RESEARCH

Serum metabolomic analysis of the effect of exercise on nonalcoholic fatty liver disease

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Abstract

Objective: Exercise benefits people with nonalcoholic fatty liver disease (NAFLD). The aim of this study was to identify a panel of biomarkers and to provide the possible mechanism for the effect of exercise on NAFLD patients via an untargeted mass spectrometry-based serum metabolomics study.
Methods: NAFLD patients were classified randomly into a control group (n = 74) and a 6-month vigorous exercise (n = 68) group. Differences in serum metabolic profiles were analyzed using untargeted ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) technology. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were used to validate the differences between these two groups, and altered metabolites were obtained by ANOVA (fold change >2, P < 0.05) and identified with the online database Metlin and an in-house database.
Results: Metabolic profiling and multiple statistical analyses of the serum samples indicated significant differences between the NAFLD patients in the control and the 6-month vigorous exercise groups. Finally, 36 metabolites were identified between the control vs exercise groups. These metabolites were mainly associated with glycerophospholipid- and sphingolipid-related pathways.
Conclusion: Our study demonstrates that glycerophospholipid and sphingolipid alterations may contribute to the mechanism underlying the effect of exercise on NAFLD patients. A LC-MS-based metabolomics approach has a potential value for screening exercise-induced biomarkers.

Key Words

- metabolomics
- ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry
- exercise
- nonalcoholic fatty liver disease

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Introduction

Nonalcoholic fatty liver disease (NAFLD) has become an important public health problem because of its high prevalence with lifestyle improvements. Currently, the principal treatment for NAFLD is lifestyle modification by diet and exercise. Several clinical trials have verified the benefit of exercise for NAFLD patients, including decreases in the intrahepatic triglyceride (IHTG) content, weight, waist circumference, body fat and blood pressure (1, 2, 3, 4). Moreover, physical activity has shown a reduction in diabetes and cardiovascular diseases (35 and 49%, respectively) among NAFLD patients (5, 6).

Metabolomics is a high-throughput technology that can measure thousands of metabolites (in biological fluids or tissues) simultaneously. The technology has the potential to identify novel biomarkers and to provide a comprehensive view of the changes in several metabolic and signaling pathways. Recently, metabolomics has been applied in both clinical and basic research for

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disease prediction, disease discrimination, drug response assessment, hypothesis generation and drug development (7, 8, 9, 10, 11). As a powerful tool for biomarker discovery, metabolomics analysis had been applied successfully to NAFLD. This approach has found markedly higher glycocholate, taurocholate and glycochenodeoxycholate levels in subjects with NAFLD (12) and has provided theoretical treatment using hydroquinone and nicotinic acid, which are inversely correlated with the histological NAFLD severity (13). Moreover, plasma lipids, such as diacylglycerol, triacylglycerol, cholesteryl ester, sphingolipid and glycerophospholipid, can distinguish NASH (non-alcoholic steatohepatitis) from steatosis (14).

Our previous randomized clinical study revealed that long-term exercise could significantly decrease the IHTG content in NAFLD patients and reduce their weight, body fat, waist circumference and blood pressure (1). However, the metabolic profile underlying the effect of exercise on NAFLD patients is uncertain, and the metabolites and related pathways that are altered during exercise are unknown. Here, we analyzed endogenous metabolites in serum samples from NAFLD patients with/without 6 months of exercise using untargeted ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) technology. Altered metabolites and metabolic pathways are established and may help explain the mechanism underlying the effect of exercise on NAFLD patients.

Materials and methods

Study protocol

This study protocol and informed consent form for the previous clinical trial were overseen and approved by a steering committee and the institutional review boards of Xiamen University and the First Affiliated Hospital of Xiamen University in China and Tulane University. All methods were carried out in accordance with the relevant guidelines and regulations (Trial registration number: NCT01418027).

The study participants and the protocol were described in our previous study (1). Briefly, 1508 participants with central obesity were recruited from community-based screening using abdominal ultrasonography from December 1, 2011, through December 25, 2013, Xiamen, China. After excluding ineligible participants (persons who had other chronic liver disease, no NAFLD, heavy alcohol consumption or other reasons), the remaining 427 participants were diagnosed by proton magnetic resonance spectroscopy. Then, a total of 220 individuals (excluding those who were eligible but declined to participate, were ineligible, had an intrahepatic triglyceride content <5% or other reasons) were selected and randomly assigned to a control group ($n=74$, no exercise), moderate exercise group ($n=73$, brisk walking for 150 min per week for 12 months) and vigorous-moderate exercise group ($n=73$, jogging for 150 min per week at 65–80% of the maximum heart rate for 6 months and brisk walking for 150 min per week at 45–55% of the maximum heart rate for another 6 months). At 08:00 h after an overnight fast, blood samples were collected from study participants after the last exercise, serum samples were stored at −80°C prior to analysis. In this present study, only the 6-month vigorous exercise intervention was considered.

