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Lipidomics Biomarkers of Diet-Induced Hyperlipidemia and Its Treatment with *Poria cocos*

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Ⓢ *Supporting Information*

ABSTRACT: Hyperlipidemia is a major cause of atherosclerotic cardiovascular disease. *Poria cocos* (PC) is a medicinal product widely used in Asia. This study was undertaken to define the alterations of lipid metabolites in rats fed a high-fat diet to induce hyperlipidemia and to explore efficacy and mechanism of action of PC in the treatment of diet-induced hyperlipidemia. Plasma samples were then analyzed using UPLC-HDMS. The untreated rats fed a high-fat diet exhibited significant elevation of plasma triglyceride and total and low-density lipoprotein (LDL) cholesterol concentrations. This was associated with marked changes in plasma concentrations of seven fatty acids (palmitic acid, hexadecenoic acid, hexanoylcarnitine, tetracosahexaenoic acid, cervonoyl ethanolamide, 3-hydroxytetradecanoic acid, and 5,6-DHET) and five sterols [cholesterol ester (18:2), cholesterol, hydroxytestosterone, 19-hydroxydeoxycorticosterone, and cholic acid]. These changes represented disorders of biosynthesis and metabolism of the primary bile acids, steroids, and fatty acids and mitochondrial fatty acid elongation pathways in diet-induced hyperlipidemia. Treatment with PC resulted in significant improvements of hyperlipidemia and the associated abnormalities of the lipid metabolites.

KEYWORDS: hyperlipidemia, Poria cocos, lipidomics, ultraperformance liquid chromatography, mass spectrometry, fatty acid metabolism

INTRODUCTION

Hyperlipidemia is a major risk factor for atherosclerotic cardiovascular disease.[1] It is caused by impaired lipid metabolism and is marked by elevation of serum total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) and relative reduction of high-density lipoprotein cholesterol (HDL-C).[2] Lipid-lowering drugs, such as statins, fibrates, and nicotinic acid, are commonly used for treatment of hyperlipidemia. However, side effects and/or poor tolerability of these drugs limits their use in some patients.[3]

Use of many natural medicines has proven safe and effective in the treatment of various disorders, especially chronic diseases.[4] *Poria cocos* (PC) is the dried sclerotium of the fungus *Poria cocos* (Schw.) Wolf (Polyporaceae), which grows around the roots of old, dead pine trees. This medicinal fungus gives edible sclerotia that have been called "tuckahoes", "Indian bread", or *Wolfiporia cocos* in North America and *Poria cocos* in Asia.[5] PC is a well-known medicinal mushroom widely used in Asian countries. About 10% of traditional Chinese medicines included in the Chinese Pharmacopoeia contain PC. PC facilitates fecal and urinary disposal of waste products and exerts diuretic, nephroprotective, and immunomodulatory activities[6−9] To our knowledge, the effect and potential mechanism of action of PC administration in the treatment of hyperlipidemia have not been previously reported.

Lipidomics is a branch of the omics field that was first introduced by Han and Gross in 2003[10] and represents a system-based study of a wide range of lipid species. Recently, a lipidomics approach has been applied as a tool for the identification of biomarkers, understanding of the mechanisms, and diagnosis of various diseases and monitoring their response to therapeutic interventions as well as drug toxicity in animal models and in clinical studies.[11−13] Although the field of lipidomics is in its infancy, it has shown promising results in the understanding of disorders of lipid metabolism including hyperlipidemia and cardiovascular disease, identification of metabolic biomarkers of diet-induced hypertriglyceridemia,[14] development of optimal animal models of human dyslipidemia,[15] and the therapeutic effects of simvastatin in patients with dyslipidemia.[16] In addition, lipidomics has been applied to evaluate the bioactivity and toxicity of natural medicines, such as Fu-Ling-Pi, Sini decoction, and *Aconiti Lateralis* radix praeparata.[17−19]

The present study was designed to define the alterations of lipid metabolites in rats with high-fat diet-induced hyperlipidemia and to explore the efficacy and mechanism of action of PC using ultraperformance liquid chromatography coupled
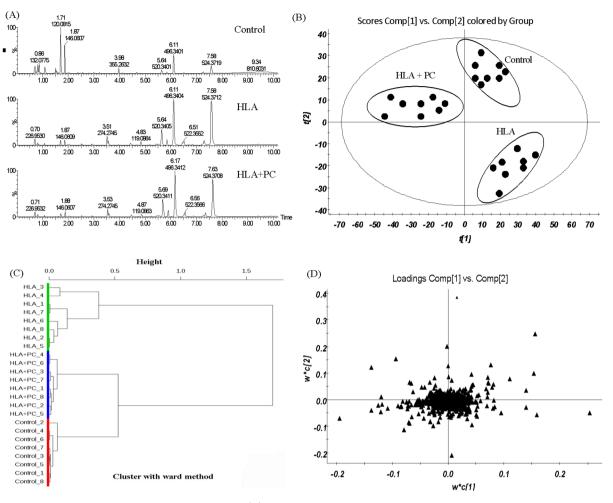
Figure 1. Lipid profiling and multivariate statistical analysis. (A) Base peak intensity chromatograms of control group, HLA group, and HLA+PC group in positive ion mode obtained from UPLC-HDMS analysis. (B) PLS-DA model for control group, HLA group, and HLA+PC group. (C) Clustering analysis of control group, HLA group, and HLA+PC group. (D) Loading plot of PLS-DA in positive ion mode from control group, HLA group, and HLA+PC group.

with quadrupole time-of-fight synapt high-definition mass spectrometry (UPLC-QTOF/HDMS). This approach has proven to be a powerful tool for the identification of lipid species in complex biological mixtures.[20,21]

MATERIALS AND METHODS

PC Sample Preparation, Animals, and Sample Collection. PC was ground to powder by a disintegrator, and the powder (1000 g) was repeatedly extracted three times with 5 L of 95% ethanol at room temperature by ultrasonic method. PC extracts were filtered, and the filtrate was concentrated to yield a dry powder. Male SD rats were randomly divided into control group, untreated hyperlipidemic group (HLA), and the PC-treated hyperlipidemic group (HLA+PC) (n = 8/ group). The control group was maintained on regular diet throughout the experimental period. The rats assigned to the hyperlipidemic groups were fed a high-fat diet for 6 weeks.[22] They were randomized to the untreated group and the PC-treated group. The PC-treated group was administered PC (250 mg/kg BW) by gastric gavage once a day. After 6 weeks, blood samples were obtained by carotid artery cannulation. Plasma was separated and stored at −80 °C. The study was approved by the Ethical Committee of Northwest University. All procedures and the care of the experimental animals were in accordance with institutional guidelines for animal use.

Plasma Lipids. Serum biochemistry including total cholesterol, triglycerides, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol levels were measured with an Olympus AU640 automatic analyzer following the manufacturer's instructions.

Sample Preparation. Lipid extractions were performed in an Ostro 96-well plate by a single-step in-well extraction as previously described.[23] The extraction of total lipids by the Ostro 96-well plate was performed as a single-step in-well extraction. One hundred microliters of plasma sample was loaded into each well of a 2000 μL Ostro preparation plate fitted onto a vacuum manifold. Three hundred microliters of methanol/chloroform (1:1, v/v) was added to each well and mixed, aspirating the mixture 10 times by a micropipette. A vacuum of 15 in. of Hg was used to the Ostro preparation plate until the solvent was drained completely. These steps were repeated with another 300 μL of methanol and chloroform with the total fraction. These steps was repeated three times to obtain the total fraction volume of 900 μL. The eluate fraction was dried by nitrogen and reconstituted with 200 μL methanol/chloroform (1:1, v/v). The sample was injected into UPLC-QTOF/MS.

