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Lipidomics biomarkers of diet-induced hyperlipidemia and its treatment with Poria cocos

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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 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 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 [palmitic acid, hexadecenoic acid, hexanoylcarnitine, tetracosahexaenoic acid, cervonoyl ethanolamide, 3-hydroxy-tetradecanoic 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 metabolisms 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, ultra-performance 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 limit 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 the 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 ten percent of traditional Chinese medicines included in the Chinese Pharmacopoeia contain PC. PC facilitates fecal and urinary disposal of the waste products and exerts diuretic, nephroprotective, and immunomodulatory activities[6,7,8,9] To our knowledge, the effect and potential mechanism of action of PC administration in the treatment of hyperlipidemia has not been previously reported.

Lipidomics is a branch of the omic field which was first introduced by Han and Gross in 2003[10] and represents a system-based study of a wide range of lipid species. Recently, lipidomics approach has been applied as a tool for identification of biomarkers, understanding of the mechanisms, and diagnosis of various diseases and monitoring their response to therapeutic interventions, and drug toxicity in animal models and in clinical studies.[11,12,13] 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 diets-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.[17,18,19]

The present study was designed to define the alterations of lipid metabolites in rats with the high fat diet-induced hyperlipidemia and to explore the efficacy and mechanism of action of PC using ultra-performance liquid chromatography coupled 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 3 times with 5 L 95% ethanol at room temperature by ultrasonic method. PC extracts were filtrated 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 six weeks.[22] They were randomized to the untreated group and PC-treated group. The PC-treated group was administered PC (250 mg/kg BW) by gastric gavage once a day. After six 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 the 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 analyser 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 Ostro 96-well plate was performed as a single-step in-well extraction. 100 μL plasma sample was loaded into each well of a 2000 μL Ostro preparation plate fitted onto a vacuum manifold. 300 μL of methanol/chloroform (1:1, v/v) was added to each well and mixed using aspirating the mixture 10x by a micropipette. A vacuum of 15'' 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 and got the total fraction volume to 900 μL. The eluate fraction was dried by nitrigen, 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^{TM} Ultra Performance LC system equipped with a Waters Xevo^{TM} G2 QTof MS. UPLC analysis was performed on a HSS T3 column. The mobile phase consisted 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) was used as follows: a linear gradient from 0–10 min, 40.0–99.0% A and 10.0–12.0 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 °C and 55 °C, respectively. A 2.0 µl sample solution was injected for each run. The MS scan ranged from 50 to 1200 m/z in positive ion mode. The cone voltage and capillary voltage 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 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 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 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 g, 385±45 g 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 control group was 0.97±0.12 mmol/L whereas HDL-C concentration in 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 rat model of diet-induced hyperlipidemic was successfully reproduced and that PC treatment was effective in alleviating dyslipidemia in rats with high fat diet-induced hyperlipidemic.

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 UPLC-HDMS method in the present study. Figure 1A showed 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 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 based on the VIP values in the loading plot of PLS-DA model (Figure 1D). 175 variables had VIP values of more than 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 eleven fatty acids, five sterol lipids, two glycerophospholipids, two sphingolipids, two glycerolipids, and one prenol lipid. Compared with the hyperlipidemic group, changes in nineteen 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 control group, twelve lipid species were below the normal levels and seven 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). PCA score plot of 22 lipid species in PC-treated group were located between untreated hypelipidemic and control groups and were 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, 20 were positively correlated with control group. The other lipid species were negatively correlated with control group, indicating normal lipid metabolism. Except for lipid species 3, 5, 10, 16 and 20, the other lipid species are positively correlated with hyperlipidemic group. These findings illustrate the marked abnormalities of metabolic profile caused by high fat diet. Nineteen lipid species in 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 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, eighteen lipid species were identified as the biomarker candidates with an AUC of 0.82 or greater. Although the AUC value for eicosenoic acid, 10,11-dihydro-12R-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 were found to be low (<80%). Fourteen out of eighteen lipid species had high AUC value (>0.80), sensitivity (>80%) and specificity (>80%) and they 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 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 eleven 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 is illustrated in Figure 6. The effects on the other pathways are shown in supplementary Figs. S1-S10. In addition, twenty-eight metabolic pathways were found to be dysregulated in diet-induced hyperlipidemia based on the analysis of the QEA algorithm of the MSEA method (Figure 5B).

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] Based on 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, fourteen lipid biomarkers were selected based on univariate or multivariate statistical analysis and ROC curve analysis. Seven fatty acyls and five sterol lipids were main biomarkers of anti-hyperlipidemic effects of PC which were in agreement with the previously identified biomarkers described in hypertriglyceridemia, hyperlipidemia, and hypercholesterolemia models.[27] Changes

