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Serum Metabolomics Study of Polycystic Ovary Syndrome Based on Liquid Chromatography−Mass Spectrometry

Xinjie Zhao,[†,⊥] Fang Xu,[‡,⊥] Bing Qi,[§,⊥] Songli Hao,[‡] Yanjie Li,[†] Yan Li,[‡] Lihong Zou,[∥] Caixia Lu,[‡] Guowang Xu,*[,†] and Lihui Hou*[,‡]

[†]Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

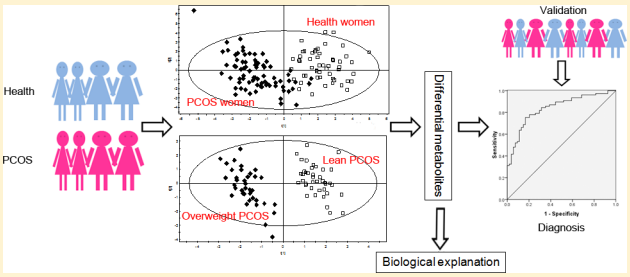
[‡]Gynaecological Clinic of First Affiliated Hospital, Heilongjiang University of Chinese Medicine, Harbin 150040, China

[§]Subsidiary Dongfang Hospital, Beijing University of Chinese Medicine, Beijing 100078, China

[∥]The First Affiliated Hospital of Medical University, Harbin 150001, China

S *Supporting Information*

ABSTRACT: Polycystic ovary syndrome (PCOS) is a complex, heterogeneous disorder, which produces in 5−10% reproductive age women. In this study, a nontargeted metabolomics approach based on ultra high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry is used to investigate serum metabolic characteristics of PCOS. PCOS women and healthy control can be clustered into two distinct groups based on multivariate statistical analysis. Significant increase in the levels of unsaturated free fatty acids, fatty acid amides, sulfated steroids, glycated amino acid and the decrease in levels of lysophosphatidylcholines, lysophosphatidylethanolamines, etc., were found. These metabolites showed abnormalities of lipid- and androgen-metabolism, increase of stearoyl-CoA desaturase (SCD) activity and accumulation of advanced glycation end-products in PCOS patients. On the basis of the binary logistic regression model, free fatty acid (FFA) 18:1/FFA 18:0, FFA 20:3, dihydrotestosterone sulfate, glycated phenylalanine, and uridine were combined as a diagnostic biomarker. The area under the curve (AUC) of combinational biomarker was 0.839 in 131 discovery phase samples and 0.874 in 109 validation phase samples. The findings of our study offer a new insight to understand the pathogenesis mechanism, and the discriminating metabolites may provide a prospect for PCOS diagnosis.



KEYWORDS: *Metabolomics, LC−MS, polycystic ovary syndrome, biomarkers*

1. INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most common female endocrine disorders; about 5−10% reproductive age women produce PCOS symptoms.[1,2] PCOS leads to female subfertility and increases risk to type 2 diabetes and cardiovascular disease.[3,4] Recent evidence suggests that women with PCOS have higher risk of developing endometrial cancer and ovarian cancer.[5−7] PCOS is a complex disease, pathogenesis is not clear, but it is believed to be the result from genetic, environmental, and lifestyle interaction.[8−10] It is relatively clear that PCOS is closely related with hormone abnormality.[9,11,12] More and more evidence displayed that insulin signaling pathways are closely related to PCOS.[3,12] PCOS may also be associated with chronic inflammation[13,14] and increased level of oxidative stress.[15,16] Diagnosis of PCOS is quite difficult since the symptoms of PCOS patients are not unified, including menstrual disturbances, elevated levels of male hormones, and polycystic ovaries.[4] Rotterdam criteria are the most widely used diagnostic standard, which can be associated with a wide range of symptoms.

Metabolomics is a power tool to study disease pathogenesis and to scan diagnostic biomarkers;[17−19] it has been successfully applied in the study of metabolic diseases, such as insulin resistance,[20] type 2 diabetes,[21] and cardiovascular disease.[18] Metabolomics studies of PCOS were also performed using nuclear magnetic resonance (NMR)[22−25] and gas chromatography−mass spectrometry (GC−MS),[23] and metabolic characteristics between different phenotypes were involved. Amino acids, creatinine, lactate, lipids, etc., were considered as the potential biomarkers of PCOS.[23−25] Compared with other analytical techniques, main advantages of liquid chromatography−mass spectrometry (LC−MS) are wide dynamic range and coverage of a wide chemical diversity; it is a complementary analytical technique to NMR and GC−MS in metabolomics studies.[26,27]

In this work, LC−MS-based metabolomics approach was used to investigate the metabolic changes of PCOS patients. Furthermore, discriminative metabolites were evaluated on the

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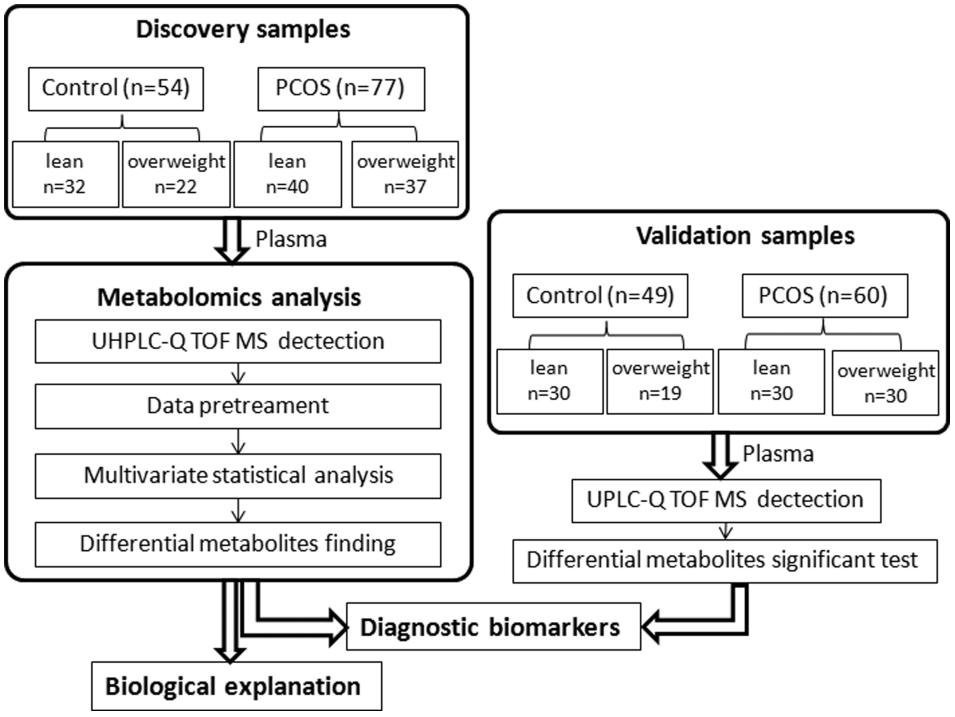

Figure 1. Flowchart of study strategy in this study.

capability to diagnose PCOS. The flowchart of study strategy is shown in Figure 1. The aim is to offer a new insight in the complex dysregulation of the metabolism in PCOS patients and to provide a new way for PCOS diagnosis.

