provide experimental evidence for early diagnosis and pathogenesis.

AIM

To investigate the serum fatty acid metabolic characteristics in lean-type MAFLD patients using targeted serum metabolomic technology.

METHODS

Between January and June 2022, serum samples from MAFLD patients and healthy individuals who were treated at Shanghai Putuo District Central Hospital were collected for serum metabolomics analysis. Principal component analysis and orthogonal partial least squares-discriminant analysis models were established, and univariate analysis was combined to screen for biomarkers of lean-type MAFLD and analyze metabolic pathways. The UPLC-Q-Orbitrap/MS content determination method was used to quantitatively detect serum palmitic acid (PA), oleic acid (OA), linoleic acid (LA), and arachidonic acid (AA) levels in lean-type MAFLD patients.

RESULTS

The levels of urea nitrogen and uric acid in lean-type MAFLD patients were higher than in healthy individuals ($P < 0.05$). Alanine transaminase and cholinesterase levels in lean-type MAFLD patients were significantly higher than in healthy individuals ($P < 0.01$), the expression levels of high-density lipoprotein and apolipoprotein A-1 in lean-type MAFLD patients were lower than in healthy individuals ($P < 0.05$), and the

expression levels of triglycerides and fasting blood glucose increased significantly (P < 0.01). A total of 65 biomarkers were screened based on "P < 0.05 and variable importance in projection > 1", which mainly affected the synthesis and metabolism of fatty acids. The levels of PA, OA, LA, and AA were significantly higher than in healthy individuals.

CONCLUSION

The metabolic profiles of lean-type MAFLD patients differed significantly from those of healthy participants, yielding 65 identified biomarkers. Notably, PA, OA, LA, and AA exhibited the most significant changes, offering valuable clinical guidance for the prevention and treatment of lean-type MAFLD.

Key Words: Lean-type metabolic-associated fatty liver disease; Targeted serum metabolomics; Fatty acids; Principal component analysis; Orthogonal partial least squares-discriminant analysis

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Core Tip: This article explores the serum targeted metabolomics of healthy individuals and metabolic-associated fatty liver disease (lean-type MAFLD), screens biomarkers and related metabolic pathways, and conducts targeted quantitative analysis of their specific biomarkers, with the aim of providing experimental evidence for the early diagnosis and pathogenesis of lean-type MALFD.

INTRODUCTION

The annual prevalence of metabolic-associated fatty liver disease (MAFLD) in China has been increasing, with the current rate exceeding 30%[1-4]. Owing to subtle early

manifestations that often go unnoticed, approximately 20% of MAFLD patients progress to metabolic-associated steatohepatitis, fibrosis, cirrhosis, and liver cancer[5,6]. MAFLD is closely associated with obesity and type 2 diabetes; however, approximately 40.8% of MAFLD patients have a body mass index (BMI) that does not meet the criteria for overweight or obesity[7-9]. This condition is commonly referred to as lean or non-obese-type MAFLD. Compared with other ethnic groups, the prevalence of metabolic disorders is higher among Asians with lower BMIs[10].

Because available studies on lean-type MAFLD are limited, its pathogenesis and treatment methods remain unclear. Patients with lean-type MAFLD are at higher risk of progressing to fatty liver inflammation and liver fibrosis, with an incidence of 30%, which is closely associated with metabolic dysfunction[11]. Metabolomics is a high-throughput detection method widely employed for disease diagnosis and mechanistic investigations[12]. In this study, we used ultra-high-performance liquid chromatography-tandem mass spectrometry with an electrospray ionization quadrupole trap analyzer to effectively identify serum metabolic markers that distinguish lean-type MAFLD patients from healthy individuals. We aimed to identify the specific metabolic pathways related to these markers and subsequently conduct targeted investigations of the metabolites and pathways that exhibit significant alterations. This study aimed to provide experimental evidence for the early diagnosis and pathogenesis of lean-type MAFLD.