208 in seven fatty acids including significant increase in palmitic acid, hexadecenoic acid, and hexanoylcarnitine,
209 tetracosahexaenoic acid as well as significant decrease in cervonoyl ethanolamide, 3-hydroxy-tetradecanoic
210 acid and 5,6-DHET were observed in diet-induced hyperlipidemic rats. These changes were completely
211 reversed by treatment with PC. A number of experimental investigations performed in the past several years
212 have revealed that unsaturated and saturated fatty acid metabolism is disturbed in diet-induced
213 hyperlipidemia.[15,23,28] For example, palmitic acid which is one of the most common saturated fatty acids, has
214 been identified as a marker of hyperlipidemia in diet-induced hyperlipidemic patients and rats.[29,30]
215 Additionally, LC-MS-based lipidomics demonstrated that the levels of plasma palmitic acid, stearic acid,
216 oleic acid, linoleic acid, linolenic acid, arachidonic acid and docosahexaenoic acid are significantly increased
217 in both schisandrin B-induced and high fat diet-induced hypertriglyceridemic mouse models.[14] Similarly,
218 fatty acids with carbon chain length from 14 to 24 carbon atoms have been shown to be altered in Watanabe
219 heritable hyperlipidemic rabbits which is a model of hypercholesterolemia.[31]
220 Hexadecenoic acid which is one of the identified lipid biomarkers is the byproduct of hydroxylation of the
221 terminal (ώ) carbon of palmitic acid. Palmitic acid ώ-hydroxylation catalyzed by cytochrome P-450 in
222 animals.[32] Hexanoylcarnitine has been identified as a biomarker for the protective effects of the Chinese
223 drug, Xin-Ke-Shu, against myocardial infarction in rats.[33] Hexanoylcarnitine is a longer-chain acyl carnitine
224 which is metabolized via medium chain fatty acid β-oxidation. Hexanoylcarnitine accumulation was
225 observed in our diet-induced hyperlipidemic rats. Treatment with PC attenuated accumulation of
226 hexanoylcarnitine in hyperlipidemic rats most likely by enhancing fatty acid oxidation and counteracting the
227 isoproterenol-induced reduction of energy production.[33]
228 Consistent with the results of the present study, in a previous study we found increased tetracosahexaenoic
229 acid in rats with chronic kidney disease and its reversal by treatment with the surface layer of PC[34]. These
230 findings indicate that fatty acids play an important pathogenic role in the dyslipidemia-associated diseases.

Indeed, free fatty acids serve as substrate for 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 non-alcoholic fatty liver disease in humans.[37] Increased blood and liver saturated fatty acids has 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 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, intestinal absorption of lipophilic nutrients, and contribute to the control of glucose, lipid and energy homeostasis.[41] Sterol lipid including cholesterol ester (18:2), cholesterol, hydroxytestosterone, 19-hydroxydeoxycorticosterone 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 high fat diet.[43]

Increased cholesterol level was due to the excessive intestinal absorption of fat and cholesterol in the 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 demonstrated increased plasma concentration of five sterol lipids and its reversal by treatment with PC in our hyperlipidaemic rats, suggesting that PC could ameliorate the perturbations of bile acid biosynthesis and cholesterol metabolism.

In conclusion, the untreated rats fed 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 metabolisms 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.

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277 **Notes**

278 The authors declare no competing financial interest.

279 ■ **ABBREVIATIONS USED**

280 95%CI, 95% confidence interval; ANOVA, one-way analysis of variance; AUC, area under the curve; FDR,
281 false discovery rate; HDL-C, deficiency of high-density lipoprotein cholesterol; HLA, hyperlipidemia; IPA,
282 ingenuity pathway analysis; LDL-C, low-density lipoprotein cholesterol; MetPA, metabolomics pathway
283 analysis; MSEA, metabolite set enrichment analysis; PC, *Poria cocos*; PLS-DA, partial least
284 squares-discriminant analysis; QEA, quantitative enrichment analysis; ROC, receiver-operating characteristic;
285 TC, total cholesterol; TG, triglycerides; UPLC-QTOF/HDMS, ultra-performance liquid chromatography
286 coupled with quadrupole time-of-fight synapt high-definition mass spectrometry.

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Table 1. Identified plasma lipid species, fold changes (FC) and *p*-values among control group, HLA group and HLA+PC group

