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Original Article

LC-MS/MS-based metabolomic profiling identifies candidate biomarkers in follicular fluid of infertile women with chronic pelvic inflammatory disease

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Abstract: Objectives: How chronic pelvic inflammatory disease (CPID), the most common cause of infertility, affects metabolic profiles of follicular fluid (FF) remains unknown. This study aimed to identify candidate biomarkers in FF of infertile women with CPID. Method: FF samples were collected from infertile women with CPID (n = 8) and healthy controls (n = 8) at the time of oocyte retrieval. Untargeted metabolomic profiling of FF samples was conducted using the liquid chromatography-tandem mass spectrometry (LC-MS/MS). Results: A total of 240 differential metabolites (104 named biochemicals and 136 unnamed biochemicals) were screened out and identified. Among them, pregnane-3,3-diol, pc(p-18:1(11z)/18:3(6z,9z,12z)), and 1-octadecanoyl-2-(4z,7z,10z,13z,16z,19z-docosahexaenoyl)-sn-glycero-3-phosphoethanolamine were markedly down-regulated, while 17,21-dihydroxypregnenolone was significantly up-regulated in infertile women with CPID. Furthermore, KEGG biological pathway analysis revealed that these metabolites were especially enriched in steroid hormone biosynthesis, glyoxylate and dicarboxylate metabolism, glucagon signaling pathway, and the tricarboxylic acid (TCA) cycle. Conclusion: FF of infertile women with CPID showed unique metabolic changes that may be involved in the pathogenesis of infertility and serve as new therapeutic targets or diagnostic biomarkers.

Keywords: Biomarker, follicular fluid, infertility, LC-MS, metabolic profiles

Introduction

Infertility means that a couple cannot become pregnant without taking contraceptive measures within one year. The incidence of infertility among reproductive-aged couples is between 10% and 15% globally, making it a global public health problem [1]. With the rapid development of society and increasing environmental pollution, more young couple are facing fertility problems [2]. Currently, in vitro fertilization and embryo transfer (IVF-ET) is widely used for the treatment of infertility. Follicular fluid (FF) is mainly derived from the sanguineous plasma and secretions of cells in the follicle wall. FF contains various metabolites, nutrients, steroids, and growth factors, which provide a microenvironment necessary for the growth, development, and survival of oocytes [3]. Change in phthalate metabolites of FF have been shown to be positively associated with the levels of ovarian reproductive hormones [4]. Therefore, the analysis of FF content may provide clinically useful biomarkers for predicting oocyte maturity, embryonic development, and fertility [5].

Metabolomics can simultaneously perform qualitative and quantitative analysis on all metabolites of various organs, tissues, or cells in a specific physiologic period or condition, so as to discover and identify differential metabolites [6]. A previous study has demonstrated that changes in the composition of FF significantly affect the quality of oocytes, and metabolomic analysis of FF has become an important index for reliable clinical prediction of in vitro fertilization (IVF) outcome [7]. Recently, nuclear magnetic resonance (NMR)-based metabolic profiling of FF has identified several novel bio-

markers for polycystic ovary syndrome (PCOS) diagnose and predicting oocyte developmental competence and pregnancy outcome [8]. Pelvic inflammatory disease is the most common cause of infertility, ectopic pregnancy, and chronic pelvic pain [9]. However, its underlying molecular mechanisms have not been fully elucidated.

In present study, we used the liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based metabolomic strategy to discover and identify candidate biomarkers of FF for infertile women with chronic pelvic inflammatory disease (CPID) specifically.

Materials and methods

Study population

A total of 16 women undergoing assisted reproduction were included and FF was collected between April 2020 and May 2021. The women were divided into two groups based on the etiology of infertility: healthy control (male factor, n = 8) and infertile women (CPID, n = 8). The diagnosis of CPID was made according to the following criteria (endometriosis biopsy shows endometritis; evidence of pelvic inflammatory disease on laparoscopy; ultrasound or other imaging examination found that fallopian tubes are thicken and filled with fluid) and exclusion criteria (other gynecological diseases such as endometriosis, leiomyoma, or PCOS) [10]. All procedures were in strict compliance with institutional guidelines and received approvals from the clinical ethics committee of our hospital. All participants had carefully read and signed informed consent forms before this research.