Materials

Ammonium acetate, formic acid and acetonitrile (all HPLC grade) were purchased from Fisher Scientific Co., and 2-chloro-L-phenylalanine was purchased from Aladdin Company (Shanghai, China). The water used in this study was prepared by a Milli-Q water purification system (Millipore).

UPLC-Q-TOF/MS condition

The metabolomics analysis was carried out in an ACQUITY UPLC (Waters Corp.) system equipped with the Waters Synapt G2 Q/TOF-MS (Waters Co., Milford, MA, USA). The optimized parameters were set as follows: capillary voltage 3 kV (positive mode)/2.5 kV (negative mode); cone voltage 40 V; extraction cone voltage 4 V; source temperature 120°C; desolvation temperature 450°C; cone gas 50 L/h; desolvation gas 800 L/h; scan rage *m/z* 50–1500 Da and both negative and positive detection modes. An external reference (lock spray) composed of a solution of 2 µg/mL of leucine enkephalin (*m/z* 556.2771 in positive ion mode and *m/z* 554.2615 in negative ion mode) was employed to control the UPLC-Q/TOF-MS system at a flow rate of 5 µL/min.

Chromatographic separations were obtained on the ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 µm). The mobile phase consisted of water (containing 5 mM ammonium acetate and 0.1% formic acid, A) and acetonitrile (B) with the following gradient elution: 0–1 min, 2% B; 1–3 min, 2–30% B; 3–5 min, 30–50% B; 5–8 min, 50–60% B; 8–10 min, 60–80% B; 10–12 min, 80–98% B and 12–13.5 min, 98% B. Then, the column

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was equilibrated with 2% B for 1.5 min. The flow rate was set at 0.4 mL/min, and the column temperature was set at 45°C. The UPLC-Q-TOF/MS system and the data acquisition and processing were conducted under control of the MassLynx software (Version 4.1, Waters Corp.).

Sample preparation

A total of 50 µL of the serum sample was added to 50 µL of an internal standard solution (2-chloro-L-phenylalanine, 60 µg/mL, prepared in methanol) and 100 µL of ice-cold acetonitrile. The mixture was vortex-mixed for 30 s and centrifuged at 19,000 ***g*** for 10 min at 4°C. Then, 5 µL of the supernatant was injected into the UPLC-Q-TOF/MS system for analysis.

Quality control (QC) samples were obtained by combining the sera from the different groups, and the analysis was repeated during sample runs with the same LC-MS method.

Statistical analysis

All raw data files obtained from the MassLynx software (version 4.1, Waters Corp.) were converted to the mzXML format using ProteoWizard tools. The XCMS software was employed to convert each data file into a matrix of detected peaks. Differences in metabolic profiles on LC-MS were determined by principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) by the SIMCA software 14.1. Biomarkers considered significant were confirmed by the *P* value (<0.05) and fold change (>2) obtained by ANOVA. Compound identification was performed with the online database Metlin (http://metlin.scripps.edu) and an in-house database. The pathway analysis and network of potential biomarkers contributing to the classification between groups were examined using METLIN (http://metlin.scripps.edu/), KEGG (http://www.kegg.com/) and MetaboAnalyst (http://www.metaboanalyst.ca/).

Results

Clinical characteristics of the subjects

A total of 220 eligible trial participants were recruited and followed up for 12 months (1). In this present work, only the control and 6-month vigorous exercise intervention groups (74 participants completed the 6-month control group and 68 participants completed the 6 months of vigorous exercise, because 5 withdrew from the trial for knee osteophytes, bone fracture or uncompleted intervention) were studied using metabolomics analysis.

Baseline characteristics in these two groups were showed in Table 1, data of which were modified from our previous study (1), and no significant differences were found in the basic anthropometric data. After 6-month vigorous exercise, body fat and cardiovascular risk factors were significantly reduced in the exercise group patients compared with the levels in the controls (data of which were modified from our previous study (1) and shown in Supplementary Table 1, see section on supplementary data given at the end of this article).

In addition to the investigated characteristics listed above, the IHTG remarkably decreased by 5.0% (95% CI, −7.2% to −2.8%; $P<0.001$) after 6 months of vigorous exercise. This decrease demonstrates the effect of exercise on NAFLD patients, because IHTG is the clinical diagnostic indicator of NAFLD.

