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Research paper

Assessment of follicular fluid metabolomics of polycystic ovary syndrome in kidney yang deficiency syndrome

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ABSTRACT

Introduction: Given the frequent use of traditional Chinese herbal medicine by women with polycystic ovary syndrome (PCOS) and the absence of robust evidence for taking Chinese herbs in accordance with different traditional Chinese medicine (TCM) syndromes (including 'Kidney Yang Deficiency syndrome' (KYDS), this study was designed to investigate the metabolomic characteristics of follicular fluid in PCOS patients with KYDS.
Method(s): A retrospective cohort study was conducted to collect follicular fluid data from 100 infertile women participating in in vitro fertilization (IVF) protocols, including 33 patients with KYDS in PCOS and 67 healthy women whose partners showed male factor infertility. Novel SWATHtoMRM metabolomics method was used for providing the broad coverage and excellent quantitative capability to discovery the human follicular fluid metabolites related to PCOS with KYDS. Based on ultra-performance liquid chromatography-mass spectrometry (UPLC-MS), an analysis platform of follicular fluid (FF) metabolomics was established; and the changes in 2 groups of metabolic profiles were observed by principal component analysis (PCA).
Result(s): Significant changes were identified in follicular fluid for example; phenylalanine, tryptophan, hemolysis phosphatidyl choline chloride, linoleic acid, oleic acid, arachidonic acid, docosahexaenoic acid, vitamin D, and 28 other metabolites. The biochemical changes involved 12 different metabolic pathways, including metabolism of fatty acids and amino acids, and biosynthesis of bile acids. Our results will provide a more objective and scientific understanding of the KYDS of PCOS.
Conclusions: According to our study, FF metabolomics will not only constitute an important tool with which to study TCM syndrome theory, but will also provide an experimental basis for the use of Chinese herbal medicine to treat individuals with PCOS with KYDS.

1. Introduction

According to the literature, the prevalence of PCOS in women of childbearing age ranges from 5% to 10%, and it is primarily caused by an abnormal endocrine state and manifests ovulatory dysfunction. [1,2] At present, the pathogenesis of PCOS remains unclear, and research on its etiology focuses on genetics [3], obesity [4], insulin resistance (IR) [5], hyperandrogenemia [6], and other elements and pathologies. Although lifestyle changes, oral contraceptives, and metformin may improve PCOS symptoms and increase to some extent the chances for pregnancy in afflicted women [7,8]; strict evidence-based medical evidence is still lacking [9], and there is an increased risk of complications and side effects [10–12]. Chinese herbs possess bioactive chemicals that can improve the reproductive endocrine environment surrounding PCOS, and it is reported that about two-fifths of the PCOS population uses Chinese herbal remedies [13,14]. However, the application of a single Chinese herbal extract is contrary to the principle of "dialectical treatment" of traditional Chinese medicine compounds [15–17]; and therefore, how to realize the objectification and standardization of TCM syndromes has become an important issue in the promotion and application of Chinese herbal medicine. Metabolomics, as a new technology of systematic "omics", embodies holistic and dynamic views, which is consistent with the theory of viscera-state and syndrome in Chinese medicine. KYDS is the basic syndrome type of PCOS. In the present study, we took IVF technology as the basic platform and follicular fluid as the research carrier to search for biomarkers

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for PCOS with KYDS from the perspective of FF metabolomics. We believe that it will be important to scientifically determine the syndrome essence of PCOS kidney Yang deficiency and thereby promote Chinese medicine worldwide.

2. Materials and methods

2.1. Study population

This study was approved by the Reproductive Medicine Ethics Committee of the Affiliated Hospital of Shandong University of TCM on September 26, 2016 (Grant No. 20,160,911,012). All Chinese women who underwent IVF/ICSI were recruited for preliminary screening via advertisements in the outpatient and inpatient departments of the Affiliated Hospital of Shandong University of TCM from October 2016 to June 2017 in Shandong, China. All participants who enrolled were provided with information about the study, and they all signed the informed consent prior to being included. All patients were conventionally treated with TCM syndrome differentiation.

All patients with PCOS included in our study were diagnosed according to the criteria from both the European Society of Human Reproduction and Embryology and the American Society for Reproductive Medicine in 2003. [18] The control group consisted of patients with infertility purely due to a male factor (azoospermia or severe oligo-asthenoteratozoospermia). Women with infertility due to poor ovarian reserve, ovulatory dysfunction, tubal factor, and endometriosis were excluded from the control group.

If any of the following conditions were noted, the patient was excluded: i.e., age ≥40 years, BMI ≥ 30 Kg/m^2, karyotyping abnormalities in both partners, congenital or acquired dysplasia of the uterus, and endometriosis or other contraindications for assisted reproductive technology.