FF sample collection and metabolite extraction

All women received a standard antagonist protocol with recombinant FSH (Gonal-F 225 IU/day) starting from cycle day 2 and GnRH antagonist (Cetrotide 0.25 mg/day) from stimulation day 6, which were continued daily up to the day of hCG administration. Follicular growth was monitored by transvaginal ultrasound. A recombinant hCG trigger was performed when there were more than two follicles with a diameter (≥ 18 mm), and oocytes were collected 34-36 h after ovulation induction. FF samples were aspirated by transvaginal ultrasound-guided follicle puncture with a fresh lumen needle. FF of multiple follicles was collected and added into a 15 ml sterile test tube from each patient separately. FF contaminated with red blood cells or flush medium was excluded from the present study. FF samples were centrifuged at 10,000×g for 10 min at 4°C and the supernatant was transferred to sterile freezing tubes and stored at -80°C until use. The frozen FF was taken out of the -80°C freezer and placed at -20°C for 1 h and then at 4°C until completely liquid. 100 μl of FF samples was added to 300 μl of precooled methanol and acetonitrile (2:1, v/v), internal standards mix 1 (IS1), and internal standards mix 2 (IS2). After vortex for 1 min and incubation for 2 h at -20°C, samples were centrifuged at 15,000×g for 20 min at 4°C. 200 μl of supernatant was transferred to each autosampler vials for LC-MS/MS analysis. One quality control (QC) sample was prepared by pooling 25 μl of supernatant from each real sample to assess the reproducibility of the whole LC-MS/MS analysis.

Untargeted LC-MS/MS analysis

The samples were analyzed using a Waters 2D ACQUITY UPLC (waters, Milford, MA, USA) combined with Q Exactive HF high resolution mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as described previously by Li *et al.* [11].

LC-MS/MS raw data from both positive and negative ions were collected using a high resolution mass spectrometer Q Exactive HF (Thermo Fisher Scientific, USA) and analyzed using the Compound Discoverer 3.1 (Thermo Fisher Scientific, USA) software, including peak extraction, retention time correction within and between groups, additive ion pooling, missing value filling, background peak labeling, metabolite identification, and finally information on compound molecular weight. The identification of metabolites was a combined result of the BGI Library (BGI inhouse-developed standard library), mzCloud and ChemSpider (HMDB, KEGG, LipidMaps) databases. Data pre-processing, statistical analysis, metabolite classification annotations, and functional annotations were performed using the self-developed metabolomics R package metaX and the metabolome bioinformatic analysis pipeline. The multivariate raw data were dimensionally reduced by principal component analysis (PCA) to analyze the groupings, trends (intra- and inter-group similarities and differences), and

Table 1. Epidemiological and clinical information of participants who provided FF samples

Value	Healthy controls (n = 8)	Patients (n = 8)	*P*
Age (years)	28.75±4.33	33.0±4.07	0.063
BMI	21.97±2.24	21.56±2.91	0.758
Primary infertility (%)	62.50	50.00	0.721
Secondary infertility (%)	37.50	50.00	
Infertility duration (years)	3.25±1.67	6.63±3.29*	0.022
Basal FSH (IU/L)	5.92±0.86	6.88±1.25	0.092
Basal LH (IU/L)	8.18±3.44	6.13±1.42	0.142
Basal E2 (pg/ml)	37.91±11.57	47.91±33.24	0.435
Basal P (ng/ml)	0.39±0.31	0.32±0.19	0.548
AMH (ng/ml)	3.76±1.39	3.89±3.21	0.921
AFC	17.38±1.69	14.63±7.63	0.337
Gn dose (IU)	1890.60±317.06	2235.90±900.68	0.324
Gn duration (days)	10.63±0.92	9.63±1.19	0.080
LH level on the trigger day (IU/L)	1.74±0.37	2.43±1.51	0.227
E2 level on the trigger day (pg/ml)	2705.05±1549.50	2168.78±1479.10	0.491
P level on the trigger day (ng/ml)	0.56±0.33	0.98±0.55	0.081
No. of oocytes retrieved	12.50±5.26	9.75±5.82	0.228
Fertilization rate (%)	77.81±14.79	72.03±9.85	0.373
Cleavage rate (%)	93.37±8.95	90.05±10.28	0.502
Clinical pregnancy rate (%)	50%	50%	

Data are presented as mean values and standard error. BIM, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; P, progesterone; AMH, anti-Mullerian hormone; AFC, antral follicle count; Gn, gonadotropin. Fertilization rate = number of fertilized embryos/total number of oocytes retrieved. Cleavage rate = number of cleavage embryos/number of two-pronuclei (2PN). *$P < 0.05$.

outliers of the observed variables in the data set (whether there was an abnormal sample). Using partial least squares method-discriminant analysis (PLS-DA), the variable importance in projection (VIP) values of the first two principal components of the model, combined with the variability analysis, the fold change (FC) and the Student's T test to screen for differential metabolites.

The heatmap was generated using MeV software. MetPA software was used for enrichment analysis of KEGG pathways. The results are presented as the mean ± the standard deviation of the mean (SD), and $P < 0.05$ was considered a significant differences. Pearson correlation coefficient was calculated using corr.test in the psych package.

Results

Clinical characteristics of infertile women with CPID and healthy controls

The relevant clinical epidemiologic information from the 16 participants is shown in **Table 1**. No statistically significant differences were found between the two groups in terms of age, body mass index (BMI), primary/secondary infertility, basal levels of follicle-stimulating hormone (FSH), estradiol (E2), luteinizing hormone (LH), or progesterone (P), anti-Mullerian hormone (AMH), antral follicle count (AFC), the dose and duration of gonadotropin (Gn), the levels of LH, E2 or P on the trigger day, number of oocytes retrieved, and the rates of fertilization, cleavage, and clinical pregnancy. However, the infertility duration was significantly higher in infertile women with CPID when compared with healthy controls (6.63±3.29 years vs 3.25±1.67 years, $P < 0.05$).