Serum metabolite profiling

The percentage coefficient of variation of the peak area in the QC samples was estimated to be 11.0–28.8% in positive ion mode and 3.3–29.2% in negative ion mode. These results collectively indicated the good repeatability, reliability and stability of this LC-MS method. The chromatogram of QC sample was show in Supplementary Fig. 1.

We investigated the metabolite profiles of individual serum samples using UPLC-Q-TOF/MS. The base peak intensity (BPI) chromatograms of the serum samples indicated that the metabolites attained suitable separation in different participants (figures not shown). After obtaining a matrix of detected peaks using the ProteoWizard tools and XCMS software, a multivariate statistical analysis was performed to determine whether the serum metabolic profiles were different among participants with or without exercise. First, principal components analysis (PCA) was used to determine the general relationships between these two groups (the PCA score plots are presented in Supplementary Fig. 2, $R^2X=0.692$, $Q^2=0.605$ in positive ion mode and $R^2X=0.663$, $Q^2=0.429$ in negative ion mode). The control and exercise groups revealed a separation trend, although the difference was not obvious. Subsequently, orthogonal partial least squares discriminant analysis (OPLS-DA) was performed to highlight the differences. The results indicate significant separations with valid model fits, as shown in Fig. 1 ($R^2X=0.707$, $R^2Y=0.6$, $Q^2=0.392$ in

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Table 1 Baseline characteristics of study participants. Data are presented as mean (S.D.) unless otherwise indicated.

Characteristics	Control	6-month vigorous exercise
	n = 74	*n* = 73
Female (No.)	46	52
Age (year)	54.0 (6.8)	53.2 (7.1)
High school education (No.)	15	23
Current cigarette smoking (No.)	18	10
Current alcohol drinking (No.)	26	20
Physical activity (METs/week)	29.1 (9.3)	31.7 (8.5)
Total energy intake (kcal/day)	2140.7 (458.0)	2124.8 (454.4)
Fat intake (%)	33.3 (6.9)	31.9 (7.3)
Waist circumference (cm)	96.1 (6.9)	95.2 (7.4)
Weight (kg)	72.1 (8.5)	71.7 (10.1)
BMI	28.0 (2.7)	27.9 (2.7)
Heart rate (/min)	81.7 (12.1)	80.8 (11.1)
Systolic blood pressure (mmHg)	134.7 (16.7)	132.1 (15.6)
Diastolic blood pressure (mmHg)	80.8 (10.6)	79.7 (9.9)
Plasma glucose (mg/dL)	103.5 (9.1)	102.6 (10.9)
Serum triglycerides, median (IQR) (mg/dL)	161.1 (126.5–225.7)	165.5 (112.4–212.4)
Serum total cholesterol (mg/dL)	232.3 (35.0)	225.6 (44.8)
HDL-C (mg/dL)	49.2 (10.8)	48.3 (9.2)
LDL-C (mg/dL)	144.4 (34.5)	137.9 (44.3)
Visceral fat (cm^2)	133.8 (43.2)	140.9 (41.9)
Subcutaneous fat (cm^2)	240.8 (80.8)	241.4 (72.6)
Body fat (%)	33.7 (7.1)	34.8 (5.3)
Intrahepatic triglyceride content (%)	17.5 (11.0)	18.4 (9.9)

$P > 0.05$ for all difference among the characters.
BMI, body mass index (calculated as the weight in kilograms divided by height in meters squared); HDL-C, high-density lipoprotein cholesterol; IQR, interquartile range; LDL-C, low-density lipoprotein cholesterol; METs, metabolic equivalents.

positive ion mode and $R^2X=0.556$, $R^2Y=0.721$, $Q^2=0.386$ in negative ion mode).