2.2. Kidney Yang deficiency syndrome

The ‘Clinic terminology of traditional Chinese medical diagnosis and treatment-Syndromes’ (GB/T 16,751.2-1997) was used to identify suitable patients for the study. Before PCOS patients entered the IVF cycle, 2 experts (deputy senior and above) differentiated the selected subjects independently on the day of pituitary down-regulation. The same subjects had to undergo 2 syndrome differentiations with respect to TCM, and if the 2 were consistent, they would be selected. The requirements for syndrome differentiation were as follows:

a) main syndromes:infertility, delayed menorrhea, amenorrhea;
b) secondary syndromes:dizziness and tinnitus; waist discomfort; reduced libido; urine in reduced volume; loose stools;
c) tongue description and pulse rate manifestations: pink tongue, white and thin coating on the tongue, thready and deep pulse.

Major syndromes are essential and can be diagnosed with 2 or more secondary syndromes, and with all tongue changes and pulse rate manifestations.

2.3. Clinical protocols

For the metabolomics analysis, the MetSizeR approach for sample size estimation was used to estimate a total sample size of 100 subjects using the following assumptions: spectra of 584 spectral bins, a target false detection rate of 5%, and an expected proportion of significant spectral bins of 20%. A total of 100 subjects were included in this study, including 33 PCOS women with KYDS, and this was set as the observational group. A total of 67 healthy female patients who underwent IVF with only male factor infertility were set as the control group. All patients received a gonadotropin-releasing hormone (GnRH) agonist for pituitary down-regulation using a long protocol for controlled ovarian hyper-stimulation (COH). Patients were monitored for serum estradiol (E_2), luteinizing hormone (LH), and progesterone (P_4) levels; and underwent serial transvaginal ultrasonographic examinations until the dominant follicles (≥17 mm sizes) were observed. Thirty-six hours after ovulation induction with recombinant human chorionic gonadotropin hormone (rhCG, 250 μg of Ovitrelle, Serono, Darmstadt, Germany), oocytes were extracted under analgesia and sedation. Intracytoplasmic sperm injection (ICSI)/IVF was performed on all patients, and follicular (FF) was collected during egg retrieval. The supernatant was collected into sterile cryovials and stored at −80 °C after centrifugation at 14,000× g for 20 min to remove cells and insoluble particles. Specimens with blood contamination were discarded.

Table 1
Characteristics of PCOS patients included in the present study.

Parameter	Observational group	Control group	*p-value*
Patients	33	67	
Age (years)	29.92 ± 4.43	29.61 ± 4.22	*0.735*
BMI (kg/m^2)	25.42 ± 5.73	24.05 ± 4.33	0.186
Cycle number	1.75 ± 0.52	1.90 ± 0.41	*0.119*
Baseline day-3 FSH (U/L)	6.53 ± 1.72	7.00 ± 2.16	*0.959*
Baseline day-3 E_2 (pg/ml)	36.7 (31, 48.50)	43 (30, 57)	*0.433*
E_2 on HCG day (pg/ml)	4800 (3176.5, 5085)	4509 (2523, 4901)	*0.198*
P on HCG day (ng/ml)	1.2 (0.86, 1.75)	1.39 (0.92, 1.99)	*0.422*
Days of Gn (d)	12 (11, 13)	12 (11, 13)	*0.976*
Gn dose (U)	2250(1800, 2637.5)	2625 (2100, 3525)	***0.031***
Retrieved oocytes (n)	15 (10.5, 21.5)	13(9, 19)	*0.149*
Frozen embryo transfer cycles with:			
Day-3 embryos, n (%)	26 (78.8%)	58 (86.6%)	*0.318*
Day-5 embryos, n (%)	7 (21.2%)	9 (13.4%)	
Transferred embryos per cycle	1.75 ± 0.53	1.80 ± 0.50	*0.646*

Note: $p < 0.05$ indicates a statistically significant difference between the 2 groups of data. The amount of Gn used for the control group was significantly greater than that for the observational group, possibly because with the latter we deliberately reduced the amount of Gn to avoid ovarian hyperstimulation syndrome (OHSS).