Metabolic differences in the FF

To obtain the differential metabolites between infertile women with CPID and healthy controls, untargeted LC-MS/MS was performed to identify metabolites in 16 FF samples under both electrospray ionization (ESI) negative and positive ion patterns. A total of 4927 compounds were included in the LC-MS/MS metabolomic

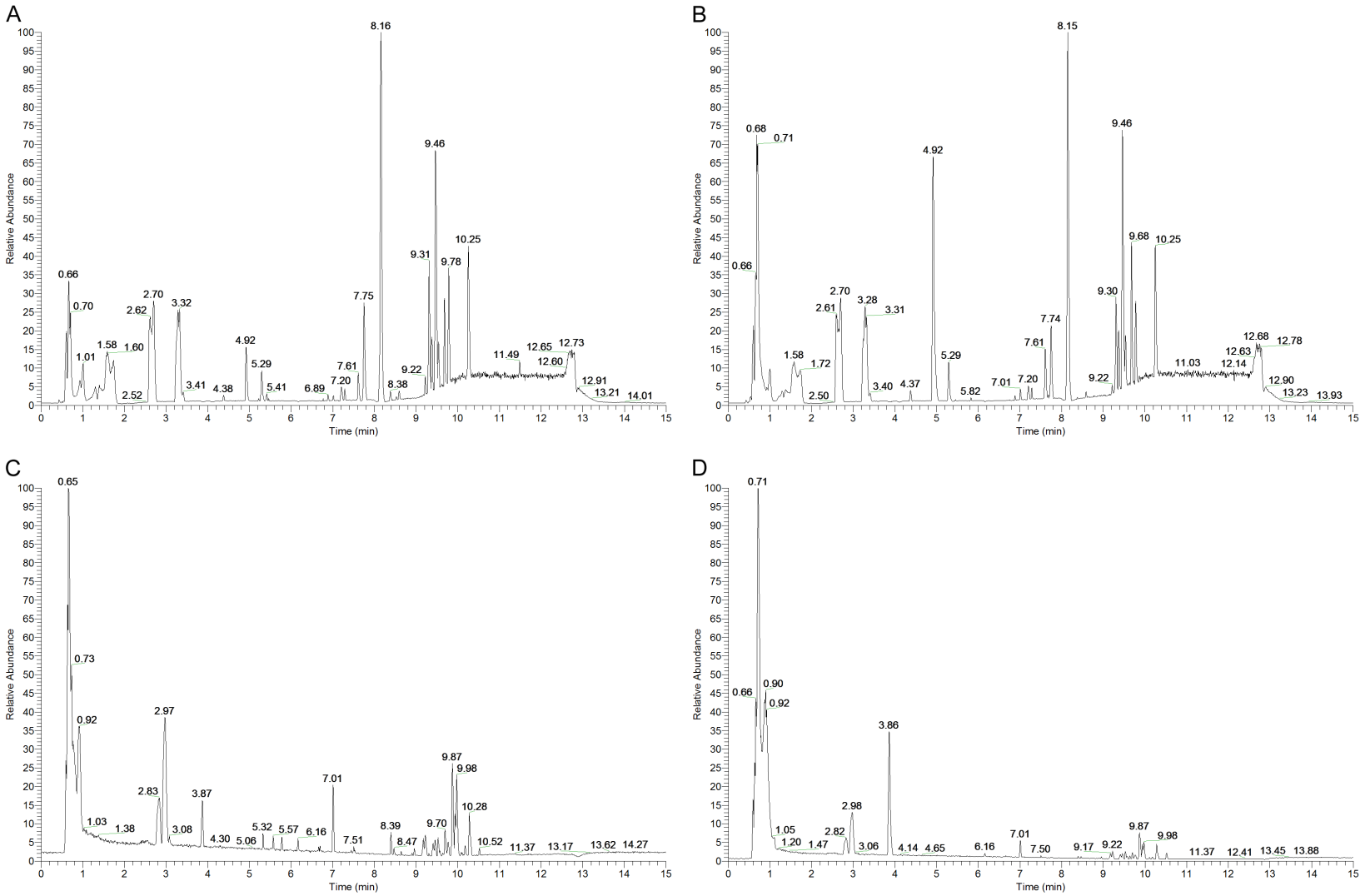

Figure 1. Typical base peak chromatograms of FF in the positive ion patterns of healthy controls (A) and infertile women with CPID (B), and in the negative ion patterns of healthy controls (C) and infertile women with CPID (D) based on LC-MS/MS are shown respectively.

