Int J Clin Exp Med 2021;14(2):789-799
www.ijcem.com /ISSN:1940-5901/IJCEM0116698

Original Article

Disorder of serum lipid metabolism in patients with postmenopausal osteoporosis based on untargeted lipidomics

Xiangyu Cao, Lijun Deng, Gaojin Zhou, Lianpeng Wang, Yajun Han, Guohua Li

Department of Orthopedics, The Second Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang Uygur Autonomous Region, China

Received June 1, 2020; Accepted November 20, 2020; Epub February 15, 2021; Published February 28, 2021

Abstract: Objective: To profile the lipid metabolic disturbances in patients with postmenopausal osteoporosis (PMOP) based on untargeted lipidomics, and investigate the pathogenesis of lipid metabolism disorder in PMOP. Methods: Patients were selected from orthopedics clinic of the Second Affiliated Hospital of Xinjiang Medical University from October 2018 to April 2019. Dual energy X-ray absorptiometry (DXA) was used to measure the bone mineral density (BMD). A total of 147 natural menopause women were scanned with DXA, including 55 in the normal group and 36 in PMOP group. Lipidomic analysis was performed by liquid chromatography-mass spectrometry (LC-MS) technique. Differential metabolites were analyzed for pathway enrichments and the correlation with BMDs. Results: Phosphatidylcholine (PC) (18:0/20:4), Triglyceride (TG) (16:0/10:0/20:4), Cardiolipin (CL) (19:0/18:2/20:0/22:6), CL (75:4), PC (36:5), and TG (54:4) expressions were significantly increased in PMOP patients, while PC (36: 2), CL (22:3/18:0/18:0/20:4), Lyso-phosphatidylcholine (LPC) (18:1), and Sphingomyelin (SM) (d16:0/18:1) expressions were reduced. Choline metabolic pathway, glycerophospholipid metabolic pathway, retrograde endogenous cannabinoid signal pathway, linoleic acid metabolic pathway, α-linolenic acid metabolic pathway and arachidonic acid metabolic pathway were involved. PC (18:0/20:4), TG (16:0/10:0/20:4), and PC (36:5) were negatively correlated with BMDs; while PC (36:2), LPC (18:1), and SM (d16:0/18:1) were positively correlated. Conclusion: There is a significant difference in plasma lipid composition between PMOP patients and normal patients. Disorders of lipid metabolism may be related to the pathogenesis of PMOP. Lipidomic analysis may provide a theoretical basis for early diagnosis, prevention and treatment of PMOP.

Keywords: Menopause, osteoporosis, plasma, non-targeted lipidome, differential lipid metabolites, pathway of lipid metabolites, BMD

Introduction

Postmenopausal osteoporosis (PMOP) is a common disease in postmenopausal women with an incidence of as high as 20~50%. Its main pathological features are characterized by reduced bone mass, degeneration of bone tissue microstructure and increased bone fragility, which are easy to cause pathological bone quality. It has become a social problem that seriously threatens the quality of life and physical health of middle-aged and elderly women [1, 2]. Studies have found that PMOP is closely related to changes in plasma high-density lipoprotein cholesterol (HDLC) levels [3], suggesting that phosphate metabolism disorders may have a certain relationship with the incidence of PMOP. Lipids serve as structural components of cell membranes and primarily function as energy storehouses. Lipids are involved in various physiological processes such as growth and development, nerve signal transduction, and the disorder of lipid metabolism is related to the occurrence and development of various diseases [4, 5]. Therefore, the analysis and research on the regulation of lipid metabolism and biological functions in vivo are of great significance for the discovery of potential biomarkers of diseases. Lipidomics is a new method to study the characteristics of lipid molecules in vivo. We used liquid chromatography-mass spectrometry (LC-MS) [6] to perform non-targeted lipidomic analysis on serum samples of naturally menopausal women for exploring the characteristics of lipid metabolism in patients with PMOP, to explore the pathogenesis

of lipid metabolic disorders in PMOP, and to provide theoretical basis for early diagnosis, prevention and treatment of PMOP.

Materials and methods

Patient samples

The patients were selected from orthopaedics clinic of the Second Affiliated Hospital of Xinjiang Medical University from October 2018 to April 2019. A total of 147 natural menopause women were scanned with DXA [7], aged 45 to 72 (57.51 ± 4.59) years. Osteoporosis was diagnosed according to the World Health Organization diagnostic criteria for osteoporosis [8]. The T value ≥ -1.0 was defined as the normal bone mass group, and the T value ≤ -2.5 is the osteoporosis group. Inclusion criteria: (I) female; (II) natural menopause for more than 1 year; (III) age ≤ 75 years old. Exclusion criteria: (I) patients received alendronate, teriparatide, raloxifene, strontium ranelate, estrogen, vitamin D, calcium, lipid-lowering drugs, and glucocorticoids within 12 months; patients received bone metabolic drugs; (II) patients with diabetes, hyperthyroidism, liver or kidney dysfunction, hypercortisolism, bone tumors, gastrointestinal diseases and other diseases; (III) patients with malignant tumors and other serious diseases; (IV) patients with bad habits such as alcoholism and smoking; (V) patients with scoliosis, thoracic kyphosis, degenerative hyperplasia of the vertebral body, compression fractures of spinal trauma; (VI) patients with history of fracture surgery; (VII) patients who had received radiation therapy. This study followed the Helsinki Declaration and was approved by the Ethics Committee of the Second Affiliated Hospital of Xinjiang Medical University. All subjects signed informed consent form.

