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The vaginal metabolomics profile with features of polycystic ovary syndrome: a matched case-control study in China

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List of abbreviations: ESI, electrospray ionization; OPLS-DA, orthogonal partial least-squares discriminant analysis; PCOS, polycystic ovary syndrome; UHPLC-MS/MS, ultra-high-performance liquid chromatography tandem mass spectrometry.

Abstract

Background: Polycystic ovary syndrome (PCOS) is the most common metabolic disorder and reproductive endocrine disease, putting women of reproductive age at increasing risk. Although serum, amniotic fluid and urine metabolism differences were obtained in PCOS, there remains a paucity of evidence for vaginal fluid. The aim was to identify different metabolites associated with PCOS in Chinese women of reproductive age and to explore whether these metabolites enriched in specific pathways.

Methods: We involved ten newly diagnosed PCOS women who attended gynecology at Zhongda Hospital and matched with ten healthy controls who conducted health check-up programs at Gulou Maternal and Child Health Center in Nanjing, China from January 1st, 2019 to July 31st, 2020. Non-targeted metabolomics based on ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) was applied to differentially screen vaginal metabolites of PCOS and healthy controls. Principal component analysis (PCA), orthogonal partial least-squares discriminant analysis (OPLS-DA) and enrichment analysis were used to observe differences, search for potential biomarkers and enrich related pathways.

Results: Among 20 participants, a total of 195 different metabolites were detected between PCOS group and healthy control group. PCOS and control groups could be well separated by vaginal fluid. Lipids and lipid-like molecules accounted for the largest number of differential metabolites. Dopamine, with an increased trend in PCOS, was the most significant differential metabolite, which may be regarded as a potential biomarker in identifying PCOS. UHPLC-MS/MS based vaginal metabolomics methods showed a significant difference between PCOS and non-PCOS healthy control group, especially linoleic acid metabolism disorder. Most differential metabolites were

enriched in linoleic acid metabolism, phenylalanine metabolism, tyrosine metabolism, nicotinate and nicotinamide metabolism or arachidonic acid metabolism pathways.

Conclusions: In this matched case-control study, significant metabolomics differences could be obtained between PCOS and healthy controls. For PCOS women of reproductive age, vaginal metabolism can be a more economical, convenient and harmless alternative to provide careful personalized health diagnosis and potential targets for therapeutic intervention.

Keywords: polycystic ovary syndrome, liquid chromatography–mass spectrometry, metabolomics, vaginal fluid, matched case-control study.

Introduction

Polycystic ovary syndrome (PCOS), a heterogeneous, female endocrine-reproductive-metabolic abnormality(Sun et al. 2021) with a prevalence of 6% to 10% globally(Bozdag et al. 2016), has become a global public health problem. Compelling evidence(Barber & Franks 2021; Fedrigon et al. 2019; Pastore et al. 2011; Wu et al. 2020) suggested that women with PCOS had significant consequences for human health, for example, they are vulnerable to hypertension(Wu et al. 2020), nephrolithiasis(Fedrigon et al. 2019), depression(Pastore et al. 2011) and pregnancy complications such as miscarriage and preterm birth(Barber & Franks 2021). However, the diagnoses of PCOS(Medical 2018; Ribbenstedt et al. 2018) were still not uniform across countries because of the unclear etiology of PCOS, which fairly does not conduce to a prediction and prevention of the disease. In practice, PCOS is a diagnosis of exclusion according to the disease guidelines(Shabbir et al. 2023). Although the interaction of susceptibility genomic variants or vaginal microbiome diversity might be indicators of PCOS(Hong et al. 2020; Hughes et al. 2006), an agreement has not been reached yet on the complex mechanisms underlying PCOS pathogenesis. Therefore, the aforementioned questions emphasize the urgency of annotating the specific pathogenesis of PCOS and further exploring a precise, efficient and multidimensional detection method and prediction for PCOS diagnosis.