2. MATERIALS AND METHODS

2.1. Human Samples

All of the PCOS patients were recruited from the Gynaecological Clinic of First Affiliated Hospital, Heilongjiang University of Chinese Medicine. Healthy women (the control group) were recruited from the Physical Examination Center of the First Affiliated Hospital, Heilongjiang University of Chinese Medicine and Physical Examination Center of the First Affiliated Hospital of Harbin Medical University. The study was approved by the Institutional Review Board of the First Affiliated Hospital, Heilongjiang University of Chinese Medicine (2010HZYLL-014) in accordance with the Declaration of Helsinki. All study participants signed an informed consent form before the start of the study.

According to the diagnostic criteria revised by the European Society of Human Reproduction and Embryology and the American Society for Reproductive Medicine at the Rotterdam in 2003,[4] PCOS patients can be diagnosed if two of the three criteria are present after excluding congenial adrenal hyperplasia, Cushing's syndrome, androgen secreting tumors, or other related disorders. The three criteria are (1) oligo- and/or anovulation; (2) clinical and/or biochemical signs of hyperandrogenism (clinical manifestations of hyperandrogenism include presence of acne, hirsutism, and androgenic alopecia); (3) polycystic ovaries by ultrasound examination: presence of 12 or more follicles in each ovary measuring 2−9 mm in diameter and/or ovarian volume >10 cm^3. All subjects had the age between 15 and 35 years and had at least 2 years of menstrual history. Control subjects and PCOS subjects were further divided into lean subgroups (BMI < 23) and overweight subgroups (BMI ≥ 23) according to the BMI cutoff in the Chinese population.[28] Obese subjects were included in the overweight subgroup.

Fasting blood samples were collected from the study subjects: during the early follicular phase of the menstrual cycle (the days 3−5) in the female control groups and PCOS subjects who had spontaneous menses. For those PCOS subjects who did not have spontaneous menses, 10 mg of dydrogesterone was prescribed for 7 days to induce withdrawal bleeding, and blood sample will be drawn during days 3−5 after withdrawal bleeding. Sera were stored at −80 °C until analysis.

2.2. Clinical Biochemical Data

Radioimmunoassay was performed by automated chemiluminescence analyzer (Abbott Laboratories, USA) to assess the serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E2), prolactin (PRL), testosterone (T), dehydroepiandrosterone sulfate (DHEAS), androstenedione (AND), and sex hormone binding globulin (SHBG). Chemiluminescence was used to analyze fasting insulin (FINS), fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL), and low density lipoprotein (LDL) by automated biochemical analyzer (Hitachi, Ltd. Japan).

2.3. Metabolomics Analysis

Sera were thawed at 4 °C. A 100 μL serum was deproteinized with 4 volume of acetonitrile including 12 internal standards, centrifuged at 14 000*g* for 12 min, and the supernatant was dried in a vacuum centrifuge. The 12 internal standards were carnitine C2:0-d3, carnitine C10:0-d3, carnitine C16:0-d3, cholic acid-d4, LPC 12:0, LPC 19:0, leucine-d3, tryptophan-d5, FFA 16:0-d3, chenodeoxycholic acid-d4, leucine-enkephalin, and lansoprazole. For the analysis, the samples were redissolved in 150 μL of acetonitrile/water (2:8) and analyzed by ultra high-performance liquid chromatography (UHPLC) (Agilent 1290 Infinity, USA), coupled to electrospray ionization (ESI)

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Table 1. Clinical Biochemical Data of the Study Groups

	lean control (n = 32)	overweight control (n = 22)	lean PCOS (n = 40)	overweight PCOS (n = 37)	PCOS and control p-value
age (years)	24.00 ± 2.57	24.59 ± 3.42	24.13 ± 3.97	24.38 ± 3.04	
BMI (kg/m2)	20.23 ± 1.70	26.57 ± 1.93[a]	20.16 ± 1.35	27.99 ± 3.69[b]	
waist−hip ratio	0.81 ± 0.06	0.86 ± 0.04[a]	0.82 ± 0.05	0.90 ± 0.07[b,d]	0.003
age of menarche (years)	13.31 ± 1.56	13.09 ± 0.97	14.20 ± 1.95[c]	13.28 ± 1.52[d]	0.014
systolic blood pressure (mmHg)	111.13 ± 10.80	113.5 ± 7.73[a]	107.30 ± 10.34	116.69 ± 15.65[b,d]	0.019
diastolic blood pressure (mmHg)	71.92 ± 8.05	69.27 ± 7.02	71.43 ± 7.28	76.33 ± 9.62[d]	
F−G score	2.13 ± 2.09	1.50 ± 1.47	3.70 ± 3.73[c]	3.36 ± 2.79[d]	0.0005
acanthosis nigricans incidence (%)	0	0	10[c]	61.5[b,d]	<0.0001
acne incidence (%)	48.7	54.5	75[c]	41[d]	
resident skin oil incidence (%)	51.3	63.6	65	92.3[b,d]	0.026
FINS (mmol/L)	8.08 ± 3.40	12.50 ± 4.48[a]	7.37 ± 5.22	17.65 ± 18.96[b]	
FPG (μIU/ml)	4.38 ± 0.45	4.64 ± 0.39[a]	4.81 ± 0.4[c]	5.14 ± 0.71[b,d]	<0.0001
HOMA-IR	1.60 ± 0.74	2.61 ± 1.04[a]	1.38 ± 1.18	4.34 ± 5.66[b]	
TG (mmol/L)	0.74 ± 0.25	1.15 ± 0.54[a]	0.97 ± 0.54[c]	1.32 ± 0.61[d]	0.011
TC (mmol/L)	3.82 ± 0.48	4.23 ± 0.94	4.33 ± 0.60[c]	4.56 ± 1.01	0.001
HDL (mmol/L)	1.49 ± 0.32	1.31 ± 0.41	1.25 ± 0.25[c]	1.00 ± 0.18[b,d]	<0.0001
LDL (mmol/L)	2.16 ± 0.51	2.73 ± 1.03[a]	2.60 ± 1.05[c]	3.08 ± 1.08	0.009
FSH (mIU/ml)	5.63 ± 1.46	4.55 ± 0.96[a]	5.89 ± 1.76	5.43 ± 1.57[d]	
LH (mIU/ml)	6.62 ± 4.69	5.26 ± 2.83	12.65 ± 7.67[c]	9.14 ± 4.45[b,d]	<0.0001
LH/FSH	1.16 ± 0.66	1.17 ± 0.58	2.19 ± 1.19[c]	1.70 ± 0.74[b,d]	<0.0001
E2 (pg/mL)	38.56 ± 17.57	35.05 ± 10.99	51.01 ± 23.67[c]	50.55 ± 21.28[d]	<0.0001
PRL (ng/mL)	14.76 ± 5.89	12.34 ± 5.07	16.47 ± 22.47	12.77 ± 6.24	
T (ng/dl)	26.35 ± 12.88	25.67 ± 12.42	54.75 ± 24.89[c]	57.91 ± 26.07[d]	<0.0001
DHEAS (ug/Dl)	209.31 ± 84.94	240.52 ± 103.14	249.36 ± 105.36[c]	234.93 ± 117.27	
AND (ng/mL)	2.93 ± 1.40	3.21 ± 1.33	4.70 ± 2.18[c]	4.34 ± 1.71[d]	<0.0001
SHBG (nmol/L)	52.13 ± 19.87	38.59 ± 21.73[a]	45.61 ± 38.57	30.16 ± 30.22	