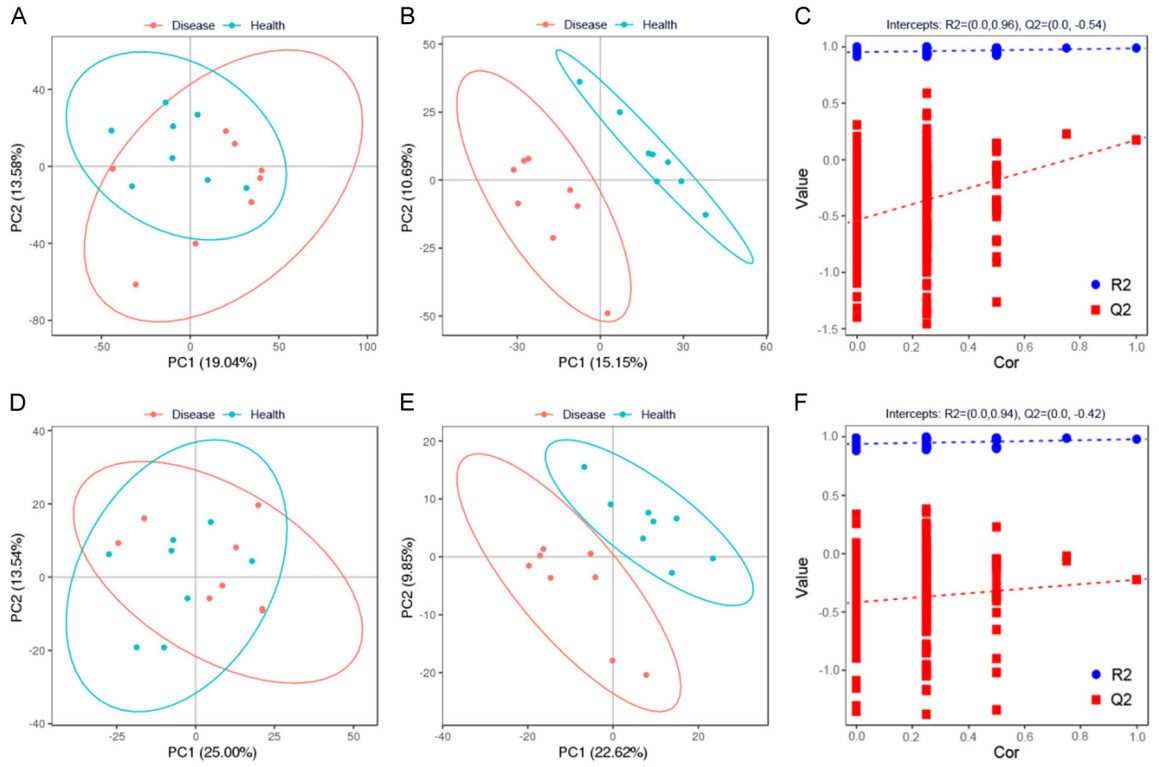

Figure 2. Principal component analysis (PCA) score plots (A), PLS-DA score plots (B), permutation plots (C) of FF in positive ion patterns of infertile women with CPID and healthy controls. PCA score plots (D), PLS-DA score plots (E), and permutation plots (F) in the negative ion patterns of infertile women with CPID and healthy controls.

analysis of metabolites between infertile women and healthy controls, of which 4041 compounds were identified in positive ion patterns and 886 compounds were identified in negative ion patterns. The base peak intensity (BPI) and total ion chromatogram (TIC) of the samples are shown in **Figure 1**. Although the PCA score plots did not have a significant separation, PLS-DA score plots and permutation plots displayed clear separation between infertile women with CIPD and healthy controls in both ESI negative and positive ion patterns, as shown in **Figure 2**.

Candidate biomarkers screening and identification

To detect the biomarkers specific for infertile women with CPID, the compound signals with variable VIP > 1, FC ≥ 1.2 or ≤ 0.83, and $P < 0.05$ were screened. This identified 214 differential metabolites (including 91 named biochemicals) in the ESI positive patterns and 26 differential metabolites (including 13 named biochemicals) in the negative patterns in the infertile group as compared to healthy control. A total of 104 differential named biochemicals were used for the following analyses, of which 44 metabolites were up-regulated and 60 metabolites were down-regulated (Supplementary Table 1). These metabolic alterations between healthy controls and the infertile group were visualized clearly in a heatmap plot (**Figure 3**). The pairwise Pearson's correlations of 104 metabolites were calculated in both healthy controls and infertile group (**Figure 4**). Of 5460 pairs of metabolites, 1016 had significant differential correlations in healthy controls (the strongest positive differential correlation was hexaric acid and citrate, and the strongest negative differential correlation was bisphenol, a diglycidyl ether and tiron free acid) and 1058 in infertility group (the strongest positive differential correlation was oxoglutaric acid and citrate, and the strongest negative differential correlation was tetrahydrocortisone and etonogestrel) ($P < 0.05$). KEGG pathway analysis revealed that such metabolites were mostly enriched in 10 metabolic pathways, including steroid hormone biosynthesis, glyoxylate and dicarboxylate metabolism, glucagon signaling pathway, tricarboxylic acid (TCA) cycle,