Metabolomics has become a research tool for elucidating biological interference with internal or external stimuli(Ribbenstedt et al. 2018) and has been used in the etiological study of PCOS and the search for biomarkers(Koivula et al. 2019). Previous studies have been conducted in serum(Escobar-Morreale et al. 2012), follicular fluid(Chen et al. 2020) or urine samples in PCOS patients(Dhayat et al. 2018) and focus on different major metabolite classes. However, most

sampling methods were invasive and fewer metabolites could be detected. Vaginal secretion metabolomics among PCOS patients, which was a more economical, convenient and harmless method, has been reported minimally. To update current knowledge on different biological samples of PCOS, we focused on Chinese PCOS female's vaginal secretion to identify correlated metabolic factors. As a supplementary analytical technhuique of nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS), most ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) non-target metabolomics technology has high sensitivity, good retention time reproducibility and extensive chemical diversity coverage.

In this study, we conducted a matched case-control study among PCOS women of reproductive age tand used UHPLC-MS/MS to analyze metabolic profiles of reproductive secretions. We aimed to reveal the correlation between clinical features and vaginal metabolites, to provide the possibility to investigate the vaginal metabolism difference in PCOS, and to determine the potential biological characteristics based on vaginal secretion swabs.

Material and methods

Study participants

This matched case-control study was reviewed and initiated by Zhongda Hospital and Gulou Maternal and Child Health Center in Nanjing, China to improve women's health and fecundability. We initially recruited 287 women at gynecology in Zhongda Hospital and 523 health check-up women which was similar to the general population in Gulou Maternal and Child Health Center from January 1, 2019 to July 31, 2020. After signing written informed consent, they completed health checkups and examinations. We included PCOS women who met the following criteria: 1) aged 20-45 years; 2) were willing to be followed until a confirmed diagnosis of PCOS, which

followed both the 2004 revised Rotterdam criteria(2004) and the Guidelines for Diagnosis of PCOS in Chinese (2020 Edition)(Endocrinology Subgroup and Expert Panel 2018). During the contemporaneous period, women recruited for the healthy control group were 1:1 matched by age (±3 years old) and ethnicity, owned the inclusion criteria as follows: 1) the ages of women were between 18-45; 2) has never suffered from PCOS, infertility or other endocrine diseases; 3) no polycystic ovary was found in B-ultrasound images; 4) had regular menstrual cycles. Women of any group would be excluded if they 1) rejected vaginal examination when participation; 2) had following complications: Cushing's syndrome, congenital adrenal hyperplasia, thyroid disease, hyperprolactinemia or androgen secreting tumors; 3) taking antibiotics, drugs or hormones within 30 days before vaginal examination; 4) lack of basic information; 5) were pregnant during participation (**Figure 1**). Finally, ten women with PCOS and ten matched controls were included in this study. This study has been approved by the Ethics Committee of Zhongda Hospital (2018ZDSYLL072-P01), all patients have provided written informed consents.

Basic information definition

Basic information was recorded based on previous studies on PCOS. For sociodemographic characteristics, age was classified into 20-24 years and ⩾25 years. Occupation was categorized into worker, civil servant and others. Educational level was classified into bachelor degree and master degree or above. For health statuses, body mass index (BMI) was calculated by dividing weight by the square of height (kg/m^2), and was binary categorized (⩽24 kg/m^2 and >24 kg/m^2)(Ji & Chen 2013). Fasting plasma glucose was tested in the morning after 8 hours of fasting(Alberti & Zimmet 1998). Menstrual period for healthy controls was recorded for at least three menstruation cycles and averaged, and for women with PCOS, they self-reported their last menstruation period

length. Menstruation period was divided into less than or equal to one month and more than one month. Self-reported dysmenorrhea was classified as yes or no. Vaginal cleanness (I°-II°, III°-IV°) and bacterial vaginosis infection (yes/no) were based on hospital laboratory testing.