UPLC-HDMS. UPLC-HDMS analysis was employed on a Waters Acquity Ultra Performance LC system equipped with a Waters Xevo G2 QTof MS. UPLC analysis was performed on a HSS T3 column. The mobile phases consisting of 10 mmol/L ammonium formate and 0.1% formic acid in 2-propanol/acetonitrile (90:10) (A) and 10 mmol/L ammonium formate and 0.1% formic acid in acetonitrile/ water (60:40) (B) were used as follows: a linear gradient from 0 to 10 min, 40.0−99.0% A and from 10.0 to 12.0 min, 99.0−40.0% A. The flow rate was set at 0.5 mL/min. The autosampler and column temperature were set at 4 and 55 °C, respectively. A 2.0 μL sample solution was injected for each run. The MS scan ranged from m/z 50 to 1200 in positive ion mode. The cone voltage and capillary voltage

Table 1. Identified Plasma Lipid Species, Fold Changes (FC), and p Values among the Control Group, HLA Group, and HLA+PC Group

no.	metabolite	VIP[a]	HLA vs control				PC vs HLA				PC vs control			
			FC[b]	p value[c]	p value[d]	FDR[e]	FC[a]	p value[b]	p value[c]	FDR[d]	FC[a]	p value[b]	p value[c]	FDR[d]
1	palmitic acid	9.8	1.29	9.86×10^{-5}	1.02×10^{-5}	1.45×10^{-4}	0.68	5.01×10^{-8}	6.97×10^{-7}	9.19×10^{-8}	0.88	7.87×10^{-3}	1.74×10^{-2}	1.73×10^{-2}
2	tyromycic acid	8.7	2.90	1.80×10^{-7}	3.63×10^{-6}	4.96×10^{-7}	0.31	3.55×10^{-9}	8.49×10^{-7}	1.30×10^{-8}	0.88	2.07×10^{-2}	2.46×10^{-3}	3.80×10^{-2}
3	LysoPC(16:0)	7.6	0.75	6.36×10^{-9}	3.62×10^{-6}	2.80×10^{-8}	1.22	4.95×10^{-6}	2.80×10^{-5}	7.78×10^{-6}	0.92	2.21×10^{-2}	2.78×10^{-2}	3.47×10^{-2}
4	cholesterol ester (18:2)	7.0	1.36	6.31×10^{-6}	8.57×10^{-6}	1.39×10^{-5}	0.64	2.75×10^{-8}	1.44×10^{-6}	5.50×10^{-8}	0.88	2.07×10^{-2}	2.46×10^{-3}	3.51×10^{-2}
5	5,6-DHET	5.4	0.48	1.60×10^{-8}	3.85×10^{-6}	5.86×10^{-8}	1.89	5.60×10^{-12}	5.51×10^{-7}	6.16×10^{-11}	0.92	2.95×10^{-1}	2.95×10^{-1}	3.41×10^{-1}
6	hexadecenoic acid	5.1	1.32	8.01×10^{-5}	5.95×10^{-6}	1.36×10^{-4}	0.84	2.44×10^{-3}	5.19×10^{-4}	3.36×10^{-3}	1.11	4.97×10^{-2}	5.97×10^{-2}	6.83×10^{-2}
7	eicosenoic acid	5.0	1.25	1.26×10^{-4}	1.14×10^{-3}	1.63×10^{-4}	0.84	2.82×10^{-3}	4.66×10^{-3}	3.65×10^{-3}	1.05	5.12×10^{-1}	3.14×10^{-1}	5.37×10^{-1}
8	cholesterol	4.9	1.63	1.93×10^{-5}	2.02×10^{-4}	3.54×10^{-5}	0.76	8.85×10^{-3}	2.89×10^{-2}	1.08×10^{-2}	1.23	2.27×10^{-1}	2.40×10^{-1}	2.77×10^{-1}
9	sphingosine 1-phosphate	4.8	1.32	8.29×10^{-5}	3.97×10^{-5}	1.30×10^{-4}	0.59	6.38×10^{-9}	2.09×10^{-6}	1.75×10^{-8}	0.79	1.05×10^{-4}	7.46×10^{-4}	7.72×10^{-4}
10	cervonoyl ethanolamide	4.6	0.39	6.66×10^{-14}	3.47×10^{-6}	1.47×10^{-12}	1.47	5.09×10^{-8}	6.02×10^{-5}	8.61×10^{-8}	0.58	1.71×10^{-7}	2.76×10^{-4}	3.76×10^{-6}
11	hydroxytestosterone	4.3	1.51	1.31×10^{-12}	4.71×10^{-5}	1.44×10^{-11}	0.55	9.64×10^{-11}	2.12×10^{-6}	4.24×10^{-10}	0.84	6.27×10^{-4}	2.03×10^{-3}	2.30×10^{-3}
12	hexanoylcarnitine	4.1	1.32	1.97×10^{-8}	2.43×10^{-3}	6.19×10^{-8}	0.53	1.39×10^{-11}	7.32×10^{-7}	1.02×10^{-10}	0.70	2.17×10^{-3}	2.27×10^{-3}	5.95×10^{-3}
13	tetracosahexaenoic acid	4.0	1.32	1.01×10^{-4}	3.97×10^{-5}	1.38×10^{-4}	0.59	8.35×10^{-9}	2.08×10^{-6}	2.04×10^{-8}	0.79	1.12×10^{-4}	8.08×10^{-4}	6.18×10^{-4}
14	10,11-dihydro-12R-hydroxy-leukotriene C4	3.8	1.23	2.12×10^{-4}	1.05×10^{-3}	2.45×10^{-4}	0.65	4.44×10^{-9}	2.97×10^{-6}	1.40×10^{-8}	0.80	1.77×10^{-4}	5.15×10^{-4}	7.80×10^{-4}
15	19-hydroxydeoxycorticosterone	3.5	1.91	4.47×10^{-12}	3.50×10^{-5}	3.28×10^{-11}	0.69	1.59×10^{-12}	7.02×10^{-7}	3.50×10^{-11}	1.32	6.03×10^{-6}	8.00×10^{-5}	6.63×10^{-5}
16	cholic acid	3.2	1.34	2.30×10^{-6}	2.50×10^{-5}	5.62×10^{-6}	0.61	1.12×10^{-8}	1.50×10^{-5}	2.46×10^{-8}	0.82	3.01×10^{-2}	2.62×10^{-2}	4.42×10^{-2}
17	2-methylbutyroylcarnitine	2.8	1.49	9.90×10^{-4}	1.21×10^{-4}	9.90×10^{-4}	0.86	6.87×10^{-2}	1.17×10^{-2}	7.20×10^{-2}	1.28	1.59×10^{-2}	1.90×10^{-2}	3.18×10^{-2}
18	12-oxo-20-carboxy-leukotriene B4	2.7	1.79	5.92×10^{-4}	1.74×10^{-4}	6.21×10^{-4}	0.75	5.15×10^{-2}	3.76×10^{-2}	5.67×10^{-2}	1.34	7.71×10^{-2}	2.87×10^{-1}	9.97×10^{-2}
19	phytosphingosine	2.5	1.27	1.74×10^{-4}	9.81×10^{-4}	2.13×10^{-4}	0.81	2.16×10^{-5}	8.99×10^{-5}	3.17×10^{-5}	1.02	7.62×10^{-1}	8.44×10^{-1}	7.62×10^{-1}
20	3-hydroxy-tetradecanoic acid	2.2	0.66	5.22×10^{-12}	3.52×10^{-6}	2.87×10^{-11}	1.34	3.90×10^{-11}	7.02×10^{-7}	2.15×10^{-10}	0.89	3.87×10^{-3}	4.16×10^{-3}	9.46×10^{-3}
21	MG(16:1)	1.9	1.29	6.94×10^{-6}	1.20×10^{-4}	1.39×10^{-5}	0.95	1.27×10^{-1}	1.37×10^{-1}	1.27×10^{-1}	1.22	1.60×10^{-3}	3.72×10^{-3}	5.02×10^{-3}
22	PE(14:1/18:2)	1.6	1.23	2.77×10^{-4}	9.83×10^{-4}	3.04×10^{-4}	0.87	3.43×10^{-2}	3.86×10^{-2}	3.97×10^{-2}	1.07	3.65×10^{-1}	7.51×10^{-1}	4.01×10^{-1}

