Identifying Lipid Metabolites Influenced by Oleic Acid Administration Using High-Performance Liquid Chromatography–Mass Spectrometry-Based Lipidomics

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ABSTRACT: Oleic acid (OA), one of the most important monounsaturated fatty acids, possesses protective properties against chronic liver disease (CLD) development, but the underlying metabolic metabolism remains unknown. HPLC–MS-based lipidomics was utilized to identify and quantify the endogenously altered lipid metabolites when hepatocytes were exposed to OA administration. The identified lipids could be grouped into 22 lipid classes; of which, 10 classes were significantly influenced by the OA treatment: lysophosphatidylcholine (LPC), phosphatidylglycerol (PG), ceramides (Cer), hexasylceramides (Hex1Cer), dihexosylceramides (Hex2Cer), cholesterol ester (ChE), and coenzyme (Co) were decreased, while diglyceride (DG), triglyceride (TG), and acyl carnitine (AcCa) were increased. In addition, as the variable importance in projection (VIP) list (VIP > 1.0 and P < 0.05) showed, 478 lipid species showed significant difference with OA administration, and these molecules could be potential biomarkers in conjunction with OA administration. In summary, our results provided a novel perspective to understand the influences of OA administration by investigating endogenous altered levels of lipid metabolites via lipidomics.

INTRODUCTION

Fatty acids (FA), as one of the most important components of lipids, are associated with growth, development, and disease. According to the number of double bonds, FA can be divided into three types, saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). All of them are linked to human health. Among them, enriched-monounsaturated fatty acid diets have positive effects on chronic liver disease (CLD) prevention. Being the predominant monounsaturated fatty acid, oleic acid (OA) has attracted increasing attention in CLD treatment.

CLD is one of the most common public health problems, and, at the early stage, it is usually linked to the liver fibrosis. Liver fibrosis may worsen into cirrhosis and finally to hepatocellular carcinoma (HCC). Meanwhile, non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) could progress to CLD via liver fibrosis. Various underlying mechanisms of liver disease progression have been demonstrated, and, among them, endoplasmic reticulum (ER) stress and lipotoxicity are generally involved in the progression of liver disease. In fact, SFAs, especially palmitic acid (PA), are toxic for the liver by inducing ER stress and lipotoxicity. However, OA is able to suppress the deleterious effects induced by SFAs.

Evidence has shown that OA could suppress PA-induced negative effects on ER stress and lipotoxicity in hepatocytes via inhibiting the activation of ribosomal protein S6 kinase 1 (S6K1) and, as a consequence, attenuate the progression of NAFLD. Similarly, OA could attenuate the PA-induced hepatic lipotoxicity in both hepatocytes and a NASH model by increasing the excretion of hepatic triglycerides and decreasing the levels of several inflammatory molecules, thus attenuating every step of NAFLD progression.

Actually, CLD is characterized as the hepatic manifestation of the metabolic syndrome and is closely associated with disordered lipid metabolism, while OA could regulate lipogenesis and lipid secretion in HepG2 cells. However,
the underlying metabolic metabolism of OA remains unclear. Consequently, we utilize lipidomics which relies on the development and application of mass spectrometry (MS) to identify and quantify the endogenous changes in lipid concentrations after OA treatment. Lipidomics, as a significant branch of metabolomics, has been dramatically developing since it was presented in 2003. It provided a new perspective to intuitively visualize the changes of lipid metabolites when exposed to physiological changes and stimulation conditions.

In summary, in this study, HPLC–MS-based lipidomics was utilized to investigate endogenous changes in lipid metabolite concentrations with OA administration, and this technique provided a novel insight revealing the effects of OA administration on lipid metabolism in hepatocytes.

**RESULTS**

**Statistical Analysis of Lipid Metabolites from OA-Treated Groups and Control Groups.** Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) score plots were per-
Figure 3. Volcano plots were obtained from all of identified molecules from the positive and negative modes. The threshold change was set as 2.0. The red points are the metabolites presented at significantly different amounts after OA treatment. Black points indicate compounds not influenced by OA treatment. The lipid metabolites to the right of the right threshold increase with OA administration, while those to the left of the left threshold decrease. FC = fold change.