Main reagents and instruments

Acetonitrile and isopropanol (chromatography pure, Merck, Germany), methanol (chromatography pure, Thermo Fisher, America), bone density measuring instrument DPX-MD (Hologic, USA), vortex oscillator TYXH-1 (Shanghai Khan-Nuo, China), high-speed freezing centrifuge TGL-16MS (Shanghai Luxiang, China), multi-sample tissue grinding Tissuelyser-48 (Shanghai Jingxin, China), high-resolution mass spectrometer Q Exactive (Thermo Fisher, America), high-performance liquid chromatograph Nexera UPLC (Shimadzu, Japan), chromatography column ACQUITY UPLC BEH C18 (100 × 2.1 mm, 1.7 μm) (Waters, American).

Bone mineral density (BMD)

All subjects were scanned with DXA under resting conditions. Lumbar spine (L_1-L_4), left femoral neck and total hip BMD and T value were measured in all subjects by DXA. The evaluation was performed by professionally trained personnel who had been professionally trained and worked for more than 5 years. The subjects lied on their back in a supine position, and the instrument was calibrated before each measurement. Two measurements were performed and the average value was taken to analyze the results.

Collection and preparation of the serum samples

Venous blood samples (2-5 ml) were obtained from each subject early in the morning after overnight fasting, which were anticoagulated with sodium heparin and stored at room temperature for 30 minutes. The supernatant was aliquated and stored at -80°C. Upon thawing, 100 μL of plasma was taken and mixed with 300 μL isopropanol, then vortexed for 30 seconds. The sample was extracted with ultrasonication for 10 min and left to stand for 30 min at -20°C. Then the mixture was centrifuged at 1200 rpm for 1 min at 4°C. Then, 300 μL supernatant was taken and put into centrifuge tube and added with 200 μL Isopropanol, vortexed for 30 s. After that, ultrasonic extraction in ice water bath was performed for 10 min. It was then reconstituted with ultrasound for 3 min and centrifuged at 4°C at 12000 rpm for 10 min. Finally, 150 μL of supernatant was taken and put into the injection vial with liner for LC-MS analysis. Quality control (QC) samples were prepared and mixed with 10 ul of all the samples, which were used to evaluate the stability of the mass spectrometry platform of the experimental process system.

Lipidomics analysis of serum samples

The ACQUITY UPLC chromatography platform was used for sample separation, and the Q Exactive mass spectrometry system was used to perform qualitative and quantitative analysis of lipid in plasma samples.

The chromatographic conditions: chromatography column ACQUITY UPLC BEH C18 (100 mm

Table 1. Comparison of BMD in different parts of normal bone mass group and osteoporosis group (g/cm^2)

Position	n	Lumbar spine (L_1-L_4)	left femoral neck	total hip
normal bone mass group	55	0.982±0.13	0.899±0.12	0.955±0.14
osteoporosis group	36	0.894±0.12	0.825±0.12	0.857±0.11
t value		-4.247	-3.715	-4.954
P value		0.000	0.000	0.000

× 2.1 mm, 1.7 μm). Mobile phase A: acetonitrile-water (60:40, V/V, containing 10 mmol/L ammonium formate, 0.1% formic acid); mobile phase B: isopropanol-acetonitrile (90:10, V/V, containing 10 mmol/L Ammonium formate, 0.1% formic acid). Linear gradient elution conditions: 0-1.5 min, 32% B; 1.5-18.5 min, 32-97% B; 18.5-22 min, 97% B. Before each injection, the column was equilibrated in the initial mobile phase for 5 min. The column temperature was 45°C, the injection volume was 5 μL, and the flow rate was 0.35 mL/min.

The MS conditions: the use of heated electrospray ionization (HESI) positive and negative ion scanning mode. Heater temperature 300°C, sheath gas flow 45 arb, auxiliary gas flow 15 arb, sweep gas flow 1 arb, spray voltage 3.5 KV, capillary temperature 320°C, S-Lens RF level 50%, MS1 scanning range: 120-1800 Da. 10 debris maps (MS2 SCAN, HCD) were collected per scan. The resolution of MS1 at M/Z 200 was 70,000, and the resolution of MS2 at M/Z 200 was 17,500.

