Study On Oxylipins Metabolomics Components of Follicular Fluid In Infertile Patients With Diminished Ovarian Reserve By UHPLC-MS-MS

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Study on oxylipins metabolomics components of follicular fluid in infertile patients with diminished ovarian reserve by UHPLC-MS-MS

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Abstract

Background: Diminished ovarian reserve (DOR) refers to the decrease of the number and quality of oocytes in the ovary, which leads to the lack of sex hormones and the decline of fertility in women. DOR can also develop into premature ovarian failure (POF), which affects women's quality of life and is an important cause of female infertility. Oxidative stress is an important cause of fertility decline in DOR patients, which significantly affects follicular microenvironment, oocyte maturation, fertilization and embryo development. Defining specific oxidized lipid components in follicular fluid (FF) of DOR infertile patients can better understand the existing knowledge of intracellular signal transduction.

Methods:

UHPLC-MS-MS technology was used to analyze oxylipins metabolic signatures in the FF of DOR patients and females with normal ovarian reserve (NOR) enrolled for the in vitro fertilization (IVF) cycle. The obtained metabolomic profiles were analyzed using the principal component analysis (PCA) and orthogonal projections to latent structure discriminant analysis (OPLS-DA). The Kyoto Encyclopedia of Genes and Genomes (KEGG) and MetaboAnalyst databases were used for pathway enrichment analysis.

Results: Fifteen oxylipins were found to be lower in FF of patients with DOR than those in NOR group, including ±20-HDoHE, ±5-iso $PGF_{2\alpha}$-VI, 12S-HHTrE, 15-deoxy-Δ12,14-PGJ_2, 1a,1b-dihomo PGE_2, 1a,1b-dihomo $PGF_{2\alpha}$, 20-COOH-AA, 20-HETE, 8S,15S-DiHETE, PGA_2, PGD_2, PGE_1, $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGJ_2. The pathway enrichment analysis revealed that the 15 different oxylipins metabolites were closely related to arachidonic acid metabolic pathway.

Conclusions: Metabolomic analysis of FF indicated that disorders of oxylipins metabolism was closely related to ovarian reserve function. Among these oxylipins metabolites, arachidonic acid metabolism undergoes significant changes that affect the development of oocytes thus causing reduced fertility in DOR patients.

Keywords: DOR, Follicular fluid, IVF, Oxylipins, Metabolomics

Trial registration: ChiCTR, ChiCTR2000038182,Registered 12 September 2020-Retrospectively registered, http://www.chictr.org.cn/ edit.aspx? pid=61313&htm=4

Introduction

Diminished ovarian reserve (DOR) refers to the decrease of the number and quality of oocytes in the ovary, which leads to the decrease of female fertility and disorder of reproductive endocrine function. The main clinical manifestation is that women under 40 years old have rare menstruation, decreased menstruation, even amenorrhea and infertility. If not treated in time, it may further develop into premature ovarian failure (POF) within 1 ~ 6 years [1]. It is estimated that the incidence of DOR among infertile women of different ages ranges from 6% to 64% [2]. In 2014, the national assisted reproductive technology data of the US Centers for Disease Control and Prevention showed that DOR patients accounted for about 30% of pregnant women [3]. Because the number of follicles in the basal sinus of DOR patients is small and the reactivity to ovulation-promoting drugs is reduced, patients with DOR underwent in vitro fertilization/intraepithelial sperm injection-embryo transfer (IVF/ICSI-ET), there are some phenomena such as poor ovarian responder (POR), decreased egg quality, decreased number of eggs obtained and high-

quality embryos, increased cycle cancellation rate and decreased cumulative pregnancy rate in the process of IVF/ICSI-ET [4].