The TOF MS-based untargeted metabolomics profiling platform detected 2030 peaks and 2047 peak features in positive mode. To lessen the workloads, ANOVA was performed first, and then the variables (fold change >2 and statistical P value <0.05) were selected and verified by the loading plots. Finally, a compound was identified according to the MS information, structure information, accurate mass, retention time and fragmentation pattern. Based on the above steps, a panel of 50 variables distinctively discriminated the control and exercise groups, including metabolites, food additives, exogenous and unknown compounds. In total, only 36 identified endogenous metabolites were selected for further investigation. These metabolites are summarized in Table 2, and the relative responses of these metabolites between the control and exercise groups are shown in Fig. 2. Among all the identified altered endogenous metabolites, most belonged to sphingolipids (ceramide and its glycosides, glucosylsphingosine and ganglioside) and glycerophospholipids (phosphatidylethanolamine, PE; phosphatidylcholine, PC; mannosyl-inositol-phosphorylceramide, MIPC; phosphatidylserine, PS; phosphatidic acid, PA; phosphatidylinositol, PI; and phosphatidylinosital biphosphate, PIP2). Of these metabolites, the PC, lipid X, 3-oxo hexacosanoic acid, 9,10-epoxy-eicosene, diglyceride, β-D-glucopyranosiduronic acid, phenolic steroid, 2-tetracosenoic acid, α-heptasaccharide and 7-β-OH-cholesterol contents were decreased after 6 months of exercise. However, the sphingolipid, PE, PI, PS and PA contents changed differently (higher or lower serum concentrations in the exercise group than in the control group) due to their diverse types of fatty acyl chains.

The biological pathways involved in the metabolism of these metabolites were analyzed using the software or websites described in the 'Methods' section. An overview of the pathways and metabolite sets enriched as biomarkers is shown in Figs 3 and 4. Glycerophospholipid metabolism and sphingolipid metabolism were most closely related, whereas inositol phosphate metabolism, arachidonic acid metabolism, glycerolipid metabolism, fatty acid metabolism and primary bile acid biosynthesis were all associated with exercise improvement in the NAFLD patients.

Discussion

NAFLD is considered a hepatic manifestation of metabolic syndrome and is strongly associated with insulin resistance (15, 16). Our previous clinical trial contributed novel

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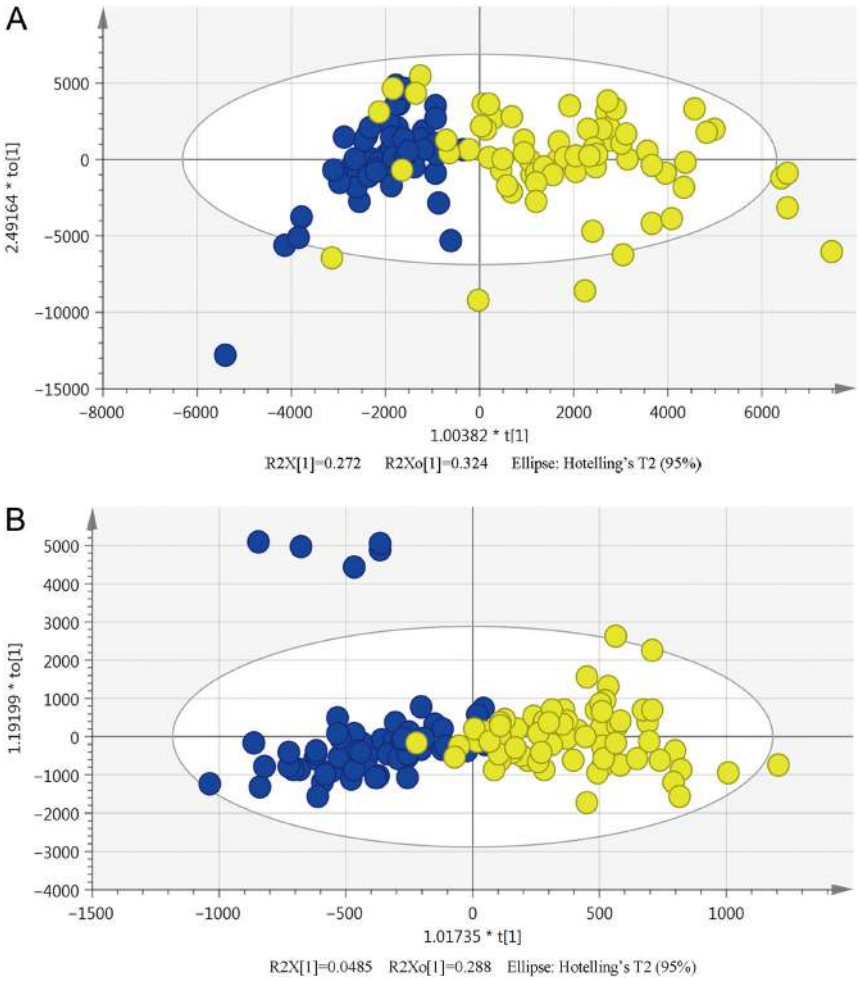

Figure 1
OPLS-DA analysis of the control (yellow color) and 6-month vigorous exercise (blue color) groups in positive (A) and negative (B) ion mode.

findings concerning the effects of long-term exercise on NAFLD in several aspects; the primary outcomes were IHTG reduction and weight loss. This present work focused on a large panel of metabolites in the serum after the exercise intervention, which was performed for the first time using an untargeted UPLC-Q-TOF/MS analysis based on a single serum sample. Making use of the full spectrum of analytes and metabolic profiling combined with pattern recognition techniques, we performed a metabolic analysis and clearly discriminated the characteristics of NAFLD patients with/without exercise. Among these identified altered metabolites, most belonged to glycerophospholipid and sphingolipids, which were associated with the glycerophospholipid and sphingolipid metabolic pathways, respectively.