2.4. Experimental procedure [19,20]

For metabolomics analysis, a SCIEX ExionLC AD ultra-performance liquid chromatography (UPLC) system equipped with a reverse-phase ACQUITY UPLC® BEH C18 column (2.1 × 100 mm, 1.7 μm) was used. The follicular fluid of 5 μL was injected at 15 °C. The column temperature was set at 40 °C and the flow rate was 0.4 mL/min. In positive mode, water with 0.1% formic acid and acetonitrile with 0.1% formic acid were used as mobile phases A and B, respectively. The elution was performed with a 12 min gradient. The gradient was kept at 95% A for 0.5 min, increased to 100% B over the next 7 min, and then returned to 95% A from 10 min to 10.1 min. The total run time was 12 min. In negative mode, water with 5 mM of ammonium acetate and acetonitrile were used as mobile phases A and B, respectively. The elution was performed with a 14 min gradient. The gradient was kept at 95% A for 0.5 min, increased to 100% B over the next 8 min, and then returned to 95% A from 12 min to 12.1 min. The total run time was 14 min. All SWATH data were acquired on a SCIEX Triple TOF 5600+ and all MRM data were acquired on a SCIEX QTRAP 5500. Nitrogen was used as the nebulizer gas (GS1 and GS2) at 55 psi. The source temperature was set at 550 °C. In positive mode, the voltage of ion spray was 5500 V. The declustering potential and collision energy were set at 60 V and 35 ± 15 V, respectively. In negative mode, the voltage of ion spray was -4,500 V. The declustering potential and collision energy were set at -60 V and -35 ± 15 V, respectively. The full scan range and the product ion scan range were all from m/z 50 to m/z 1200. The raw SWATH data were converted to mzXML files using the “msconvert” program from ProteoWizard. Multiple data files were grouped and processed by SWATHtoMRM. A large-scale set of MRM transitions were produced

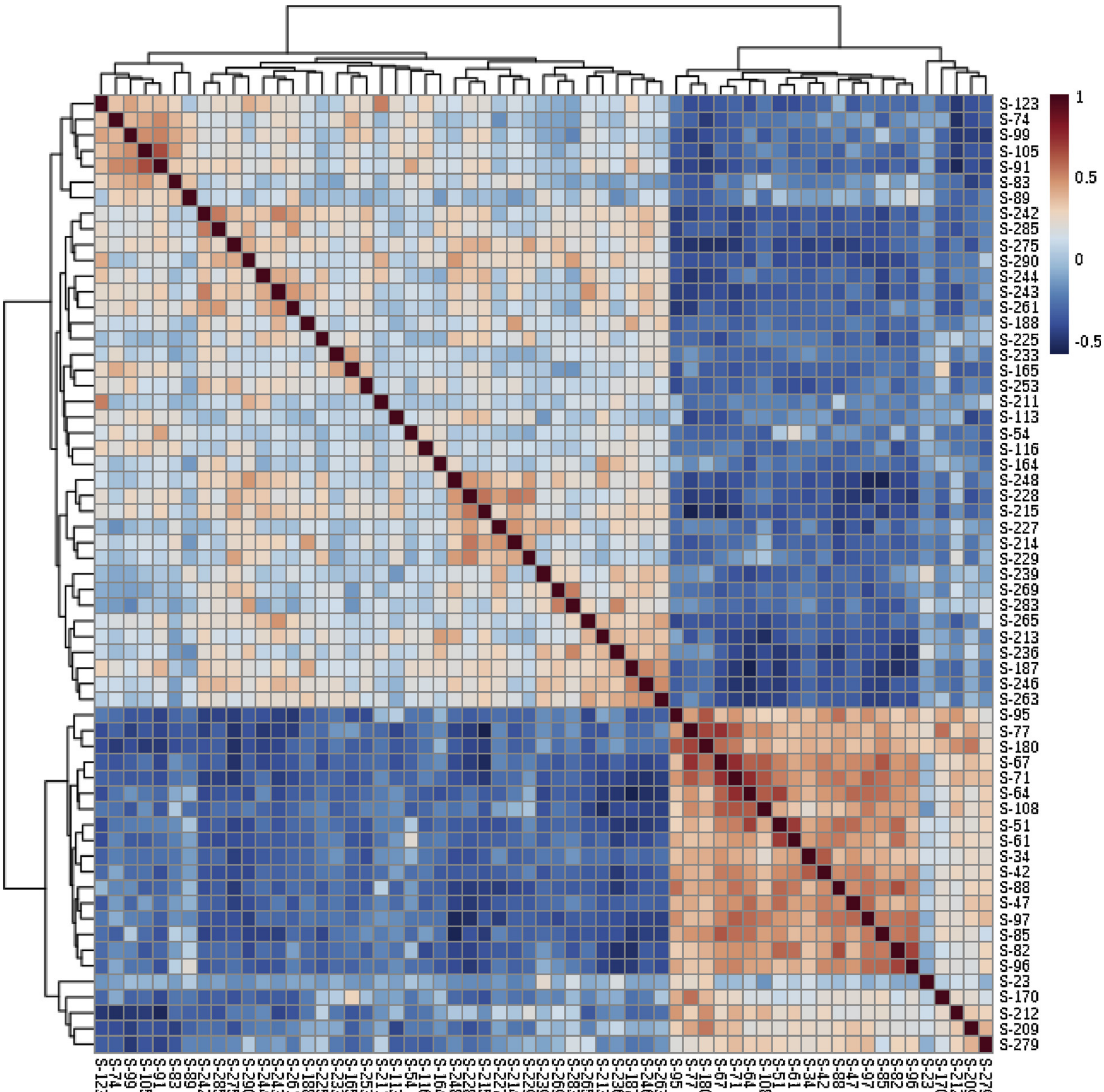

Fig. 1. A. Heatmap of different compounds under positive ion mode. Fig. 1 **B.** Heatmap of different compounds under negative ion mode.

and a scheduled MRM method was then constructed using Analyst TF 1.7.1 software to maximize the number of measured MRM transitions in each analysis.