Oxidative stress (OS) refers to the phenomenon that excessive reactive oxygen species (ROS) is produced after being stimulated in vivo and in vitro, which exceeds the antioxidant capacity in the body, and there is a serious imbalance between oxidation and antioxidant system in the body, resulting in oxidative stress damage to cells and tissues [5]. ROS mainly includes superoxide anion($O_2^-\cdot$), hydrogen peroxide (H2O2), hydroxyl radical ($\cdot OH$), etc. Under physiological conditions, ROS in vivo participates in intercellular signal transmission as an important second messenger, and regulates gene expression to maintain cell homeostasis. However, excessive ROS accumulation in tissue cells will cause damage to proteins, nucleic acids and lipids, resulting in mutation or deletion of nuclear DNA and mitochondrial DNA, lipid peroxidation of cell membrane leading to changes in membrane fluidity, protein oxidative damage leading to inactivation of important enzymes, and finally inducing apoptosis and tissue structure damage [6]. As the sensitive target of ROS attack, the cell membrane rich in polyunsaturated fatty acids is easily damaged by oxidative stress. ROS reacts with phospholipids, enzymes and membrane receptor-related macromolecules on the membrane surface to produce malondialdehyde (MDA), 4-Hydroxynonenal, isomeric prostaglandins and other active products, namely lipid peroxides [7]. OS plays an important role in the physiological and pathological process of female reproductive system, which is one of the important reasons for the decline of female reproductive function. In recent years, research reports show that oxidative stress can lead to ovarian endocrine dysfunction, oocyte quality decline, granulosa cell apoptosis, and then follicular atresia, which is an important reason for the decline of fertility of DOR patients, and significantly affects the follicular microenvironment, oocyte maturation, fertilization and embryo development [8-10].

Follicular fluid (FF) is the place where oocytes exchange materials and

metabolize energy with surrounding cells. Its metabolite composition can reflect the level of FF environment and indirectly reflect the level of oocyte metabolism. It consists of secretions produced by peripheral granulosa cells and serum diffused by local capillaries, and also contains plasma components crossing the blood follicular barrier, mainly including hormones, growth factors, interleukins, anti-apoptosis factors, proteins, sugars, amino acids, active oxygen and antioxidant enzymes [11-12]. It affects oocyte maturation, follicular wall rupture, fertilization and early embryo development [13-14]. Oxidative stress markers in FF are closely related to the growth, development and maturation of oocytes. Infertility caused by DOR has always been an unavoidable problem in the development of reproductive medicine, and the quality of oocytes is also reflected in the changes of related metabolites in FF. Therefore, it is necessary to study the oxylipins metabolomics in FF of infertile patients with DOR.

Metabolomics, as an integral part of system biology, imitates the research ideas of genomics and proteomics, makes quantitative analysis of metabolites in organisms, and finds out the relative relationship between metabolites and physiological and pathological changes [15]. In recent years, metabolomics has been used to detect potential biomarkers in FF. The study of FF metabolomics is of great significance to evaluate and predict the potential of in vitro fertilization and embryo development of oocytes. Merhi et al. [16] used metabolomics to find biomarkers of ovarian reserve function, the results showed that advanced glycation end-products (AGE) in FF was positively correlated with the level of anti-Mullerian hormone (AMH), which could be used as an important biological index for predicting ovarian reserve in patients receiving ART treatment. Another metabolomics study showed that glucose concentration in FF of DOR patients decreased significantly, while lactate and progesterone concentrations increased significantly. At the same time, glucose uptake, lactic acid production, platelet-type phosphofructokinase gene expression in granulosa cells and cumulus cells increased significantly, while progesterone concentration decreased significantly, suggesting that decreased aerobic metabolism,

increased anaerobic metabolism and high progesterone stimulation of DOR oocytes may be important reasons for their quality decline and early embryo dysplasia [17]. However, the metabolomics of oxylipins in FF of DOR patients has not been reported.

In this study, we used UHPLC-MS-MS technology to detect oxylipins in FF of DOR infertile patients with target metabolomics, to identify the metabolites of oxidative lipids in FF of DOR infertile patients, to screen potential biomarkers, and to explore the possible mechanism of oocyte quality decline in DOR, which has far-reaching significance for exploring the pathogenesis of DOR.

Materials and methods

Sample collection and preparation

Patients with DOR (n= 20) and normal ovarian reserve (NOR) women (n = 20, as control group) were recruited from the Reproductive Medicine Center of Shanghai first maternity and infant hospital, Tongji university school of medicine, from May 2020 to January 2021. The study was approved by the ethics Committees of Shuguang hospital affiliated to Shanghai university of traditional Chinese medicine (No.2020-833-40-01) and registered in China clinical trial registration center (ChiCTR2000038182). All subjects signed an informed consent form prior to the study. Patients with DOR were diagnosed based on diagnostic criteria [18]: (1) the age is 20 ~ 40 years old. (2) requires one of the following three features to be met: 1) Follicle stimulating hormone (FSH): 10 IU/L ~ 30 IU/L with or without FSH/ luteinizing hormone (LH) ≥ 3; 2) serum levels of AMH: 20 ~ 25 years old (AMH≤3.0 ng/ml), 26 ~ 30 years old (AMH≤2.5 ng/ml), 31 ~ 35 years old (AMH≤1.5 ng/ml), 36 ~ 40 years old(AMH≤1.0 ng/ml); 3) the number of basic antral follicle count (bAFC) ≤ 5 on the 2nd to 5th day of menstruation.(3) accompanied by rare menstruation, even amenorrhea, infertility and other clinical manifestations. DOR can be diagnosed by meeting any one of (1), (3) and (2). The control group consisted of subjects with infertility purely due to tubal factor. Women with infertility due to endometriosis, polycystic ovarian syndrome, ovulatory dysfunction, and male factor were