[a] $p < 0.05$ overweight control compared with lean control. [b] $p < 0.05$ overweight PCOS compared with lean PCOS. [c] $p < 0.05$ lean PCOS groups compared with lean control. [d] $p < 0.05$ overweight PCOS groups compared with overweight control.

quadrupole time-of-flight mass spectrometry (Q-TOF MS) (Agilent 6540, USA) in both positive and negative ion modes.

For the ESI positive ion mode, the metabolites separation was performed on a 2.1 × 100 mm ACQUITYTM 1.7 μm C8 column (Waters, Ireland), and the mobile phase contained water with 0.1% formic acid (A) and acetonitrile (B). The gradient program was 95% A for 1 min, changed to 100% B linearly within 24 min and held for 4 min, finally back to 95% A. For the ESI negative ion mode, the metabolite separation was performed on 2.1 × 100 mm ACQUITYTM 1.8 μm T3 column (Waters, Ireland), and the mobile phase contained 6.5 mM ammonium bicarbonate water solution (C) and 6.5 mM ammonium bicarbonate in 98% methanol and water (D). The gradient program was 95% C for 1 min, changed to 95% D linearly within 18 min and held for 4 min, and finally back to 95% C. Flow rate was 0.35 mL/min, and the column temperature was kept at 40 °C in positive ion mode and 50 °C in negative ion mode. A 3 μL aliquot of each sample was injected onto the column. The quality control samples prepared by mixing 10 μL of each sample were analyzed after each 8 serum samples.

Mass spectrometry detections were operated in either the positive or negative ion mode (full scan mode from m/z 80−1000) with a gas temperature at 350 °C, drying gas flow rate at 11 L/min, capillary voltage at 4000 V, and fragmentor voltage at 230 V.

2.4. Data Collection and Data Analysis

All the ion features were extracted and aligned by using MassHunter workstation (Agilent, USA) and exported to an Excel table (Microsoft, USA) for further data analysis. The missing values were removed by the modified 80% rule.[29] The intensity of each retained peak was normalized using one of the internal standards.

For the multivariate statistical analysis, the SIMCA-P software was used (version 11.0; Umetrics, Umea, Sweden). Principal component analysis (PCA) and orthogonal signal correction (OSC) partial least-squares-discriminant analysis (PLS-DA) were performed after the Pareto scaling (mean centering and scaled to square root of variance). The S-plot was used to define metabolites exerting a major influence on the group membership. The predictive ability of the model was assessed by the internal validation using the 7-fold cross-validation and response permutation testing.

All the clinical data and metabolomics analysis results were computed using SPSS17.0 version software. The t test was performed on measurement data, and $p < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

3.1. Comparison of Clinical Characteristics and Biochemical Data

The information of study subjects and biochemical data is summarized in Table 1. There were no significant differences in age and BMI between control group and PCOS group. The waist−hip ratio of the PCOS patients was evidently higher than that of control women (p = 0.003), and the main contribution came from overweight PCOS patients. As the waist−hip ratio primarily reflects the distribution of visceral adipose tissue,[30] it can be implied that the adipose tissue is distributed differently in the PCOS patients and the corresponding control group. In

