Serum metabolomic profiling reveals potential biomarkers in assessing the management of women with polycystic ovary syndrome: a randomized controlled trial

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Abstract

Background: As one of the most common endocrinal disorders for women at childbearing age, the diagnostic criteria of polycystic ovary syndrome (PCOS) have been defined differently among different international health organizations. Phenotypic heterogeneity of PCOS also brings about difficulties for its diagnosis and management assessment. Therefore, more efficient biomarkers representing the progression of PCOS are expected to be integrated into the monitoring of management process using metabolomic approaches.

Methods: In this prospective randomized controlled trial, 117 PCOS patients were enrolled from December 2016 to September 2017. Classical diagnostic parameters, blood glucose, and metabolome were measured in these patients before and at 2 months and 3 months of different medical interventions. The receiver operating characteristic (ROC) curves were built based on multivariate statistical analysis using data at baseline and 3 months' management, and combinational biomarkers with appreciable sensitivity and specificity were selected, which then validated with data collected at 2 months.

Results: A set of metabolites including glutamic acid, aspartic acid, 1-methylnicotinamide, acetylcarnitine, glycerophosphocholine, and oleamide were filtered out with high performance in representing the improvement through 3-month management of PCOS with high sensitivity and specificity in ROC analysis and validation with other two groups showed an appreciable area under the curve over 0.96.

Conclusions: The six metabolites were representative of the remission of PCOS through medical intervention, making them a set of potential biomarkers for assessing the outcome of PCOS management.

Trial Registration: ClinicalTrials.gov, NCT03264638.

Keywords: Polycystic ovary syndrome; Metabolomic approach; Management evaluation; Serum metabolomic

Background

Polycystic ovary syndrome (PCOS) is one of the most common diseases among women of childbearing age, which is also one of the major causes of infertility due to ovulatory malfunction, a manifestation of the disrupted reproductive endocrine system. However, the heterogenic etiology and diversified symptoms and signs of PCOS bring about a large number of difficulties in its diagnosis and management evaluation.[1] Meanwhile, the diagnostic criteria of PCOS have undergone many alternations by different international health organizations, resulting in the fluctuation of its prevalence, ranging from 8% to 13%.[2] Based on its definition, the most commonly evaluated clinical parameter, androgen, does not appear in every case. PCOS has been the hot spot in the reproductive endocrinology research field for years, but the knowledge of its pathogenesis and causation-orientated treatments remains limited.[3] Though genome-wide association studies have revealed a part of the genetic foundation of PCOS, the detailed correlations between gene and phenotypes have yet been delineated, and easily accessible parameters with higher sensitivity and specificity are expected to be integrated into assessing the progression and management outcomes of PCOS.

Metabolome refers to the collection of small metabolites within cells, tissues, and biofluids which can directly reflect the microenvironment under the influence of genetic regulation and external exposure.[4] As the end product

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of genome and transcriptome, metabolomics have been recognized as better representatives that closely reflect the actual phenotype, as a result of both external environment changes and inherited material. The advancement of liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) allows researchers to evaluate all the metabolites non-invasively in a hypothesis-free way, with great accuracy and efficiency.[5,6]

Besides ovulatory disorder, PCOS has been closely related to alternated metabolism, since there was an increased incidence of developing obese and impaired insulin sensitivity in the PCOS population, accompanied by a higher risk of cardiovascular disease and type 2 diabetes as long-term complications.

Metabolome has been extensively studied to evaluate the process of endocrinological disorders such as insulin resistance and type 2 diabetes mellitus, whereas the metabolic profile in PCOS needs further excavation and more refined analysis.[7,8] To provide new insights on the association between metabolomic profiles and PCOS progression or remission, we characterized the metabolism fingerprint associated with PCOS by applying an LC-MS/MS metabolomic approach to serum samples obtained from three different intervention groups at baseline, 2nd month, and 3rd month, and non-targeted multivariate statistical analysis was performed to filter out the most relevant candidate for potential biomarker.