excluded from the control group.

All subjects underwent ovarian micro-stimulation according to our established protocols. Once more than one follicle was larger than 16 mm or three follicles were larger than 18 mm, 5000~10,000 IU human chorionic gonadotropin was intramuscularly administered, and the mature follicles (diameter≥18 mm) were aspirated using 17-gauge Cook needles. Oocytes were subsequently retrieved. After the laboratory separated the oocytes, FF obtained from 3 dominant follicles was pooled in a 15 ml centrifuge tube and centrifuged at 30,000g for 15 min to remove insoluble particles and cells. The supernatant was packed in a 2ml freezing tube, and then stored at -80 °C ultra-low temperature storage box until further study and avoid repeated freezing or thawing.

Serum hormone measurement and antral follicle calculation

Serum FSH, LH, estrogen (E_2) and progesterone (P) were detected by radio-immunoassay on the 2nd day of menstruation, and the number of bAFC was counted by ultrasound on the 2nd to 5th day of menstruation.

Metabolites Extraction

1000 μL aliquot of each individual sample was precisely transferred to an Eppendorf tube. After the addition of the isotopically-labelled internal standard mixture, the samples were vortexed for 30 s and sonicated for 5 min in the ice-water bath. The sample was further purified with SPE. The SPE cartridges were equilibrated with 1 mL of methanol and 1 mL of water. After loading a sample (supernatant obtained following the procedure described above), the cartridge was washed with 1 mL of 5% MeOH/H2O (v/v). The flow-through fraction was then discarded. Finally, the samples were eluted with 1 mL of MeOH, and then the eluent was evaporated to dryness under a gentle stream of nitrogen and were reconstituted in 100 μL of 30% ACN/H2O (v/v). After centrifugation (15 min, 12000 rpm, and 4 °C), the clear supernatant was subjected to UHPLC-MS/MS analysis.

UHPLC-MRM-MS analysis

The UHPLC separation was carried out using an EXIONLC System (Sciex), equipped with a Waters ACQUITY UPLC BEH C18 column (150 × 2.1 mm, 1.7

μm, Waters). The mobile phase A was 0.01% formic acid in water, and the mobile phase B was 0.01% formic acid in acetonitrile. The column temperature was set at 50 °C. The auto-sampler temperature was set at 4 °C and the injection volume was 10 μL.

A SCIEX 6500 QTRAP+ triple quadrupole mass spectrometer (Sciex), equipped with an IonDrive Turbo V electrospray ionization (ESI) interface, was applied for assay development. Typical ion source parameters were: Curtain Gas = 40 psi, IonSpray Voltage = -4500 V, temperature = 500 °C, Ion Source Gas 1 = 30 psi, Ion Source Gas 2 = 30 psi.

The MRM parameters for each of the targeted analytes were optimized using flow injection analysis, by injecting the standard solutions of the individual analytes, into the API source of the mass spectrometer. Several most sensitive transitions were used in the MRM scan mode to optimize the collision energy for each Q1/Q3 pair. Among the optimized MRM transitions per analyte, the Q1/Q3 pairs that showed the highest sensitivity and selectivity were selected as ‘quantifier’ for quantitative monitoring. The additional transitions acted as ‘qualifier’ for the purpose of verifying the identity of the target analytes. SCIEX Analyst Work Station Software (Version 1.6.3) and Multiquant 3.03 software (Version 20.2) were employed for MRM data acquisition and processing.

Data collection, processing and statistical analysis

The SPSS 25.0 software was used for statistical analysis of the data. The baseline characteristics of the study population are described. The continuous variables with a normal or near-normal distribution are expressed as the means ± standard deviations (SDs) and were analyzed by Student’s t test, otherwise, a Mann–Whitney U test was used. The classification data are statistically described using frequency (composition ratio) and were analyzed using the chi-square test. Statistical significance was defined as $P < 0.05$.