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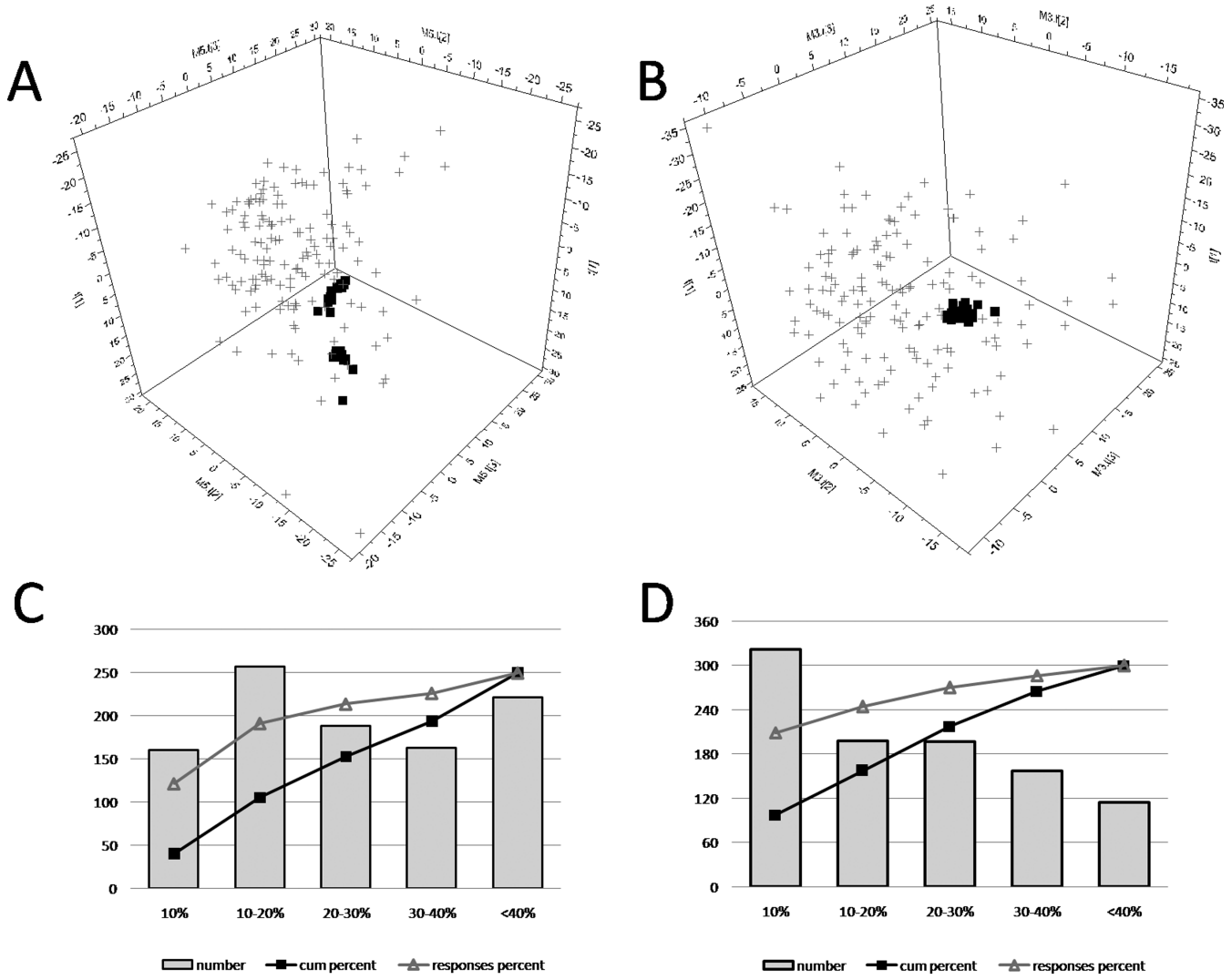

Figure 2. Three-dimensional score scatter plot of PCA (A,B) and the distribution of RSD% for all metabolites in QCs (C,D): (A) normalized data; (B) internal standards calibration data; (C) normalized data; (D) internal standards calibration data. Columns represent the percentage of the peak number within the specified RSD% range. Lines show the cumulative percentages of the peak area within the specified RSD% range.

this study, age of menarche in PCOS group was older than that of control group, and that was mainly due to some lean PCOS patients who had later age of menarche. Age of menarche in women with PCOS is associated with genetic/environmental factors and also with BMI.[31] Systolic blood pressures of the PCOS patients, especially overweight PCOS patients, were significantly higher than control women, and diastolic blood pressures of overweight PCOS patients were also significantly higher than overweight control women, which intimated high risk of hypertension in PCOS group.

From the clinical biochemical data, fasting blood glucose (FBG) and blood lipids of PCOS patients showed significant differences compared with those of control subjects, especially in overweight PCOS patients. Disorders of hormone metabolism were also appearing in PCOS patients, the serum levels of LH, LH/FSH, E2, T, and AND were significantly increased. In lean PCOS patients, the serum levels of LH and LH/FSH increased more significantly compared with the overweight PCOS patients. The clinical biochemical results indicated a more dysregulation of glucose and lipids in overweight PCOS patients and a more pronounced alteration in the hypothalamic-pituitary-ovarian (HPO) in lean PCOS patients.

3.2. Analysis of the Metabolite Fingerprint in Serum by UHPLC Q-TOF MS

Totally 131 sera in the discovery phase were analyzed with RPLC Q-TOF MS in both positive ion mode and negative ion mode. Eighteen QC samples were run (one QC after each 8 serum samples). After aligning peaks from PCOS patients and healthy controls and removing the zero-values using the modified 80% rule, 989 ions in positive ion mode and 802 ions in negative ion mode were obtained.

In large-scale nontargeted LC−MS metabolomics measurements, the reproducibility of analysis may be influenced by source contamination or the maintenance and cleaning of the mass spectrometer. Normalization is a most commonly preprocessing method to decrease systematic change. However, normalization of the data may cause the nonsystematic, compound-dependent variability.[32] In this study, internal standards were used to calibrate the response of metabolite ions. Isotope-labeled carinitines were used to calibrate the metabolites in positive ion mode, isotope labeled FFA C16:0-

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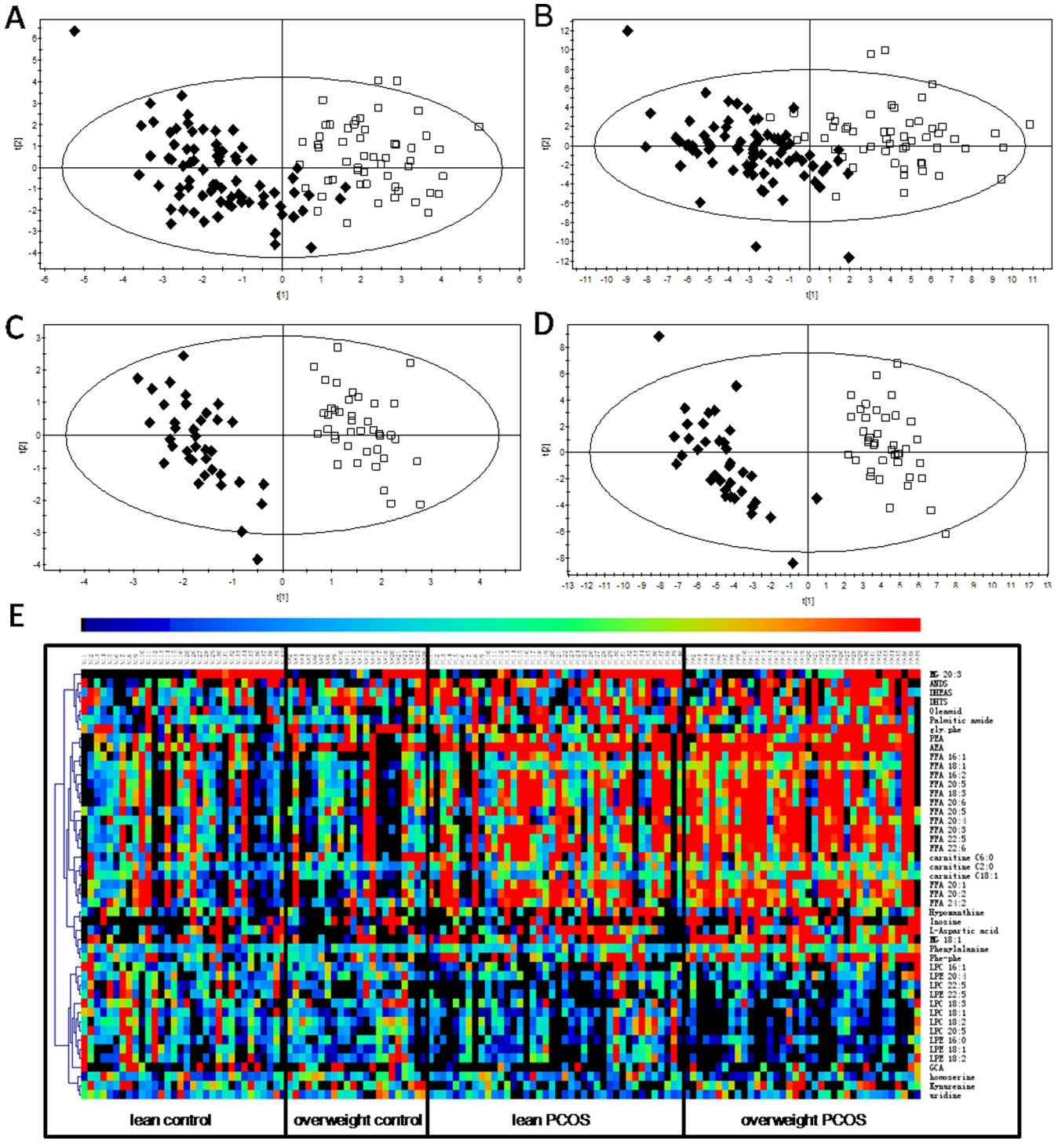