MATERIALS AND METHODS

Study participants

Between January 2022 and June 2022, 20 patients diagnosed with lean-type MAFLD were recruited from the gastroenterology department of the Central Hospital of Putuo District, Shanghai. Additionally, 20 apparently healthy volunteers were included in the control group after physical examinations. General information and clinical data, including complete blood count, liver function, renal function, and lipid profiles, were recorded and analyzed. The research protocol was approved by the Ethics Committee

of the Central Hospital of Putuo District (Affiliated to the Putuo Hospital of Shanghai University of Traditional Chinese Medicine; approval number PTEC-R-2020-29-1). All the enrolled patients provided informed consent before participating in the study.

Diagnostic criteria

The clinical diagnostic criteria for MAFLD were outlined by the Chinese Society of Hepatology, Chinese Medical Association, in the "2010 Guidelines for the Diagnosis and Treatment of Nonalcoholic Fatty Liver Disease"[11]. The diagnostic criteria for MAFLD were as follows: (1) No history of excessive alcohol consumption or an equivalent ethanol intake of < 140 g per week for men (< 70 g per week for women); (2) Exclusion of specific diseases such as viral hepatitis, drug-induced liver disease, total parenteral nutrition, hepatolenticular degeneration, and autoimmune liver diseases that can cause fatty liver; and (3) Histopathological changes in liver biopsy specimens consistent with the pathological diagnosis criteria for fatty liver disease.

MAFLD is defined by: (1) Liver imaging findings consistent with the diagnosis of diffuse fatty liver and exclusion of other causes; and (2) Manifestations related to metabolic syndrome with persistently elevated levels of serum alanine transaminase (ALT), aspartate transaminase, or gamma-glutamyl transferase for > 6 months. Individuals with abnormal enzyme profiles and fatty liver on imaging whose profiles and liver appearance return to normal or improve to normal levels after weight loss and improvement in insulin resistance fulfill the diagnostic criteria for MAFLD. The diagnostic criteria for lean-type MAFLD are as follows: BMI < 23, 23 ≤ BMI < 28, and BMI ≥ 28 define lean-type, overweight-type, and obese-type MAFLDs, respectively.

Inclusion criteria

Patients who met the following inclusion criteria were included in the study: (1) Aged between 16 and 75 years, regardless of sex; (2) Those meeting the diagnostic criteria for lean-type MAFLD according to Western medical standards[13]; (3) Those with complete

and reliable information on various data, biochemical tests, and specimen collection; and (4) Those who provided consent to participate.

Exclusion criteria

The exclusion criteria were as follows: (1) Presence of concomitant liver-extrinsic fibrotic diseases, including systemic lupus erythematosus, rheumatic diseases, renal failure, chronic obstructive pulmonary disease; (2) Presence of severe primary diseases related to cardiovascular, cerebrovascular, urinary, renal, hematopoietic systems, malignant tumors, other serious complications, or psychiatric disorders; (3) Presence of thyroid disorders, including hyperthyroidism, hypothyroidism, subclinical hypothyroidism, and Hashimoto's thyroiditis; and (4) Patients with incomplete clinical data relevant to this study.

Materials

Ultra-high-performance liquid chromatography (Ultimate 3000, Thermo); high-resolution mass spectrometry (Orbitrap Elite, Thermo); cryogenic high-speed centrifuge (1730R, GENE GmbH, Germany); ultrapure water system (Milli-Q, Merck Biotechnology Shanghai Co., Ltd.); multi-tube vortex oscillator (VX-II, Beijing Tajin Technology Co., Ltd.); methanol (HPLC grade, China National Pharmaceutical Group Chemical Reagent Co., Ltd., Batch No.: O0621152); methyl tert-butyl ether (analytical grade, China National Pharmaceutical Group Chemical Reagent Co., Ltd., Batch No.: 20210227); formic acid (chromatography grade, Shanghai ANPEL Scientific Instrument Co., Ltd., Batch No.: D1290265); ammonium acetate (chromatography grade, Shanghai ANPEL Scientific Instrument Co., Ltd., Batch No.: BCBR1129V); isopropanol (chromatography grade, Shanghai ANPEL Scientific Instrument Co., Ltd., Batch No.: V589K144); acetonitrile (chromatography grade, Shanghai ANPEL Scientific Instrument Co., Ltd., Batch No.: K3021728).