Figure 4. Heat map of endogenous metabolites between OA-treated groups (n = 7) and control groups (n = 7). The heat map was obtained from all identified lipids from the positive and negative modes. Red-filled and blue-filled lines indicate increased and decreased levels of lipids, respectively. The fold change is indicated.
formed to determine the differences between OA-treated groups and control groups (Figure 1). Obviously, a distinct separation between OA-treated groups and control groups was observed in the PCA score plots, and the same trend was detected in OPLS-DA score plots. A high repeatability of each group was also detected. In addition, 200 times of chance permutations were carried out to evaluate the predictability of the OPLS-DA model (Figure 2). The $R^2$ and $Q^2$ values were 0.93 and 0.607, respectively, and the y intercept was $-0.508$. The $R^2$ reflected the difference of the principal components, and the closer $R^2$ gets to 1, the more different the principal component gets. $Q^2$ represented the predictability of the OPLS-DA model. Only when $Q^2 > 0.5$, the OPLS-DA model possessed predictability. The negative y intercept indicated that the model was not overfitting. Taken together, OPLS-DA analysis presented significant separation and positive predictability.

More than 1000 different lipid compounds were identified in the OA-treated and control groups. Volcano plots based on all of the identified lipid metabolites are presented in Figure 3. As reported, the parameters of significance threshold and fold change set at $P < 0.05$ and 1.2, respectively, were enough to identify metabolites with significant difference. In our results, a large number of lipids were present at significantly higher or lower amounts for the fold change set at 2.0, which was more valid, indicating that OA administration significantly regulated lipid metabolism.

The heat map obtained from all of the identified lipid metabolites was used to directly visualize the trend of lipid metabolite changes after OA treatment and assess their hierarchical cluster. As the heat map result showed, a large proportion of lipid metabolites was decreased after OA treatment (Figure 4).

The variable importance in projection (VIP) list (VIP > 1.0) was obtained from OPLS-DA, showing the components most influenced by the OA treatment.

**Identification of Lipid Metabolites by HPLC–MS.** The identified lipids influenced by OA treatment were grouped into 22 classes belonging to the four categories including phospholipids, sphingolipids, neutral lipids, and “fatty acyl and other lipids”. The 22 classes were as follows: phospholipids: lysophosphatidylethanolamine (LPE), lysophosphatidylcholine (LPC), phosphatidylglycerol (PG), phosphatidylglycerols (PI), phosphatidylserines (PS), phosphatidylethanolamines (PE), phosphatidylcholines (PC), and cardiolipin (CL); sphingolipids: hexosylceramides (Hex1Cer), trihexosylceramides (Hex3Cer), ceramides (Cer), gangliosides (GM3), sphingomyelins (SM), glycero phosphocholines (SPH), and dihexosylceramides (Hex2Cer); neutral lipids: cholesterol ester (ChE), diglyceride (DG), and triglyceride (TG); and fatty acyl and other lipids: N-acetylglactosamine (AEA), FA, acyl carnitine (AcCa), and coenzyme (Co). The summary signs for the identified lipid classes between OA-treated groups and control groups are presented in Table 1.

**Significant Changes of Lipid Metabolites in OA-Treated Groups.** The statistical results obtained from Table 1 followed by t-tests were used for comparing differences in the summary signals for the compounds in each of the 22 identified lipid classes between OA-treated groups and control groups. Differences were detected in 10 of the 22 classes (Figure 5). First, LPC and PG were significantly decreased when comparing with control groups. Second, Hex1Cer, Hex2Cer, and Cer presented similar trends, and they were dramatically reduced after OA treatment. As components of neutral lipids, ChE was notably decreased, conversely, DG and TG were both increased when comparing with control groups, and TG presented significant difference, while the change in absolute of DG was relatively small. Furthermore, AcCa and Co as constituents of fatty acyl compounds showed an opposite tendency. The content of AcCa was clearly elevated by OA treatment, while Co was significantly decreased in OA-treated groups.