No.	metabolites	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	9.86E-05	1.02E-05	1.45E-04	0.68	5.01E-08	6.97E-07	9.19E-08	0.88	7.87E-03	1.74E-02	1.73E-02
2	Tyromycic acid	8.7	2.90	1.80E-07	3.63E-06	4.96E-07	0.31	3.55E-09	8.49E-07	1.30E-08	0.88	2.07E-02	2.46E-03	3.80E-02
3	LysoPC(16:0)	7.6	0.75	6.36E-09	3.62E-06	2.80E-08	1.22	4.95E-06	2.80E-05	7.78E-06	0.92	2.21E-02	2.78E-02	3.47E-02
4	Cholesterol ester (18:2)	7.0	1.36	6.31E-06	8.57E-06	1.39E-05	0.64	2.75E-08	1.44E-06	5.50E-08	0.88	2.07E-02	2.46E-03	3.51E-02
5	5,6-DHET	5.4	0.48	1.60E-08	3.85E-06	5.86E-08	1.89	5.60E-12	5.51E-07	6.16E-11	0.92	2.95E-01	2.95E-01	3.41E-01
6	Hexadecenoic acid	5.1	1.32	8.01E-05	5.95E-06	1.36E-04	0.84	2.44E-03	5.19E-04	3.36E-03	1.11	4.97E-02	5.97E-02	6.83E-02
7	Eicosenoic acid	5.0	1.25	1.26E-04	1.14E-03	1.63E-04	0.84	2.82E-03	4.66E-03	3.65E-03	1.05	5.12E-01	3.14E-01	5.37E-01
8	Cholesterol	4.9	1.63	1.93E-05	2.02E-04	3.54E-05	0.76	8.85E-03	2.89E-02	1.08E-02	1.23	2.27E-01	2.40E-01	2.77E-01
9	Sphingosine 1-phosphate	4.8	1.32	8.29E-05	3.97E-05	1.30E-04	0.59	6.38E-09	2.09E-06	1.75E-08	0.79	1.05E-04	7.46E-04	7.72E-04
10	Cervonoyl ethanolamide	4.6	0.39	6.66E-14	3.47E-06	1.47E-12	1.47	5.09E-08	6.02E-05	8.61E-08	0.58	1.71E-07	2.76E-04	3.76E-06
11	Hydroxytestosterone	4.3	1.51	1.31E-12	4.71E-05	1.44E-11	0.55	9.64E-11	2.12E-06	4.24E-10	0.84	6.27E-04	2.03E-03	2.30E-03
12	Hexanoylcarnitine	4.1	1.32	1.97E-08	2.43E-03	6.19E-08	0.53	1.39E-11	7.32E-07	1.02E-10	0.70	2.17E-03	2.27E-03	5.95E-03
13	Tetracosahexaenoic acid	4.0	1.32	1.01E-04	3.97E-05	1.38E-04	0.59	8.35E-09	2.08E-06	2.04E-08	0.79	1.12E-04	8.08E-04	6.18E-04
14	10,11-Dihydro-12R-hydroxy-leukotriene C4	3.8	1.23	2.12E-04	1.05E-03	2.45E-04	0.65	4.44E-09	2.97E-06	1.40E-08	0.80	1.77E-04	5.15E-04	7.80E-04
15	19-Hydroxydeoxycorticosterone	3.5	1.91	4.47E-12	3.50E-06	3.28E-11	0.69	1.59E-12	7.02E-07	3.50E-11	1.32	6.03E-06	8.00E-05	6.63E-05
16	Cholic acid	3.2	1.34	2.30E-06	2.50E-05	5.62E-06	0.61	1.12E-08	1.50E-05	2.46E-08	0.82	3.01E-02	2.62E-02	4.42E-02
17	2-Methylbutyroylcarnitine	2.8	1.49	9.90E-04	1.21E-04	9.90E-04	0.86	6.87E-02	1.17E-02	7.20E-02	1.28	1.59E-02	1.90E-02	3.18E-02
18	12-Oxo-20-carboxy-leukotriene B4	2.7	1.79	5.92E-04	1.74E-04	6.21E-04	0.75	5.15E-02	3.76E-02	5.67E-02	1.34	7.71E-02	2.87E-01	9.97E-02
19	Phytosphingosine	2.5	1.27	1.74E-04	9.81E-04	2.13E-04	0.81	2.16E-05	8.99E-05	3.17E-05	1.02	7.62E-01	8.44E-01	7.62E-01
20	3-Hydroxy-tetradecanoic acid	2.2	0.66	5.22E-12	3.52E-06	2.87E-11	1.34	3.90E-11	7.02E-07	2.15E-10	0.89	3.87E-03	4.16E-03	9.46E-03
21	MG(16:1)	1.9	1.29	6.94E-06	1.20E-04	1.39E-05	0.95	1.27E-01	1.37E-01	1.27E-01	1.22	1.60E-03	3.72E-03	5.02E-03
22	PE(14:1/18:2)	1.6	1.23	2.77E-04	9.83E-04	3.04E-04	0.87	3.43E-02	3.86E-02	3.97E-02	1.07	3.65E-01	7.51E-01	4.01E-01

[a] VIP was obtained from PLS-DA; [b] FC was calculated based on 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 vs control, between PC vs HLA or between PC vs control, while a FC value less than zero indicates a lower intensity of the lipid species between HLA vs control, between PC vs HLA or between PC vs 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 Benjamini Hochberg method.

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; the 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; the impact is the pathway impact value calculated from pathway topology analysis.