Metabolomics study

Serum handling: This study included 20 patients with lean-type MAFLD and 20 healthy volunteers. Morning fasting venous blood (10 mL) was collected, left to stand at 4 °C for 2 hours, and then centrifuged at 3000 rpm for 15 minutes. The upper serum layer was then collected. Serum samples (50 μL) were mixed with 200 μL of methanol, vortexed for complete extraction. After low-temperature centrifugation at 14000 rpm and 4 °C for 10 minutes, the supernatant was transferred to a sample vial for analysis.

Chromatographic conditions: Column: C_{18} chromatographic column (Hypergod C_{18}, 100 mm × 2.1 mm, 1.9 μm); flow rate: 0.3 mL/minutes; column temperature: 40 °C; mobile phase composition: A: Pure water + 0.1% formic acid and B: Acetonitrile + 0.1% formic acid. Gradient elution program: 0-2 minutes, 95% A; 2-12 minutes, 5%-95% A; 12-15 minutes, 5%-95% A; 15-17 minutes, 5%-95% A.

Mass spectrometric conditions: Positive ion mode: Heater temperature: 300 °C; sheath gas flow rate: 45 psi; auxiliary gas flow rate: 5 L/minutes; sweep gas flow rate: 0.3 L/minutes; electrospray voltage: 3.0 kV; capillary temperature: 350 °C; S-Lens RF level: 30%. Negative ion mode: Heater temperature 300 °C; sheath gas flow rate: 45 psi; auxiliary gas flow rate: 5 L/minutes; sweep gas flow rate: 0.3 L/minutes; electrospray voltage: 3.2 kV; capillary temperature: 350 °C; S-Lens RF level: 60%.

Fatty acid targeted metabolomics

Chromatographic conditions: Column: Waters Acquity UPLC BEH C_8 column (2.1 mm × 100 mm, 1.7 μm); column temperature: 40 °C; flow rate: 0. 35 mL/minutes; mobile phase: Water (0.1% formic acid): Acetonitrile (0.1% formic acid); gradient elution program: 1 minutes, 50% B; 1-5 minutes, 50%-80% B; 5-6.5 minutes, 80%-95% B; 6.5-10 minutes, 95% B.

Tandem mass spectrometry (MS/MS detection): The serum concentrations of fatty acids and their metabolites were determined using ultra-high-performance liquid

chromatography (Waters H-Class) coupled with triple quadrupole mass spectrometry (AB SCIEX 6500). MS/MS data were collected using deuterated arachidonoylethanolamide (AEA-d8), deuterated oleoylethanolamide (OEA-d4), deuterated linoleoylethanolamide (LEA-d4), and deuterated oleic acid (OA-d9) as internal standards. Analytes, including AEA, 2-arachidonoyl glycerol ester, palmitoylethanolamide (PEA), OEA, LEA, 2-arachidonoylglycerol (2-AG), 1-palmitoyl glycerol (1-PG), 1-oleoyl glycerol (1-OG), and 1-linoleoyl glycerol (1-LG), were detected in positive electrospray ionization mode, whereas arachidonic acid (AA), stearic acid, palmitic acid (PA), OA, and linoleic acid (LA) were detected in negative mode. The optimized operational conditions were as follows: Ion spray voltage of + 5500 V in positive mode and -4500 V in negative mode, ion source temperature of 550 °C. Nitrogen was used as the collision gas. The ion pairs and related internal standards for multiple reaction monitoring are presented in Table 1.

Sample preparation: A total of 30 µL of serum sample was combined with mixed internal standards, followed by successive additions of 500 cL of methyl tert-butyl ether, 150 µL of methanol, and 140 µL of ultrapure water. The mixture was vortexed for 1 minutes and then centrifuged at 4 °C for 10 minutes (3000 rpm). The upper layer was collected, concentrated, and dried, followed by reconstitution with 100 µL of acetonitrile. The resulting supernatants were transferred to sample vials for further analysis.