[a]VIP was obtained from PLS-DA. [b]FC was calculated on the basis of mean ratios for HLA vs control, PC vs HLA, or PC vs control. FC with a value greater than zero indicates a higher intensity between HLA abd control, between PC and HLA, or between PC and control, whereas a FC value less than zero indicates a lower intensity of the lipid species between HLA and control, between PC and HLA, or between PC and control. [c]p values are calculated from a one-way ANOVA. [d]p values are calculated from nonparametic test Mann–Whitney U test. [e]FDR value was obtained from the adjusted p value using the Benjamini Hochberg method.
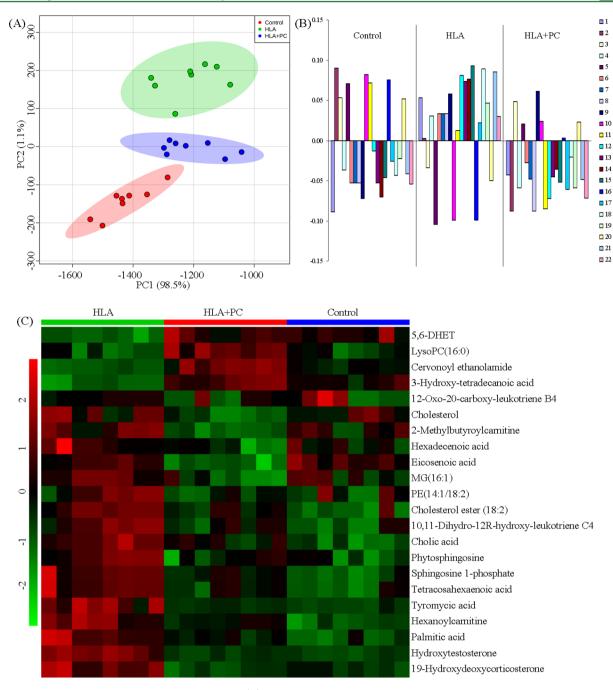
Figure 2. Lipidomic profiling of 22 identified lipid species. (A) PCA of two components of lipid species from control group, HLA group, and HLA+PC group. (B) Correlation coefficient analysis among control group, HLA group, and HLA+PC group with corresponding 22 lipid species in the different groups. Numbers are consistent with Table 1. (C) Heatmap of 22 lipid species among control group, HLA group, and HLA+PC group. Red and green indicate increased and decreased levels, respectively.

were set at 45 V and 2.5 kV, respectively. The desolvation gas was set at 900 L/h at a temperature of 550 °C; the cone gas was set at 50 L/h, and the source temperature was set at 120 °C. All of the acquisitions were operated by Waters MassLynx v4.1 software.

Pattern Recognition Analysis and Data Processing. The precision and reproducibility were verified as described in detail previously.[24] The original data were imported to Markerlynx XS for peak detection and alignment. The data were normalized to the summed total ion intensity per chromatogram, and the resultant data matrices were introduced to the EZinfo 2.0 for partial least-squares discriminant analysis (PLS-DA). Lipid species were obtained from PLS-DA, and the potential biomarkers were selected according to the VIP values, which reflected the contribution of each variable in the

three groups. Heatmap, fold changes (HLA/control, HLA+PC/HLA, or HLA+PC/control) and receiver-operating characteristic (ROC) curves from the identified lipid species were analyzed by Metaboanalyst 3.0 or Medcalc 12.7. Ingenuity pathway analysis (IPA) was carried out on metabolomics pathway analysis (MetPA) for pathway analysis and visualization metabolomics. Lipid metabolic pathways in HLA were analyzed by the quantitative enrichment analysis (QEA) algorithm described in the metabolite set enrichment analysis (MSEA) from Lipid Maps. Visualization of lipid pathways was obtained by Metscape running on Cytoscape 3.0. One-way analysis of variance (ANOVA) and the Mann−Whitney U test were used to calculate the statistical significance by SPSS 19.0. False discovery rate (FDR) correction was calculated to reduce the risk of a false positive
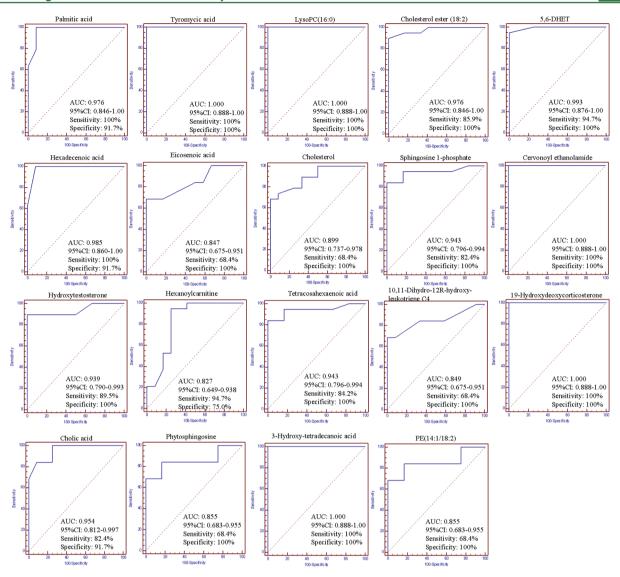
Figure 3. PLS-DA-based ROC curves of the 19 lipid species for biomarker selection of anti-hyperlipidemic effects of PC. The associated AUC, 95% CI, sensitivities, and specificities are indicated.

by the adjusted p values (<0.05) based on the Benjamini Hochberg method.

RESULTS AND DISCUSSION

Physiological and Clinical Chemistry Data. The body weights of control, untreated hyperlipidemic, and PC-treated hyperlipidemic groups at week 6 were 350 ± 38, 385 ± 45, and 355 ± 48 g, respectively. Compared to the normal control group, the body weight of the untreated hyperlipidemic rats was slightly increased, but the difference did not reach statistical significance. Compared to the untreated hyperlipidemic group, body weight was reduced in the PC-treated group.