Additionally, changes in LPC (18:0) and PG (16:0_18:1) (Figure 6), Cer (d18:1_22:0) and Cer (d18:1_24:0) (Figure 7), Hex1Cer (d18:1_22:0), Hex1Cer (d18:1_23:0), Hex1Cer (d18:1_24:0), and Hex1Cer (d18:1_24:1) (Figure 8), and ChE (22,6) (Figure 9) were shown as representative lipids involved in liver disease development. These were all significantly decreased with OA administration.

**DISCUSSION**

In this study, lipidomics based on HPLC–MS was used to identify and quantify lipid compounds. As the results showed, more than 1000 lipid species were identified and grouped into four lipid categories, containing phospholipids, sphingolipids, neutral lipids, and “fatty acyl and other lipids”. Among them, some lipid classes were significantly influenced by OA treatment.

Phospholipids remained generally relatively constant with OA administration, except for LPC and PG, which were significantly decreased after OA treatment. LPC was regarded as the death effector of lipoapoptosis in hepatocytes, stimulating the development of NAFLD, and, as a consequence, the content of LPC in the liver of patients with NAFLD was significantly higher than that of control groups. Also, the content of LPC in the liver of patients with NASH seemed to be higher than in NAFLD groups. Furthermore, LPC (18:0) is considered as a biomarker of NAFLD progression and was detected by LC-MS-based lipidomics. Berberine showed therapeutic effects on NAFLD via modulating the lipid metabolism. Particularly, LPC (18:0) was significantly decreased after berberine treatment. Similarly, as the VIP list showed, LPC (18:0) was significantly decreased after OA treatment. Therefore, the decreased levels of LPC after OA treatment may potentially excellently explain the effect of OA administration on CLD treatment. The content of PG was dramatically increased in the mice model of NAFLD, and PG (16:0_18:1) was regarded as the potential biomarker of NAFLD. Conversely, PG (16:0_18:1) was significantly decreased with OA administration. Hence, the attenuation of PG would be one of the potential lipid targets of OA administration on NAFLD prevention, and PG (16:0_18:1) could be a potential biomarker of OA on NAFLD prevention.

Sphingolipid changes were detected after OA treatment, and different tendencies were presented among the classes of sphingolipids, but Cer, Hex1Cer, and Hex2Cer were all significantly reduced with control groups. As reported, the content of Cer was significantly elevated in the diet-induced NAFLD mice model. Myricin-induced inhibition of Cer synthesis did rescue the hepatocyte disarray and reduced lipid accumulation, inflammation, and fibrosis, indicating that inhibited Cer synthesis would attenuate NAFLD development. Similar results were observed in mice with NAFLD where myricin inhibited ceramide de novo synthesis and subsequently reduced hepatotoxic lipid accumu-
| Lipid species | Con-7 | Con-6 | Con-5 | Con-4 | Con-3 | Con-2 | Con-1 | OA-7 | OA-6 | OA-5 | OA-4 | OA-3 | OA-2 | OA-1 |
|--------------|-------|-------|-------|-------|-------|-------|-------|------|------|------|------|------|------|------|
| LPE          | 5.42  | 1.07  | 4.69  | 1.07  | 4.00  | 1.07  | 3.72  | 1.07  | 3.92  | 1.07  | 3.66  | 1.07  | 3.39  | 1.07  |
| LPC          | 1.42  | 10.00 | 1.49  | 10.00 | 1.40  | 10.00 | 1.31  | 10.00 | 1.23  | 10.00 | 1.15  | 10.00 | 1.07  | 10.00 |
| PG           | 1.27  | 10.00 | 1.27  | 10.00 | 1.27  | 10.00 | 1.27  | 10.00 | 1.27  | 10.00 | 1.27  | 10.00 | 1.27  | 10.00 |
| PS           | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| PE           | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| PI           | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| SM           | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| G1M          | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| G2M          | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| CL           | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| C12           | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| C14           | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| C16           | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| C18           | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| C20           | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| C22           | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| C24           | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 | 1.07  | 10.00 |
| FA           | 3.41  | 10.00 | 3.41  | 10.00 | 3.41  | 10.00 | 3.41  | 10.00 | 3.41  | 10.00 | 3.41  | 10.00 | 3.41  | 10.00 |
| ChE          | 1.88  | 10.00 | 1.88  | 10.00 | 1.88  | 10.00 | 1.88  | 10.00 | 1.88  | 10.00 | 1.88  | 10.00 | 1.88  | 10.00 |