Glycerophospholipids alone provided the highest discriminatory power of all metabolite classes in separating these two groups, including PE, PC, PS, PA and PI, which were mostly associated with the glycerophospholipid metabolic pathway based on the pathway map searching function of the KEGG website. Several studies have shown that the plasma PE, PC, PS and PI concentrations can distinguish NAFLD patients and healthy people, because the levels are higher in patients with simple steatosis or nonalcoholic steatohepatitis than that in healthy controls (17). Additionally, an animal experiment found a significant difference in the amount of these phospholipids after chronic exercise and/or intake of a high-fat diet, because these metabolites are involved in insulin sensitivity/resistance in skeletal muscle (18). The PC and/or PE contents of blood or various tissues have been implicated in metabolic disorders, such as atherosclerosis, insulin resistance and obesity. PC is synthesized by the Kennedy or CDP-choline pathway, whereas PE is synthesized via the CDP-ethanolamine and phosphatidylserine decarboxylase pathways. Additionally, approximately 30% of PC is biosynthesized by PE under phosphatidylethanolamine N-methyltransferase (PEMT) catalysis in the liver (19). Some studies have demonstrated an important role for the PC to PE ratio as a key determinant of liver health, and changes in this ratio have been linked to the development of NAFLD in humans

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Table 2 Discriminative metabolites between control and 6-month vigorous exercise groups.

Metabolites	*m/z*	Rt (s)	FDR (adjust *P* value)
2,3-Diacylglucosamine 1-phosphate (lipid X)	748.3725	223	6.55E-03
2-Tetracosenoic acid	331.3416	602	8.03E-09
3-Oxo hexacosanoic acid	375.3675	605	3.23E-08
7-β-OH-cholesterol	423.3257	790	3.38E-04
9,10-Epoxy-eicosene	312.3274	605	1.50E-06
Ceramide (Cer, 18:2/21:0)	200.8521	815	4.26E-04
Diglyceride (DG, 18:4/18:0)	617.5131	822	3.54E-04
Fatty acid (FA, 30:4)	425.3737	777	4.27E-04
Galalpha^{1}-^{3}Galalpha^{1}-^{3}Galalpha^{1}-^{3}Galalpha1-^{3}Galalpha^{1}-^{4}Galbeta^{1}-^{4}Glcbeta-Ceramide (Cer, 18:1/24:1)	889.9884	814	2.39E-15
Galalpha^{1}-^{4}Galbeta-Ceramide (Cer, 18:1/26:0)	1024.7338	652	4.13E-08
Ganglioside GT3(18:1/12:0)	558.6124	38	8.87E-07
Glucosylsphingosine	506.3375	606	1.99E-04
Manalpha^{1}-^{3}Manbeta^{1}-^{4}Glcbeta-Ceramide (Cer, 18:1/18:0)	1069.7627	648	2.41E-08
Manalpha^{1}-^{3}Manbeta^{1}-^{4}Glcbeta-Ceramide (Cer, 18:1/26:1)	670.468	683	9.82E-08
Mannosyl-inositol-phosphorylceramide (MIPC, 20:0/24:0)	1084.755	577	2.38E-08
NeuAcalpha^{2}-^{3}Galbeta^{1}-^{3}(NeuAcalpha$^{2-6}$)(Fucalpha$^{1-4}$)GlcNAcbeta^{1}-^{3}Galbeta^{1}-^{4}Glcbeta-Ceramide (Cer, 18:1/26:1)	696.7718	243	8.45E-03
NeuAcalpha^{2}-^{6}Galbeta^{1}-^{3}GalNAcbeta^{1}-^{3}Galalpha^{1}-^{3}Galbeta^{1}-^{4}Glcbeta-Ceramide (Cer, 18:1/22:0)	881.0084	815	2.76E-10
Palmitoleoyl CoA	500.651	38	7.91E-08
Phenolic steroid	279.1725	232	4.05E-10
Phosphatidic acid (PA, 16:0/0:0)	411.233	825	6.38E-04
Phosphatidic acid (PA, 16:0/18:3)	669.4582	789	2.96E-03
Phosphatidyl ethanolamine (PE, 17:1/20:1)	758.5694	787	1.45E-02
Phosphatidyl ethanolamine (PE, 18:4/18:3)	751.5056	694	1.87E-04
Phosphatidyl ethanolamine (PE, 20:2/18:1)	787.6034	735	1.18E-05
Phosphatidyl ethanolamine (PE, 20:4/18:1)	783.5723	798	4.23E-10
Phosphatidylcholine (PC, 18:2/2:0)	620.3525	661	2.65E-05
Phosphatidylcholine (PC, 18:4/18:0)	782.5689	777	9.59E-09
Phosphatidylcholine (PC, 18:4/18:0)	782.5688	797	2.50E-05
Phosphatidylcholine (PC, 20:1/16:1)	786.5999	723	4.98E-06
Phosphatidylinosital biphosphate (PIP2, 18:3/18:1)	1036.5028	798	4.71E-13
Phosphatidylinositol (PI, 18:4/18:1)	877.4902	815	1.80E-03
Phosphatidylinositol (PI, 20:5/14:1)	670.468	683	1.58E-03
Phosphatidylserine (PS, 16:0/0:0)	462.2683	302	5.46E-05
Prostaglandin (PG, 12:0/15:0)	670.468	683	7.32E-14
α-Heptasaccharide	1220.4838	241	2.62E-04
β-D-Glucopyranosiduronic acid	471.2408	310	4.22E-04