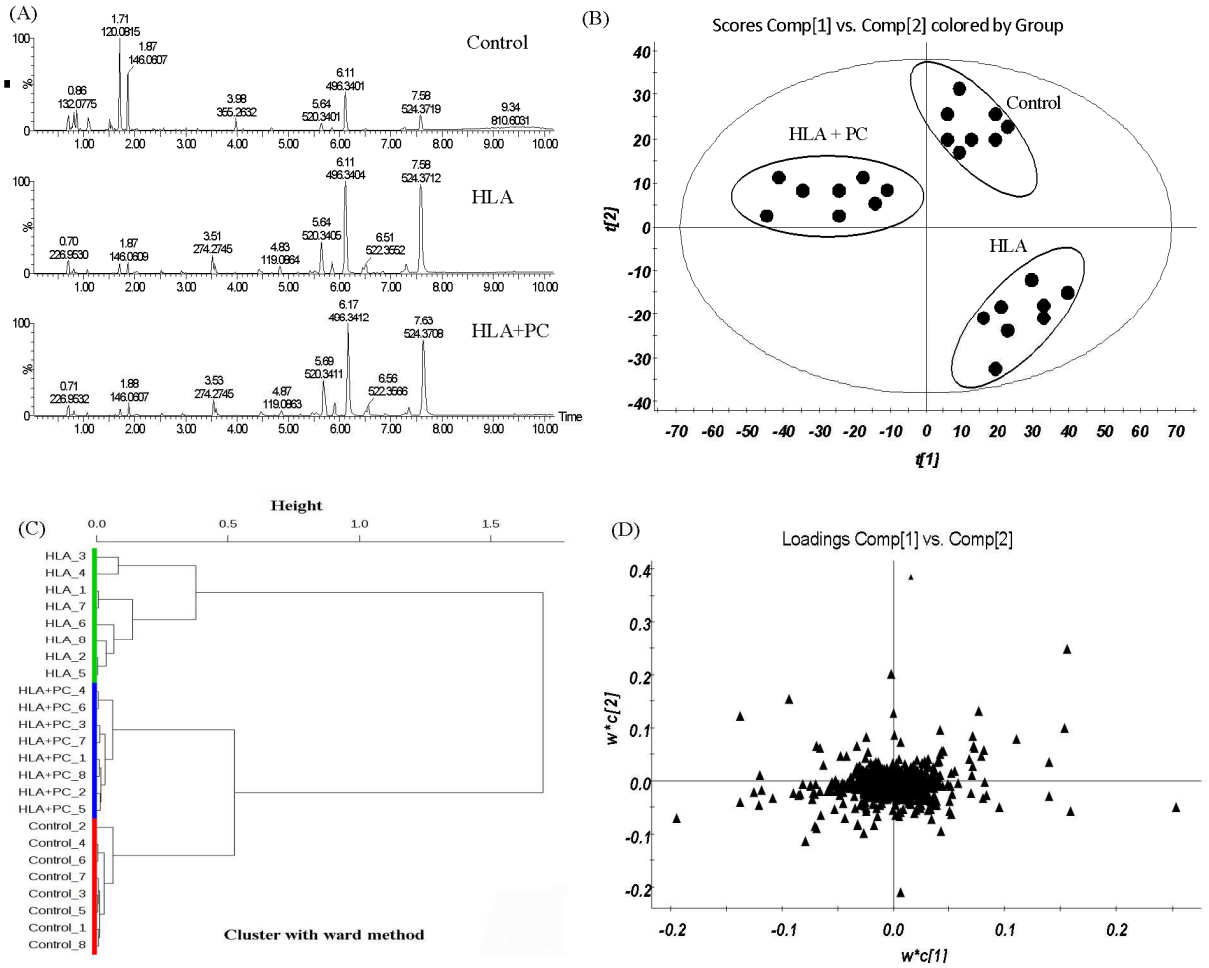


Figure 1. Lipid profiling and multivariate statistical analysis. (A) Base peak intensity chromatograms of control group, HLA group and HLA+PC group in positive ion mode obtained from UPLC-HDMS analysis. (B) PLS-DA model for control group, HLA group and HLA+PC group. (C) The clustering analysis of control group, HLA group and HLA+PC group. (D) Loading plot of PLS-DA in positive ion mode from the control group, HLA group and HLA+PC group.