Data processing

Using Lipid Search software (Thermo Fisher, USA) to extract, identify the LC-MS detection data and align the peaks, which were merged into a two-dimensional matrix. MetaboAnalyst 4.0 software was used for statistical analysis and metabolic pathway enrichment analysis. Using principle component analysis (PCA) and orthogonal-partial least squares-discriminant analysis (OPLS-DA) to conduct multivariate statistical analysis. T-test and fold change analysis were used to compare differences between groups. OPLS-DA model variable weight value (VIP) > 1 and t test *P* value < 0.05 were used to screen differential lipid metabolites. VIP was used to measure the response strength of each metabolite expression, and T test was used to verify whether the differences in metabolites were significant. Based on KEGG (https://www.kegg.jp/) database, the pathway enrichment analysis of different lipid metabolites was carried out, and the signal pathway satisfying the threshold of $P < 0.05$ was regarded as the signal pathway with significant enrichment. Pearson correlation analysis was carried out to analyze the correlation between different lipid metabolites and BMD in osteoporosis patients, and $P < 0.05$ was considered to be correlated.

Result

BMD determinations

Dual energy X-ray absorptiometry (DXA) was used to measure bone mineral density (BMD) in lumbar vertebrae (L_1-L_4), left femoral neck and total hip in 147 natural menopause women, including 55 in the normal bone mass group with $T \geq -1.0$ and 36 in the osteoporosis group patients with $T \leq -2.5$. The BMDs of the osteoporosis group in lumbar vertebrae (L_1-L_4), left femoral neck and total hip were significantly lower than those of the normal bone mass group ($P < 0.01$) (**Table 1**).

Quality control of plasma specimen lipidomics data

During the mass spectrometry process, a QC sample was inserted into every eight formal samples. The QC sample was used to evaluate the stability of the system's mass spectrometry platform throughout the experiment. The QC sample base peak chromatogram (BPC) of the strongest ions in the chromatogram of each time point was described continuously, and BPC was compared with the spectrum overlap. The response intensity and retention time of each chromatographic peak basically overlapped, which indicated that the lipid components of each group were well separated (**Figures 1** and **2**).

Multivariate statistical analysis

The PCA model (**Figure 3**) and the OPLS-DA model (**Figure 4**) of positive and negative ion model data were established. The OPLS-DA model can distinguish between the normal bone mass group and the osteoporosis group, and there was no obvious overlap between the two groups. The permutation test of the OPLS-DA model (**Figure 5**) indicated that the OPLS-DA