Methods

Ethical approval

This trial was approved by the Ethics Committee of Peking Union Medical College Hospital, Peking Union Medical College (PUMCH), Chinese Academy of Medical Science (No. ZS-1222). All participants provided written informed consent.

Study design and participants

This was a single-center, prospective, open-label, parallel-group, and randomized clinical trial. Baseline and follow-up examinations were conducted at the clinics of PUMCH in Beijing, China, from December 2016 to September 2017. Participants were recruited by advertisement. Women aged 18 to 39 years and diagnosed with PCOS based on Rotterdam consensus (two of three criteria: oligo-/anovulation, hyperandrogenemia, and sonographic polycystic ovary morphology [PCOM]) were eligible. Diagnosis of oligo-/anovulation was based on a menstrual pattern of oligo-/amenorrhea (cycle $>$ 35 days) and/or a low mid-luteal serum progesterone concentration. Hyperandrogenemia was diagnosed either clinically (acne/hirsutism) or biochemically (testosterone $\geq$ 0.75 ng/mL). Polycystic ovaries were $\geq$12 follicles (2–9 mm) and/or an ovarian volume of $>$10 mL under ultrasonography. Exclusion criteria were medical or surgical treatment of PCOS within 3 months, thyroid disease, hyperprolactinemia, active liver disease, history of cardiac or renal failure, hormone medication, alcohol use, and regular smoking.

Interventions

Participants were randomly assigned to one of three intervention groups in 1:1:1 ratio by researchers. The random numbers were generated by computer and assigned to each eligible patient uniquely. All drugs were taken orally for 21 consecutive days followed by 7 days of non-utilization of drugs. Group A took 7 g Dingkundan daily; Group B took one tablet of Diane-35 daily; Group C took both 7 g Dingkundan and one tablet of Diane-35 daily. The intervention lasted for 3 months. Physical examination body mass index (BMI), clinical biochemical (total testosterone, fasting glucose, glucose 1 h, glucose 2 h, hemoglobin A1c, fasting insulin, insulin 0.5 h, insulin 1 h, insulin 2 h, total cholesterol [TC], triglyceride [TG], high-density lipoprotein cholesterol [HDL-C], and low-density lipoprotein), and metabolomic analysis were performed at baseline, 2-month, and 3-month treatment as the primary outcomes.

Sample size calculation

The estimated decrease of testosterone in the Dingkundan group was 0.014 $\pm$ 0.200 ng/mL, with a two-sided alpha of 5% and a power of 80%, and taking a 15% drop-out rate into consideration; 110 patients were expected to be recruited for this experiment.

Clinical biochemical analysis

Measurements of testosterone (T) and sulfated-dehydroepiandrosterone (DHEAS) were performed by chemiluminescence immunoassay using Beckman Coulter DXI 800 (Minnesota, USA) .

Metabolite extraction

High performance liquid chromatography grade methanol was pre-chilled at −80°C before use. The pre-chilled methanol was added to 100 μL serum samples up to 80% (v/v). Samples were placed at −80°C for 1 h and then centrifuged at 14,000 *g* for 30 min. Supernatants were carefully transferred to new Eppendorf tubes. Subsequently, all supernatants were dried and stored in a −80°C freezer for future analysis.

Metabolomics analysis

Samples were re-dissolved using 60 μL of 80% (v/v) methanol. After centrifugation for 20 min, supernatants were transferred to sample vials for LC-MS/MS analysis. Metabolites were profiled using Q Exactive mass spectrometer coupled with ultrahigh performance liquid chromatography system (Thermo, Waltham, MA USA). In the analysis, data-dependent acquisition in positive and negative ion mode was performed. Ethylene bridged hybrid amide column (2.1 mm $\times$ 100.0 mm) was used for LC separation with a flowrate of 250 μL/min. In positive ion mode, 95% and 50% of acetonitrile with 5 mmol/L ammonium formate and 0.1% formic acid were used as mobile phases. In negative ion mode, 95% and 50% of acetonitrile with 5 mmol/L ammonium acetate at pH 9.0 (adjusted using ammonium hydroxide) were