P values were calculated by ANOVA analysis.

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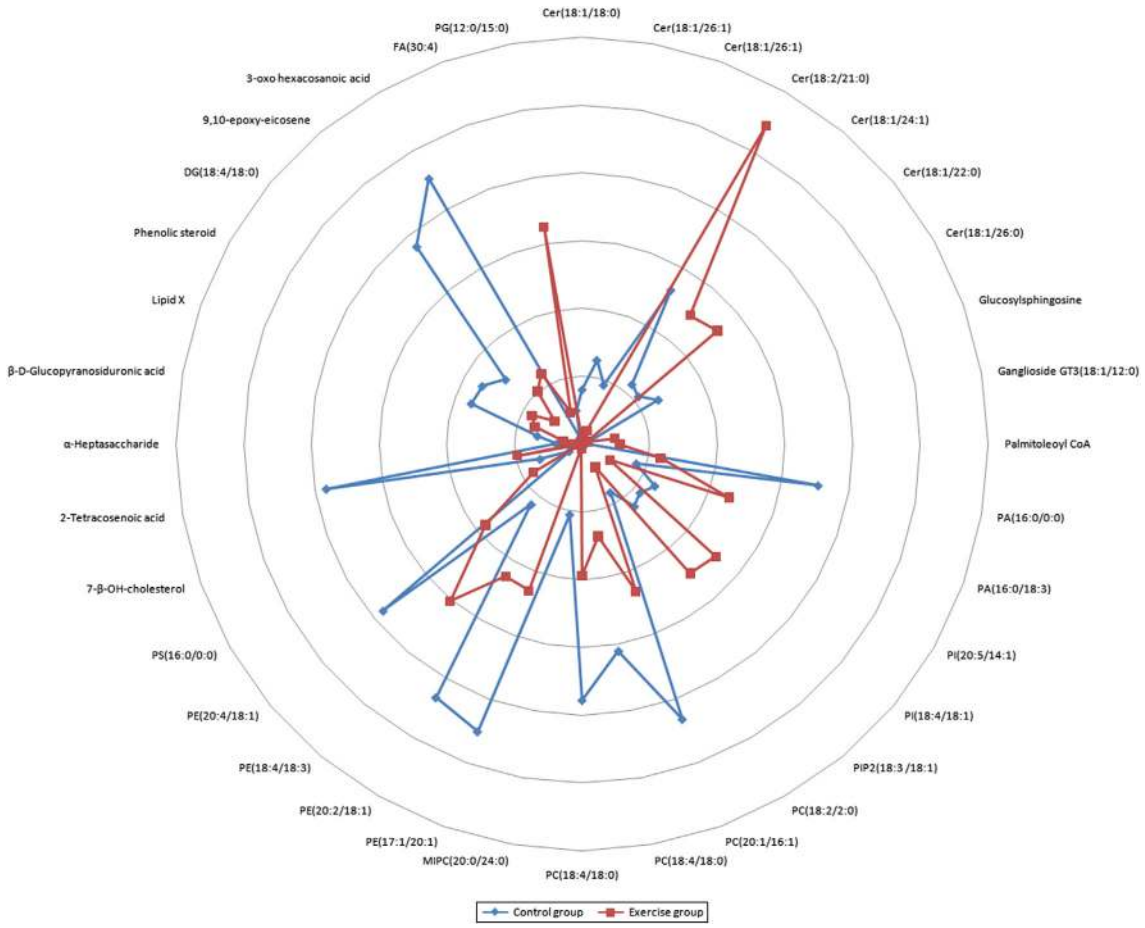

Figure 2
Radar graph of altered metabolites in serum samples from patients in the control and 6-month vigorous exercise groups.