Study procedure

All participants were recruited in a female reproductive health program and carried out basic information records the first time reached the clinics or health center. After signing written informed consent, they completed health examinations and were recorded for menstrual intervals. For healthy controls, they were followed up every month for exact menstruation length. During the gap of menstruation period, health workers would confirm that women were not sexually active or had vaginal irrigation in the previous 48 hours before vaginal swab collection. Swabs were collected from all participants at the lithotomy site from their posterior fornix by rotating three times. Collected swabs were stored in a drying tube with identification labels and then transferred into a special collection container at 4°C. Furthermore, they were placed in a -80 ℃ refrigerator until metabolites detecting procedure was conducted.

UHPLC-MS/MS, data processing and identification

All samples were thawed at 4°C for extraction, which would be conducted with an additional 1 mL of extract solution (ACN:MeOH 50:50 (v/v), with isotopically-labelled internal standard mixture). The mixture was vortex-mixed for 30s and then incubated in ice water for 30 min. The samples were homogenized at 35 Hz for 4 min, sonicated in ice water for 5 min, incubated at -40°C for 1 h, and centrifuged at 12,000 rpm for 15 min at 4°C. A 750 μL aliquot supernatant of each sample was prepared in EP tubes. The resulting supernatant was vaporized without heating, and a 100 -μL aliquot of extract solution (ACN: MeOH 50:50 (v/v)) was added to make up the dried

sample. Next, the solution was vortexed for 30 s, sonicated in ice water for 10 min, and centrifuged at 4°C for 15 min. The supernatant was moved into a fresh glass vial for UPLC-MS analysis. All samples were kept inside an auto-sampler with a temperature of 4°C during the analysis.

The UHPLC system (Vanquish, Thermo Fisher Scientific) was used to conduct UHPLC-MS/MS analysis with a UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm) combined to Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo) with electrospray ionization (ESI). Quality control (QC) samples were used to monitor the reliability and stability. Peaks were normalized and filtered to remove noise based on relative standard deviation, and data with no more than 50% null values or no more than 50% in all groups were retained. The parameter cutoff was set to 0.5.

Statistical Analysis

We described baseline information in terms of means (standard deviation) and counts (percentages). Pairwise student's t-test and Fisher exact test were used to analyze data between groups. Pearson and Spearman correlation were used for correlation analysis. These analyses were conducted using R statistical software (version 4.1.3). For metabolomics analysis, principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) were conducted using SIMCA software (version 16.0.2). A two-sided p value < 0.05 was considered statistically significant.

Results

In this study, ten PCOS and ten health control women were finally included. All of them were Han ethnicity. After matching with age, the average ages of PCOS and health control women were 24.20±2.35 and 25.20±3.39 years, respectively. The proportions of participants with more entended

menstruation periods and higher vaginal pH were statistically higher in PCOS women ($p<0.05$). The sociodemographic characteristics and health status of the study population were presented in **Table 1**.

All samples were successfully measured by UHPLC-MS/MS. A total of 859 metabolites were detected in the study population. Among them, 195 differential metabolites were found between PCOS and healthy control, containing 122 metabolites in positive ion mode (75 compounds showed upward trends while 47 compounds showed downward trends) and 77 metabolites in negative ion mode (51 compounds showed upward trend while 26 compounds showed downward trend), in which four of them were detected both in ESI positive and negative modes. The differential compounds were categorized into seven major classes: alkaloids and derivatives, benzenoids, lipids and lipid-like molecules, organic acids and derivatives, organic oxygen compounds, organoheterocyclic compounds, phenylpropanoids and polyketides.

In unsupervised PCA score plots of the metabolite results (**Supplement Figure 1**), an overall view of the two groups was initially revealed and all sample data were wrapped in the Hotelling *T*-squared ellipse. Meanwhile, both PCOS and control groups could be clearly separated, and QC samples were closely assembled to show the stability of the test results. Similarly, OPLS-DA score plots showed the same separation between the two groups with well robustness and no overfitting (**Figure 2**).