Standard and internal standard preparation: Precisely weighed amounts of AEA, LEA, PEA, OEA, 2-AG, 1-LG, 1-PG, 1-OG, AEA-d8, OEA-d4, and LEA-d4 were prepared at concentrations of 1250, 500, 250, 125, 50, 25, 12. 5, 5, 1, and 0. 5 ng, respectively. OA, PA, AA, and LA standards were prepared at concentrations of 5, 10, and 20 µg/mL, respectively, including internal standards AA-d8 (10 µg/mL) and OA-d9 (1 µg/mL).

Data processing and statistical methods: Peak alignment, retention time correction, and peak area calculations were performed using LC-MS software. Accurate molecular weights and MS/MS spectra were used for the identification and database retrieval of the metabolites. Unsupervised principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were used for multidimensional statistical analysis. Enrichment analysis of significantly altered metabolic pathways was performed using the Kyoto Encyclopedia of Genes and Genomes database. All other data were analyzed using SPSS 22. 0 statistical software (IBM Corp., Armonk, NY, United States). Normally distributed quantitative data are expressed as the mean ± SD. Comparisons of quantitative data among groups were performed using analysis of variance (ANOVA) if the data satisfied the normality and homogeneity of variance assumptions; otherwise, the Wilcoxon non-parametric test was used. A significance level of $P < 0.05$ was considered statistically significant.

RESULTS

Clinical data analysis

Twenty lean-type MAFLD patients and 20 healthy individuals were included in this study. No significant differences in general characteristics, including sex, age, or BMI were observed between lean-type MAFLD patients and healthy individuals (Table 2). Routine blood tests revealed a notable difference in white blood cell counts between lean-type MAFLD patients and healthy controls ($P < 0.01$). Renal function test results revealed significant discrepancies in urea nitrogen, uric acid, and creatinine levels were observed between lean-type MAFLD patients and healthy controls ($P < 0.01$). Liver function tests, including cholinesterase and ALT levels, significantly differed between lean-type MAFLD and healthy controls ($P < 0.01$). The glucose metabolism results demonstrated a significant difference in fasting blood glucose levels between lean-type MAFLD patients and healthy controls ($P < 0.01$). Blood lipid analysis revealed marked variations in high-density lipoprotein (HDL), triglycerides, and apolipoprotein A1 (APOA-1) levels between lean-type MAFLD patients and healthy controls ($P < 0.01$).

LC-MS quality control results

The total ion chromatogram of quality control samples from serum samples of lean-type MAFLD patients is shown in Figure 1. The overlapping chromatograms indicated excellent instrument stability and consistent retention times, validating the reliability of the obtained analytical data.

PCA analysis

Data from patient serum metabolomics were analyzed using the Compound Discover software, followed by normalization. An unsupervised PCA model was built using Simca-P 14.0 to compare the overall profiles of individual samples under both positive and negative detection modes. In both positive and negative modes, samples from lean-type MAFLD patients clustered together and were clearly differentiated from those in the control group (Figure 2). This indicates a pronounced distinction in metabolite levels between lean-type MAFLD patients and healthy controls. The PCA model for the positive mode showed an R^2X of 0. 698 and Q^2 of 0. 497, whereas for the negative mode, R^2X was 0. 6794 and a Q^2 was 0. 566. These results confirmed the ability of this PCA data model to elucidate variations in metabolites among samples.

OPLS-DA analysis

To comprehensively analyze and compare the differences in metabolites between lean-type MAFLD patients and healthy controls, the OPLS-DA method was used to analyze the collected data. In both positive and negative modes, the lean-type MAFLD patients and healthy controls were completely separated in the OPLS-DA plots (Figure 3). The corresponding R^2Y and Q^2 values in the positive and negative modes were 0.957 and 0.962 and 0.954 and 0.921, respectively. To further validate the robustness of the model, a permutation test with 200 iterations was conducted, which revealed no signs of data overfitting. This confirmed the good fit and predictive ability of the model, with statistically significant results.