applied as mobile phase A and B. Electrospray ionization voltages of 3.5 kV in positive mode and 2.5 kV in the negative mode were used, respectively. MS scan with 70,000 resolution and MS/MS scans of the top 10 the most intense precursors with 17,500 resolutions were applied. Mass range of 70 to 1050 m/z was used to acquire data in positive ion mode and 80 to 1200 m/z for negative mode was used. Ten microliters of serum from each of all the samples were mixed for the sample quality control (QC) to assess the repeatability of samples and validating the stability of the whole analysis. Before the experiment, five QC samples were successively tested for balancing the columns, and subsequently, one QC sample was acquired for every 15 samples to further monitor the stability of the LC-MS/MS analysis.

Metabolite identification

Metabolites were identified using Tracerfinder 3.2 (Thermo Fisher, Waltham, MA USA) based on an in-house MS/MS library. The in-house MS/MS library was established using either chemical standards or biological samples. Two levels of metabolite identifications were achieved, one confirmed by MS/MS and the rest matched using precursor mass. Therefore, the ones without MS/MS confirmation were assigned as metabolite candidates. For metabolites having MS/MS confirmation, only the ones with LS score > 30 were considered as confidently identified. Otherwise, metabolites had only tentative assignments. The in-house software "MetaInt" was incorporated for high-throughput data analysis.

Statistical and pathway analysis

Metabolites were normalized by the sum of the area of identified peaks before subsequent statistical analysis. Partial least squares discriminate analysis (PLS-DA) was performed using SIMCA 14 (Umetrics, Umeå, Sweden). Student *t*-test results and one-way analysis of variance (ANOVA) results with false discovery rate (FDR) adjustment were acquired from Metaboanalyst 4.0 (www.metaboanalyst.ca). The Human Metabolome Database (www.HMDB.ca), The Small Molecule Pathway Database (SMPDB), and Metaboanalyst (www.metaboanalyst.ca) were used for pathway analysis.

Results

Comparison of clinical characteristics and biochemical data at baseline

The patients recruited for this study were diagnosed based on the 2003 Rotterdam diagnostic criteria (two out of three manifestations including oligo- or anovulation, clinical and/or biochemical hyperandrogenism, or polycystic morphological changes on ultrasound, after exclusion of other relevant diseases). The eligible patients were assigned to three groups randomly based on random numbers generated by the computer and assigned to each of them uniquely. Their baseline characters of three groups are summarized in Table 1, including the classic clinic laboratory results relevant to PCOS and metabolic status of patients, for instance, the diagnostic criteria for chemical hyperandrogenism (total testosterone and DHEAS), BMI, blood glucose and insulin level through 2 h oral glucose tolerance test, and other parameters describing lipid metabolism (low-density lipoprotein cholesterol, HDL-C, TC, and TG). No statistical difference was found in all listed parameters among three randomly assigned PCOS patient groups (one-way ANOVA).

Improvement of classic clinical parameters between baseline and end of 3-month management

After 3-month management using Dingkundan, Diane-35, and Dingkundan combined with Diane-35, information of classic clinical parameters and relevant metabolism characters applied to assess the improvement of PCOS status was listed in Table 2, describing the net changes of factors listed in baseline.

Table 1: Demographic and clinical characteristics of the women with polycystic ovary syndrome at baseline ($n = 117$).