For potential biomarker searching, the Euclidean distance matrix for the quantitative value of differential metabolite was calculated among all different compounds and the most significant 20 metabolites which changed remarkably, as shown in a heat map (**Figure 3**). Among them, the majority were lipids and lipid-like molecules, followed by phenylpropanoids and polyketides,

organic acids and derivatives, benzenoids and alkaloids and derivatives. Among the greatest differential metabolites, dopamine (DA) was the top significant metabolite in vaginal secretions. DA, isocitric acid, 5,6-dimethoxysterigmatocystin, m-coumaric acid, oxoadipic acid, l-cysteine, 20-hydroxyeicosatetraenoic acid, 3,4-dihydroxymandelaldehyde, 3-o-feruloylquinic acid, 5,7-dihydroxy-2-phenyl-6,8-bis[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]-4h-chromen-4-one and sedoheptulose showed upward trend in PCOS group, while n-cyclopropyl-trans-2-cis-6-nonadienamide, 4,8 dimethylnonanoyl carnitine, 11-dehydro-thromboxane B2, 3,3-dimethylglutaric acid, 24-epibrassinolide, dioscoretine, 2-(3,4-dihydroxyphenyl)-3,4-dihydro-2h-1-benzopyran-3,5,7-triol, caprylic acid and 8-hydroxyoctanoate showed downward trend.

To expound on metabolic and regulatory pathways, all differential metabolites were searched in Kyoto Encyclopedia of Genes and Genomes (KEGG). The pathways were filtered based on bubble size and color in bubble plots (**Figure 4**). The differential metabolites were enriched in five main pathways: linoleic acid metabolism, phenylalanine metabolism, tyrosine metabolism, nicotinate and nicotinamide metabolism and arachidonic acid metabolism. Hit metabolites in each pathway are shown in **Table 2,** with 15 showing upward trends and 3 showing downward trends (**Table S1**). We also found four compounds of the twenty most significant metabolites in pathways. Respectively, DA, 11-dehydro-thromboxane B2, m-coumaric acid and 3,4-dihydroxymandelaldehyde were enriched in tyrosine metabolism, arachidonic acid metabolism and phenylalanine metabolism.

Correlation analyses were performed between differential metabolites hit in the main five pathways and sociodemographic characteristics and health indicators. As shown in **Table S1**, menstruation period, dysmenorrhea, vaginal cleanness and pH had same correlation trend and were

significantly positively correlated with all hit metabolites except p-hydroxyphenylacetic acid, prostaglandin G2 and 11-Dehydro-thromboxane B2. Vaginal cleanness was positively correlated with 3,4-dihydroxymandelaldehyde, N1-Methyl-2-pyridone-5-carboxamide, phenylacetic acid and 4-hydroxybenzoic acid, while educational level, BMI and fasting plasma glucose was not correlated with any hit metabolites in top five pathways.

Discussion

As a high-resolution and high-throughput sensitive technology, non-targeted metabolomics analysis has been widely used to measure metabolite differences and explore biomarkers(Johnson et al. 2016). Most of the UHPLC-MS/MS have the advantages of a comprehensive analysis range, reliable qualitative results, low detection limit and short analysis time. We certified that PCOS and control group had distinct metabolic perturbations, most of the top 20 metabolites were lipids and lipid-like molecules. All differential metabolites were targeted in five main pathways, the most important of which was linoleic acid metabolism. Many abnormal metabolic disorders were related to menstruation period, while a few were related to vaginal cleanness. The subtle changes in vaginal microenvironment were partly explained, which may contribute to the occurrence and development of PCOS, and can be used as potential biomarkers for diagnosis. Meanwhile, the related metabolic pathways provided a theoretical basis for the study of the pathogenesis of PCOS in the future. It also proved that the vaginal fluid metabolomics tested by UHPLC-MS/MS showed great practicability in disease diagnosis and mechanism research.