2.5. Data processing and statistics analyses

In total, 100 follicular fluid samples were analyzed in replicates using UPLC-TOF and UPLC-QTRAP. Data was processed using the PeakView software and the MarkerView software for peak detection, extraction of MS^2 peaks and chromatograms, and MS^1 and MS^2 peak grouping. According to the "80% rule", peak present in more than 80% samples of either group were kept for further analysis. In large-scale metabolomics measurements, the reproducibility of the analysis may be influenced by source contamination or the maintenance and cleaning of the mass-spectrometer. Normalization is a common preprocessing method to decrease systematic change. In our study, peak areas were normalized to metabolites with CV values over 15% in QC samples were removed from the data table. Principal component analysis (PCA) was required to find different variation features on the MarkerView software. The Student's *t*-test was used for statistical comparisons. The contributing list of metabolites was determined by p-values below 0.05. The predictability of the model was determined by internal validation with 7-fold cross-validation and response permutation testing. The differential Metabolites with high contribution score were identified by accurate mass, isotope patterns and mass spectrometric fragmentation patterns, which were used to search databases, including KEGG, PubChem compound, METLIN, the Madison Metabolomics Consortium Database and the Human Database.

3. Results

3.1. Baseline data

Our clinical baseline data–including age, BMI, number of cycles, baseline FSH, baseline estradiol (E_2), E_2 and progesterone (P) on the day of HCG administration, gonadotropin (Gn) dose, number of eggs retrieved, days of Gn administration, number of cleavage embryos or blastocysts, and number of transferred embryos per cycle–are shown in