TC, TG, and LDL-C concentrations in the control group were 2.61 ± 0.31, 0.64 ± 0.07, and 1.67 ± 0.18 mmol/L, respectively. TC, TG, and LDL-C concentrations in hyperlipidemic group were 4.58 ± 0.57, 0.99 ± 0.11, and 3.12 ± 0.36 mmol/L, respectively. HDL-C concentration in the control group was 0.97 ± 0.12 mmol/L, whereas HDL-C concentration in the hyperlipidemic group was 0.57 ± 0.07 mmol/L. Treatment with PC significantly improved the TC, TG, LDL-C, and HDL-C abnormalities. The TC, TG, LDL-C, and HDL-C concentrations were 3.64 ± 0.41, 0.84 ± 0.09, 2.28 ± 0.34,

and 0.77 ± 0.08 mmol/L in the PC-treated hyperlipidemic group, respectively. These data indicated that the rat model of diet-induced hyperlipidemia was successfully reproduced and that PC treatment was effective in alleviating dyslipidemia in rats with high-fat diet-induced hyperlipidemia.

Selection and Identification of Important Differential Lipid Species. Method reproducibility was confirmed by six replicated determinations of each plasma sample for all analyses. Extracted peaks of 10 ions including 3.56_318.3003, 4.94_373.3133, 3.97_373.2732, 6.73_686.4443, 4.68_424.2186, 0.76_780.4463, 7.56_815.0174, 3.57_318.3999, 4.92_266.2839, and 5.51_357.2783 were chosen for the method validation. The RSD values of the retention time and peak area were below 0.64 and 2.83%, respectively. The data demonstrated good reproducibility of the UPLC-HDMS method in the present study. Figure 1A shows typical base peak intensity chromatograms including control, untreated hyperlipidemic, and PC-treated hyperlipidemic groups.

To evaluate whether PC can modify the lipid profile in diet-induced hyperlipidemia, the two-predictive component PLS-DA model [R2X(cum) = 0.961, Q2(cum) = 0.751] was
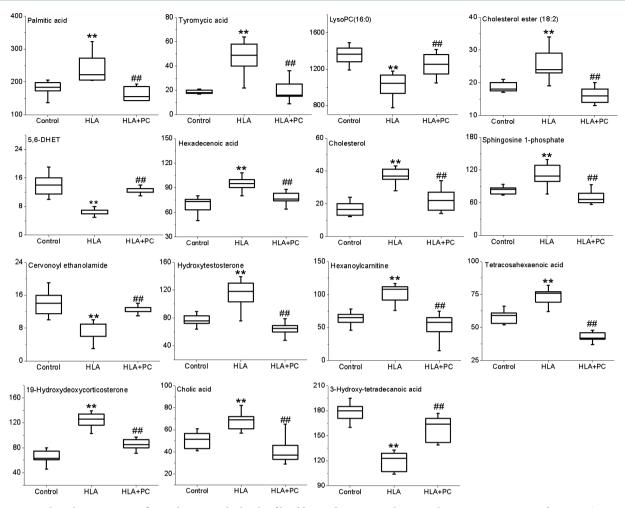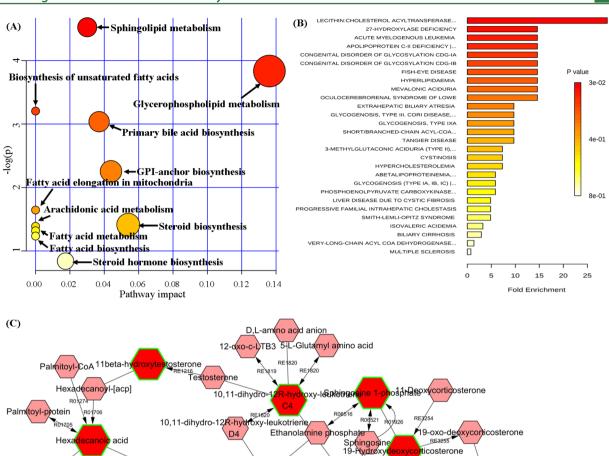
Figure 4. Box plots showing 15 significant changes in the levels of lipid biomarkers among the control group, HLA group, and HLA+PC group. The statistical significance between the two groups is marked. (**) $p < 0.01$ significant difference compared with control group; (##) $p < 0.01$ significant difference compared with HLA group. Y-axis: normalized relative intensity.

conducted. It showed satisfactory discriminating capacity by lipidomics data with 3289 variables from three groups (Figure 1B). As can be seen from the clustering analysis, the PC-treated group is positioned between the untreated hyperlipidemic group and the control group (Figure 1C). Therefore, the result demonstrated the efficacy of PC in improving diet-induced hyperlipidemia.

To find the significantly altered lipid species, initially, variables were selected on the basis of the VIP values in the loading plot of the PLS-DA model (Figure 1D). One hundred and seventy-five variables had VIP values of >1.5. Xenobiotics and different fragment ions from the same lipid species were excluded. A total of 45 ions were selected in this study, and 22 differential lipid species were identified between hyperlipidemic and control groups (Table 1). These lipid classes included 11 fatty acids, 5 sterol lipids, 2 glycerophospholipids, 2 sphingolipids, 2 glycerolipids, and 1 prenol lipid. Compared with the hyperlipidemic group, changes in 19 lipid species were completely reversed in the PC-treated group based on the FC values and one-way ANOVA, Mann−Whitney U test, and FDR ($p < 0.05$). Compared with the control group, 12 lipid species were below normal levels and 7 lipid species were restored to normal or near-normal levels in the PC-treated group based on the one-way ANOVA, Mann−Whitney U test, and FDR ($p > 0.05$). The PCA score plot of 22 lipid species in the PC-treated

group was located between those of untreated hyperlipidemic and control groups and was much closer to control values (Figure 2A), which is consistent with the result of the heatmap analysis (Figure 2C). In addition, Figure 2B shows the result of the correlation coefficient analysis between lipid species and their corresponding groups. Lipid species situated in the upper panel were positively correlated, whereas those situated in the opposite panel are negatively correlated with the corresponding group. The lipid species 2, 3, 5, 10, 11, 16, and 20 were positively correlated with control group. The other lipid species were negatively correlated with the control group, indicating normal lipid metabolism. Except for lipid species 3, 5, 10, 16, and 20, the other lipid species are positively correlated with the hyperlipidemic group. These findings illustrate the marked abnormalities of metabolic profile caused by a high-fat diet. Nineteen lipid species in the PC-treated group showed the same tendencies observed in the control group. The results demonstrated the efficacy of PC treatment in ameliorating diet-induced hyperlipidemia, thus demonstrating the anti-hyperlipidemic effects of PC.