Figure 3. Scores plots of OSC PLS-DA model separating PCOS women from control women (A,B) and separating overweight PCOS women from lean PCOS women (C,D): (A) in ESI positive ion mode, R2Y = 0.781, Q2 = 0.707; (B) in ESI negative ion mode, R2Y = 0.711, Q2 = 0.626; (C) in ESI positive ion mode, R2Y = 0.964, Q2 = 0.896; (D) in ESI negative ion mode, R2Y = 0.939, Q2 = 0.907. No overflitting was found according to the permutation validation in all models. (E) Heat map of differential metabolites corresponding to Table 2.

d3 was used only for negative ion mode, other internal standards were used for both ion modes. QC samples were used to evaluate the reproducibility of metabolomics analysis.[32] The PCA score plot using positive ion mode normalized data is shown in Figure 2A. It can be seen that QC samples are not tightly clustered together, but migrated with the injection time. After being calibrated with internal standards, the QC samples were tightly located in the middle (Figure 2B). The result means that the reproducibility was improved with internal standards calibration. Furthermore, the reproducibility of the metabolite ions was also evaluated with relative standard deviation (RSD%) in the 18 QC samples (shown in Figure 2C,D). In the positive ion mode, among the 989 metabolite ions, 62% of ions had RSD% less than 30%, and the responses sum of those ions was about 86%. After internal standards calibration, 72.5% of ions had RSD% less than 30%, the

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Table 2. Differential Metabolites of Control and PCOS Women

metabolite	accurate mass	error	PCOS and control[a]	class	pathway
DHEAS[c]	[e]367.1600	5.7	↑	sulfated steroids	androgen metabolism
ANDS	[e]369.1753	4.7	↑	sulfated steroids	androgen metabolism
DHTS	[e]369.1752	4.5	↑	sulfated steroids	androgen metabolism
oleamide[c]	[d]282.2790	−2.4	↑	fatty acid amide	fatty acid amide metabolism
palmitic amide	[d]256.2633	−2.9	↑	fatty acid amide	fatty acid amide metabolism
PEA[c]	[d]300.2895	−2.5	↑	fatty acid amide	fatty acid amide metabolism
AEA[c]	[d]348.2894	−2.5	↑	fatty acid amide	fatty acid amide metabolism
carnitine C2:0[c]	[d]204.1228	−3.8	↑	carnitine	beta oxidation of fatty acids
carnitine C6:0[c]	[d]260.1856	−2.2	↑	carnitine	beta oxidation of fatty acids
carnitine C18:1	[d]426.3576	−1.7	↑[b]	carnitine	beta oxidation of fatty acids
FFA 16:1[c]	[e]253.2189	8.5	↑[b]	free fatty acid	lipid metabolism
FFA 16:2	[e]251.2029	7.1	↑	free fatty acid	lipid metabolism
FFA 18:1[c]	[e]281.2500	7.0	↑[b]	free fatty acid	lipid metabolism
FFA 18:3	[e]277.2190	8.0	↑	free fatty acid	lipid metabolism
FFA 20:1	[e]309.2813	6.4	↑	free fatty acid	lipid metabolism
FFA 20:2	[e]307.2658	6.8	↑	free fatty acid	lipid metabolism
FFA 20:3	[e]305.2501	6.6	↑[b]	free fatty acid	lipid metabolism
FFA 20:4[c]	[e]303.2347	7.5	↑[b]	free fatty acid	lipid metabolism
FFA 20:5	[e]301.2184	5.4	↑[b]	free fatty acid	lipid metabolism
FFA 20:6	[e]299.2006	−1.6	↑	free fatty acid	lipid metabolism
FFA 22:5	[e]329.2503	6.7	↑[b]	free fatty acid	lipid metabolism
FFA 22:6	[e]327.2346	6.8	↑	free fatty acid	lipid metabolism
FFA 24:2	[e]363.3292	7.9	↑	free fatty acid	lipid metabolism
MG 18:1	[d]357.2999	−1.6	↑	free fatty acid	lipid metabolism
MG 20:3	[d]381.2975	−7.8	↑	free fatty acid	lipid metabolism
hypoxanthine[c]	[d]137.0457	−4.6	↑	purine derivatives	purine metabolism
inosine	[d]269.0879	−2.6	↑	nucleosides	purine metabolism
L-aspartic acid[c]	[e]132.0286	−8.0	↑	amino acid	aspartate metabolism
Gly.Phe[c]	[e]326.1255	4.8	↑[b]	aromatic amino acids	phenylalanine and tyrosine metabolism
phenylalanine[c]	[e]164.0721	5.7	↑[b]	aromatic amino acids	phenylalanine and tyrosine metabolism
Phe−Phe	[d]313.1547	−1.7	↑[b]	aromatic amino acids	phenylalanine and tyrosine metabolism
LPC 16:1	[d]494.3241	−1.1	↓	lysophosphatidylcholines	lipid metabolism
LPC 18:1	[d]522.3553	−1.3	↓[b]	lysophosphatidylcholines	lipid metabolism
LPC 18:2	[d]520.3397	−1.2	↓[b]	lysophosphatidylcholines	lipid metabolism
LPC 18:3	[d]518.3239	−1.5	↓	lysophosphatidylcholines	lipid metabolism
LPC 20:5	[d]542.3215	−5.8	↓[b]	lysophosphatidylcholines	lipid metabolism
LPC 22:5	[d]570.3551	−1.5	↓	lysophosphatidylcholines	lipid metabolism
LPE 16:0[c]	[d]454.2927	−1.5	↓	lysophosphatidylethanolamines	lipid metabolism
LPE 18:1	[d]480.3082	−1.7	↓	lysophosphatidylethanolamines	lipid metabolism
LPE 18:2	[d]478.2929	−1.0	↓	lysophosphatidylethanolamines	lipid metabolism
LPE 20:4	[d]502.2927	−1.3	↓	lysophosphatidylethanolamines	lipid metabolism
LPE 22:5	[d]528.3089	−0.2	↓	lysophosphatidylethanolamines	lipid metabolism
glycocholic acid[c]	[e]464.3025	2.7	↓	bile acids	bile acid metabolism
homoserine[c]	[e]118.0510	4.9	↓	amino acid	methionine metabolism
kynurenine[c]	[d]192.0652	−4.5	↓	amino acid	tryptophan metabolism
uridine[c]	[e]243.0600	−7.0	↓	pyrimidine nucleosides	pyrimidine metabolism