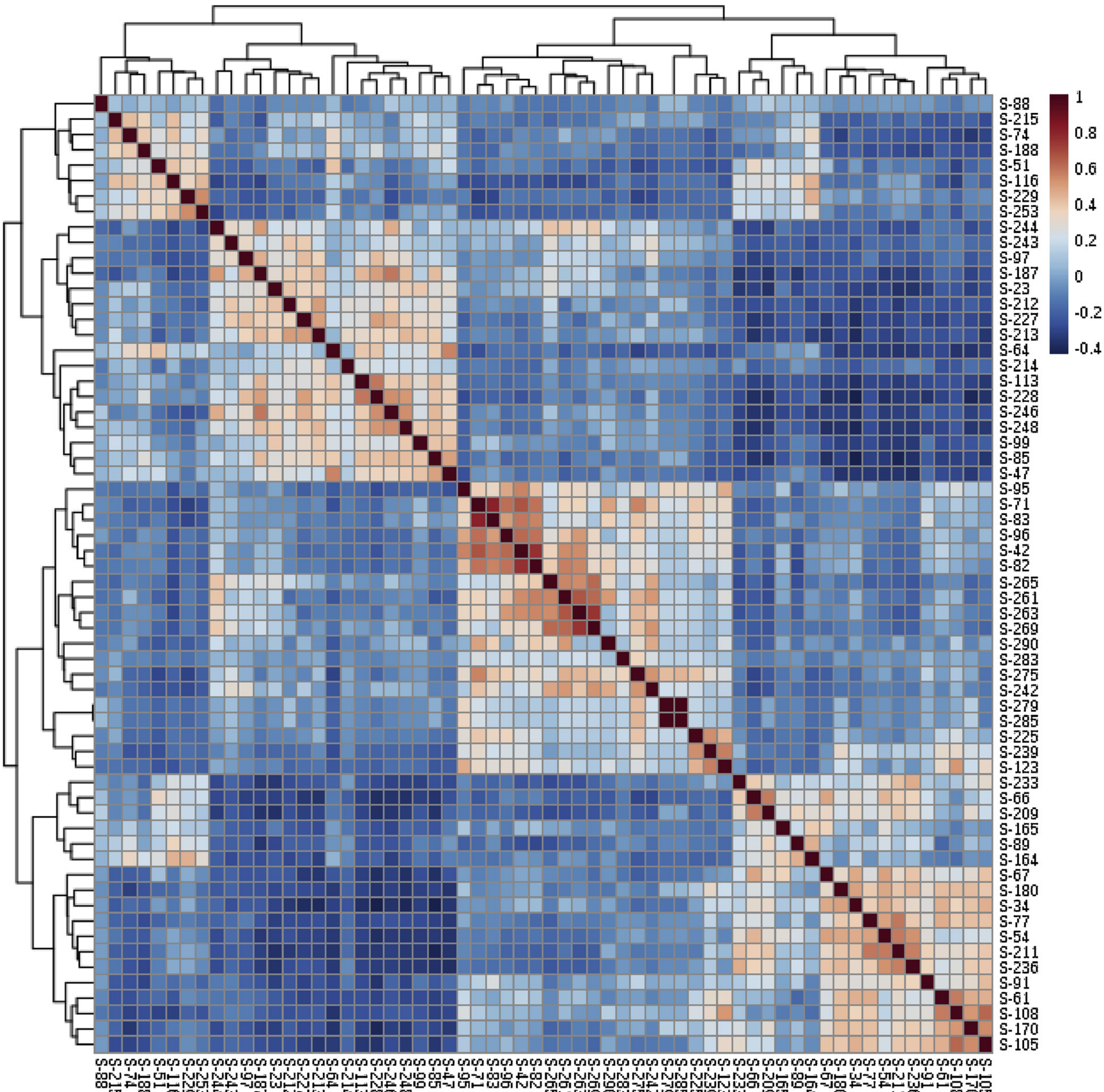

Fig. 1. (*continued*)

Table 1. In order to prevent ovarian hyperstimulation syndrome, the amount of gonadotropin in the PCOS group was relatively less than the control group. However, because the number of basal antral follicles were significantly higher than that in the control group, the number of eggs obtained in the PCOS group was relatively higher.

3.2. Metabolomic analysis

The follicular fluid samples of the 2 groups were collected in the positive and negative ion modes using UPLC-MS. The total ion flow diagram of the 2 groups under the positive ion model is shown in Fig. 2A, and the total ion flow diagram of the 2 groups under the negative ion model is shown in Fig. 2B. The relationship between the chromatographic retention time deviation and retention time of the sample composition of follicular fluid under the positive and negative ion modes is shown in Fig. 3A and Fig. 3B, respectively. It can be seen from Figs. 3A and 3B that the chromatographic behavior is acceptable and the retention time is not offset.

Using the MetaboAnalyst metabolomics software, we created a heat map of the compounds showing significant differences between the observational group and the control group (Fig. 1A, positive ion mode; Fig. 1B, negative ion mode).

The follicular fluid samples of the observational group and the control group were comprehensively analyzed by means of non-targeted metabolomics. After peak extraction, peak matching, and statistical analysis, the PCA scoring figures of the 2 groups are shown in Fig. 4A (positive ion mode) and Fig. 4B (negative ion mode). Figs. 4A and 4B show that 2 groups in the PC1 dimension have an acceptable separation, indicating that the metabolites in the follicular fluid of PCOS patients with KYDS have changed.

We derived a total of 28 different compounds in follicular fluid upon analysis (shown in Table 2), including the metabolic pathways in which they specifically participate. These differentiated substances involved 12 metabolic pathways, including tyrosine metabolism, cholesterol biosynthesis, fatty acid metabolism, lipid metabolism, tryptophan metabolism, retinoic acid metabolism, vitamin D3 metabolism, and vitamin E metabolism. These results indicated that the metabolic pathways of PCOS patients with kidney Yang deficiency changed to differing degrees.

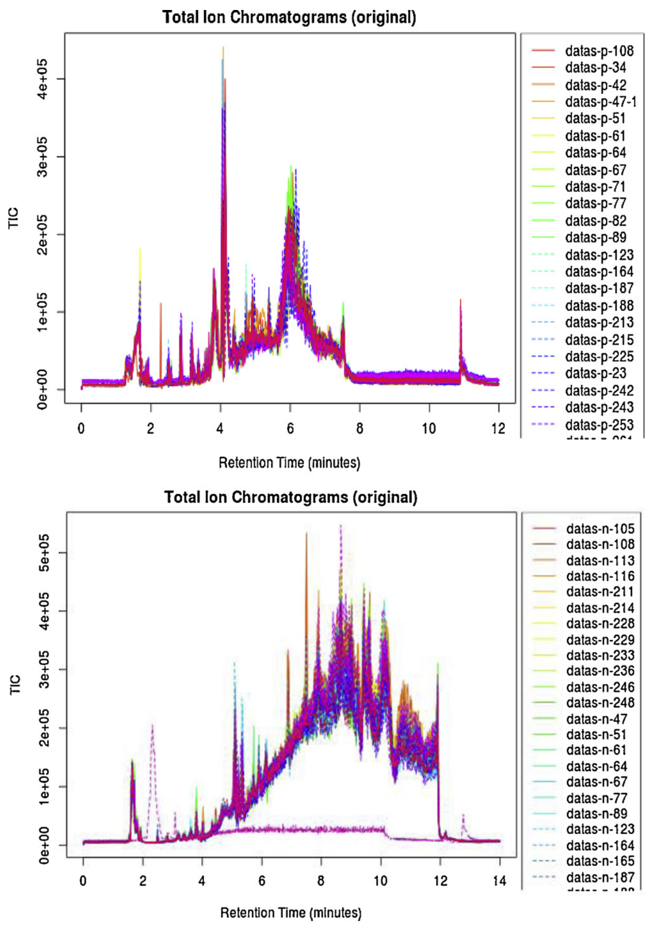

Fig. 2. A. Total ion flow in the sample of all subjects under positive ion mode. Fig. 2 **B**. Total ion flow in all subjects' follicular fluid samples under negative ion mode.