ROC Curve Analysis and Biomarker Selection. To further find potential biomarkers of anti-hyperlipidemic effects of PC, PLS-DA-based ROC curves were performed. Although 2-methylbutyroylcarnitine, 12-oxo-20-carboxy-leukotriene B4, and MG(16:1) were reversed by PC treatment compared with

Figure 5. Lipid metabolic pathway analysis of identified differential lipid species. (A) Summary of IPA with MetPA including sphingolipid metabolism, glycerophospholipid metabolism, biosynthesis of unsaturated fatty acids, primary bile acid biosynthesis, GPI-anchor biosynthesis, fatty acid elongation in mitochondria, steroid biosynthesis, arachidonic acid metabolism, fatty acid metabolism and biosynthesis, and steroid hormone biosynthesis from significantly differential lipid species. The size and color of each circle are based on pathway impact value and *p* value, respectively. (B) QEA performed using MSEA. (C) Visualization of the remarkably disturbed metabolic pathways by MetScape analysis. The differential lipid species are shown by red hexagons. Hexagons with green lines indicate significant changes of the identified lipid species in HLA had statistical significance. The size of hexagons showed the FC of the differential lipid species in HLA relative to control. In addition, pink hexagons show metabolites participating in the metabolic pathway but not identified in the current study. Anti-hyperlipidemic effects of PC were associated with androgen and estrogen biosynthesis and metabolism, arachidonic acid metabolism, bile acid biosynthesis, C21-steroid hormone biosynthesis and metabolism, de novo fatty acid biosynthesis, glycerophospholipid metabolism, glycosphingolipid metabolism, leukotriene metabolism, monounsaturated fatty acid β-oxidation, ω-3 fatty acid metabolism, saturated fatty acid β-oxidation, and squalene and cholesterol biosynthesis.

untreated group, they did not reach statistical significance ($p > 0.05$). Therefore, they could not be considered as potential biomarker candidates of lipid-lowering effects of PC. The area under the curve (AUC), 95% confidence interval (95%CI), sensitivities, and specificities of 19 lipid species are shown in Figure 3. In addition to the commonly accepted standard biomarker, cholesterol, 18 lipid species were identified as

biomarker candidates with an AUC of ≥ 0.82. Although the AUC values for eicosenoic acid, 10,11-dihydro-12*R*-hydroxy-leukotriene C4, phytosphingosine, and PE(14:1/18:2) were high, they were excluded from the subsequent validation study because their sensitivity or specificity was found to be low (<80%). Fourteen of 18 lipid species had high AUC value (>0.80), sensitivity (>80%), and specificity (>80%), and they

Table 2. Ingenuity Pathway Analysis with MetPA from Differential Lipid Species[a]

pathway name	total metabolites	hits	p	−log(p)	Holm p	FDR	impact	details
sphingolipid metabolism	21	2	0.0108	4.52	0.87	0.87	0.030	Figure 6
glycerophospholipid metabolism	30	2	0.0216	3.83	1.00	0.87	0.136	Figure S1
biosynthesis of unsaturated fatty acids	42	2	0.0406	3.20	1.00	0.97	0.000	Figure S2
primary bile acid biosynthesis	46	2	0.0479	3.03	1.00	0.97	0.036	Figure S3
GPI-anchor biosynthesis	14	1	0.1048	2.25	1.00	1.00	0.043	Figure S4
fatty acid elongation in mitochondria	27	1	0.1932	1.64	1.00	1.00	0.000	Figure S5
steroid biosynthesis	35	1	0.2435	1.41	1.00	1.00	0.053	Figure S6
arachidonic acid metabolism	36	1	0.2496	1.38	1.00	1.00	0.000	Figure S7
fatty acid metabolism	39	1	0.2676	1.31	1.00	1.00	0.000	Figure S8
fatty acid biosynthesis	43	1	0.2910	1.23	1.00	1.00	0.000	Figure S9
steroid hormone biosynthesis	70	1	0.4319	0.83	1.00	1.00	0.017	Figure S10

[a]"Total" is the total number of differential lipid species in the pathway; "hits" is the actually matched number from the user differential lipid species; the raw p is the original p calculated from the enrichment analysis; the "Holm p" is the p value adjusted by Holm−Bonferroni method; "impact" is the pathway impact value calculated from pathway topology analysis.
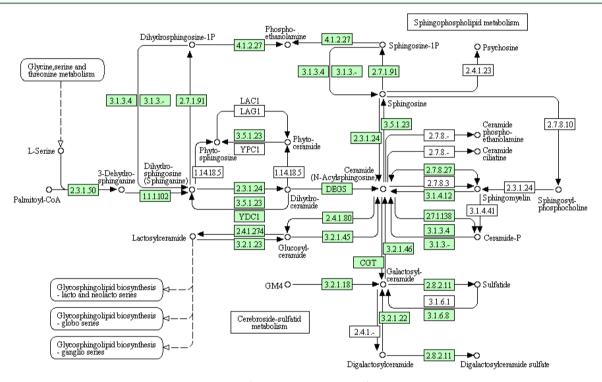

Figure 6. Overview of sphingolipid metabolism with MetPA (reference map by KEGG). Green boxes represent enzymatic activities with putative cases of analogy in rats.

could be considered as potential biomarkers of lipid-lowering effects of PC. These lipid species included seven fatty acids and four sterol lipids, one prenol lipid (tyromycic acid), one glycerophospholipid [LysoPC(16:0)], and one sphingolipid (sphingosine 1-phosphate). Figure 4 illustrates the difference in the level of the key lipid biomarkers among the control group and the untreated and PC-treated hyperlipidemic groups. Therefore, these results indicated that fatty acids and sterol lipids represent potential biomarkers of the lipid-lowering effects of PC.

Perturbed Metabolic Network in Hyperlipidemia and Its Response to PC Therapy. High-fat diets trigger an imbalance between the lipid synthesis and degradation and cause excessive lipid accumulation in hepatocytes. To determine possible metabolic pathways and networks influenced by a high-fat diet, IPA was performed with MetPA, a web-based tool for pathway analysis and visualization of

metabolomics. The differential lipid species were analyzed by MetPA. The IPA revealed dysregulation of 11 lipid pathways associated with sphingolipid metabolism, glycerophospholipid metabolism, primary bile acid biosynthesis, GPI-anchor biosynthesis, mitochondrial fatty acid elongation, fatty acid metabolism and biosynthesis, arachidonic acid metabolism, and steroid biosynthesis in rats with diet-induced hyperlipidemia (Figure 5A and Table 2). As an example, the detailed results from the biological pathway analysis of sphingolipid metabolism are illustrated in Figure 6. The effects on the other pathways are shown in Figures S1−S10. In addition, 28 metabolic pathways were found to be dysregulated in diet-induced hyperlipidemia on the basis of the analysis of the QEA algorithm of the MSEA method (Figure 5B) and cytoscape software (Figure 5C).

The LIPID MAPS consortium has defined lipids as hydrophobic or amphipathic compounds that originate entirely or in part by carbocation-based condensations of isoprene

group or by carbanion-based condensations of ketoacyl group.[25] On the basis of this definition, lipids can be divided into eight categories: fatty acid, sphingolipid, glycerolipid, glycerophospholipid, saccharolipid, prenol lipid, sterol lipid, and polyketide.[26]