[a]Arrows show increase or decrease in the PCOS women compared with that in health women. [b]Significant difference between lean PCOS and overweight PCOS women. [c]Validated with standard sample. [d]Obtained accurate mass in positive ion mode. [e]Obtained accurate mass in negative ion mode.

responses sum of those ions was 90%. It is noteworthy that after being calibrated, 322 ions had RSD% less than 10%, the responses sum of those ions was 70%. So the calibration data with multiple internal standards were more suitable for nontargeted LC−MS approach. The reproducibility of analytical platform was good for the metabolomics study.

3.3. Differential Metabolites of PCOS and Control Subjects

To excavate the metabolic fingerprint changes in PCOS patients, OSC PLS-DA was performed. As can be seen from the scores plot of the PLS-DA model (Figure 3A in positive ion mode and Figure 3B in negative ion mode), the control subjects were clearly separated from the PCOS subjects. To further study the metabolic fingerprint difference of lean PCOS patients and overweight PCOS patients, we used the same OSC PLS-DA method, the clear separation was shown in Figure 3C,D, and the S-plot was used to find potentially interesting metabolites. All selected metabolites were filtered by t test. The metabolites with a significant difference ($p < 0.05$) were kept.

These metabolites were identified following our recently published strategy; accurate mass and mass spectrometric

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Table 3. Comparison of the Fatty Acid Patterns

	lean control (n = 32)	overweight control (n = 22)	lean PCOS (n = 40)	overweight PCOS (n = 37)	PCOS and control degree of change	PCOS and control p-value
total FFA	188.94	183.78	189.40	219.81[b,d]	1.09	0.050
sum SFA	80.93	79.83	74.57	82.57[b]	0.97	
sum MUFA	48.23	44.94	50.95	61.35[b,d]	1.19	0.002
sum PUFA	59.79	59.01	63.88	75.89[b,d]	1.17	0.005
SFA%	43.51%	44.24%	40.25%[c]	38.08%[d]	0.89	<0.001
MUFA%	25.31%	24.15%	26.54%	27.75%[d]	1.09	<0.001
PUFA%	31.19%	31.61%	33.22%[c]	34.17%[d]	1.07	<0.001
steroyl-CoA desaturase						
16:1/16:0	0.19	0.15	0.22[c]	0.26[b,d]	1.36	<0.001
18:1/18:0	1.36	1.25	1.53[c]	1.67[b,d]	1.21	<0.001

[a] $p < 0.05$ overweight control compared with lean control. [b] $p < 0.05$ overweight PCOS compared with lean PCOS. [c] $p < 0.05$ lean PCOS groups compared with lean control. [d] $p < 0.05$ overweight PCOS groups compared with overweight control.