Table 1. Summary Signals for the Identified Lipid Classes between OA-Treated Groups and Control Groups.
translation; hence, the inhibition of Cer synthesis was considered as a novel target for NAFLD prevention. Furthermore, the concentration of total and some certain Cer was assessed in the patients with NAFLD, and the results showed that the level of total Cer was significantly increased and Cer (d18:1_16:0), Cer (d18:1_18:0), Cer (d18:1_22:0), and Cer (d18:1_24:0) were markedly higher than in the reference group. The total content of Cer was decreased with OA administration, and Cer (d18:1_22:0) and Cer (d18:1_24:0) were significantly decreased after OA treatment. Hence, OA may exhibit a protective effect on NAFLD development via decreasing the concentration of Cer. Thus, Cer (d18:1_22:0). Besides, accumulated Hex1Cer was positively associated with the risk of liver diseases, such as chronic hepatitis C (CHC), NASH, 

Figure 5. Statistical histogram of 22 lipid classes detected after OA treatment. The blue histograms are control groups, and the red histograms are OA-treated groups. T-tests were carried out subsequently. P < 0.05 was considered statistically significant. *P < 0.05, **P < 0.01, ***P < 0.001 vs the controls. LPE: lysophosphatidylethanolamine, LPC: lysophosphatidylcholine, PG: phosphatidylglycerol, PI: phosphatidylinositol, PS: phosphatidylserine, PE: phosphatidylethanolamine, PC: phosphatidylcholine, and CL: cardiolipin; Hex1Cer: hexosylceramides, Hex3Cer: trihexosylceramides, Cer: ceramides, SM: sphingomyelin, GM3: gangliosides, SPH: sphingosine, Hex2Cer: dihexosylceramides; ChE: cholesterol ester, DG: diglyceride, and TG: triglyceride; and fatty acyl and other lipids: AEA: N-acyl ethanolamine, FA: fatty acids, AcCa: acyl carnitine, and Co: coenzyme.

Figure 6. Boxplots of representative lysophosphatidylcholine (LPC) and phosphatidylglycerol (PG) species found significantly decreased in OA-treated groups comparing with control groups. The blue histograms are control groups, and the red histograms are OA-treated groups. T-tests were carried out subsequently. P < 0.05 was considered statistically significant. *P < 0.05, **P < 0.01, ***P < 0.001 vs the control.
and cholangiocarcinoma (CCA). Evidence showed that Hex1Cer (d18:1_22:0), Hex1Cer (d18:1_23:0), Hex1Cer (d18:1_24:0), and Hex1Cer (d18:1_24:1) were obviously increased in CHC, NASH, and CCA, and these Hex1Cers could be the indicator for these liver diseases.26−28 As the statistical histogram results and the VIP list presented, Hex1Cer was attenuated with OA administration, and Hex1Cer (d18:1_22:0), Hex1Cer (d18:1_23:0), Hex1Cer (d18:1_24:0), and Hex1Cer (d18:1_24:1) were all significantly reduced after OA administration. Similarly, Hex2Cer accumulation was a potential lipid biomarker of alcoholic liver disease,29 and decreased Hex2Cer was detected in OA-treated groups. The above results indicated that OA administration possessed the property of CLD prevention probable via attenuating the synthesis of Cer, Hex1Cer, and Hex2Cer, and several specific lipids.

DG and TG, components of neutral lipids, were closely associated with liver diseases, particularly TG, as the neutral lipids’ core of lipid droplets (LDs) was closely associated with ER homeostasis.30 The ER was the major site for lipid synthesis in hepatocytes, and ER stress would accelerate NAFLD, while *Schisandra chinensis* extracts possessed a protective property on NAFLD prevention via inhibiting ER stress.31,32 Furthermore, as reported, TG-rich LDs would ameliorate ER stress as well. Evidence demonstrated that an increased level of TG storage in LDs alleviated tunicamycin-induced ER stress.33 Similarly, increased TG storage in LDs
could protect human cardiomyocytes from PA-induced ER stress, and OA always was used as a stimulant for TG accumulation.\textsuperscript{34} Hence, taken together, OA may provide contributions to ER stress attenuation via increasing the TG storage in LDs, and TG-rich LDs. ChE, another component of neutral lipids, was detected in the liver with NASH.\textsuperscript{35} As our results showed, the total content of ChE declined with OA administration, and ChE (22:6), one of ChE with n-6 PUFAs, was significantly decreased in OA-treated groups. Therefore, ChE could be another lipid metabolite of OA administration for NAFLD prevention, and ChE (22:6) could be a potential biomarker of OA administration on NAFLD prevention.