Fatty Acyl Lipid Metabolism. In this study, 14 lipid biomarkers were selected on the basis of univariate or multivariate statistical analysis and ROC curve analysis. Seven fatty acyls and five sterol lipids were the main biomarkers of anti-hyperlipidemic effects of PC and were in agreement with the previously identified biomarkers described in hyper-triglyceridemia, hyperlipidemia, and hypercholesterolemia models.[27] Changes in seven fatty acids including a significant increase in palmitic acid, hexadecenoic acid, hexanoylcarnitine, and tetracosahexaenoic acid as well as significant decrease in cervonoyl ethanolamide, 3-hydroxy-tetradecanoic acid and 5,6-DHET were observed in diet-induced hyperlipidemic rats. These changes were completely reversed by treatment with PC. A number of experimental investigations performed in the past several years have revealed that unsaturated and saturated fatty acid metabolism is disturbed in diet-induced hyperlipidemia.[15,23,28] For example, palmitic acid, which is one of the most common saturated fatty acids, has been identified as a marker of hyperlipidemia in diet-induced hyperlipidemic patients and rats.[29,30] Additionally, LC-MS-based lipidomics demonstrated that the levels of plasma palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, arachidonic acid, and docosahexaenoic acid are significantly increased in both schisandrin B-induced and high-fat diet-induced hypertrigly-ceridemic mouse models.[14] Similarly, fatty acids with carbon chain length from 14 to 24 carbon atoms have been shown to be altered in Watanabe heritable hyperlipidemic rabbits, which is a model of hypercholesterolemia.[31]

Hexadecenoic acid, which is one of the identified lipid biomarkers, is the byproduct of hydroxylation of the terminal (ω) carbon of palmitic acid. Palmitic acid ω-hydroxylation is catalyzed by cytochrome P-450 in animals.[32] Hexanoylcarnitine has been identified as a biomarker for the protective effects of the Chinese drug Xin-Ke-Shu against myocardial infarction in rats.[33] Hexanoylcarnitine is a longer chain acyl carnitine, which is metabolized via medium-chain fatty acid β-oxidation. Hexanoylcarnitine accumulation was observed in our diet-induced hyperlipidemic rats. Treatment with PC attenuated accumulation of hexanoylcarnitine in hyperlipidemic rats most likely by enhancing fatty acid oxidation and counteracting the isoproterenol-induced reduction of energy production.[33]

Consistent with the results of the present study, in a previous study we found increased tetracosahexaenoic acid in rats with chronic kidney disease and its reversal by treatment with the surface layer of PC.[34] These findings indicate that fatty acids play an important pathogenic role in dyslipidemia-associated diseases. Indeed, free fatty acids serve as substrate for the formation of reactive lipid moieties and development of oxidative stress leading to mitochondrial damage and cell death.[35] A recent study demonstrated increased peroxidation of polyunsaturated fatty acids and significant reduction of polyunsaturated fatty acid levels in diet-induced hyperlipidemic rats.[36] In fact, elevated polyunsaturated fatty acid peroxidation due to oxidative stress and the consequent depletion of the polyunsaturated fatty acids have been implicated in the pathogenesis of nonalcoholic fatty liver disease in humans.[37] Increased blood and liver saturated fatty acids have been shown to accelerate β-oxidation and increase acetyl-CoA generation.

Part of the acetyl-CoA derived from the TCA cycle is used to generate energy, and the rest participates in the generation of cholesterol and ketone bodies, leading to increased blood cholesterol and ketone bodies.[25] In fact, excessive intake of saturated fatty acids has been shown to increase hepatic HMG-CoA reductase activity and cholesterol synthesis.[38] In addition, expression of genes involved in fatty acid oxidation is up-regulated in ApoE*3 Leiden transgenic mice with high cholesterol intake, providing indirect evidence for acceleration of fatty acid oxidation.[39] Our results are consistent with a previous study in rats in which increased intake of lipids resulted in increased plasma fatty acids.[40] These results demonstrated the efficacy of PC in alleviating abnormal fatty acid metabolism in hyperlipidemic rats.

Sterol Lipid Metabolism. Sterol lipids have different physiological functions. For example, bile acids regulate cholesterol homeostasis and intestinal absorption of lipophilic nutrients and contribute to the control of glucose, lipid, and energy homeostasis.[41] Sterol lipids including cholesterol ester (18:2), cholesterol, hydroxytestosterone, 19-hydroxydeoxycor-ticosterone, and cholic acid were significantly increased in the plasma of our diet-induced hyperlipidemic rats. The previous study demonstrated significant increase in plasma total bile acid and steroid concentrations with chronic increase in the high-fat diet intake.[42] Similarly, bile acids and steroids are significantly elevated in hyperlipidemic rabbits.[31] The previous study demonstrated significant increase in plasma cholesterol concentration in hamsters fed a high-fat diet.[43] Increased cholesterol level was due to the excessive intestinal absorption of fat and cholesterol in hyperlipidemic hamsters, causing the competitive inhibition of phytosterols and reduction of hepatic campesterol.[44] Additionally, plasma cholesterol ester (18:2) and cholesterol concentrations are significantly elevated in C57Bl6 mice fed a high-fat diet.[45] Plasma bile acids including ursodeoxycholic acid and chenodeoxycholic acid have been shown to be elevated in rats with atherosclerosis,[46] leading to distortion of energy homeostasis, glucose metabolism, and impaired cholesterol metabolism.[47] The present study demon-strated increased plasma concentration of five sterol lipids and its reversal by treatment with PC in our hyperlipidemic rats, suggesting that PC could ameliorate the perturbations of bile acid biosynthesis and cholesterol metabolism.

In conclusion, the untreated rats fed a high-fat diet exhibited significant elevation of plasma triglyceride and total and LDL cholesterol concentrations. This was associated with marked changes in plasma concentrations of seven fatty acids and five sterols. These changes represented disorders of biosynthesis and metabolism of the primary bile acids, steroids, and fatty acids and mitochondrial fatty acid elongation pathways in diet-induced hyperlipidemia. Treatment with PC resulted in significant improvements of hyperlipidemia and the associated abnormalities of the lipid metabolites.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b05350.

Figures S1−S10 (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

95%CI, 95% confidence interval; ANOVA, one-way analysis of variance; AUC, area under the curve; FDR, false discovery rate; HDL-C, high-density lipoprotein cholesterol; HLA, hyperlipidemia; IPA, ingenuity pathway analysis; LDL-C, low-density lipoprotein cholesterol; MetPA, metabolomics pathway analysis; MSEA, metabolite set enrichment analysis; PC, *Poria cocos*; PLS-DA, partial least-squares discriminant analysis; QEA, quantitative enrichment analysis; ROC, receiver-operating characteristic; TC, total cholesterol; TG, triglycerides; UPLC-QTOF/HDMS, ultraperformance liquid chromatography coupled with quadrupole time-of-flight synapt high-definition mass spectrometry

■ REFERENCES

(1) LaRosa, J. C. Prevention and treatment of coronary heart disease: who benefits? *Circulation* **2001**, *104*, 1688−1692.

(2) Alwaili, K.; Alrasadi, K.; Awan, Z.; Genest, J. Approach to the diagnosis and management of lipoprotein disorders. *Curr. Opin. Endocrinol., Diabetes Obes.* **2009**, *16*, 132−140.

(3) Wilkinson, M. J.; Laffin, L. J.; Davidson, M. H. Overcoming toxicity and side-effects of lipid-lowering therapies. *Best Pract. Res. Clin. Endocrinol. Metab.* **2014**, *28*, 439−452.

(4) Harvey, A. L.; Edrada-Ebel, R.; Quinn, R. J. The re-emergence of natural products for drug discovery in the genomics era. *Nat. Rev. Drug Discovery* **2015**, *14*, 111−129.

(5) Lindner, D. L.; Banik, M. T. Molecular phylogeny of Laetiporus and other brown rot polypore genera in North America. *Mycologia* **2008**, *100*, 417−430.