Metabolites in human vagina are influenced by bacterial metabolism of human-derived nutrients. As samples were collected from vaginal swabs among women of reproductive age, it is

242 considered that metabolites within vaginal microecology vary with differential metabolic activities
243 in vivo. The results suggest that there was potential metabolic heterogeneity between PCOS patients
244 and non-PCOS healthy controls. In this study, differential metabolites in vaginal secretions in
245 childbearing age women were identified, including 122 in positive ion mode and 77 in negative ion
246 mode. Compared with previous studies, the difference in vaginal secretion samples was much
247 greater than that in blood or follicular fluid samples(Saller et al. 2014). The complex environment
248 like different compositions of vaginal and cervical mucosa microbiome may be served as a reason,
249 which was involved with various vaginal microbiota abundance(Wang et al. 2021). In view of the
250 similar metabolism of vaginal flora and human cells, it is difficult to distinguish. However, there is
251 a lack of metabolic enzymes for the degradation of benzene ring compounds in the human body,
252 and the degradation of these phenyl compounds usually depends on flora. It is generally considered
253 that the compounds containing phenyl in human metabolites are mainly produced by microbial
254 metabolism, like 5,6-Dimethoxysterigmatocystin, m-Coumaric acid, 3,4-
255 Dihydroxymandelaldehyde, etc.

256 The DA was detected as the most differential significant metabolites between PCOS and
257 healthy controls among women of reproductive age, with a much higher level in PCOS group than
258 in healthy controls. A study showed a similar increased trend of DA in the human granulosa cells of
259 women with PCOS(Gómez et al. 2011) as our study. It was previously detected in human blood and
260 follicular fluid of ovulatory follicles of the human ovary(Saller et al. 2014). However, DA detected
261 in vaginal swabs has been reported minimally, which was revealed for the first time the presence of
262 DA in human vaginal swabs. Meanwhile, DA is associated with cellular uptake and metabolism-
263 dependent generation of reactive oxygen species(Saller et al. 2014). The first detection of DA in

vaginal secretion reveals a method which was more convenient, economical and rapid than sampling in follicular fluid, and it is possible to screen for PCOS by detecting DA in vaginal secretion swabs.

Linoleic acid metabolism is the most influenced pathway by enrichment analysis of differential metabolites. Both linoleic acid and bovinic acid hit in the pathway were up-regulated compared with that of healthy control, which was similar to previous research in plasma metabolism(Escobar-Morreale et al. 2012; Zhao et al. 2012). Linoleic acid metabolism was associated with seborrhea, abnormal corneocyte desquamation and its storage in sebaceous follicle, suggesting that linoleic acid metabolism may be associated with PCOS obesity phenotype(Downing et al. 1986). In addition, linoleic acid may inhibit maturation and development of oocyte, and the enrichment of differential metabolites in linoleic acid metabolism may lead to the increase of immature oocytes and ovulatory disorder, suggesting that it may be related to the clinical symptoms of PCOS(Marei et al. 2010). Linoleic acid shows strong pro-inflammatory activity(Toborek et al. 2002) and is also a potential chronic low-grade inflammatory marker of PCOS(Escobar-Morreale et al. 2011; Ojeda-Ojeda et al. 2013). The role of linoleic acid in the human body has been already clear, while the relationship between linoleic acid metabolism and vaginal flora still needs to be further verified in the future.

Phenylalanine metabolism plays an important role in oocyte development and ovulation(Jóźwik et al. 2017), and phenylalanine may be converted to tyrosine. Results showed increased tyrosine metabolism in PCOS patients with normal BMI. Tyrosine enrichment was detected in vaginal secretion which shows consistency with previous studies of plasma metabolites(Zhang et al. 2014). The increase of tyrosine is related to insulin resistance and ovulation dysfunction(Fong et al. 2011). Ovulation dysfunction was improved by lowering aromatic amino acids level like phenylalanine and tyrosine(Tang et al. 2019). These results indicate/illustrate that

tyrosine metabolism and phenylalanine metabolism are closely related to the pathogenesis of PCOS.