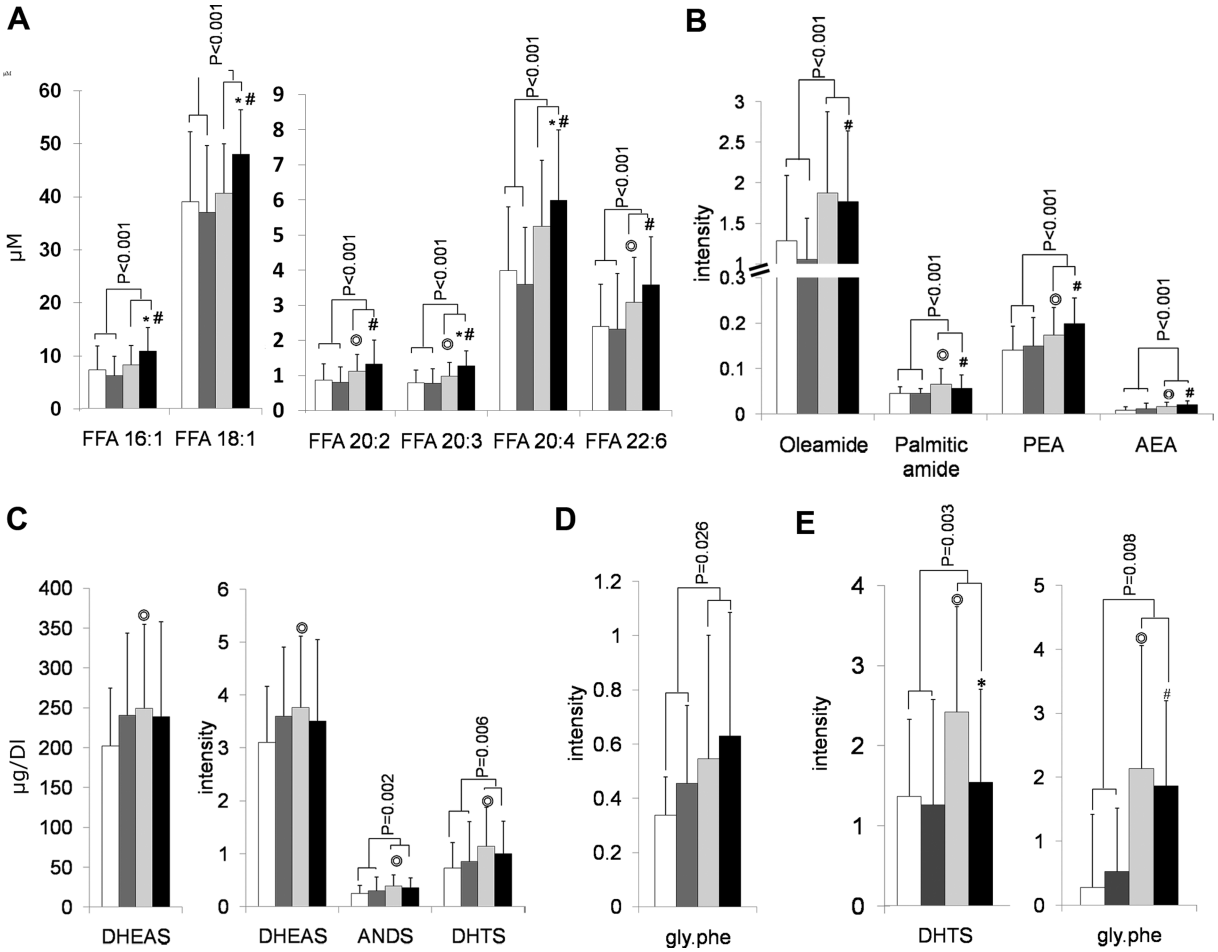

Figure 4. Comparison of the intensities of differential metabolites: (A–D) in discovery samples and (E) in validation samples. △$p < 0.05$ overweight control compared with lean control. *$p < 0.05$ overweight PCOS compared with lean PCOS. ◎$p < 0.05$ lean PCOS groups compared with lean control. #$p < 0.05$ overweight PCOS groups compared with overweight control.

fragmentation patterns were utilized to search databases including KEGG, PubChem compound, METLIN, and HMD,[33] then the metabolites were validated with available standards. The results were summarized in Table 2, and their corresponding contents were shown in the heat map (Figure 3E). It can be seen that the metabolite contents in the upper half increased in PCOS group including sulfated steroids, carnitines, fatty acid amides (FAAs), several unsaturated FFAs, and amino acids. In the lower half, the metabolites contents decreased in the PCOS group including lysophosphatidylcholines (LPCs), lysophosphatidylethanolamine (LPEs), glycocholic acid, uridine, etc.

FFAs are a class of important metabolites. In our study, 28 fatty acids were measured by RP UHPLC Q-TOF MS. The concentrations of all fatty acids were calibrated by isotope-labeled internal standard d3-palmitic acid. It is observed from Table 3 that the levels of total FFAs in PCOS subjects show a significant increase compared with the control subjects (p =

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0.05); especially in overweight PCOS group, the levels of total FFAs were significantly increased compared with other groups (Figure 4A). Increased circulating free fatty acids involved many adverse metabolic effects including insulin resistance, type 2 diabetes, hypertension, dyslipidemia, etc.[34,35]

In FFAs pattern, the percentage increase of monounsaturated fatty acid (MUFA) and percentage decrease of saturated fatty acid (SFA) were detected in PCOS group. The ratio of the MUFA species to their SFA, FFA 16:1/FFA 16:0, and FFA 18:1/FFA 18:0, were used to evaluate stearoyl-CoA desaturase activity (SCD).[36] It was observed that the ratios of the MUFA species to their SFAs increased to 1.36 and 1.21 times in PCOS subjects. The notable increase was observed not only in overweight PCOS subjects but also in lean PCOS subjects, which represented the increases of SCD activity. The SCD activity was closely related with body adiposity, ploy-unsaturated fatty acid (PUFAs), and cholesterol intake and also interrelated to hormonal imbalance, such as insulin, leptin, sex hormone, etc.[37−40] Hsieh et al.[41] reported SCD mRNA level increased significantly in estradiol and testosterone treated animals. Marks et al.[42] demonstrate ovarian hormones were involved with SCD and affected MUFA biosynthesis. Higher SCD activity was reported to involve in diabetes, cardiovascular disease, obesity, hypertension, neurological diseases, immune disorders, and cancer.[36,43,44] Until now, not many studies have shown that the relationship between SCD and PCOS, but the SCD activity changes in PCOS groups indicated FFAs metabolism change and may be closely related to the disorder of hormone metabolism and the total cholesterol increasing. That is a probable reason why PCOS patients more easily to get diabetes and hypertension, also in lean PCOS patients.

The serum levels of ploy-unsaturated fatty acid (PUFAs) in PCOS subjects were found significantly higher than those of control subjects (Table 2), such as FFA C20:2, FFA C20:3, FFA C20:4, and FFA C22:6, which were so-called the precursors of eicosanoids. Eicosanoids exert complex control over many body systems in inflammation and immunity and as messengers in the central nervous system.[45] The increasing of PUFAs, like FFA C20:4, may be related with inflammation.

The decrease in levels of LPCs, LPEs and the increase in levels of FAAs, carnitines, and monoglyceride were detected in PCOS groups (Table 2). LPC is well-known to induce an inflammatory response and apoptosis and tumor cell invasiveness.[46,47] Recent studies have shown that LPCs may play an important role in glucose metabolism,[48] which may also be considered as marker molecules of insulin resistance.[20,49] FAAs were found increased in PCOS patients, the comparison of signal intensities was shown in Figure 4B. Endogenous FAAs are considered as a class of endogenous signaling molecules.[50] Anandamide (AEA) is an endogenous agonist of the cannabinoid CB1 and CB2 receptors,[51] and oleamide was studied as a chemical messenger signaling sleep,[52] which can induce vasorelaxation.[53] Palmitoylethanolamide (PEA) was incapable of binding to cannabinoid receptors; it exhibits anti-inflammatory activity.[54] Adipocytes are able to secrete a large quantity of PEA. PEA was correlated with secretion of IL-6 and leptin, which might become a potentially interesting candidate molecule in the prevention of obesity-associated insulin resistance.[55,56]

Some of the aforementioned differential metabolites showed significant differences between lean PCOS group and overweight PCOS group, like FFA 16:1, FFA 20:4, LPC 18:1, etc. (Table 2). These results suggested that abnormal lipid metabolism was more significant in overweight PCOS group.