Differential metabolic pathway screening

Following OPLS-DA analysis, differential metabolites were selected based on a combination of variable importance in projection (VIP) values and *t*-tests. The selection criteria were set to satisfy VIP > 1 and $P < 0.05$. The selected metabolites were cross-referenced against the HMDB database. Ultimately, 65 potential differential metabolites were identified in lean-type MAFLD patients. Among these, 33 and 32 were identified in positive and negative modes, respectively (Figure 4). Metabolic pathway enrichment analysis was conducted using MetaboAnalyst 5.0 for the identified metabolites. The 65 metabolites were primarily associated with pathways involving unsaturated fatty acid biosynthesis, LA metabolism, fatty acid degradation, and ether lipid metabolism. Notably, significant differences were found in the pathways of unsaturated fatty acid biosynthesis and LA metabolism (Figure 5). To gain a deeper understanding of the significance of fatty acid metabolism in lean-type MAFLD patients, targeted metabolomic analysis of fatty acids was performed in these patients.

Fatty acid targeted metabolomics standard curve and chromatogram

Different concentrations of mixed fatty acid standards were sequentially injected with concentration (μg/mL) as the x-axis and peak area as the y-axis to construct the standard curve. The linear ranges and correlation coefficients (*r*) are listed in Table 3. The results demonstrate that the standard curve exhibited excellent linearity and was suitable for the quantitative detection of fatty acids in the samples. The multiple reaction monitoring chromatograms for the samples and standard materials are shown in Figures 6 and 7, and the results indicate no interference from other matrices in the content detection of the samples.

Changes in fatty acid content in lean-type MAFLD patients

The changes in fatty acid content between lean-type MAFLD patients and healthy individuals are shown in Table 4. The levels of PA, OA, LA, and AA in lean-type

MAFLD patients were 3.41 ± 0.84, 2.63 ± 1.45, 2.42 ± 1.18, and 2.45 ± 1.21 μg/mL, respectively. These levels were significantly higher than those in the control group ($P < 0.05$).

DISCUSSION

Obesity and type 2 diabetes were prevalent among MAFLD patients. However, approximately 40. 8% of MAFLD patients exhibit a BMI below the overweight or obese standards (BMI < 23), referred to as lean-type MAFLD. According to epidemiological surveys, lean-type MAFLD accounts for 25.8% of all MAFLD cases globally, whereas in China, lean-type MAFLD represents 44.3% of all MAFLD cases[14]. Owing to its subtle onset, mild symptoms, or lack of specificity, diagnosis remains challenging and is often diagnosed during routine liver function and imaging examinations. Current research predominantly focuses on obese-type MAFLD, with limited studies on lean-type patients. This study used serum metabolomics technology to investigate characteristic biomarkers in lean-type MAFLD patients and perform quantitative analysis.

In this study, the BMI of lean-type MAFLD patients included was < 23 kg/m^2, which was higher in both BMI and body mass than that in lean healthy participants. Compared with healthy controls, lean-type MAFLD patients exhibited significantly elevated white blood cell counts ($P < 0.01$). Additionally, significant differences were observed in liver and kidney function and lipid profile results ($P < 0.01$), such as urea nitrogen, uric acid, creatinine, cholinesterase, ALT, fasting blood glucose, HDL, triglycerides, and APOA-1 levels.

ALT is distributed in the hepatic cytoplasm, where increased intracellular triglycerides in the liver cells provide sufficient reactive substrates for lipid peroxidation, thereby affecting the activity of antioxidant enzymes. This leads to increased oxidative stress in the body. When liver cells are damaged, intracellular enzymes are released into the blood, causing ALT to spill over from liver cells into the extracellular space, resulting in increased peripheral blood ALT levels. Therefore, ALT levels reflect the integrity of liver cells. In this study, ALT and triglyceride levels in

lean-type MAFLD patients were significantly higher than those in healthy lean controls ($P < 0.01$). Excessive accumulation of lipids within liver cells is considered a crucial factor that leads to hepatocyte degeneration and inflammation. Therefore, effective lipid transport and reduction in lipid synthesis may be crucial for the prevention and treatment of fatty liver disease. APOA-1, the primary apolipoprotein in HDL, is involved in cholesterol transport[15]. Mice with APOA-1 deficiency cannot form normal HDL particles, resulting in ineffective cholesterol transport to liver tissues and cholesterol accumulation[16], which is consistent with our finding that APOA-1 levels were lower in lean-type MAFLD patients than in lean healthy controls, whereas triglyceride levels were significantly increased ($P < 0.05$). Cholinesterase is an enzyme secreted by the liver cells into the bloodstream. A cross-sectional analysis of 5384 individuals indicated elevated serum cholinesterase concentrations in patients with fatty liver, which is consistent with our results[17].