Characteristics	Group A ($n = 38$)	Group B ($n = 40$)	Group C ($n = 39$)	P^*
Age (years)	27.5 ± 3.4	27.2 ± 3.5	26.7 ± 6.4	0.783
BMI (kg/m^2)	26.1 ± 5.4	25.5 ± 5.8	26.4 ± 5.9	0.764
Total T (ng/mL)	0.81 ± 0.27	0.72 ± 0.20	0.74 ± 0.24	0.220
Fasting glucose (mmol/L)	5.1 ± 0.4	5.1 ± 0.5	5.2 ± 0.7	0.570
Glucose 1 h (mmol/L)	7.9 ± 2.1	8.2 ± 3.0	8.0 ± 2.5	0.866
Glucose 2 h (mmol/L)	6.6 ± 1.3	7.0 ± 2.3	7.0 ± 2.4	0.700
HbA1c (%)	5.3 ± 0.3	5.3 ± 0.4	5.2 ± 0.4	0.596
Fasting insulin (μIU/mL)	16.320 ± 8.770	18.030 ± 15.820	18.780 ± 13.120	0.703
Insulin 1 h (μIU/mL)	115.700 ± 67.070	124.200 ± 74.410	114.820 ± 76.310	0.860
Insulin 2 h (μIU/mL)	94.380 ± 61.670	90.930 ± 77.960	75.869 ± 68.930	0.473
TC (mmol/L)	4.66 ± 1.19	4.65 ± 0.92	4.49 ± 0.99	0.731
TG (mmol/L)	3.18 ± 12.51	1.34 ± 1.03	1.36 ± 0.99	0.460
HDL-C (mmol/L)	1.48 ± 0.57	1.31 ± 0.33	1.37 ± 0.72	0.463
LDL-C (mmol/L)	2.90 ± 0.84	2.81 ± 0.16	2.78 ± 0.75	0.821

Data are represented as mean ± standard error of mean; Group A: Dingkundan; Group B: Diane-35; Group C: Dingkundan combined with Diane-35. *Between groups determined by one-way analysis of variance. BMI: Body mass index; HbA1c: Hemoglobin A1c; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; Total T: Total testosterone; TC: Total cholesterol; TG: Triglyceride.

Table 2: Absolute changes of tested parameters from baseline to 3 months of the women with polycystic ovary syndrome ($n = 117$).

Characteristics	Group A ($n = 38$)	Group B ($n = 40$)	Group C ($n = 39$)	P^*
BMI (kg/m^2)	0.1 (0.9)	−0.0 (1.2)	−0.3 (0.9)†	0.242
Total T (ng/mL)	−0.04 (0.24)	−0.14 (0.16)*	−0.14 (0.17)†	0.033
LH (IU/L)	−0.10 (5.33)	−4.93 (6.18)†	−5.23 (6.30)†	0.010
FSH (IU/L)	−0.03 (1.73)	0.45 (2.64)	0.77 (2.58)	0.348
Fasting glucose (mmol/L)	−0.1 (0.3)†	−0.3 (0.3)†	−0.2 (0.5)†	0.139
Fasting insulin (μIU/mL)	−0.77 (6.91)	−0.95 (12.33)	−3.39 (11.79)	0.492
2h-OGTT glucose (mmol/L)	−0.40 (1.30)	0.30 (1.80)	−0.03 (1.50)	0.169
2h-OGTT insulin (μIU/mL)	−8.12 (79.91)	2.09 (60.92)	6.40 (48.35)	0.592
TC (mmol/L)	−0.27 (0.52)†	0.22 (0.81)	0.45 (0.78)†	0.000
TG (mmol/L)	0.28 (0.59)†	0.62 (0.86)†	0.42 (1.04)†	0.245
LDL-C (mmol/L)	−0.25 (0.40)†	−0.25 (0.65)†	−0.01 (0.71)	0.143
HDL-C (mmol/L)	−0.18 (0.56)	0.35 (0.50)†	0.24 (0.83)	0.002

Data are represented as mean (standard error of mean); Group A: Dingkundan; Group B: Diane-35; Group C: Dingkundan combined with Diane-35. *Between treatment groups determined by one-way analysis of variance. $^\dagger P < 0.050$ for the comparison between baseline and 3 months. BMI: Body mass index; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; OGTT: Oral glucose tolerance test; TC: Total cholesterol; TG: Triglyceride; Total T: Total testosterone.

In Group B, with Diane-35, one of the conventional treatments of PCOS, the results showed a significant decrease in total testosterone levels compared with the Dingkundan group, indicating an improvement of high androgen status after 3-month management. Compared with Group B, the combo of traditional Chinese medicine Dingkundan and combined oral contraceptive pills Diane-35 (Group C) showed an additional effect on BMI reduction. However, for lipid metabolism, there were significant increases in TC and HDL-C levels in Group B and Group C, which diverted from the trend observed in Group A.