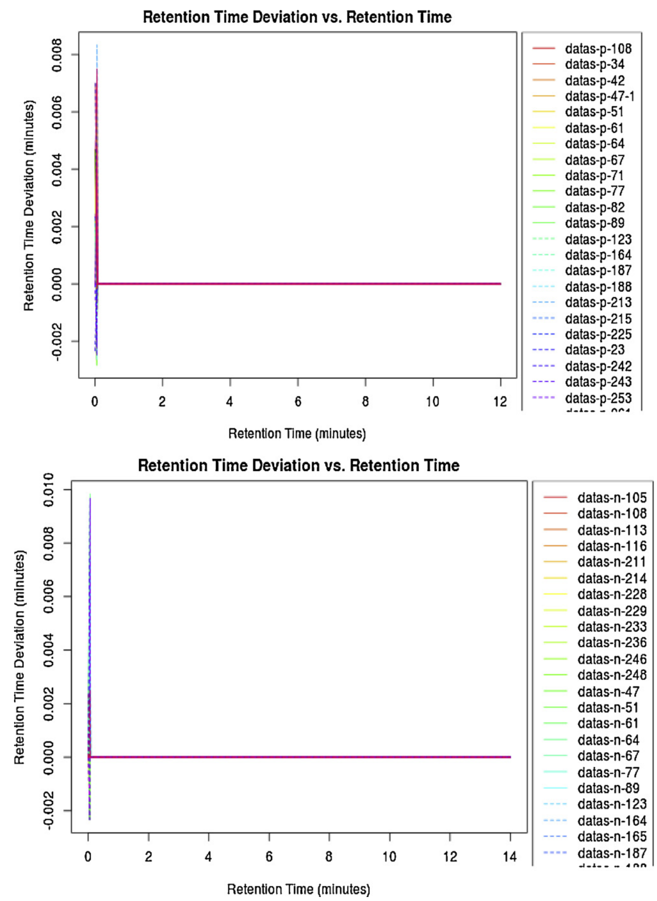

Fig. 3. A. The relationship between retention time and retention time of the follicular fluid sample under positive ion mode. Fig. 3 **B**. The relationship between retention time and retention time of the follicular fluid sample under negative ion mode.

4. Discussion

According to statistics from the World Health Organization, infertility, cardiovascular diseases, and tumors are listed as the 3 major diseases affecting human life and health; and 1 in 6 couples of childbearing age around the world now suffers from infertility [21]. In the past 30 years, at least 7 million babies have been born thanks to ART, especially *in vitro* fertilization [22]. Despite this, the pregnancy rate of "test-tube babies" still hovers between 30% and 40% [23]. PCOS, a disease routinely treated with assisted reproductive technology, is a major cause of infertility. Some scholars believe that the use of Chinese herbs is beneficial for the PCOS population [24–27], but these studies did not follow the basic principles of "treatment based on syndrome differentiation" and were not "people-oriented" [16,17,28]. Therefore, it is necessary to correctly identify a patient's specific "syndrome" in the formulation of Chinese herbal medicine, and use it to produce a prescription that achieves a better therapeutic effect. KYDS is a basic syndrome type of PCOS, and its objectification and standardization are the preconditions for treatment based on syndrome differentiation of PCOS, and for the improvement of its clinical symptoms and pregnancy outcome. Therefore, in the present study–after egg aspiration–we used the residual follicular fluid for metabolomic analysis. The purpose of this study was to explore the biomarkers of PCOS with KYDS, promote its objectification and standardization, and guide clinical practice more accurately, so as to promote the worldwide application of Chinese herbal medicine.