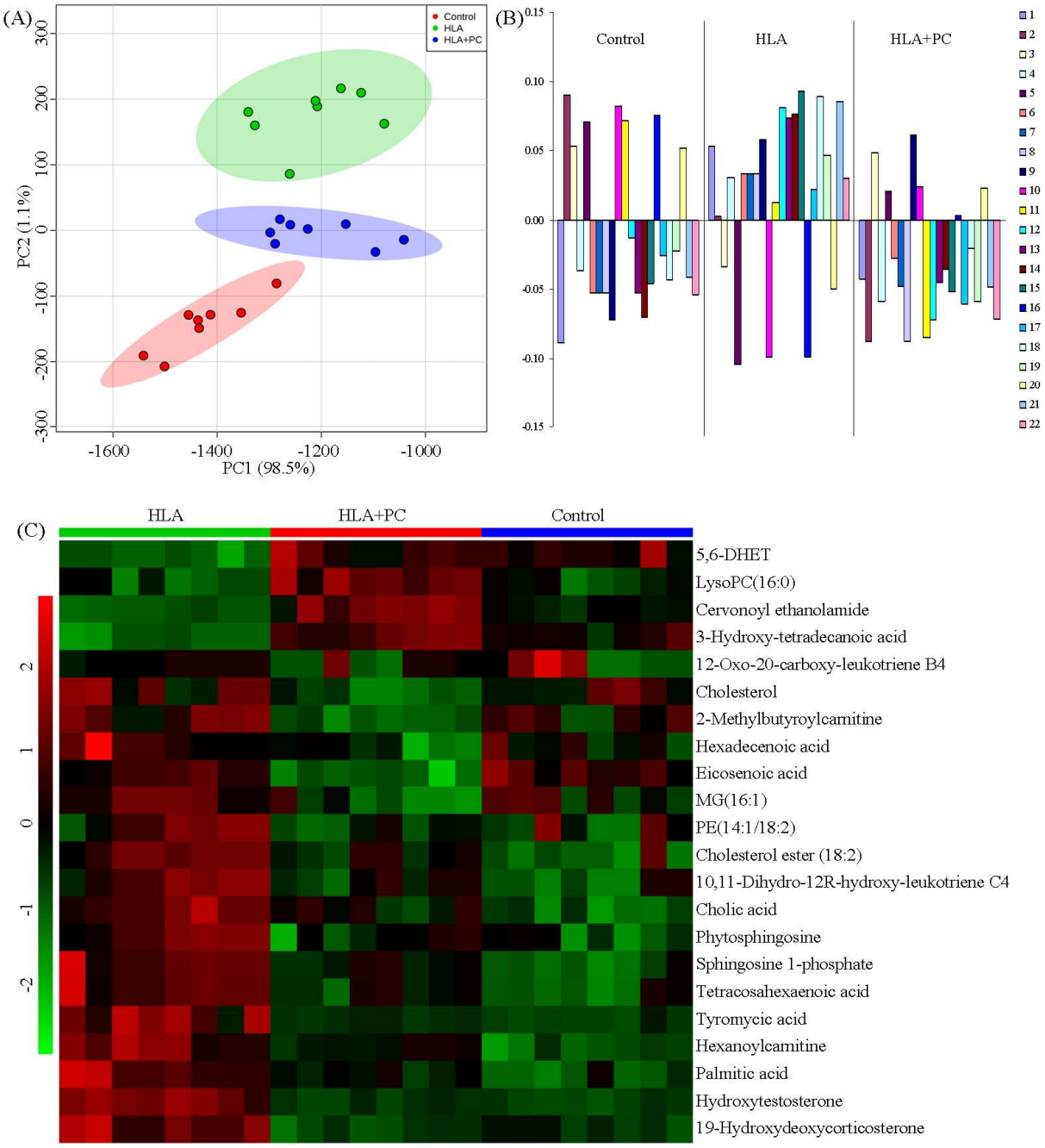


Figure 2. Lipidomic profiling of 22 identified lipid species. (A) PCA of two components of lipid species from control group, HLA group and HLA+PC group. (B) Correlation coefficient analysis among control group, HLA group and HLA+PC group with corresponding 22 lipid species in the different groups. Numbers consist 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.

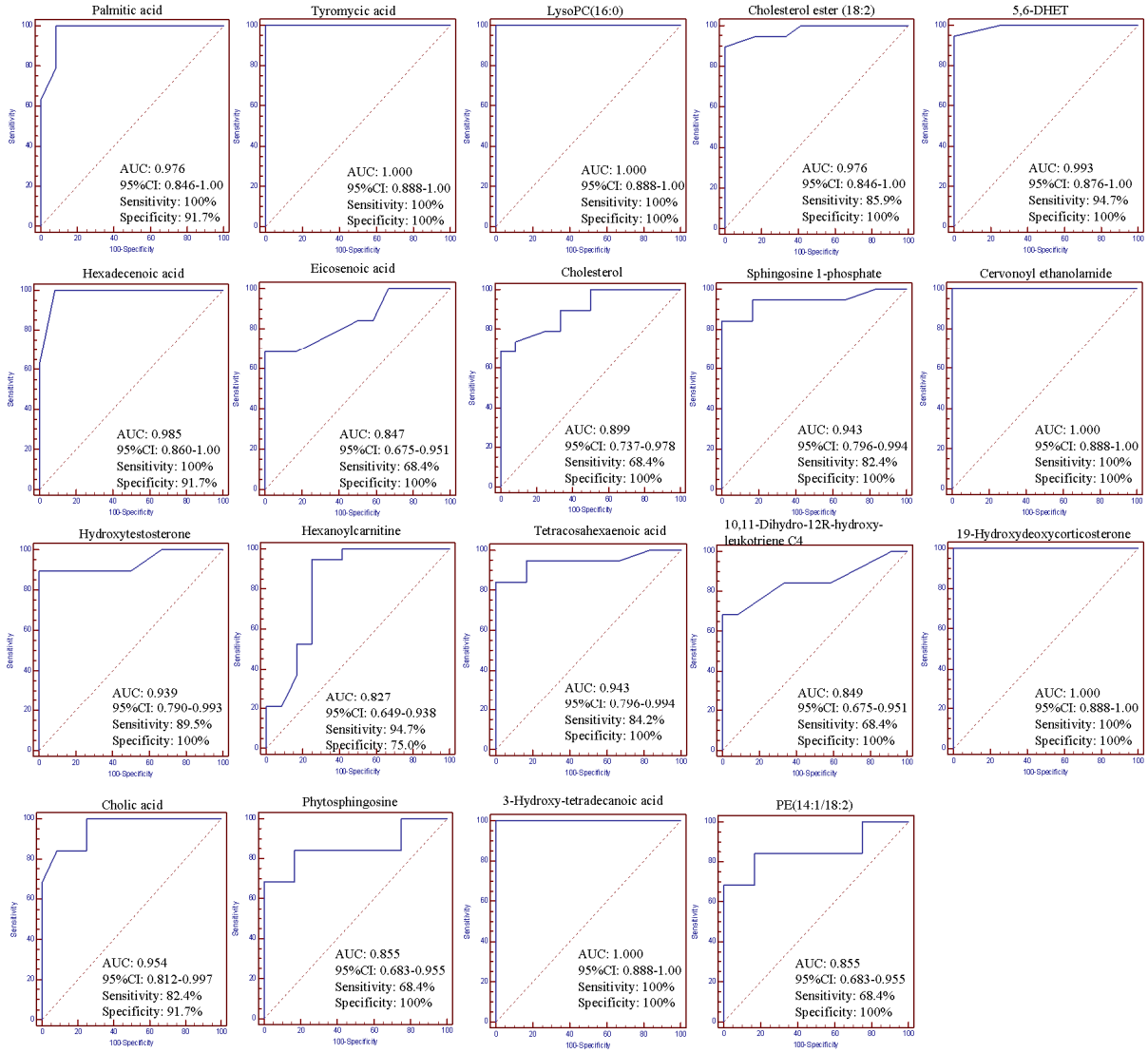


Figure 3. PLS-DA-based ROC curves of the 19 lipid species for biomarker selection of antihyperlipidemic effects of PC. The associated AUC, 95% CI, sensitivities and specificities were indicated.