Metabolic pathway enrichment using the metabolomic profile in PCOS patients before and after management

In this research, >600 small molecules in blood serum were detected using LC-MS/MS and non-targeted analysis was performed, and multivariate statistical analysis PLS-DA was performed to find the potential interesting metabolites. The concentration of metabolites was normalized based on log transformation and auto-scaling before PLS-DA, and a total of 93 metabolites have been identified as relevant with variable importance in projection (visual infusion phlebitis) scores > 1 and FDR < 0.05.

Relevant pathways were enriched using Metaboanalyst, which were plotted with *P* value illustrated with the color gradient in Figure 1. The most significantly different pathways after 3-month treatment were mainly related to amino acid metabolism, especially for one participant, aspartate.

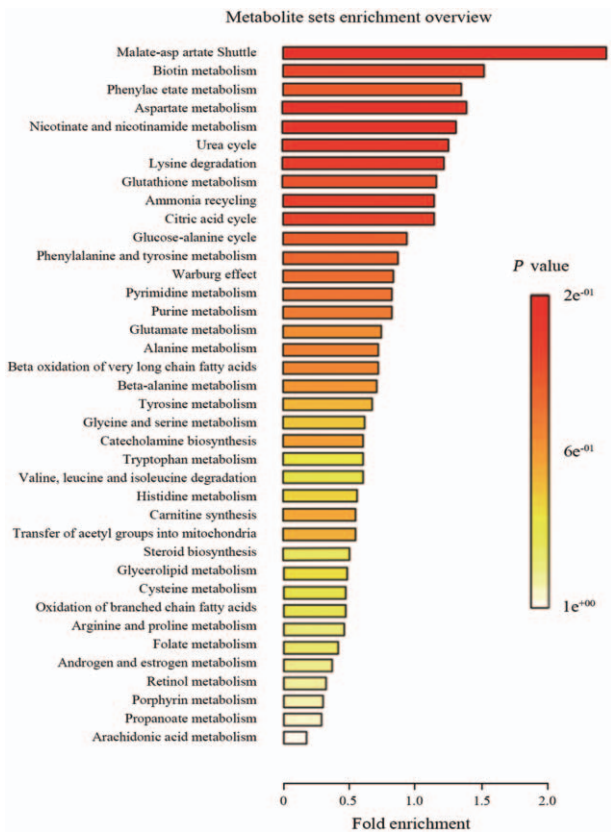

Figure 1: The fold enrichment of metabolites.

Comparing metabolic fingerprints before and after 3 months' management

From all the metabolites detected in patient serum in Group C, differential metabolites with detectable concentrations were investigated to understand the potential underpinning metabolic pathways contributing to the alleviation of symptoms. The metabolites were combined randomly based on PLS-DA, and the receiver operating characteristic (ROC) curves were constructed to assess the sensitivity and specificity of the combinational biomarkers. The final combinational biomarkers, namely, glutamic acid, aspartic acid, 1-methylnicotinamide, acetylcarnitine, glycerophosphocholine, and oleamide, were selected with a relatively small number of metabolites and high area under the curve (AUC), and their relative intensity was plotted separately [Figure 2]. Aspartic acid, glycerophos-