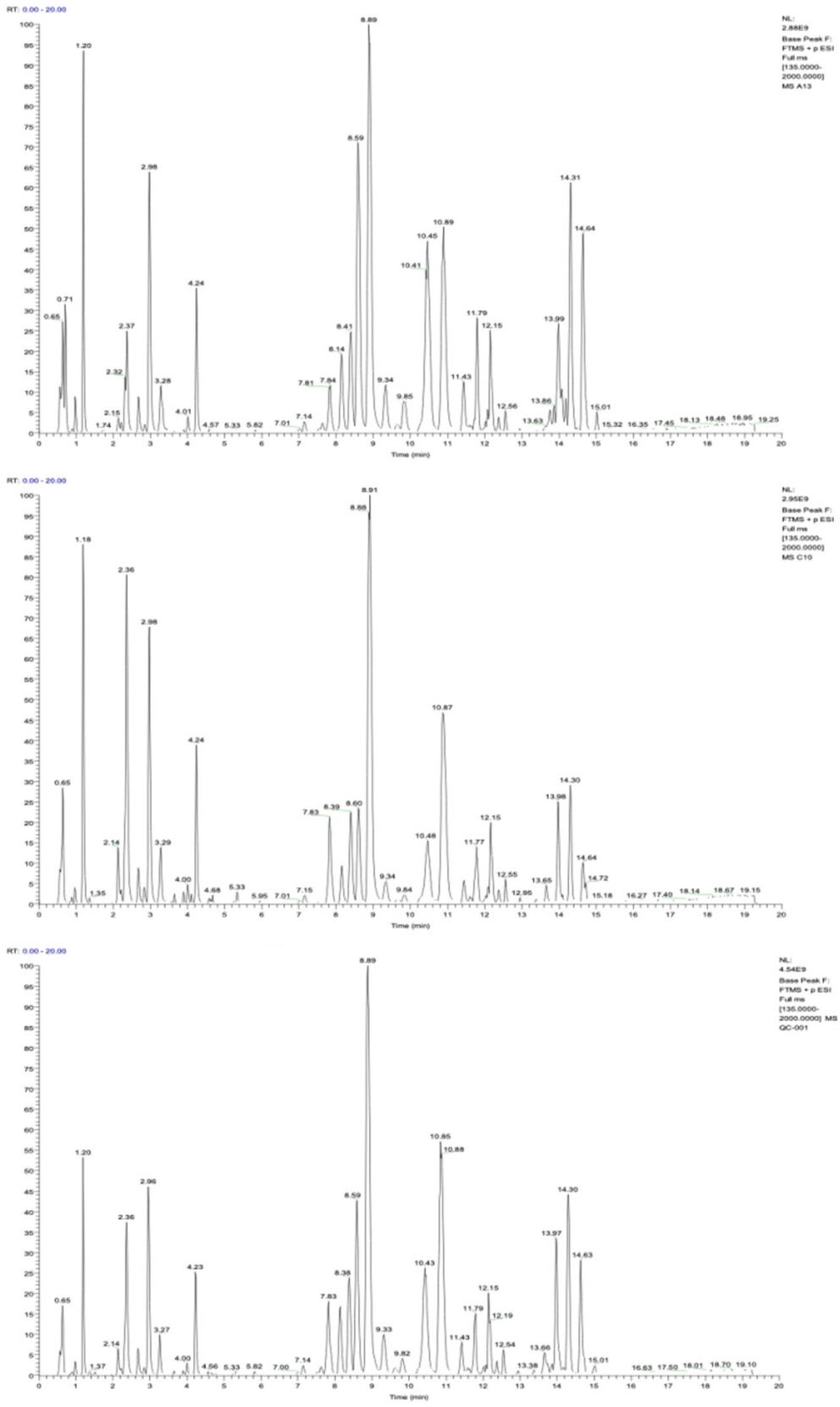

Figure 1. Overlap of BPC in positive ion mode.

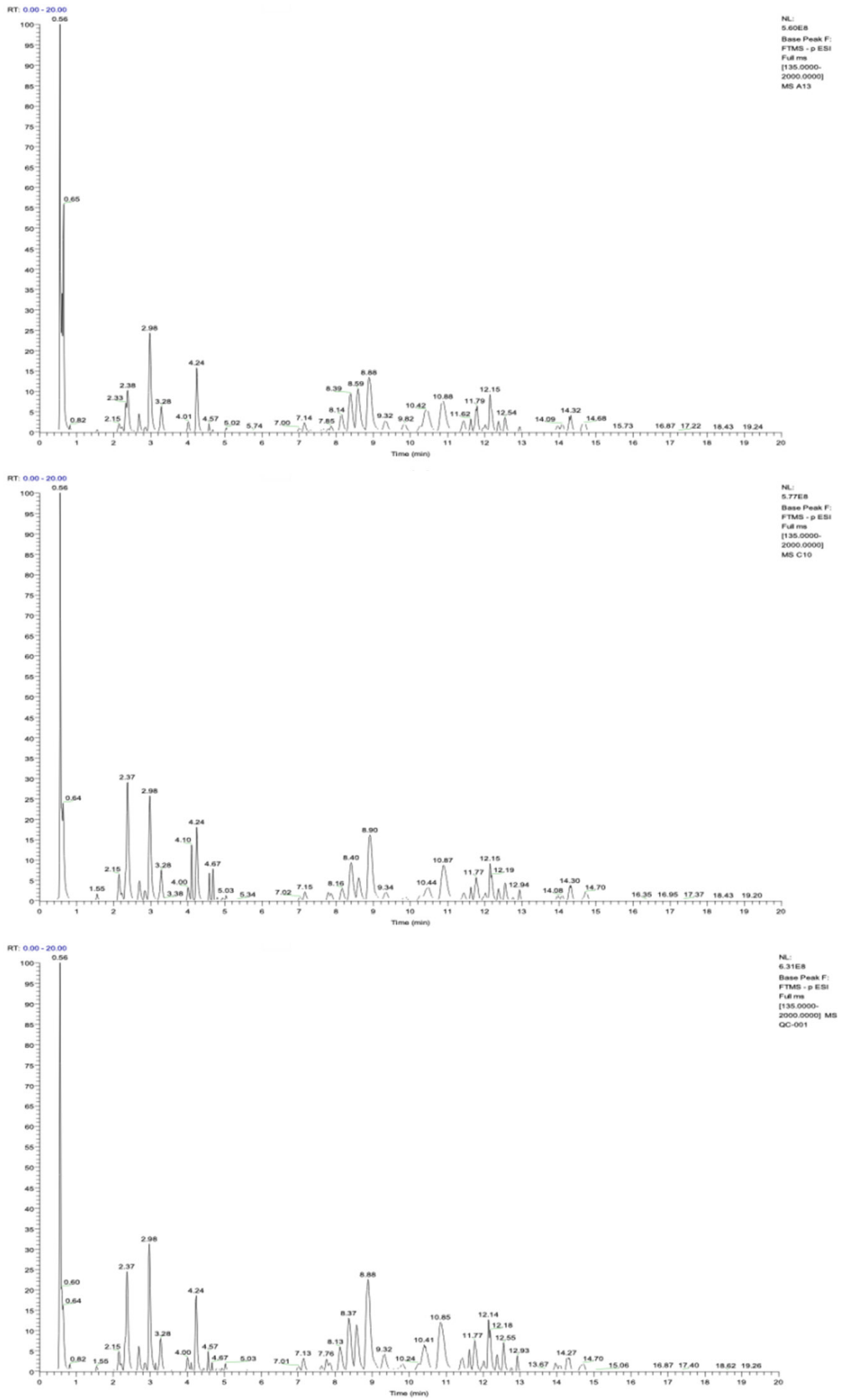

Figure 2. Overlap of BPC in negative ion mode.