Metabolomics, a high-throughput detection method, can reflect disease status based on the overall biochemical phenotype of an organism. This enables the examination of changes in endogenous metabolites at the macroscopic level following biological stimuli. By analyzing comprehensive metabolic profiles, it is possible to identify disease-associated metabolites and reveal their metabolic pathways. In our study, serum metabolomics and liquid chromatography coupled with triple quadrupole mass spectrometry were used to investigate specific biomarkers in lean-type MAFLD patients. Using "VIP > 1 and $P < 0.05$" as selection criteria, 65 potential biomarkers specific to lean-type MAFLD patients were identified in both positive and negative modes. These biomarkers were primarily concentrated in pathways related to fatty acid, AA, and ether lipid metabolism.

Additionally, quantitative lipidomic analysis of the four specific biomarkers revealed a significant increase in the serum levels of PA, OA, LA, and AA in lean-type MAFLD patients ($P < 0.05$), which were 2.23-, 2.09-, 1.86-, and 1.98-times higher, respectively, than those in healthy individuals. Fatty acids are fundamental components of triglycerides. Currently, approximately 60 types of fatty acids have been discovered in

the plasma and tissues; however, only a few of them can be absorbed and utilized by the human body[18]. The homeostasis of body fatty acids ensures normal functioning.

Fatty acids play a crucial role in lipid metabolism; however, relevant literature concerning their role in lean-type MAFLD patients is lacking. Current research indicates a close association between fatty acids and metabolic disorders, in which elevated levels of PA, palmitoleic acid, and LA are positively correlated with the onset and progression of MAFLD[19]. Studies conducted by Gambino *et al*[20] compared the changes in serum free fatty acids between MAFLD patients and healthy controls, and demonstrated a significant increase in serum levels of free LA, OA, and AA. Puri *et al*[21] conducted plasma lipidomics research in MAFLD and non-alcoholic steatohepatitis patients, reported significantly higher levels of PA, OA, and LA than in healthy individuals, whereas AA showed no significant change. These findings align broadly with our research findings and, to a certain extent, reflect the serum levels of PA, OA, LA, and AA in lean-type MAFLD patients.

AA, a crucial fatty acid in the cell membrane, is involved in cellular signal transduction during various inflammatory responses[22]. Abnormalities in fatty acid metabolism disrupt the balance between the release and uptake of fatty acids in serum, leading to increased fatty acid generation and reduced re-esterification capability. Eventually, this causes the accumulation of serum fatty acids, resulting in lipotoxicity and subsequent damage to the cardiovascular, endocrine, and digestive systems[23]. These studies indicated that abnormal fatty acid metabolism in lean-type MAFLD patients significantly increases the likelihood of developing metabolic disorders. Based on the existing research, we believe that elevated levels of PA and OA in the serum are directly associated with the occurrence of lean-type MAFLD, whereas increased levels of LA and AA are linked to the progression of MAFLD. In addition, PA promotes hepatic stellate cell activation, increases extracellular matrix deposition in MAFLD rats[24], induces podocyte apoptosis[25], and contributes to inflammation[26]. OA, a monounsaturated fatty acid and the preferred substrate for synthesizing triglycerides and cholesterol esters, can induce hepatic cell steatosis and enhance tumor

invasiveness[27]. LA is an essential fatty acid that cannot be synthesized in the body and must be obtained from dietary sources. LA is converted into AA in the body through the action of enzymes such as Δ-6 desaturase (Figure 8).

CONCLUSION

The results of serum-targeted metabolomics analysis elucidate that fatty acid metabolism was impaired in lean-type MAFLD patients. The characteristic biomarkers screened in this study may provide potential insights into the treatment of lean-type MAFLD. However, the inclusion of a smaller sample size is a major limitation of our study, warranting further investigation.

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