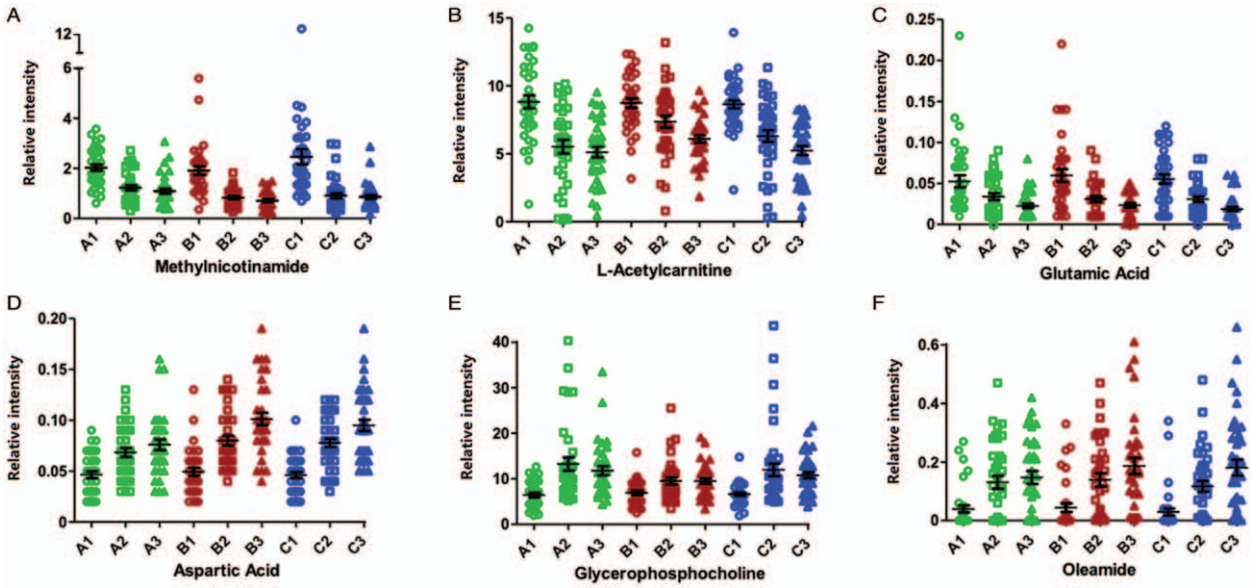

Figure 2: Abundance of the set of six metabolites after various medications. (A) Methylnicotinamide, (B) acetylcarnitine, (C) glutamic acid, (D) aspartic acid, (E) glycerophosphocholine, and (F) oleamide. Groups A1, B1, and C1 are from serums before drug treatment. Groups A2, B2, and C2 as well as Groups A3, B3, and C3 are the ones after taking 2-month and 3-month medicine, respectively.