Disorder of hormone metabolism is a notable feature of PCOS.[4] On the basis of our nontargeted LC−MS metabolomics approach, some sulfated steroids could be measured, such as DHEAS, dihydrotestosterone sulfate (DHTS), and androsterone sulfate (ANDS). As can be seen in Figure 4C, the level of DHEAS shows a significant increasing in the lean PCOS group compared with that in lean control group, which was comparable to the results measured by radioimmunoassay analysis. DHEAS is a useful marker for adrenal androgen, and elevated DHEAS levels is highly correlated with male reproductive phenotype in the women with PCOS,[57] but not correlated with insulin resistance.[58,59] Some studies suggest that serum DHEAS was inversely proportional to BMI.[57] In our study, except DHEAS, the levels of serum DHTS and ANDS showed a significant increase in PCOS group compared with control group. Combined with clinical biochemical data, PCOS women display the androgen excess, especially in the lean PCOS group. LC−MS method provides a sensitive and easy approach for androgen excess monitoring.

In addition, we also found metabolic abnormalities of some amino acids, like phenylalanine and aspartic acid (Table 2). It is noteworthy that a glycated amino acid, glycated phenylalanine, was detected as increased in PCOS subjects (Figure 4D). Glycated proteins and glycated amino acids were reported as accumulated in diabetes.[60] Glycated hemoglobin (HbA1c) has been used to measure the average serum glucose concentration over prolonged periods of time.[61] The Maillard reaction is also an intermediate step in the production of advanced glycation end-products (AGEs). The accumulation of AGEs has been implicated in age-related diseases, like diabetes, cardiovascular disease, stroke, and Alzheimer's disease.[62] It is believed AGEs play a role in proinflammatory and oxidant mediators in the vascular complications of diabetes mellitus.[63] Diamanti-Kandarakis et al. demonstrated that the AGE proteins and testosterone levels had a high correlation and that the levels of AGEs increased in normoglycaemic women with polycystic ovary syndrome.[64,65] In our study, the increasing of glycated amino acid illustrated accumulation of AGEs in PCOS patients and may associate with abnormal glucose metabolism and hormone metabolism.

3.4. Distinguish PCOS Patients from Control Subjects Using Combinational Metabolite Biomarker

PCOS is a complex disease with no uniform diagnostic criteria, which results in difficulty in diagnosis. LC−MS metabolomics approach can show more comprehensive phenotype of diseases, and it has been successfully applied in finding diagnostic biomarkers. Here, differential metabolites of PCOS (Table 2) were investigated to know the possibility of potential biomarkers for PCOS diagnostics. Considering the complexity of PCOS, we focused on combinational biomarkers for reflecting the wide variety of metabolic characteristics. All differential metabolites of PCOS were randomly combined using binary logistic regression model, and receiver operating characteristic (ROC) curve was used to evaluate sensitivity and specificity of the combinational biomarker. Finally, a combinational biomarker containing FFA 18:1/FFA 18:0, FFA 20:3, DHTS, glycated phenylalanine, and uridine showed good sensitivity and specificity. The area under the curve (AUC) of the combinational biomarker was 0.839 (Supporting Information Figure S1A). For the lean subjects, the AUC was 0.818

(Supporting Information Figure S1B), and the AUC was 0.860 for overweight subjects (Supporting Information Figure S1C).

3.5. Validation of the Combinational Metabolic Biomarker

To validate the combinational biomarkers of PCOS, another batch of serum samples was collected including 30 lean controls, 19 overweight controls, 30 lean PCOS patients, and 30 overweight PCOS patients. The information of these subjects and biochemical data were given in Supporting Information Table S1. The metabolites were measured using UPLC Q-TOF MS (Waters) with the same method as described in section 3.2. With the different samples and instruments from the discovery phase, FFA 18:1/FFA 18:0, FFA 20:3, DHTS, glycated phenylalanine, and uridine showed significant differences between control group and PCOS group. After handled with binary logistic regression model, the AUC of combinational biomarker was 0.874 (Supporting Information Figure S1D). The result was similar and slightly better than that in the previous discovery phase samples. Specifically, the AUC of lean subjects was 0.906 (Supporting Information Figure S1E); it was much better than 0.818, the AUC of discovery phase samples. The increasing of AUC was mainly due to the serum levels of DHTS and glycated phenylalanine, which increased more pronounced in lean PCOS group (Figure 4E). They were probably related to the different hormone levels of two batch samples.

4. CONCLUSIONS

In the present work, based on LC−MS metabolomics approach, the metabolic changes of PCOS patients were investigated. The differential metabolites revealed metabolism disorder of PCOS in lipid metabolism and androgen metabolism, increases of SCD activity, and accumulation of AGEs in PCOS patients. Furthermore, PCOS patients and healthy control could be distinguished using a combinational biomarker, which was composed of five metabolites defined by using the binary logistic regression.

These results suggest that LC−MS metabolomic approach was a useful tool to investigate the metabolic abnormality of PCOS women. The discriminating metabolites may be useful to understand the pathogenesis mechanisms of PCOS and provide a good prospect for PCOS diagnosis.

■ ASSOCIATED CONTENT

Supporting Information

Clinical biochemical data of the study group and ROC curves. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*(L.H.) Tel: +86-451-82124304. Fax: +86-451-82119168. E-mail: houlihui2007@sina.com.
*(G.X.) Tel/Fax: +86-411-84379530. E-mail: xugw@dicp.ac.cn.

Author Contributions

⊥These authors (X.Z. and F.X.) contributed equally to this work.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PCOS, polycystic ovary syndrome; LH, luteinizing hormone; PRL, prolactin; DHEAS, dehydroepiandrosterone sulfate; SHBG, sex hormone binding globulin; FBG, fasting blood glucose; TG, triglyceride; LDL, low density lipoprotein; MUFA, monounsaturated fatty acid; SCD, stearoyl -CoA desaturase; FAA, fatty acid amide; PEA, palmitoylethanolamide; ANDS, androsterone sulfate; LPE, lysophosphatidylethanolamine; BMI, body mass index; FSH, follicle-stimulating hormone; E2, estradiol; T, testosterone; AND, androstenedione; FINS, fasting insulin; TC, total cholesterol; HDL, high density lipoprotein; SFA, saturated fatty acid; PUFA, ploy-unsaturated fatty acid; AEA, anandamide; DHTS, dihydrotestosterone sulfate; LPC, lysophosphatidylcholine; AGEs, advanced glycation end-products

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