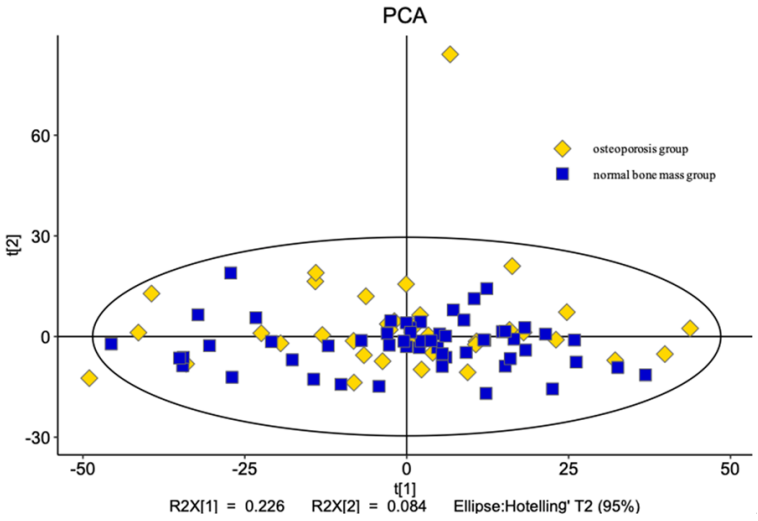

Figure 3. PCA diagram of untargeted lipidomics in osteoporosis group and normal bone mass group.

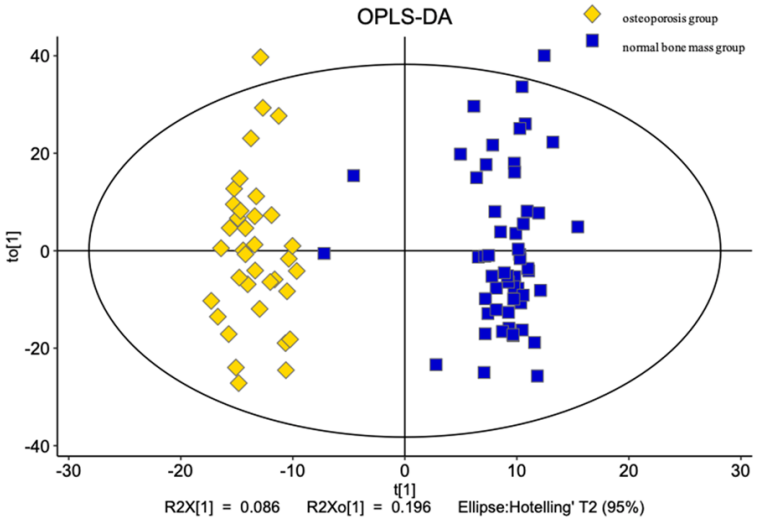

Figure 4. OPLS-DA diagram of non-targeted lipidomics in osteoporosis group and normal bone mass group.

model had not been overfitted and the model was reliable. VIP value > 1 and t test *P* value < 0.05 were used as the screening criteria for different lipid metabolites. VIP was used to measure the response strength of each metabolite expression, and t test was used to verify whether the different metabolites were significant.

Screening of significantly different lipid molecules

The t-test was used to compare the expression levels in detected lipid components (**Figure 6**). In the volcanic diagram, the red origin represents significantly up-regulated differential metabolites, the blue origin represents significantly down-regulated differential metabolites, and the gray point represents insignificant differential metabolites. A total of 10 differential lipid metabolites were screened with VIP > 1 and P < 0.05 as the screening criteria for differential lipid metabolites (**Figure 7**; **Table 2**). The expression of different lipid metabolites by hierarchical cluster analysis (Hierarchical Clustering, HC) yields the difference of lipid metabolites heat map (**Figure 7**); the abscissa represents sample name, ordinate represents the difference of lipid metabolites, color from green to red represents metabolites expression abundance from low to high, and the more red color indicates that difference in metabolites expression abundance was higher. The 10 kinds of different lipid metabolites included phosphatidylcholine (PC), triglycerides (TG), Cardiolipin (CL), lysophosphatidylcholine (LPC), and sphingomyelin (SM). PC (18:0/20:4), TG (16:0/10:0/20:4), CL (19:0/18:2/20:0/22:6), CL (75:4), PC (36:5), Tand G (54:4) in the osteoporosis group were significantly higher than those of the normal bone mass group, while PC (36:2), CL (22:3/18:0/18:0/20:4), LPC (18:1), and SM (d16:0/18:1) were significantly lower. The difference was statistically significant (P < 0.01).