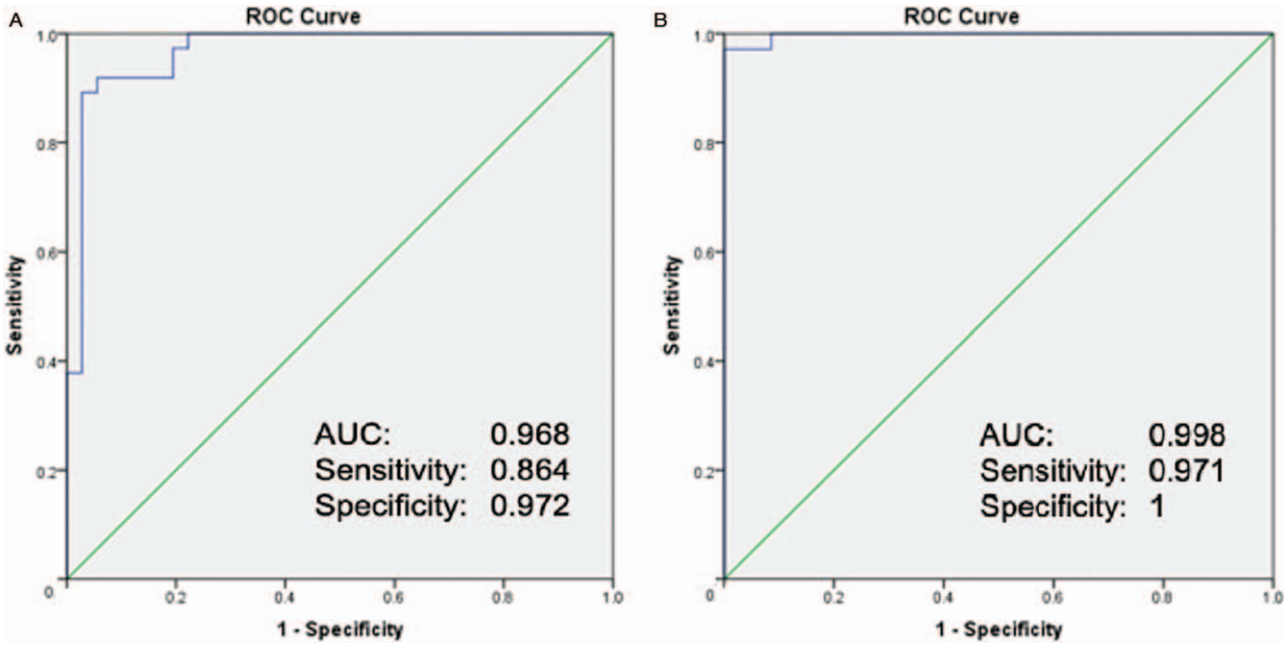

Figure 3: ROC curves of the set of six metabolites. Methylnicotinamide, acetylcarnitine, glutamic acid, aspartic acid, glycerophosphocholine, and oleamide were used to evaluate drug efficacy in the group of (A) herbal medicine, (B) western drug. ROC: Receiver operating characteristic.

phocholine, and oleamide showed a gradually increasing trend during management, whereas methylnicothinamide, L-acetylcarnitine, and glutamic acid decreased after treatment.

Validation of the combinational biomarkers

The combinational biomarkers including glutamic acid, aspartic acid, 1-methylnicotinamide, acetylcarnitine, glycerophosphocholine, and oleamide were validated in the batch of serum sample of Group A and Group B collected after 3-month management, and the results were plotted in Figure 3. AUC for the three circumstances were all >0.96, suggesting these set of metabolites may be as efficient as blood testosterone level in monitoring the efficacy of PCOS management.

In addition, these combinational biomarkers have also been applied to assess the management efficiency of medical intervention at 2nd month of management. We applied these six biomarkers to re-evaluate the clinical

progress at 2nd month of treatment using Dingkundan, Daine-35, and Dingkundan plus Daine-35, respectively [Figure 4], from which the best drug efficiency has been seen in Group C, suggesting that the combo of herbal medicine and western drug was more efficient in treating PCOS than the western drug alone.

Discussion

In the present study, these six metabolites were filtered out based on the multivariate ROC curve, and their sensitivity and specificity were evaluated with AUC over 0.96, suggesting this combinational biomarker has no less efficiency than serum concentration alternation of testosterone for reflecting the progression of PCOS management. Their performance in assessing the efficiency was demonstrated using data collected from the 2nd month.

In these six metabolites, two of them are amino acids. Aspartic acid is a non-essential amino acid involved in the synthesis of other amino acids, for example, asparagine and arginine. It also participates in tricarboxylic acid (TCA) cycles and urea cycles.[9] Aspartic acid also serves an important role in the malate-aspartate shuttle, in which the electron generated from glycolysis is translocated across the semipermeable mitochondrial inner membrane.[10] Notably, aspartic acid also has a role in neural signal transfer.[11] The other non-essential amino acid selected is glutaric acid, serving as an indispensable precursor for the biosynthesis of gamma-aminobutyric acid in neurons.[12] It also serves as the amino acid donor in ketoglutarate synthesis, an intermediate in the TCA cycle, providing pyruvate for further oxidation within mitochondria.[13] The changes of aspartic acid and glutaric acid illustrated an alternation in the amino acid metabolism pattern of PCOS patients.

Methylnicotinamide is a member involved in the nicotinamide metabolism pathway and is mainly produced in the liver with anti-inflammatory properties.[14] Methylnicotinamide was predicted to be the endogenous activator of prostacyclin, which is involved in the physiological regulation of thrombogenesis and the inflammatory process in the cardiovascular system (CVS).[15] The decrease of methylnicotinamide during treatment conforms with the expectation of reducing the long-term risk of complication in CVS.