(6) Wang, Y. Z.; Zhang, J.; Zhao, Y. L.; Li, T.; Shen, T.; Li, J. Q.; Li, W. Y.; Liu, H. G. Mycology, cultivation, traditional uses, phytochemistry and pharmacology of *Wolfiporia cocos* (Schwein.) Ryvarden et Gilb.: a review. *J. Ethnopharmacol.* **2013**, *147*, 265−276.

(7) Zhao, Y. Y.; Lei, P.; Chen, D. Q.; Feng, Y. L.; Bai, X. Renal metabolic profiling of early renal injury and renoprotective effects of *Poria cocos* epidermis using UPLC Q-TOF/HSMS/MS^E. *J. Pharm. Biomed. Anal.* **2013**, *81−82*, 202−209.

(8) Zhao, Y. Y.; Li, H. T.; Feng, Y. L.; Bai, X.; Lin, R. C. Urinary metabonomic study of the surface layer of *Poria cocos* as an effective treatment for chronic renal injury in rats. *J. Ethnopharmacol.* **2013**, *148*, 403−410.

(9) Lu, Y. T.; Kuan, Y. C.; Chang, H. H.; Sheu, F. Molecular cloning of a *Poria cocos* protein that activates Th1 immune response and allays Th2 cytokine and IgE production in a murine atopic dermatitis model. *J. Agric. Food Chem.* **2014**, *62*, 2861−2871.

(10) Han, X.; Gross, R. W. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: a bridge to lipidomics. *J. Lipid Res.* **2003**, *44*, 1071−1079.

(11) Zhao, Y. Y.; Cheng, X. L.; Lin, R. C.; Wei, F. Lipidomics applications for disease biomarker discovery in mammal models. *Biomarkers Med.* **2015**, *9*, 153−168.

(12) Zhao, Y. Y.; Cheng, X. L.; Lin, R. C. Lipidomics applications for discovering biomarkers of diseases in clinical chemistry. *Int. Rev. Cell Mol. Biol.* **2014**, *313*, 1−26.

(13) Zhao, Y. Y.; Miao, H.; Cheng, X. L.; Wei, F. Lipidomics: Novel insight into the biochemical mechanism of lipid metabolism and dysregulation-associated disease. *Chem.−Biol. Interact.* **2015**, *240*, 220−238.

(14) Kwan, H. Y.; Hu, Y. M.; Chan, C. L.; Cao, H. H.; Cheng, C. Y.; Pan, S. Y.; Tse, K. W.; Wu, Y. C.; Yu, Z. L.; Fong, W. F. Lipidomics identification of metabolic biomarkers in chemically induced hyper-triglyceridemic mice. *J. Proteome Res.* **2013**, *12*, 1387−1398.

(15) Yin, W.; Carballo-Jane, E.; McLaren, D. G.; Mendoza, V. H.; Gagen, K.; Geoghagen, N. S.; McNamara, L. A.; Gorski, J. N.; Eiermann, G. J.; Petrov, A.; Wolff, M.; Tong, X.; Wilsie, L. C.; Akiyama, T. E.; Chen, J.; Thankappan, A.; Xue, J.; Ping, X.; Andrews, G.; Wickham, L. A.; Gai, C. L.; Trinh, T.; Kulick, A. A.; Donnelly, M. J.; Voronin, G. O.; Rosa, R.; Cumiskey, A. M.; Bekkari, K.; Mitnaul, L. J.; Puig, O.; Chen, F.; Raubertas, R.; Wong, P. H.; Hansen, B. C.; Koblan, K. S.; Roddy, T. P.; Hubbard, B. K.; Strack, A. M. Plasma lipid profiling across species for the identification of optimal animal models of human dyslipidemia. *J. Lipid Res.* **2012**, *53*, 51−65.

(16) Chen, F.; Maridakis, V.; O'Neill, E. A.; Hubbard, B. K.; Strack, A.; Beals, C.; Herman, G. A.; Wong, P. The effects of simvastatin treatment on plasma lipid-related biomarkers in men with dyslipidaemia. *Biomarkers* **2011**, *16*, 321−333.

(17) Miao, H.; Li, M. H.; Zhang, X.; Yuan, S. J.; Ho, C. C.; Zhao, Y. Y. The antihyperlipidemic effect of Fu-Ling-Pi is associated with abnormal fatty acid metabolism as assessed by UPLC-HDMS-based lipidomics. *RSC Adv.* **2015**, *5*, 64208−64219.

(18) Wu, S.; Chen, S.; Dong, X.; Tan, G.; Li, W.; Lou, Z.; Zhu, Z.; Chai, Y. Lipidomic profiling reveals significant alterations in lipid biochemistry in hypothyroid rat cerebellum and the therapeutic effects of Sini decoction. *J. Ethnopharmacol.* **2015**, *159*, 262−273.

(19) Cai, Y.; Gao, Y.; Tan, G.; Wu, S.; Dong, X.; Lou, Z.; Zhu, Z.; Chai, Y. Myocardial lipidomics profiling delineate the toxicity of traditional Chinese medicine *Aconiti Lateralis* radix praeparata. *J. Ethnopharmacol.* **2013**, *147*, 349−356.

(20) Zhao, Y. Y.; Wu, S. P.; Liu, S.; Zhang, Y.; Lin, R. C. Ultra-performance liquid chromatography-mass spectrometry as a sensitive and powerful technology in lipidomic applications. *Chem.−Biol. Interact.* **2014**, *220*, 181−192.

(21) Zhao, Y. Y.; Lin, R. C. UPLC−MS^E application in disease biomarker discovery: the discoveries in proteomics to metabolomics. *Chem.−Biol. Interact.* **2014**, *215*, 7−16.

(22) Miao, H.; Chen, H.; Zhang, X.; Yin, L.; Chen, D. Q.; Cheng, X. L.; Bai, X.; Wei, F. Urinary metabolomics on the biochemical profiles in diet-induced hyperlipidemia rat using ultraperformance liquid chromatography coupled with quadrupole time-of-flight SYNAPT high-definition mass spectrometry. *J. Anal. Methods Chem.* **2014**, *2014*, 184162.

(23) Miao, H.; Chen, H.; Pei, S.; Bai, X.; Vaziri, N. D.; Zhao, Y. Y. Plasma lipidomics reveal profound perturbation of glycerophospholi-pids, fatty acids, and sphingolipids in diet-induced hyperlipidemia. *Chem.−Biol. Interact.* **2015**, *228*, 79−87.

(24) Zhao, Y. Y.; Cheng, X. L.; Wei, F.; Bai, X.; Lin, R. C. Application of faecal metabonomics on an experimental model of tubulointerstitial fibrosis by ultra performance liquid chromatography/high-sensitivity mass spectrometry with MS^E data collection technique. *Biomarkers* **2012**, *17*, 721−729.

(25) Fahy, E.; Subramaniam, S.; Brown, H. A.; Glass, C. K.; Merrill, A. H., Jr.; Murphy, R. C.; Raetz, C. R.; Russell, D. W.; Seyama, Y.; Shaw, W.; Shimizu, T.; Spener, F.; van Meer, G.; VanNieuwenhze, M. S.; White, S. H.; Witztum, J. L.; Dennis, E. A. A comprehensive classification system for lipids. *J. Lipid Res.* **2005**, *46*, 839−862.