Pathway enrichment analysis of differential lipid metabolites

Based on KEGG (https://www.kegg.jp/) database, the pathway enrichment analysis of different lipid metabolites was carried out, and the signal pathway satisfying the threshold of P < 0.05 was regarded as the signal pathway with significant enrichment. The results of pathway enrichment analysis of 10 metabolites showed

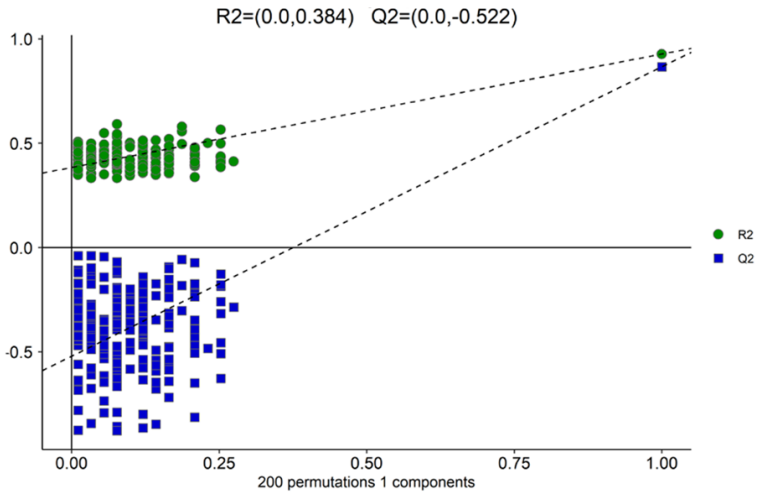

Figure 5. Permutation test of OPLS-DA models.

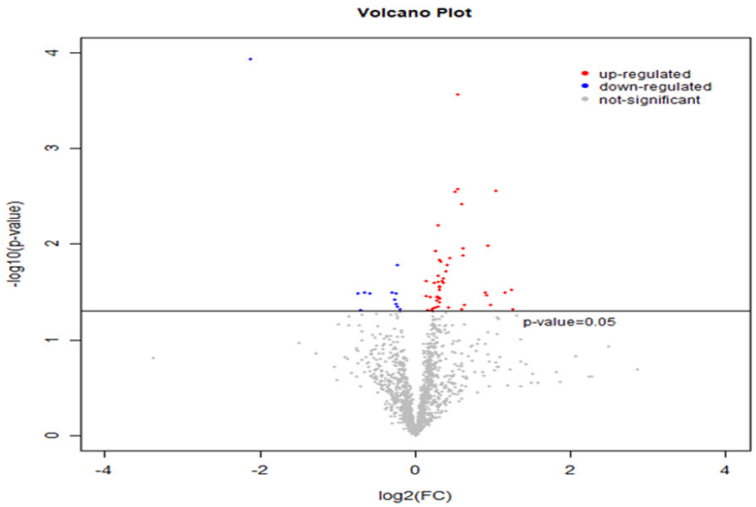

Figure 6. Volcano diagram of differential lipid molecules.

that the metabolic pathways containing more than one biomarker were choline metabolism, glycerophospholipid metabolism, retrograde endocannabinoid signaling pathway, linoleic acid metabolism, α-linolenic acid metabolism, and arachidonic acid metabolism. Ten different lipid metabolites were metabolized by 6 metabolic pathways. Enrichment analysis was performed to draw the bubble diagram of the metabolic pathway (**Figure 8**). The ordinate was the name of the metabolic pathway and the abscissa was the enrichment factor (Rich factor = significant difference in the number of metabolites/total number of metabolites in the signal pathway). The greater the enrichment factor was, the greater the enrichment degree was. The color from red to green indicated that the *P* value decreased successively. The larger the point, the higher the number of metabolites in the fuset pathway.

Analysis of correlation between different lipid metabolites and BMD in osteoporosis patients

Pearson correlation analysis showed that PC (18:0/20:4), TG (16:0/10:0/20:4) and PC (36:5) were negatively correlated with lumbar spine (L_1-L_4), left femoral neck and total hip BMD. While PC (36:2), LPC (18:1), SM (D16:0/18:1) were positively correlated, and CL (19:0/18:2/0/22:6) and CL (22:3/18:0/18:0/20:4) had no correlation (**Table 3**).

Discussion

Postmenopausal female osteoporosis has gradually increased with the aging of the social population, and has become a social problem affecting the quality of life and physical and mental health of middle-aged and elderly women [2]. PMOP occurs mainly due to women's postmenopausal ovarian function decline, estrogen level decline, bone absorption higher than bone formation, resulting in bone loss, osteoporosis, low back pain, length shortening, hunchback and fragility fractures and other complications [9]. Studies have found that postmenopausal women with hypercholesterolemia can accelerate the loss of bone mass and cause osteoporosis [10]. Therefore, studying the changes in lipid metabolites and lipid metabolism disorders in PMOP patients, early detection of PMOP high-risk groups and early diagnosis are of great significance for the prevention and treatment of PMOP.