AcCa and Co as components of fatty acyl compounds were increased and decreased with OA administration, respectively. ACa possessed neuroprotective properties, especially in Alzheimer’s disease (AD) treatment. For example, the levels of AcCa were gradually declined from healthy subjects to subjects with mild cognitive impairment, subjective memory complaint, and up to AD.\textsuperscript{36} However, the effects of ACa on liver disease require further research. Co as the component of acyl-CoA:cholesterol acyltransferase 1 could take part in cholesterol synthesis and stimulate the accumulation of cholesterol, which could promote the progression of liver fibrosis,\textsuperscript{37} but more specific knowledge of the effect of Co with OA administration on liver disease prevention is needed.

In addition, there were 478 lipid species detected in the VIP list, which were significantly influenced by OA treatment. These molecules would be potential biomarkers with OA administration. Detailed information about the majority of these compounds on CLD development requires further research.

\section*{CONCLUSIONS}
An untargeted lipidomics based on HPLC−MS analysis was utilized to investigate changes in endogenous metabolites with OA administration. In total, numerous lipid species influenced by OA treatment were identified, and these identified lipids belonged to 22 lipid classes grouped into four categories. Decreased concentrations of LPC, PG, Cer, Hex1Cer, Hex2Cer, and ChE as well as the increased content of TG were detected with OA administration. Moreover, the 478 lipid species with significant difference after OA treatment could be potential biomarkers of OA administration. Among them, LPC (18:0), PG (16:0_18:1), Cer (d18:1_22:0), Cer (d18:1_24:0), Hex1Cer (d18:1_22:0), Hex1Cer (d18:1_23:0), Hex1Cer (d18:1_24:0), Hex1Cer (d18:1_24:1), and ChE (22:6) were clearly increased in CLD, while these lipid species were significantly decreased after OA treatment. Therefore, this study provided a novel perspective to understand the effects of OA administration on lipid metabolism through investigating endogenous changed lipids via HPLC-MS-based lipidomics.

\section*{MATERIALS AND METHODS}

\textbf{Reagents.} OA and methanol were purchased from Sigma Aldrich Co. (St. Louis, U.S.A.), and OA was dissolved in dimethylsulfoxide (DMSO) to obtain a stock concentration of 280 mM. Chloroform was obtained from the office of laboratory and equipment management of Nanjing Agricultural University. Methanol and chloroform were of chromatographic grade. Potassium hydroxide (KOH) was purchased from Lingfeng Chemical Reagent Co. (Shanghai, China).

\textbf{Cell Culture and Cell Treatments.} The human hepatocytes HL-7702 were cultured in RPMI 1640 medium (Hyclone, Logan, USA) containing 10% fetal bovine serum (FBS, Cegrogen, South America) and 1% penicillin− streptomycin solution (Hyclone, Logan, U.S.A.) at 37 °C with 5% CO₂. Hepatocytes at a concentration of 5 × 10⁵ cells/cm² were treated with 70 μM OA in DMSO or with the same concentration of DMSO which were termed as control groups. When treated with OA, the 280 mM OA was diluted to 70 mM, and 20 μL of 70 mM OA was added to the growth medium of 20 mL; therefore, the cells were treated with the final concentration of 70 μM OA. In the control groups, 20 μL of DMSO was added to the growth medium for 20 mL. After 12 h, cells were collected for lipid metabolite extraction.