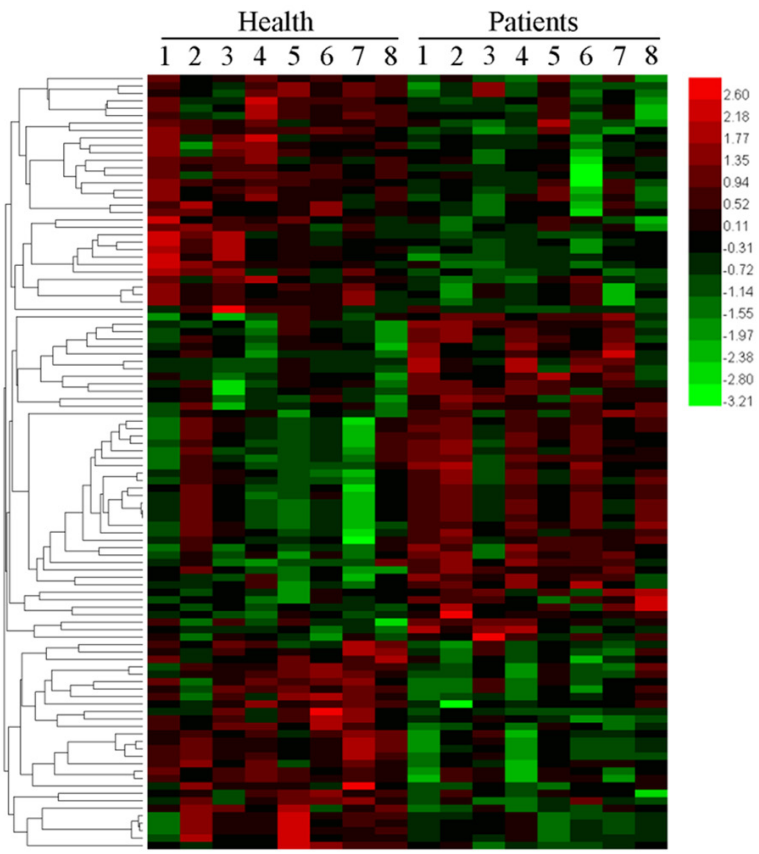

Figure 3. Heatmap plots of 104 differential metabolites with VIP > 1, FC ≥ 1.2 or ≤ 0.83, and $P < 0.05$ in FF of infertile women with CPID compared to healthy controls.

central carbon metabolism in cancer, biosynthesis of amino acids, carbon metabolism, bile secretion, alamine, aspartate and glutamate metabolism, and 2-oxocarboxylic acid metabolism (**Figure 5**). The peak intensities of 12 candidate biomarkers of the two groups are shown in **Figure 6**. Among these, pregnane-3,3-diol, pc(p-18:1(11z)/18:3(6z,9z,12z)), 1-octadecanoyl-2-(4z,7z,10z,13z,16z,19z-docosahexaenoyl)-sn-glycero-3-phosphoethanolamine,3-(icosanoyloxy)-4-(trimethylammonio) butanoate, hippurate, ho-dpeg8-oh, 3-oxolauric acid, adrenaline, and afegostat were markedly down-regulated in infertile women. However, 17, 21-dihydroxypregnenolone, (5-benzyl-3,6-dioxo-2-piperazinyl) acetic acid, and 9-decenoylcarnitine were up-regulated in infertile women with CPID.

Discussion

Previous studies have indicated that FF provides a critical microenvironment for oocyte development and maturation, which may be altered by several diseases such as pelvic inflammatory disease [12, 13]. In the present study, we conducted an untargeted metabolomic analysis of FF to investigate the differential metabolites between infertile women with CPID and healthy controls. A total of 4927 metabolites were identified by both positive and negative ion patterns. After comparison and screening, 240 differential metabolites (VIP > 1, FC ≥ 1.2 or ≤ 0.83, and $P < 0.05$) were finally found using the METLIN metabolite database. Among them, 104 differential metabolites (named biochemicals) were enriched in various important biologic processes and pathways including steroid hormone metabolism, which were strongly linked to infertility.

Studies have found that the levels of FF steroids, particularly androgens (testosterone (T) and dihydrotestosterone), estrogens (E2 and estrone), and P, have a very important impact on ovarian folliculogenesis and maturation [13, 14]. Lamb *et al.* reported that higher levels of E2 may be used as predictive biomarkers for fertilization outcome after intracytoplasmic sperm injection [15]. Furthermore, some studies have also suggested that using the steroid ratios in FF, such as the P/E2, P/T, E2/T, and cortisol/cortisone may serve as the best indicators of oocyte maturity [16-18]. However, the majority of these earlier studies only measured a few of the steroids by immunoassays. Recently, LC-MS/MS-based metabolomic analysis of steroids in FF of infertile women with CPID revealed that lower levels of pregnenolone, lower ratios of androstenedione (A4)/dehydroepiandrosterone (DHEA) and T/DHEA, as well as greater ratios of E2/T and estrone/A4 were significantly associated with outcome of IVF treatment (live birth) [19]. However, other studies have not confirmed these findings [20, 21]. Pacella *et al.* suggested that steroid hormone (E2, P4, and AMH) levels