With the decrease of estrogen levels and redistribution of body fat in postmenopausal women, the lipid composition in the serum also changes accordingly. In this study, 147 postmeno-

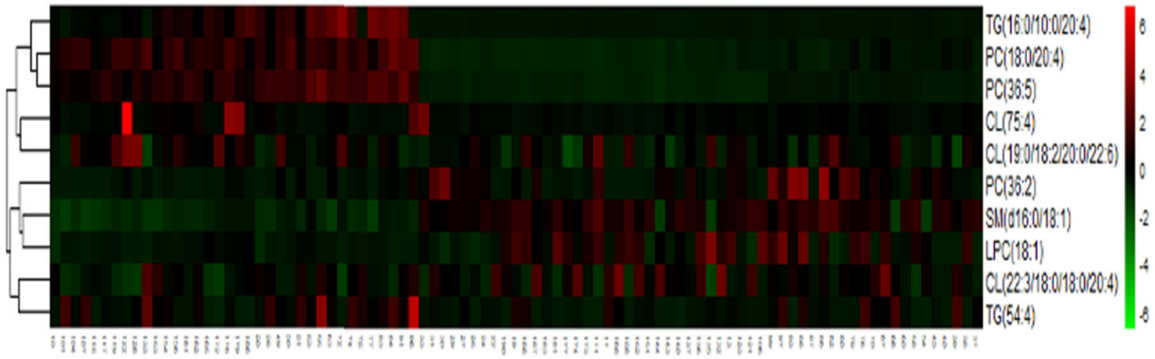

Figure 7. Heat map of differential lipid metabolites based on untargeted lipidomics.

Table 2. Differential lipid metabolites based on untargeted lipidomics

Ion mode	(m/z)	retention time	lipid	VIP	fold change	*P* value
neg	854.5917	10.53523	PC (18:0/20:4)	21.72073	6.685092	1.35E-34
pos	788.6763	12.40055	TG (16:0/10:0/20:4)	3.332531	17.80423	7.84E-14
neg	772.5336	9.263217	CL (19:0/18:2/20:0/22:6)	1.14956	1.184764	0.025369
neg	748.5336	10.08751	CL (75:4)	1.291081	2.214669	0.031807
pos	786.6007	10.40383	PC (36:2)	1.005257	0.227457	0.000115
pos	780.5538	9.141374	PC (36:5)	3.031224	5.780065	1.79E-36
neg	766.5336	10.97835	CL (22:3/18:0/18:0/20:4)	1.645997	0.854103	0.044867
pos	900.8015	14.57399	TG (54:4)	1.292158	2.050635	0.002779
neg	566.3463	3.367196	LPC (18:1)	6.851338	0.400457	2.04E-05
neg	747.5658	8.444172	SM (d16:0/18:1)	31.87923	0.266177	1.48E-19

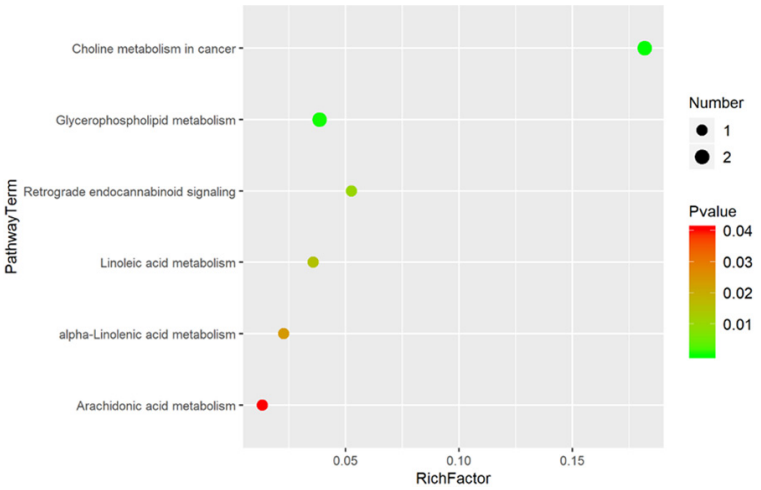

Figure 8. The bubble diagram of pathway enrichment analysis of differential lipid metabolites.

pausal women were measured for BMD, 55 normal bone mass and 36 osteoporotic serum samples were selected, and the composition of lipid metabolites of the serum samples was comprehensively analyzed by LC-MS lipidomics qualitatively and quantitatively. There are significant changes in lipid metabolites and metabolic pathways in serum samples, and a total of 10 significantly different lipid molecules were detected, including PC (18:0/20:4), TG (16:0/10:0/20:4), CL (19:0/18:2/20:0/22:6), CL (75:4), PC (36:5), and TG (54:4), which were higher in patients with osteoporosis than those with normal bone mass ($P < 0.05$). Among them, PC (36:2), CL (22:3/18:0/18:0/20:4), LPC (18:1), and SM (d16:0/18:1) were significantly lower than those with normal bone mass. Pathway enrichment analysis showed that these 10 differential lipid metabolites passed through the choline metabolism pathway, glycerophospholipid metabolism pathway, retrograde endocannabinoid signaling pathway, linoleic acid metabolism path-