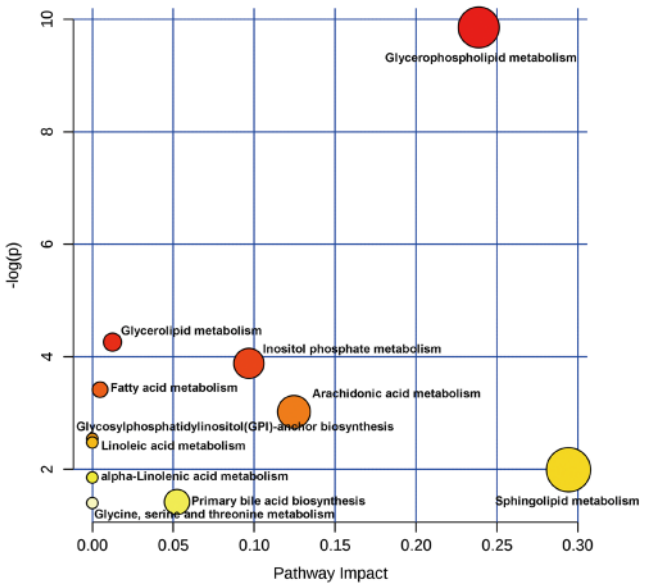

Figure 3
Overview of pathways established by the MetaboAnalyst website.

(20), and their contents of skeletal muscle respond to exercise, was closely related with insulin sensitivity (21). Moreover, phospholipids play a vital role in the exercise-induced increase in insulin sensitivity. Skeletal muscle undergoes remodeling in response to exercise (22), and in particular, unbalanced PC and PE synthesis can regulate muscle insulin sensitivity by choline/ethanolamine phosphotransferase 1 as the terminal enzyme in the Kennedy pathway of phospholipid synthesis (23, 24). Therefore, changes in these phospholipids may be the underlying metabolic mechanism responsible for the effect on NAFLD through insulin resistance improvement.

Ceramides and related sphingolipids represent minor components of the accumulated lipids. These lipids are a family of lipid molecules that circulate in the serum and accumulate in skeletal muscle and are related to insulin resistance and glucose homeostasis. As mediators of insulin resistance, cell death and inflammation (25), ceramides and related sphingolipids can interfere with insulin signaling (26), which suggests that they are important

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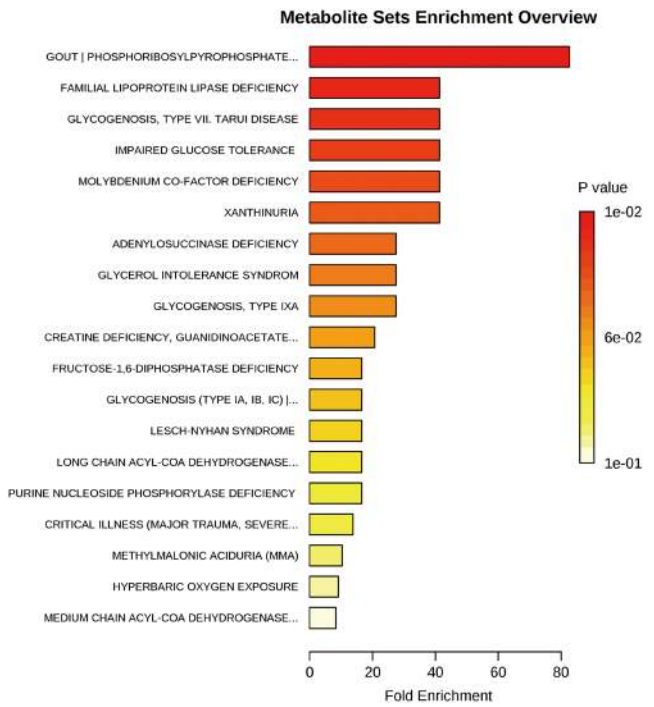

Figure 4
Metabolite overview of the set of enriched biomarkers.