The material basis of cellular activity is protein, but in fact, most cellular activities are closely related to or regulated by metabolites–such as signal transduction, energy transfer, and intercellular communication–and metabolomics provides direct results of molecular regulation in metabolic pathways. Although genomics and proteomics can be used to study metabolic pathways regulated by molecules, metabolomics can directly investigate biologically phenotypic pathways of metabolism [29]. It allows for analysis of all low-molecular weight compounds (not only individual metabolites), and provides information about the functional integrity of the entire organism–which is consistent with the integrity and overall systems approach of TCM. The emerging field of metabolomics provides a new approach to understanding the molecular mechanism(s) subserving various diseases, allows final quality control of Chinese medicine, and fosters the development of novel and ideal drugs. Metabolomics has also been used increasingly to evaluate the efficacy of many herbal and traditional Chinese medicine prescriptions [30–33]. Currently, there are many metabolic profiling tools, including ^{1}H NMR spectroscopy, HPLC/MS, CE/MS, and GC/MS [34–37]; but compared with HPLC, UPLC/MS exhibits a better chromatographic peak resolution, shorter analytical time, and higher sensitivity; and is considered to possess a broader catalog of applications to metabolomics [38–40].

Follicular fluid is composed of plasma proteins that cross the blood-egg barrier and accumulate together with granulocytes, oocytes, and membrane cell secretions in and around the follicle and into the follicular antrum. It is principally composed of steroid hormones, polysaccharides, proteins, and metabolites [41]. Simultaneously, follicular fluid also acts as the information transmitter between oocytes and surrounding follicular cells. It is the locus for material exchange and energy metabolism, the microenvironment upon which oocytes depend

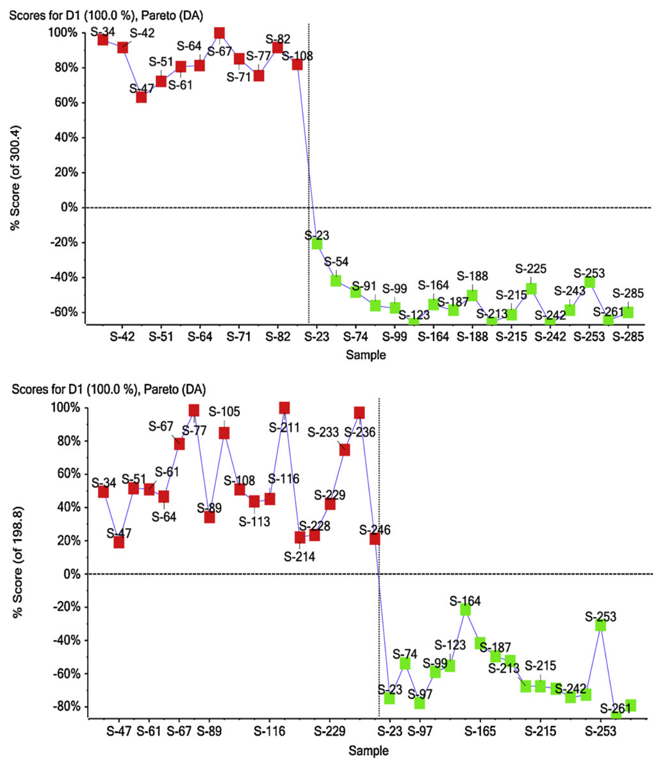

Fig. 4. A. PCA score of different metabolites between the observational and control groups (positive ion mode). Fig. 4 **B.** PCA score of different metabolites between the observational and control groups (negative ion mode).

for survival; and is crucial to the development of oocytes. Pantasri found that the composition of follicular fluid is not the same as plasma, and that the microenvironment of follicular fluid has its own unique lipid profile that provides appropriate conditions for the growth and development of oocytes [42]. The proportion and content of fatty acids in follicular fluid are affected by fatty acids in plasma, but they are not completely identical. Subsequent studies showed that if fatty acid β-oxidation was inhibited, free fatty acids would accumulate in the follicular fluid due to a reduction in decomposition, leading to endoplasmic reticulum stress, and affecting the development potential of oocytes and lowering blastocyst formation [43]. Rago studied patients with PCOS-related infertility and found that palmitic acid and oleic acid levels in plasma and follicular fluid of obese PCOS were higher than those in non-obese patients; and that supplementing PCOS-fatty acids and inositol improved their reproductive outcome [44]. In addition, glucose concentrations in follicular fluid can also reflect the developmental potential of oocytes [45]. For example, in the infertile mouse model, Zhao observed abnormal changes such as impaired oocyte development and meiotic maturation disorders in diabetic mice [46]. A recent and intensely studied research topic within metabolomics is the evaluation of oocyte quality by using amino acid levels in follicular fluid [29]. High levels of homocysteine in the follicular fluid of infertile women constituted adverse factors affecting IVF outcomes, while high levels of proline, leucine, and isoleucine in follicular fluid were associated with successful pregnancy [47].

We identified in the present study a total of 28 metabolites that showed significant changes (including phenylalanine, hemolytic phosphatidylcholine, and linoleic acid). These biochemical changes involved 12 metabolic pathways related to fatty acid metabolism, amino acid metabolism, and biosynthesis of bile acids. The information garnered from our study will help us to better understand the mechanism(s) underlying PCOS, which can lead to reproductive dysfunction. It also allows us to determine a PCOS syndrome type with a characteristic metabolite group, and to better direct us to improved treatment modalities based upon syndrome differentiation.