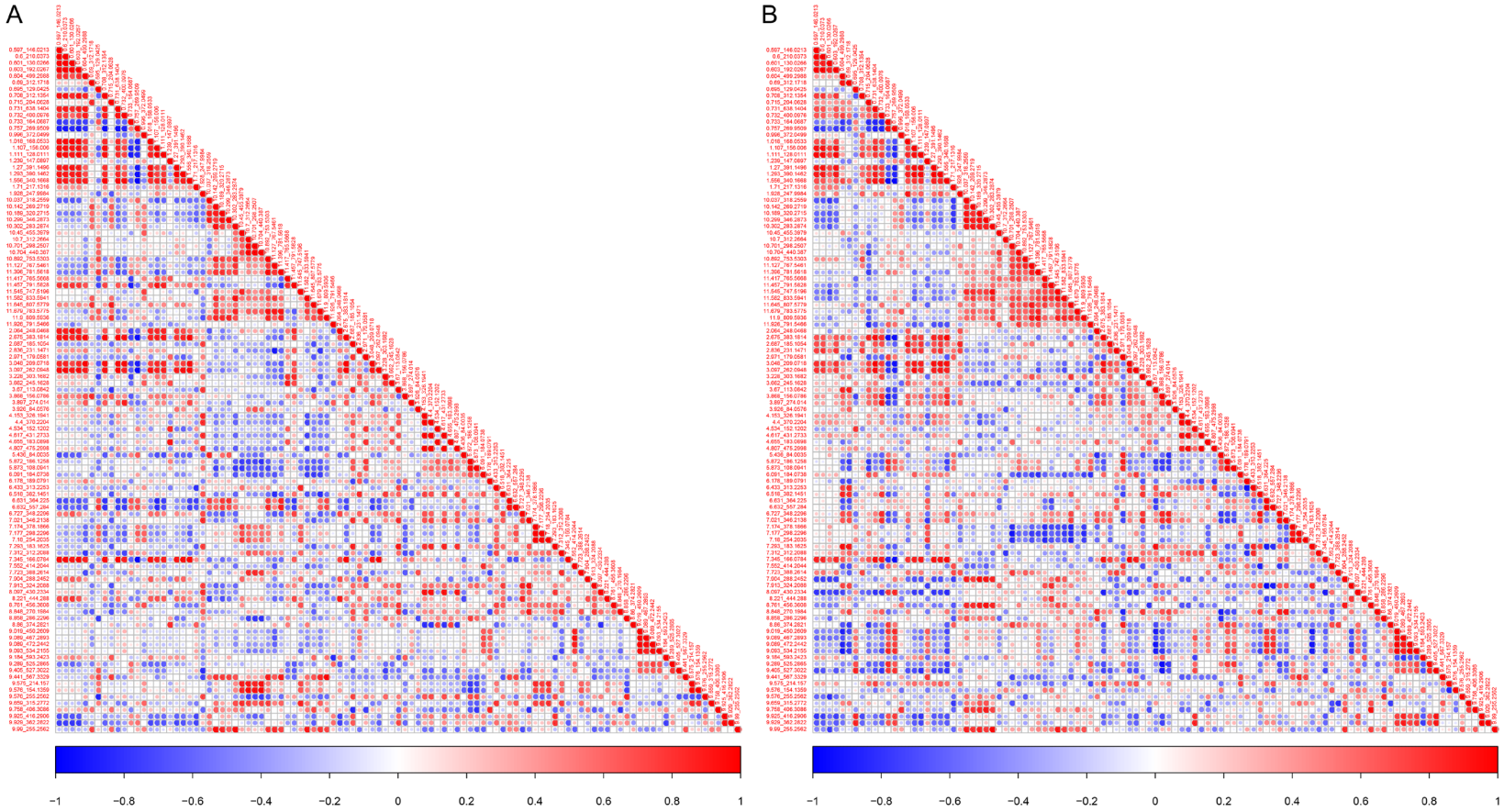

Figure 4. Pearson correlation analysis of 104 differential metabolites in FF of healthy controls (A) and infertile women with CPID (B).