\textbf{Lipid Metabolite Extraction from Cells.} Cells were collected with 1.5 mL of phosphate buffer saline (PBS, Hyclone, Logan, U.S.A.) and transferred to a glass tube. The cells were mixed with four volumes of the organic phase made up with chloroform and methanol at a volume ratio of 2:1. Subsequently, the mixture was adequately vibrated several times and centrifuged at 3000 rpm for 15 min. After that, the mixture was separated in three parts: the top layer was the aqueous phase, the middle layer was the protein phase, and the lower layer was the organic phase. The lower organic layer was collected by a glass syringe to a new glass tube, avoiding picking any protein up, and then dried by a nitrogen gas. The dried lipid extract was stored at −80 °C until further analysis. At last, the protein phase was dissolved with 0.1 M KOH at 4 °C overnight, and the protein concentration was determined using the BCA protein quantitation kit (Beyotime, Shanghai, China). The redissolution volume of dried lipid metabolite extraction was normalized based on the protein concentration. The method of lipid metabolite extraction was carried out according to the protocol provided by Tsinghua University.\textsuperscript{18}

\textbf{HPLC Methods for Lipid Metabolites.} Orbitrap QHEF coupled with Ultimate 3000 HPLC (Thermo, CA) was used for lipidomics analysis. Lipid extracts were analyzed by HPLC using a Cortecs C18 column (2.1 × 100 mm, Waters) both in positive and negative modes. The elution solvent system contained a mix of mobile phase A (acetonitrile (ACN):H₂O (60:40), 10 mM ammonium acetate) and mobile phase B (isopropanol:ACN (90:10), 10 mM ammonium acetate), which were utilized both in positive and negative modes. The detailed elution gradient was as follows: 0 min, 37% B; 1.5 min, 37% B; 2 min, 45% B; 2.5 min, 52% B; 3 min, 58% B; 4 min, 66% B; 5 min, 70% B; 6 min, 75% B; 10 min, 98% B; 15 min, 98% B; 20 min, 22.1 min 37%B; and 25 min, 37% B. The dried lipid metabolite extraction preparations were redissolved, and the redissolutions of OA treated and control samples were injected into the column randomly for analysis, following the parameter setting above. The QC samples, consisting of a mixture of OA-treated samples and control samples, were inserted before or after OA-treated samples and control samples. They were used to evaluate the stability of the instrument in order to provide repeatable and high-quality data. The data of QC samples are in the original data and may be downloaded from the website for reference. In the analysis, PC (14:0_14:0) was added in the samples as internal standards at a concentration of 2 μg/mL. The method was carried out essentially as in the protocol provided by Tsinghua University.\textsuperscript{18}

\textbf{MS Methods for Lipid Metabolites.} A Q Exactive Orbitrap mass spectrometer (Thermo, CA) was used for lipid
metabolites analysis. Mass range (m/z) was set as 240–2000 for the positive mode and 200–2000 for negative mode. The spray voltage was different between positive mode and negative mode, which was 3.2 and 2.8 kV, respectively. The capillary temperature was kept at 320 °C for all analyses. Furthermore, the remaining parameters were as follows: sheath gas flow rate (arb), 35; aux gas flow rate (arb), 10; topN, 10; NCE, 15/30/45; and duty cycle, 1.2 s. The method of MS analysis was performed according to the protocol provided by Tsinghua University.38

Data Analysis. Lipids were identified according to matching precursor and characteristic fragment masses. A S ppm and 10 ppm mass tolerance was used for precursors and fragments, respectively. A 0.25 min retention time shift was allowed for quantitation.38 For statistical analysis, PCA, an unsupervised pattern recognition model, and OPLS-DA, a supervised pattern recognition model, were utilized to construct the predictive models to assess the differences between OA-treated groups and control groups. The VIP list (VIP > 1.0 and \( \text{FDR} < 0.05 \)) obtained from OPLS-DA was used to determine significantly changed metabolites after OA treatment. Volcano plots and heat maps were generated using MetaboAnalyst 3.0, an online data analysis system. These statistics compensate for analyzing many metabolites at the same time and were based on the conversion from the \( p \) value to FDR (\( p \) value) (https://www.metaboanalyst.ca/faces/ModuleView.xhtml). A t-test was used for comparing the differences between OA-treated groups and control groups. There were seven biological parallels for both OA-treated and control groups, and the significance level was set at \( p < 0.05 \).

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Notes

The authors declare no competing financial interest.

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