Correlation has been found between nicotinate and nicotinamide metabolism and lipid metabolism(Yang et al. 2014). Oral gavage for nicotinamide adapted hyperandrogenism in rat model(Nejabati et al. 2020). However, it has been proved that there remains optimum dose of nicotinamide, or would be harmful to homeostasis of glucose and damage insulin resistance(Cantó et al. 2012). Nicotinamide acts as the substrate of N-methyltransferase, which leads to the generation of N1-Methylnicotinamide. Hence the further formation of N1-Methyl-2-pyridone-5-carboxamide. Studies have shown that the production of N1-Methylnicotinamide in cumulus cells of patients with PCOS was significantly increased(Nejabati et al. 2020). The upstream and downstream products of N1-Methylnicotinamide were tested to be increased, which indicates that nicotinate and nicotinamide metabolism can also be detected in vaginal secretions, as in tissues and serum.

Linoleic acid can be converted into arachidonic acid by acyl-CoA 6-desaturase, elongation of very long chain fatty acids protein 5 and acyl-CoA (8-3)-desaturase. Arachidonic acid is then converted to a series of short-lived metabolites. In arachidonic acid metabolism, arachidonic acid and its cyclooxygenase-generated metabolites have the ability to regulate different ovarian functions and luteolysis(Husein & Kridli 2003; Medan et al. 2003). Studies have shown that the increase of arachidonic acid and linoleic acid in follicular fluid significantly decreased the ability of oocytes to form nucleus and fertilize, which may be related to the low fertility of patients with PCOS. However, the level of arachidonic acid changed in different tissues. In PCOS rat model, arachidonic acid in serum was up-regulated while down-regulated in ovarian tissue(Huang et al. 2018). Arachidonic acid derived metabolites, such as prostaglandins and thromboxane, have been shown to play an important role in oocyte maturation, cumulus expansion and ovulatory(Li et al. 2019). In this study,

11-Dehydro-thromboxane B2 and prostaglandin G2, which were downstream products of thromboxane B2 and upstream products of prostaglandin A2, prostaglandin B2, prostaglandin C2 and prostaglandin E2, respectively, furtherly indicated the important role of arachidonic acid metabolism in PCOS patients.

Limitations

First, we used stricter inclusion criteria for both PCOS and healthy controls, which means PCOS women should be newly diagnosed and meet both Rotterdam criteria and Chinese Guidelines for Diagnosis of PCOS to ensure typical PCOS cases and healthy controls were followed up for three months for regular menstruation and to exclude complications, which limited the study sample size. Second, although age match and ethnicity match were conducted through our study to eliminate more baseline covariate differences among groups, potential covariates between groups were still inevitable. Third, as PCOS women had irregular menstrual cycles, time in swab collection was hard to 2.

Conclusions

Based on this reproductive age case-control study, we found significant metabolomics differences between PCOS and healthy controls. Vaginal metabolites, especially DA, can be regarded as a potential biomarker in PCOS screening, and linoleic acid metabolism can be identified as the most influenced pathway. This study highlights the need for vaginal secretion metabolism in reproductive age and for careful personalized health diagnosis and potential targets for therapeutic intervention.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors have no competing interests to declare.

Author Contributions

All authors contributed to manuscript editing and read and approved the final manuscript. Yan Xuan and Xiang Hong contributed to **concept and design**. Yan Xuan, Xiang Hong, Tao Yan, and Xu Zhou contributed to **acquisition, analysis, or interpretation of data.** Yan Xuan and Xiang Hong contributed to **drafting of the manuscript**. Xiang Hong and Bei Wang contributed to **critical review of the manuscript for important intellectual content.** Yan Xuan, Tao Yan contributed to **statistical analysis.** Bei Wang contributed to **obtained funding**. Pengfei Qin and Danhong Peng contributed to **administrative, technical, or material support**. Bei Wang contributed to **supervision.**

Human Ethics

This study was conducted in accordance with ethical procedures and approved by the Institutional Ethics Committees of Zhongda Hospital (Approval notice: 2018ZDSYLL072-P01), abided by the Declaration of Helsinki. All participants were given information about the study and written informed consent was obtained from all study participants for participation in the study.

Data Availability

The datasets used and/or analyzed during the current study are available in the supplementary files.

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