(26) Chong, W. P.; Goh, L. T.; Reddy, S. G.; Yusufi, F. N.; Lee, D. Y.; Wong, N. S.; Heng, C. K.; Yap, M. G.; Ho, Y. S. Metabolomics

profiling of extracellular metabolites in recombinant Chinese hamster ovary fed-batch culture. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 3763−3771.

(27) Chen, H.; Miao, H.; Feng, Y. L.; Zhao, Y. Y.; Lin, R. C. Metabolomics in dyslipidemia. *Adv. Clin. Chem.* **2014**, *66*, 101−119.

(28) Zhang, Q.; Wang, G.; A, J.; Ma, B.; Dua, Y.; Zhu, L.; Wu, D. Metabonomic profiling of diet-induced hyperlipidaemia in a rat model. *Biomarkers* **2010**, *15*, 205−216.

(29) Bai, D.; Song, J. N. Plasma metabolic biomarkers for syndrome of phlegm and blood stasis in hyperlipidemia and atherosclerosis. *J. Trad. Chin. Med.* **2012**, *32*, 578−583.

(30) Zhang, Q.; Wang, G. J.; A, J. Y.; Wu, D.; Zhu, L. L.; Ma, B.; Du, Y. Application of GC/MS-based metabonomic profiling in studying the lipid-regulating effects of *Ginkgo biloba* extract on diet-induced hyperlipidemia in rats. *Acta Pharmacol. Sin.* **2009**, *30*, 1674−1687.

(31) Ooga, T.; Sato, H.; Nagashima, A.; Sasaki, K.; Tomita, M.; Soga, T.; Ohashi, Y. Metabolomic anatomy of an animal model revealing homeostatic imbalances in dyslipidaemia. *Mol. BioSyst.* **2011**, *7*, 1217−1223.

(32) Roman, L. J.; Palmer, C. N. A.; Clark, J. E.; Masters, B. S. S. Expression of rabbit cytochromes P-450 which catalyze the ω-hydroxylation of arachidonic acid, fatty acid and prostaglandins. *Arch. Biochem. Biophys.* **1993**, *307*, 57−65.

(33) Liu, Y. T.; Jia, H. M.; Chang, X.; Cheng, W. H.; Zhao, X.; Ding, G.; Zhang, H. W.; Cai, D. Y.; Zou, Z. M. Metabolic pathways involved in Xin-Ke-Shu protecting against myocardial infarction in rats using ultra high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. *J. Pharm. Biomed. Anal.* **2014**, *90*, 35−44.

(34) Zhao, Y. Y.; Feng, Y. L.; Bai, X.; Tan, X. J.; Lin, R. C.; Mei, Q. Ultra performance liquid chromatography-based metabonomic study of therapeutic effect of the surface layer of *Poria cocos* on adenine-induced chronic kidney disease provides new insight into anti-fibrosis mechanism. *PLoS One* **2013**, *8*, e59617.

(35) Tarantino, G.; Colao, A.; Capone, D.; Conca, P.; Tarantino, M.; Grimaldi, E.; Chianese, D.; Finelli, C.; Contaldo, F.; Scopacasa, F.; Savastano, S. Circulating levels of cytochrome C, gamma-glutamyl transferase, triglycerides and unconjugated bilirubin in overweight/obese patients with non-alcoholic fatty liver disease. *J. Biol. Regul. Homeost. Agents.* **2011**, *25*, 47−56.

(36) Song, X.; Wang, J.; Wang, P.; Tian, N.; Yang, M.; Kong, L. ^{1}H NMR-based metabolomics approach to evaluate the effect of Xue-Fu-Zhu-Yu decoction on hyperlipidemia rats induced by high-fat diet. *J. Pharm. Biomed. Anal.* **2013**, *78−79*, 202−210.

(37) Araya, J.; Rodrigo, R.; Videla, L. A.; Thielemann, L.; Orellana, M.; Pettinelli, P.; Poniachik, J. Increase in long-chain polyunsaturated fatty acid n−6/n−3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clin. Sci.* **2004**, *106*, 635−643.

(38) Wang, M.; Wang, Y.; Zhao, M.; Wang, F.; Zhao, C. Metabolic profiling analysis of fatty acids from hyperlipidemic rats treated with *Gynostemma pentaphyllum* and atorvastatin based on GC/MS. *Anal. Methods* **2014**, *6*, 8660−8667.

(39) Kleemann, R.; Verschuren, L.; van Erk, M. J.; Nikolsky, Y.; Cnubben, N. H.; Verheij, E. R.; Smilde, A. K.; Hendriks, H. F.; Zadelaar, S.; Smith, G. J.; Kaznacheev, V.; Nikolskaya, T.; Melnikov, A.; Hurt-Camejo, E.; van der Greef, J.; van Ommen, B.; Kooistra, T. Atherosclerosis and liver inflammation induced by increased dietary cholesterol intake: a combined transcriptomics and metabolomics analysis. *Genome Biol.* **2007**, *8*, R200.

(40) Toye, A. A.; Dumas, M. E.; Blancher, C.; Rothwell, A. R.; Fearnside, J. F.; Wilder, S. P.; Bihoreau, M. T.; Cloarec, O.; Azzouzi, I.; Young, S.; Barton, R. H.; Holmes, E.; McCarthy, M. I.; Tatoud, R.; Nicholson, J. K.; Scott, J.; Gauguier, D. Subtle metabolic and liver gene transcriptional changes underlie diet-induced fatty liver susceptibility in insulin-resistant mice. *Diabetologia* **2007**, *50*, 1867−1879.

(41) Li, T.; Chiang, J. Y. Bile acid signaling in liver metabolism and diseases. *J. Lipids* **2012**, *2012*, 754067.

(42) Hofmann, A. F. The continuing importance of bile acids in liver and intestinal disease. *Arch. Intern. Med.* **1999**, *159*, 2647−2658.

(43) Gu, S.; A, J.; Wang, G.; Zha, W.; Yan, B.; Zhang, Y.; Ren, H.; Cao, B.; Liu, L. Metabonomic profiling of liver metabolites by gas chromatography-mass spectrometry and its application to characterizing hyperlipidemia. *Biomed. Chromatogr.* **2010**, *24*, 245−252.

(44) Tilvis, R. S.; Miettinen, T. A. Serum plant sterols and their relation to cholesterol absorption. *Am. J. Clin. Nutr.* **1986**, *43*, 92−97.

(45) Castro-Perez, J. M.; Roddy, T. P.; Shah, V.; McLaren, D. G.; Wang, S. P.; Jensen, K.; Vreeken, R. J.; Hankemeier, T.; Johns, D. G.; Previs, S. F.; Hubbard, B. K. Identifying static and kinetic lipid phenotypes by high resolution UPLC-MS: unraveling diet-induced changes in lipid homeostasis by coupling metabolomics and fluxomics. *J. Proteome Res.* **2011**, *10*, 4281−4290.

(46) Zhang, F.; Jia, Z.; Gao, P.; Kong, H.; Li, X.; Chen, J.; Yang, Q.; Yin, P.; Wang, J.; Lu, X.; Li, F.; Wu, Y.; Xu, G. Metabonomics study of atherosclerosis rats by ultra fast liquid chromatography coupled with ion trap-time of flight mass spectrometry. *Talanta* **2009**, *79*, 836−844.

(47) Houten, S. M.; Watanabe, M.; Auwerx, J. Endocrine functions of bile acids. *EMBO J.* **2006**, *25*, 1419−1425.