For the metabolomics analysis, the missing values in the raw data were filled by half of the minimum value. Additionally, the overall normalization method was used for data analysis. The multi-variate analysis, principal

component analysis (PCA), and orthogonal projections to latent structure discriminant analysis (OPLS-DA) were performed using SIMCA software (V16.0.2, Sartorius Stedim Data Analytics AB, Umea, Sweden). PCA was used to show the distribution of the original data. OPLS-DA was used to further observe the separation between two groups and to further understand the variables responsible for classification. The variable importance in the projection (VIP) of the first principal component obtained in the OPLS-DA analysis was obtained. The metabolites with a VIP > 1.0 and *P*-value < 0.05 in the univariate analysis were considered significantly different. Moreover, the OPLS-DA model quality was evaluated with standard parameters (R^2Y and Q^2). The Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www. Genome. jp/kegg/) and the MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) database were used for pathway enrichment analysis.

Results

Clinical characteristics and Ovulation outcomes

The average age, body mass index (BMI), duration of infertility, type of infertility, total number of bAFC, serum AMH, bFSH, bLH, bE2 and P levels on day 2, the numbers of oocytes retrieved, the numbers of MII oocytes, the numbers of fertilizations and high-quality embryos of the participants are shown in Table 1. There was no significant difference in BMI, duration of infertility, type of infertility, serum bLH, bE2 and P levels on day 2 between DOR and NOR group ($P > 0.05$). The average age in the DOR group was significantly older compared to that in the NOR group ($P < 0.05$). Compared with NOR patients, serum bFSH levels were significantly higher in patients with DOR ($P < 0.05$). However, total number of bAFC, serum AMH levels and the number of retrieved oocytes, the numbers of MII oocytes, the numbers of fertilizations and high-quality embryos were significantly lower in patients with DOR compared to that in the NOR group ($P < 0.05$). This was an indication that the ovarian function of the DOR group was significantly low.

Table 1 The demographic and clinical characteristics of patients with DOR and NOR

Item	DOR group (n= 20)	NOR group (n= 20)	*P* value
Age (year)	35.350±4.017	30.350±3.514	0.001
BMI (kg/m^2)	21.050±2.404	20.876±1.780	0.797
Duration of infertility(year)	4.350±3.014	2.900±1.586	0.211
Type of infertility, n (%)			0.342
Primary	9(45.00)	11(55.00)	
Secondary	12 (60.00)	8(40.00)	
bFSH(mIU/ml)	11.268±3.991	6.331±1.084	0.000
bLH (mIU/ml)	3.668±2.105	3.783±1.151	0.830
bE2 (pg/ml)	55.025±43.448	43.116±14.850	0.529
P (ng/ml)	0.512±0.249	0.569 ± 0.163	0.253
bAFC (n)	2.850±1.461	12.700±3.229	0.000
AMH (ng/mL)	0.709±0.344	3.900±1.189	0.000
Oocytes retrieved (n)	3.500±1.433	14.200±4.884	0.000
MII oocytes (n)	2.850±1.496	10.350±4.146	0.000
Fertilizations (n)	2.750±1.410	9.800±4.686	0.000
High-quality embryos(n)	0.750±0.786	2.650±2.777	0.012

Multivariate analysis of metabolites

In this study, 103 peaks were detected and 68 metabolites were left after relative standard deviation de-noising. Then, the missing values were filled up by the median value. The final dataset containing the information of peak number, sample name and normalized peak area was imported to SIMCA software for multivariate analysis. Data was scaled and logarithmic transformed to minimize the impact of both noise and high variance of the variables. After these transformations, PCA and OPLS-DA, were performed to comprehensively compare the FF metabolomic profiles between DOR and NOR and to reveal the degree of diversity between the DOR and NOR group. Sixteen quality control (QC) samples were included to evaluate the stability and

repeatability of the system, and the QCs were clustered together and separated from the samples from the study subjects, which indicated the correctness of the PCA (Fig. 1a). The PCA score plot showed clear differences between DOR and NOR, and one outlier was located beyond 95% Hotelling's T-squared ellipse (Fig. 1b). As shown in Fig. 1b, most DOR samples clustered on the left, whereas all NOR clustered on the right. However, one DOR samples located on the right were also observed in the PCA score plots.