5. Limitations

There were some potential limitations of this study when interpreting our conclusions. First, the number of patients in this study was relatively small. Second, it is indispensable that in further studies, a larger number of PCOS subjects with KYDS in prospective validation

Table 2
Differential metabolites between the 2 groups and the metabolic pathways involved.

No.	m/z	Adduct	Identity	Metabolic pathways
1	115.0397	M-H[-]	2-Oxoisovalerate	Leucine and isoleucine degradation
2	129.0556	M-H[-]	6-Oxohexanoate	Leucine and isoleucine degradation
3	164.0707	M-H[-]	L-Phenylalanine	Tyrosine metabolism
4	191.0176	M+HCOO[-]	2-Oxoglutarate	Leucine and isoleucine degradation
5	279.232	M-H[-]	Linoleate	Fatty acid metabolism
6	281.2472	M-H[-]	Oleic acid	Fatty acid metabolism
7	299.2562	M + CH3COO[-]	Hexadecanal	Glycosphingolipid metabolism
8	303.2321	M-H[-]	Arachidonate	Lipid metabolism
9	327.232	M-H[-]	Docosahexaenoic acid	Fatty acid metabolism
10	347.2591	M + CH3COO[-]	13,14-Dihydroretinol	Retinoic acid metabolism
11	357.2783	M-H2O-H[-]	Lithocholic acid	Cholate biosynthesis
12	391.2836	M-H[-]	Hyodeoxycholate	Cholate biosynthesis
13	419.3117	M + Cl[-]	Vitamin D3	Vitamin D3 metabolism
14	426.3627	M-H[-]	Stearoylcarnitine	Carnitine shuttle system
15	431.3114	M + Cl[-]	5-Dehydroepisterol	Cholesterol biosynthesis
16	445.3308	M+HCOO[-]	25-Hydroxyvitamin D3	Vitamin D3 metabolism
17	447.3469	M+HCOO[-]	27-Hydroxycholesterol	Cholate biosynthesis
18	474.3591	M + ACN-H[-]	3alpha,7alpha,12alpha-Trihydroxy-5beta-cholestan-26-al	Cholate biosynthesis
19	491.372	M+HCOO[-]	13'-Hydroxy-alpha-tocopherol	Vitamin E metabolism
20	492.3715	M + ACN-H[-]	5beta-Cholestane-3alpha,7alpha,12alpha,23,25-Pentol	Cholate biosynthesis
21	205.0978	M+H[+]	Tryptophan	Tryptophan metabolism
22	496.3411	M+H[+]	LysoPC (16:0)	Lipid metabolism
23	518.3193	M+H[+]	LysoPC (18:3)	Lipid metabolism
24	520.3319	M+H[+]	LysoPC (18:2)	Lipid metabolism
25	522.3562	M+H[+]	LysoPC (18:1)	Lipid metabolism
26	524.3678	M+H[+]	LysoPC (18:0)	Lipid metabolism
27	542.3196	M+H[+]	LysoPC (20:5)	Lipid metabolism
28	544.3394	M+H[+]	LysoPC (20:4)	Lipid metabolism

experiments should be obtained to verify the present results based on predefined targeted metabolomics. Third, FF can merely be obtained from patients performing oocyte retrieved. Therefore, our findings can only provide indirect evidence for the pathogenesis of PCOS with KYDS.

6. Conclusions

In the present study, the special physiologic and pathologic status of PCOS with KYDS were explored with the help of an IVF technology platform, with follicular fluid as the research carrier, and the metabolomic analysis method was based on UPLC/MS. Some potential biomarkers such as phenylalanine, hemolytic phosphatidylcholine, and linoleic acid were identified. Combined with the results of bioinformatics analysis, the changes in the follicular fluid metabolite group suggested that the metabolic pathways for fatty acids, amino acid metabolism, and biosynthesis of bile acids are related to PCOS with KYDS. Our work demonstrated that metabolomics methods constitute a powerful tool with which to study the essence of Chinese medicine's syndrome theory; and also provided experimental evidence for treatments based on syndrome differentiation of PCOS with KYDS, and its promotion and application.

Authors' contributions

Zhengao Sun and Jingyan Song participated in the design of this study. Shan Xiang carried out the concepts, design, and definition of intellectual content. Zhengao Sun and Yi Yang carried out data analysis and manuscript preparation. Jingyan Song and Yi Yang provided assistance for data acquisition, data analysis, and statistical analysis. Zhengao Sun and Shan Xiang performed manuscript review. All authors have read and approved the content of the manuscript.

Declarations

Authors: All research done by the authors.
Financial support: no.

Declaration of Competing Interest

None.

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