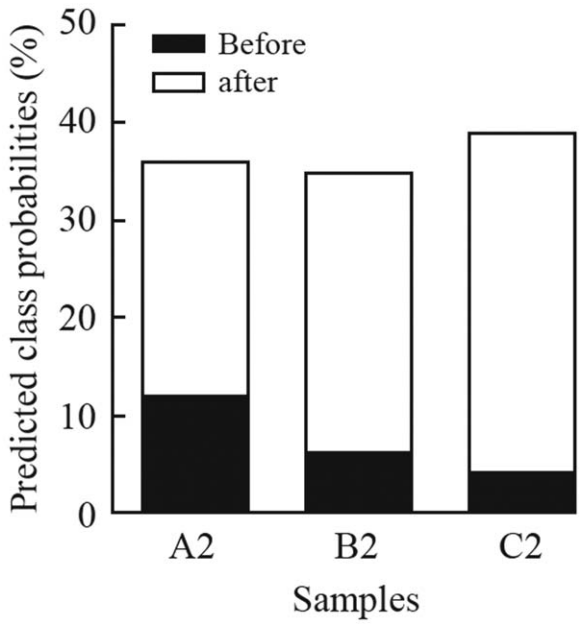

Figure 4: The predicted class probabilities for (A) herbal medicine, (B) western drug, (C) combo drugs after 2-month treatment using the set of six metabolites.

In the metabolome data, several long-chain fatty acids were significantly decreased with a lower level of several acyl-carnitines.[16] These evidences indicate a reduced lipid synthesis. On the other hand, increased glycerophosphocholine is observed, which means medication increases hydrolysis of phosphatidylcholine.[17]

Interestingly, oleamide is found to be elevated by 3.7-fold and 4.2-fold after medication of herbal and western drugs, respectively. The combined drug treatment even increased oleamide level by six-folds. It has been known that oleamide induces sleep in animals. This may lead to a side effect in treatment.[18]

As a complex syndrome, PCOS is a combination of endocrine disorder, reproductive malfunction, and metabolism abnormality. The diagnostic criteria of PCOS vary from the institute due to its sophisticated etiology and diversified manifestation, causing a fluctuated prevalence in women at childbearing age. For instance, PCOS was defined by the European Society of Human Reproduction and Embryology in Rotterdam as a combination of two out of three criteria, namely, an irregular menstrual cycle, a sign of hyperandrogenism, and PCOM[19]; whereas the Androgen Excess Society focused more on androgen-excess, defining PCOS as clinical or biochemical hyperandrogen combined with oligo-/anovulation or PCOM.[20]

The phenotypic heterogeneity and unknown pathogenic mechanism bring in large difficulty for the accurate diagnosis and progress assessment of patients at risk. Accompanied by endocrinological disorder, PCOS patients also show metabolic disorder, and insulin resistance is observed in 60% to 80% of PCOS patients.[21] The metabolomics approach has been successfully applied to investigate the pathogenesis and potential diagnostic biomarkers in other types of metabolic disorientation, such as type 2 diabetes, cardiovascular diseases, and insulin resistance.[7,8,22] Thus, using a metabolomic approach to profile the fingerprint of PCOS metabolic alternation may provide insights into the potential diagnostic and prognostic biomarkers.

In this way, we found six metabolites with great potential for assessing the PCOS management process of medical treatment. However, analysis of metabolomics is a relatively complicated process, which may lead to certain difficulty in its popularization, and further trial with larger sample size is expected to validate their effect before applying them in clinical practice.

With the metabolomic approach, LC-MS/MS was applied to find out the differential changes of certain metabolites, from which several ROC curves were constructed using

PLS-DA, and the one with relatively fewer metabolites and higher sensitivity and specificity with these six combinational biomarkers was selected, namely, glutamic acid, aspartic acid, 1-methylnicotinamide, acetylcarnitine, glycerophosphocholine, and oleamide. Their high fidelity property for monitoring the management process was proved with AUC no less than 0.96 through the other two groups, and it was applicable for evaluating the management efficiency in the 2nd-month data. A larger experiment setup is expected to further validate these six metabolites' utility in monitoring management efficiency in PCOS.

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Conflicts of interest

None.

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