players in NAFLD. Bioinformatics strategies and gene array analyses have demonstrated a strong association between the hepatic ceramide levels and hepatic fat content (27, 28). For instance, the ceramide concentration was much higher in obese individuals (29), and a supervised aerobic exercise intervention significantly reduced plasma ceramide in obese individuals with T2DM, possibly due to its ability to improve insulin resistance (30, 31). Moreover, lifestyle-induced weight loss resulted in a lower serum ceramide concentration (32), which led to significant alterations in the regulation of ceramide biosynthesis genes in the liver, especially CER (ceramide synthase) and SPTLC (serine palmitoyltransferase) expression (33). Interestingly, weight loss was the primary outcome of the effect on NAFLD patients in our clinical trial, and the effect of the exercise intervention on the IHTG content reduction was most likely mediated by weight loss, because it was no longer significantly associated with the IHTG content reduction after adjustment for weight loss. Therefore, the significant alterations in the sphingolipid content after exercise in the NAFLD patients were unsurprising. In our study, we found that some sphingolipids were decreased in the serum after exercise, which was in accordance with previous reports, whereas others were increased unexpectedly. This discrepancy may be due to different sphingolipid species associating with either protective or harmful features of glucose tolerance and insulin sensitivity (33). These different species may represent novel therapeutic targets for nonalcoholic liver disease in the future.

Several studies have highlighted the significance of exercise for NAFLD, but these studies have not assessed the metabolic profiles or related pathways. To the best of our knowledge, this study is the first to explore the metabolomics characteristics of NAFLD patients with/without long-term exercise, and the clinical efficiency-based metabolomics study is more convincing than normal animal experiments. Although plasma contains a multitude of lipids that may have utility as biomarkers for NAFLD and lipids are most likely responsible for the exercise effect (14), and metabolic disorders of lipid has been implicated in the pathogenesis of NAFLD, the types of lipids involved are poorly understood. Our present data clearly revealed significant changes in certain key lipids and captured comprehensive alterations mostly in glycerophospholipids and sphingolipids. NAFLD improvement by exercise is very likely via regulation of glycerophospholipids and sphingolipids metabolism, these targeted pathways involved provide novel insights into the mechanism of the exercise effect.

Although our present work found some novel results and helped fill part of the gap in this aspect of the influence of exercise on NALFD, some limitations should still be a cause for concern. First, the specific disease stages of the participants selected in our clinical trial were not diagnosed, and all subjects were included in the same category, although NAFLD includes steatosis, nonalcoholic steatohepatitis and cirrhosis. A few studies showed significant differences in the lipid content among these stages, which meant that intragroup differences were not sufficiently evaluated in our work. Moreover, all participants attended a health education session and might have adopted a low-fat diet automatically. These factors may contribute to the contradictions between our present work and other studies. In addition, the clinical serum samples were collected after a long-term exercise intervention; therefore, this experiment will be difficult to repeat in other labs. Evidence has shown that exercise training induces changes in phospholipid species, while how exercise training induces these changes and what roles these phospholipids play in the functional changes of exercise-trained skeletal muscle are not fully understood, since skeletal muscle is the major contributor to insulin-stimulated glucose tolerance and whole-body energy expenditure (34). Despite these limitations, the current findings identified molecular species that could

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provide the underlying metabolic mechanism of the effect of exercise on NAFLD in humans, and another feasible method for determining the improvement of NAFLD by quantitative analysis of these altered metabolites using thin-layer chromatography (TLC), imaging mass spectrometry (IMS) (18) and other useful analytical tools such as nuclear magnetic resonance (NMR), liquid chromatography-mass spectrometry(LC-MS) and gas chromatography-mass spectrometry (GC-MS). This topic has not been studied previously and may provide a basis for other similar studies.

This work demonstrated that alternation of the glycerophospholipid and sphingolipid profiles partly explained the beneficial effects of exercise training on NAFLD patients through improvement of insulin resistance. However, the effects of this training and the related genes and/or proteins need further clarification in targeted studies based on our present metabolomics study.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/EC-19-0023.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Study concept and design: Jia Li, Yan Zhao, Zhong Chen, Xuejun Li; acquisition of data: Jia Li, Yan Zhao; analysis and interpretation of data: Jia Li, Yan Zhao; drafting of the manuscript: Jia Li; critical revision of the manuscript for important intellectual content: Xuejun Li, Zhong Chen; statistical analysis: Jia Li, Yan Zhao; obtained funding: Xuejun Li; administrative and technical support: Caoxin Huang, Zheng Chen, Xiulin Shi; study supervision: Xuejun Li, Zhong Chen.

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