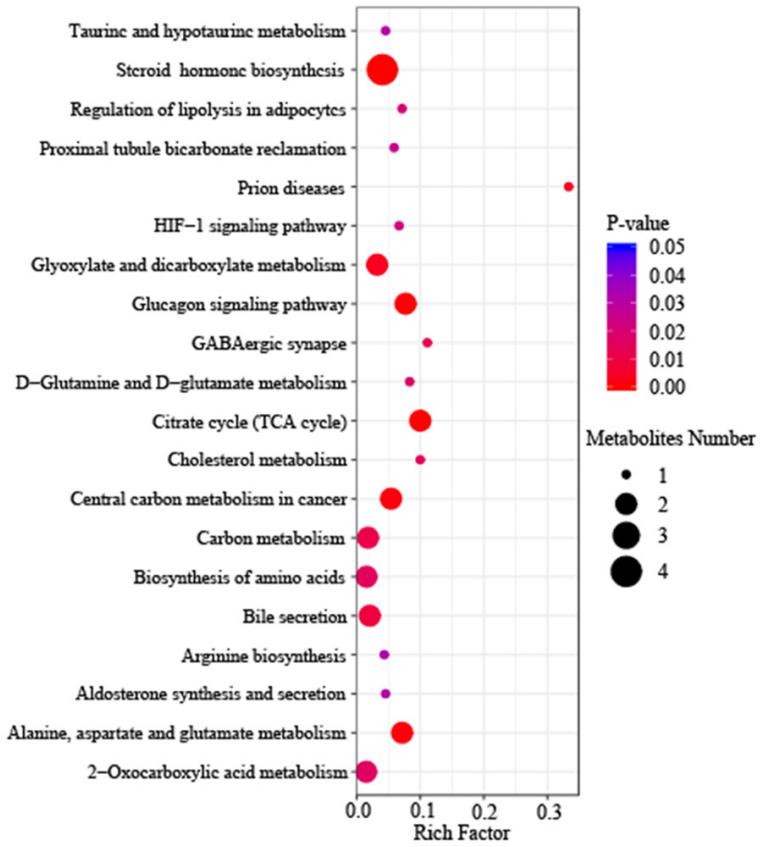

Figure 5. Overview of metabolic pathway analysis based on 104 differential metabolisms.

are markedly changed in FF of women with advanced maternal age [22]. Indeed, older women show more reduction in the antioxidant candidate and more reactive oxygen species (ROS) accumulate in FF, which could cause changes in concentrations of intra-follicular hormones and a decline in fertility [23]. Additionally, extensive epidemiological and experimental animal studies have shown that inflammation triggered by infection or other immunogens, has the ability to impair ovarian steroid hormone production [24, 25]. In the present study, the FF levels of pregnane-3,3-diol were significantly down-regulated and 17,21-dihydroxypregnenolone was up-regulated in infertile women with CPID compared to healthy controls. Pregnane-3,3-diol is one of the progesterone metabolites, and previous studies suggested that progesterone and its metabolites have a variety of effects on regulation of gonadotropin secretion and estrogen receptors. Oocyte maturation [26, 27]. 17,21-dihydroxypregnenolone can significantly stimulate cortisol secretion, which is inhibited by cyproterone acetate. Elevated levels of cortisol were associated with areduced likelihood of pregnancy in women undergoing IVF [28]. We speculated that the disturbance in pregnane-3,3-diol and 17, 21-dihydroxypregnenolone may play a role in pathogenesis in infertile women with CPID.

Pc(p-18:1(11z)/18:3(6z,9z,12z)) and 1-octadecanoyl-2-(4z,7z,10z,13z,16z,19z-docosahexaenoyl)-sn-glycero-3-phosphoethanolamine, the metabolites of glycerophospholipids, were also found to be significantly lower in infertile women with CPID. Glycerophospholipids are major structural components of biological membranes and precursors of many informational molecules, which are well-known to regulate a wide variety of biologic processes, such as cell proliferation, migration, signal transduction, apoptosis, immunity, and inflammation [29, 30]. Phosphatidylcholine (PC), prostaglandin (PG), lysophosphatidylcholine (LPC), and lysophosphatidic acid (LPA) are types of bioactive glycerophospholipids and have been well studied in the previous literature [31-33]. LPC is derived from PC by the phospholipase A2 (PLA2) enzyme superfamily that catalyzes the hydrolysis of the sn-2 ester bond of PC [31, 34]. LPC is significantly increased while PC is decreased in psoriasis plasma [31]. LPC has been shown to be involved in inflammation-associated diseases [31] and exerts its biological effects through regulation of different signaling pathways including NF-κB and cyclooxygenase type 2 (COX-2) expressions which are a key pro-inflammatory mediator [33, 35]. PG is produced from arachidonic acid (AA) through the actions of PLA2 superfamily and COX-2 [36]. LPA has been observed in various fluids such as FF and has also been demonstrated to regulate the levels of PG and the activity of COX-2 [30, 31]. Current evidence