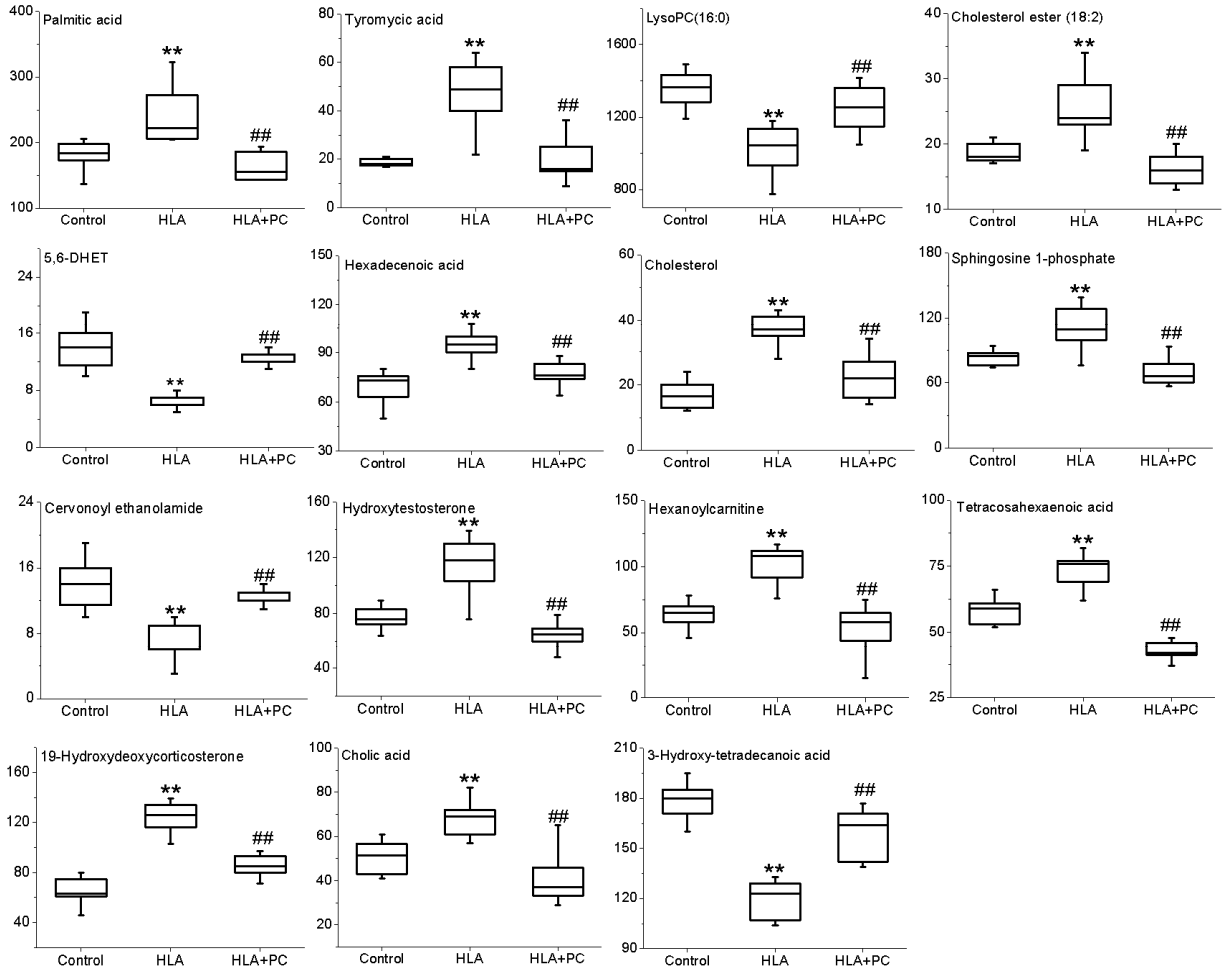


Figure 4. Box plots showing fifteen significant changes in the levels of lipid biomarkers among the control group, HLA group and HLA+PC group. The statistical significance between the two groups is marked. $^{**}p<0.01$ significant difference compared with control group; $^{\#\#}p<0.01$ significant difference compared with HLA group. Y-axis: normalized relative intensity.