Table 3. Correlation analysis between the differential lipid metabolites and BMD

Differential lipid metabolites	lumbar (L_1-L_4) BMD		left femoral neck BMD		total hip BMD	
	r value	*p* value	r value	*p* value	r value	*p* value
PC (18:0/20:4)	-0.849	0.000	-0.524	0.000	-0.558	0.000
TG (16:0/10:0/20:4)	-0.638	0.000	-0.393	0.000	-0.418	0.000
CL (19:0/18:2/20:0/22:6)	-0.201	0.056	-0.066	0.537	-0.133	0.209
CL (75:4)	-0.229	0.029	-0.194	0.065	-0.193	0.067
PC (36:5)	-0.826	0.000	-0.541	0.000	-0.556	0.000
TG (54:4)	-0.249	0.017	-0.067	0.529	0.023	0.832
PC (36:2)	0.361	0.000	0.298	0.004	0.399	0.000
CL (22:3/18:0/18:0/20:4)	0.187	0.076	0.145	0.171	0.164	0.120
LPC (18:1)	0.392	0.000	0.419	0.000	0.391	0.000
SM (d16:0/18:1)	0.679	0.000	0.388	0.000	0.436	0.000

way, α-linolenic acid metabolism pathway, and arachidonic acid metabolic pathway for metabolism. Pearson correlation analysis of 10 differential lipid metabolites found that PC (18:0/20:4), TG (16:0/10:0/20:4), PC (36:5) and lumbar spine (L_1-L_4), left femoral neck and total hip BMD were negatively correlated; PC (36:2), LPC (18:1), and SM (d16:0/18:1) were positively correlated; CL (19:0)/18:2/20:0/22:6) and CL (22:3/18:0/18:0/20:4) had no correlation, and CL and BMD had no correlation. This may be related to the different levels of BMD in different parts. This result shows that there is a significant difference between the lipid composition of PMOP patients and those with normal bone mass. Differential lipid metabolites are metabolized through different lipid metabolism pathways, and their differentially expressed lipid metabolites may participate in the pathogenesis of PMOP. From the perspective of lipid metabolite changes, PC, TG, CL, and SM lipids have changed through different metabolic pathways. PC is the main component of cell membrane and is metabolized by the choline pathway. Among the glycerophospholipids, LPC is the main component of oxidized low density lipoprotein (ox-LDL). Ox-LDL can promote bone marrow mesenchymal stem cells (mesenchymal stem cell, MSC) to differentiate into adipocytes and inhibit their differentiation into osteoblasts, accelerating the occurrence of osteoporosis [11]. TG belongs to glycerolipids, and the main components are very low density lipoprotein (VLDL) and chylomicrons [12]. TG (16:0/10:0/20:4) and TG (54:4) are significantly increased in patients with osteoporosis, and the abnormal accumulation of fatty and lipid peroxidase can promote the expression of interleukin-6 (IL-6), IL-1β, IL-8 and other inflammatory factors, and induce oxidative stress in the body. The production of reactive oxygen species (ROS) increases, and ROS causes oxidative damage to lipids, proteins and DNA, which inhibits osteoblast-mediated bone formation, leading to osteoporosis [13]. CL is a kind of mitochondrial cardiolipin; abnormal metabolism can lead to tricarboxylic acid cycle disorder and cell apoptosis and necrosis [14]. SM belongs to sphingomyelin, which generates ceramides (cer) under the action of sphingomyelin hydrolase. The cer can lead to the production of reactive oxygen species (ROS) and oxygen-containing free radicals, thereby inhibiting osteoblast-mediated bone formation and leading to the occurrence of osteoporosis [15]. These differential lipid metabolites form fat emboli in the peripheral blood of PMOP patients through different metabolic pathways, resulting in thrombosis, increase in the number of adipocytes in the bone marrow cavity, compression of blood vessels in the bone marrow cavity, disturbance of bone marrow microcirculation, decreased bone metabolism and bone mass loss, leading to osteoporosis [16]. Increased blood lipid levels in postmenopausal women can also lead to abnormal differentiation of osteoclasts in the bone marrow cavity and excessive activation, resulting in bone loss and leading to osteoporosis [17]. Tarakida et al. [18] found that hypercholesterolemia in postmenopausal women can accelerate bone loss and cause osteoporosis. Griffith et al. [19] also proved that lipid components accumulate in blood vessels around bones, which can promote the reduction of BMD in rats. Kim et al. [20] found that BMD of the lumbar spine, pelvis and femoral neck was

correlated with TG and TG/HDLC in patients with hyperlipidemia. Clinical observations have also found that PMOP patients often have lipid metabolism disorders, meanwhile, lipid metabolism disorders have been improved to a certain extent after relevant treatment [3]. These research conclusions are consistent with our research results that lipid metabolism disorders play a very important role in the pathogenesis of PMOP. However, the current research conclusions on the correlation between dyslipidemia and PMOP are not uniform, and some studies have not found its correlation [21], which may be due to the influence of the study population, different races and different genetic backgrounds on lipid metabolism and BMD.

In summary, the results of this study indicate that abnormal lipid metabolism may be involved in the pathogenesis of PMOP. Lipid metabolism disorders are an important cause of PMOP. Early detection of lipid metabolism disorders in postmenopausal women, early prevention and treatment can decrease the occurrence rate of complication such as back pain, kyphosis and fragility fractures.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Guohua Li, Department of Orthopedics, The Second Affiliated Hospital of Xinjiang Medical University, Urumqi 830063, Xinjiang Uygur Autonomous Region, China. Tel: +86-0991-4609011; Fax: +86-0991-4609011; E-mail: xwrobert@163.com

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