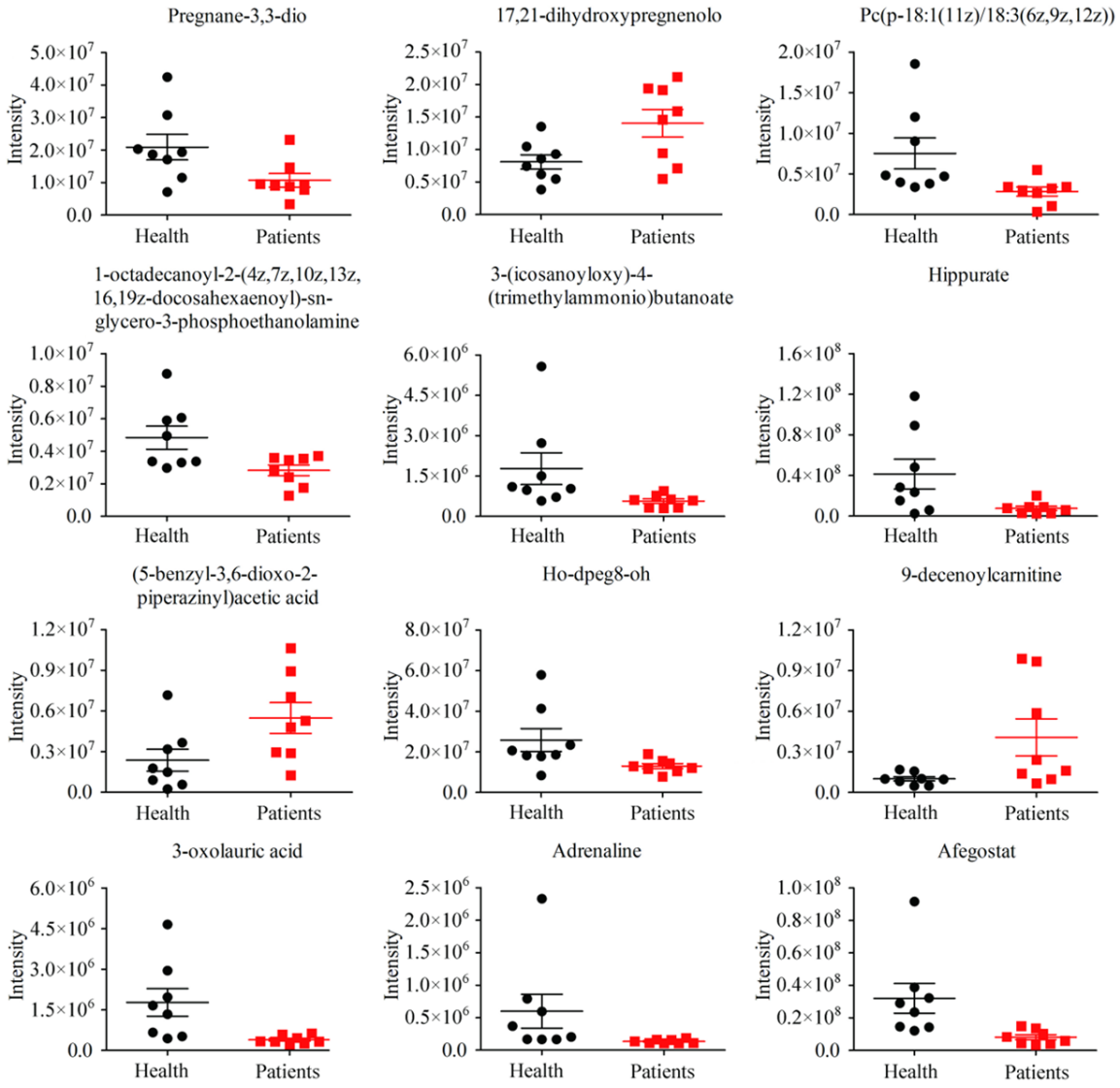

Figure 6. 12 typical metabolites with significant alterations in FF of infertile women with CPID compared to healthy controls.

shows that PG as well as LPA play an important role in reproductive processes, including ovulation and implantation [30, 37]. Achache *et al.* reported that the expression levels of PG were significantly decreased in the secretory endometrium of patients with repeated IVF failure and were strongly correlated with decreased implantation rates [36]. Intracervical application of seminal plasma with high levels of PG has shown a great candidate to increase clinical pregnancy rate [36]. Similarly, a recent study had shown that LPA and PG were lower in vaginal specimens of women with recurrent implantation failure, which leaded to deferred implantation and embryo crowding [30]. LPA receptor LPA3-deficient mice had a significantly decreased litter size of embryos, delayed implantation, and changed embryo spacing [37]. LPA3-deficient mice given exogenous PG have significantly more implantation, although this rescue did not completely restore embryo spacing compared to wild-type controls [37]. These findings in an animal model clearly demonstrated the positive effects of LPA signaling on PG biosynthesis, which are critically involved in regulating female fertility.

In summary, we employed LC-MS/MS-based FF metabolomics to investigate the pathophysiology of infertile women with CPID. The profiles,

including a number of steroids/steroid derivatives such as pregnane-3,3-diol and 17,21-dihydroxypregnenolone, glycerophospholipids such as Pc(p-18:1(11z)/18:3(6z,9z,12z)) and 1-octadecanoyl-2-(4z,7z,10z,13z,16z,19z-docosahexaenoyl)-sn-glycero-3-phosphoethanolamine, and other novel metabolites, are dramatically altered in FF of infertile women with CPID, which provide the ability to uncover new biomarkers associated with women's infertility. A main limitation of this study was the small sample size, Further research therefore is still needed to confirm the changes in the candidate biomarkers observed in this study using targeted metabolomics and larger samples, and to illustrate their molecular mechanisms in infertile women with CPID.

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Disclosure of conflict of interest

None.

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