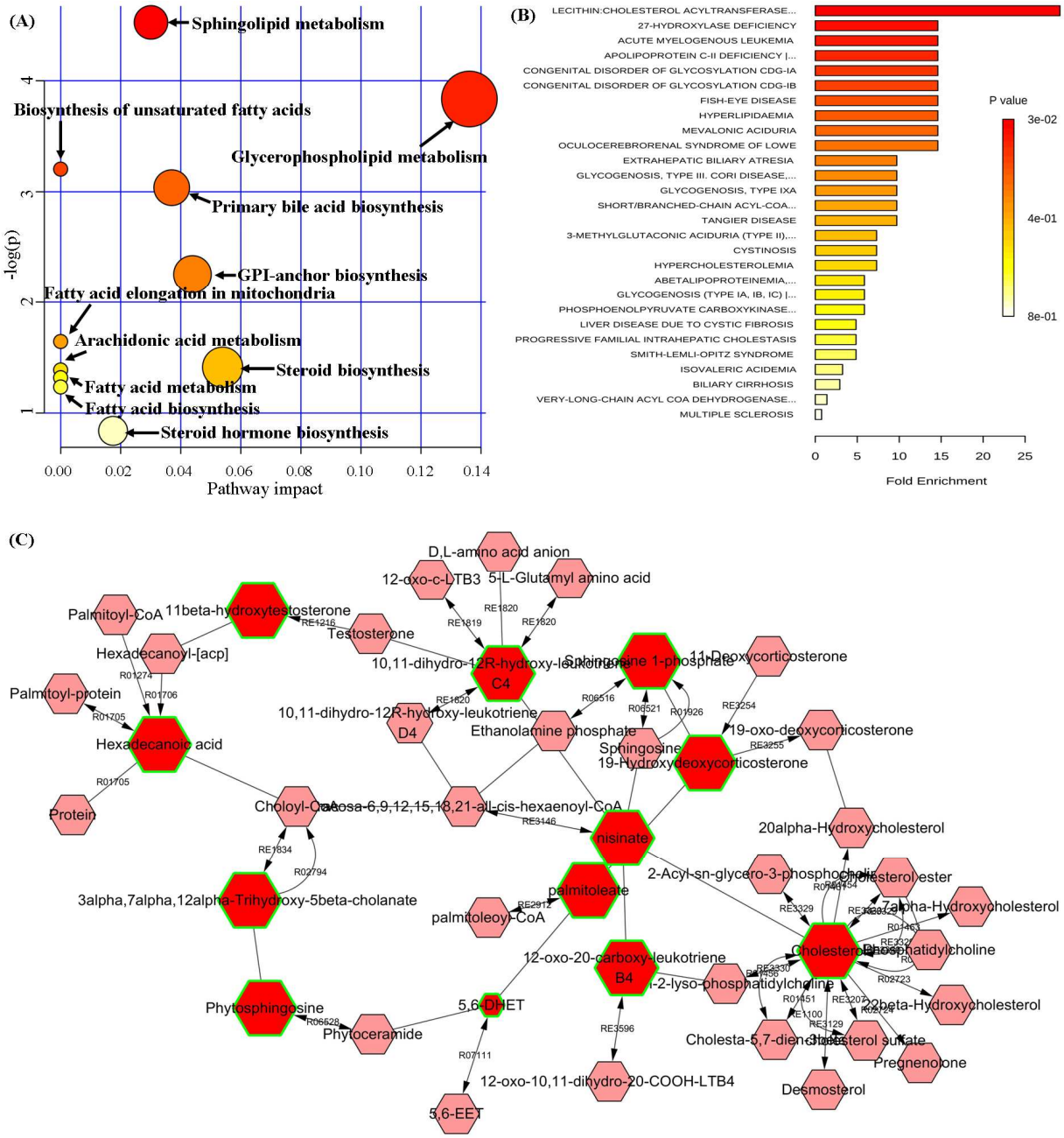


Figure 5. lipid metabolic pathway analysis of identified differential lipid species. (A) Summary of IPA with MetPA including sphingolipid metabolism, glycerophospholipid metabolism, biosynthesis of unsaturated fatty acids, primary bile acid biosynthesis, GPI-anchor biosynthesis, fatty acid elongation 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 was 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 were shown by red hexagons. Hexagons with green lines means that the significantly 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 showed metabolites participating in the metabolic pathway but not been identified in the current study. antihyperlipidemic 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, mono-unsaturated fatty acid beta-oxidation, omega-3 fatty acid metabolism, saturated fatty acids beta-oxidation, squalene and cholesterol biosynthesis.

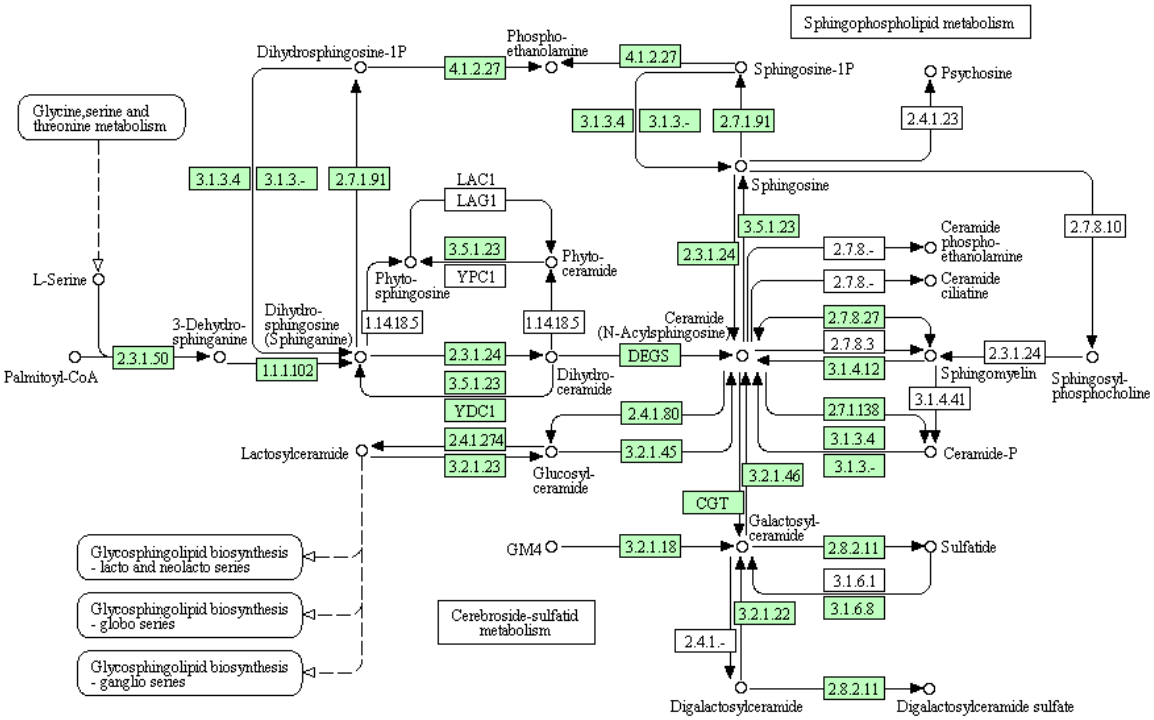
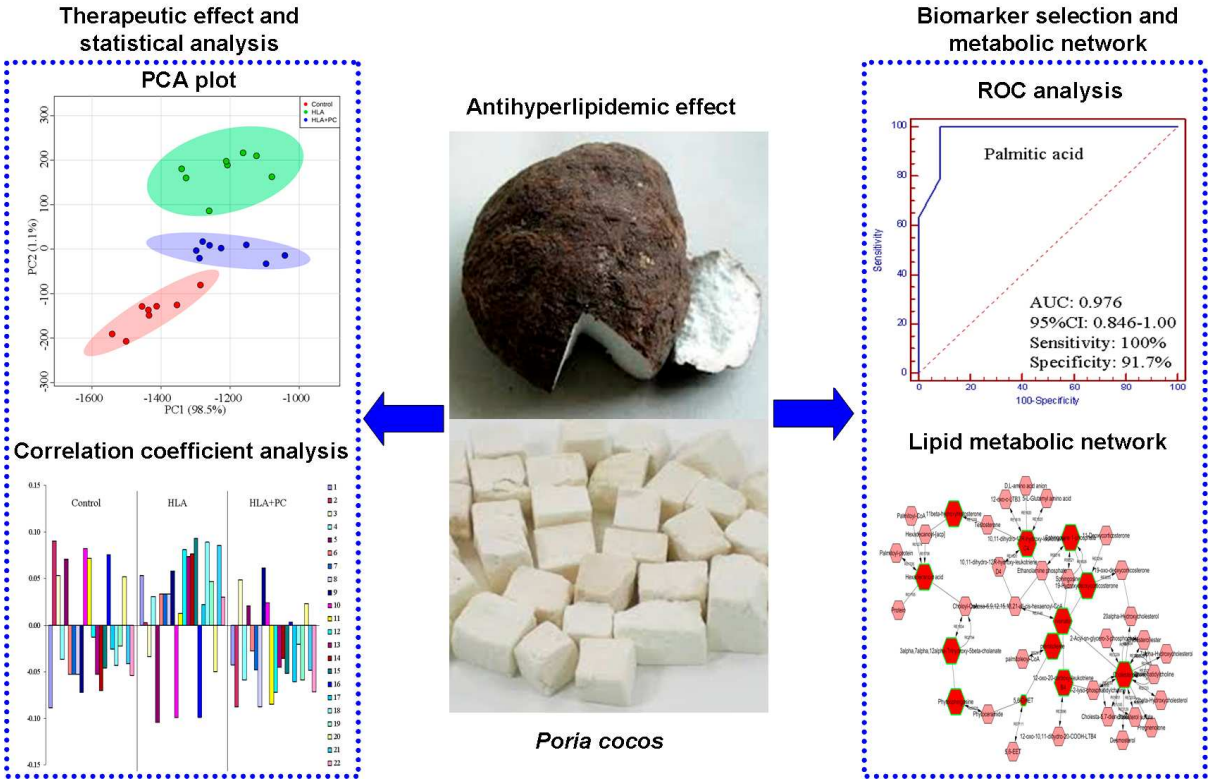
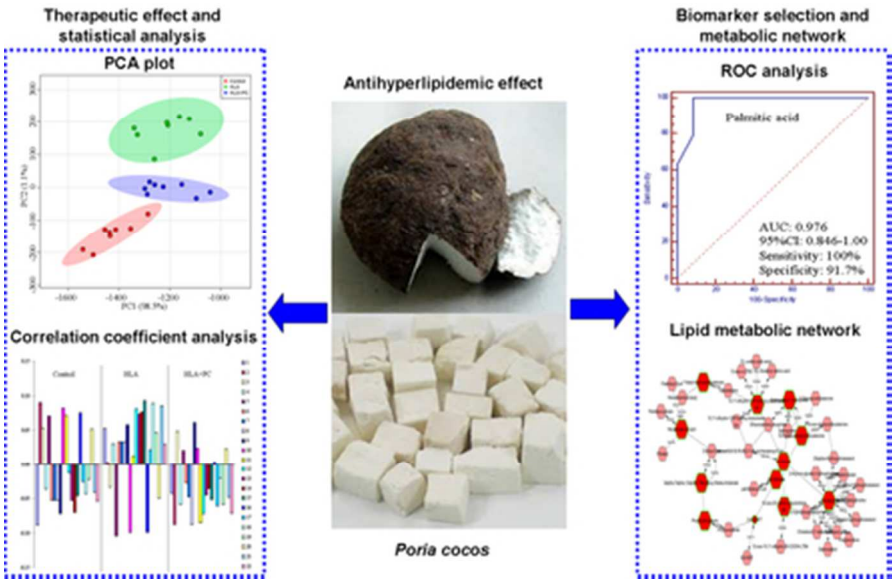


Figure 6. Overview of sphingolipid metabolism with MetPA. The reference map by KEGG. The green boxes represent enzymatic activities with putative cases of analogy in rats.

Graphic for manuscript





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