An OPLS-DA was performed to further compare the FF metabolomic profiles between DOR and NOR. The OPLS-DA score plot showed significant differences in the FF metabolomic profiles between DOR and NOR samples (Fig.1c). As shown in Fig.1c, all NOR samples clustered on the right, whereas most DOR clustered on the left. However, one DOR sample located on the right. This result indicated the existence of significant differences in the FF metabolomic profiles between patients with DOR and NOR. Additionally, the permutation test yielded $R^2Y(cum)$ and $Q^2(cum)$ values of 0.36 and −0.8, respectively, and these results indicated the lack of overfitting and the good predictive ability of the OPLS-DA model (Fig. 1d), which indicated the suitability of the model for subsequent optimization analyses.

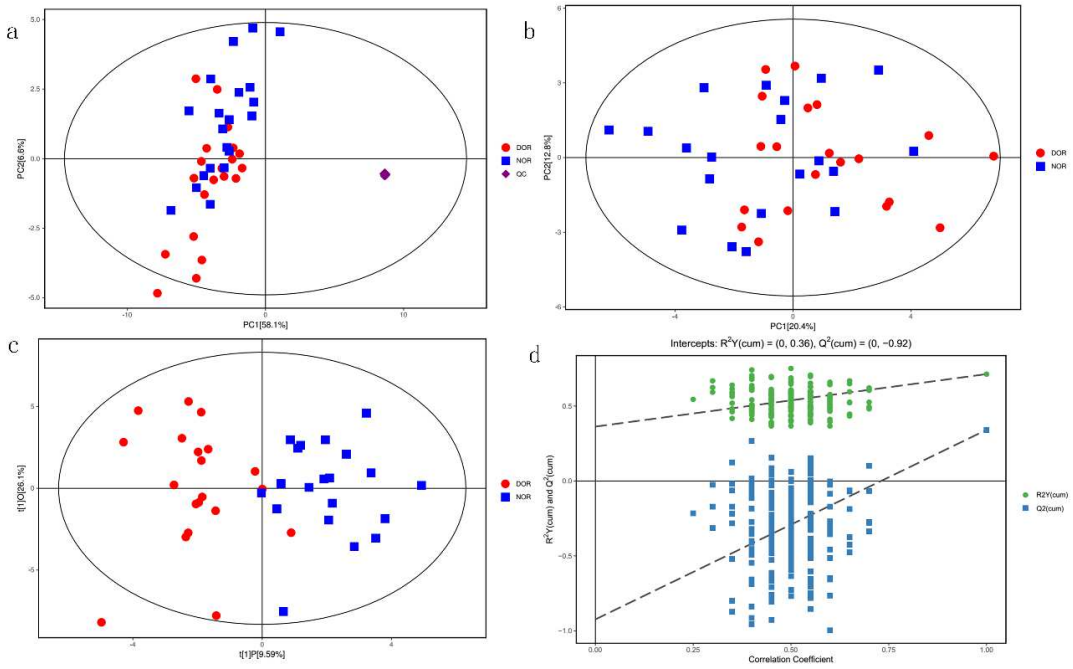


Fig. 1 PCA score plots, OPLS-DA score plot, and corresponding validation plot of OPLS-DA results derived from the metabolomics profiles of FF between DOR and NOR. a PCA score plot with QC. b PCA score plot without the QC samples. c OPLS-DA score plot. d Permutation test of the OPLS-DA model.

Identification of significantly different metabolites and pathways associated with DOR

In our study, 15 metabolites of FF with VIP values > 1 in the OPLS-DA analysis and *P*-values < 0.05 in the univariate analysis were found between DOR and NOR samples (Table 2), which indicated that these 15 metabolites were associated with ovarian reserve function. Specifically, 15 metabolites were all reduced and no metabolites were increased in DOR compared with NOR group. These 15 metabolites associated with ovarian reserve function were then summarized in a volcano plot (Fig. 2a): the 15 down-regulated metabolites (blue spots) were located on the left, but no up-regulated metabolites were found in FF of DOR compared with NOR group. Furthermore, a heatmap was used to classify the upregulated and downregulated metabolites in patients of DOR compared with NOR (Fig. 2b).

Table 2 Differential metabolites in FF samples between DOR and NOR groups

Compounds	VIP	P-value	Fold change	Type
±20-HDoHE	1.46468	0.00481	0.68185	Down
±5-iso $PGF_{2\alpha}$-VI	1.05784	0.01656	0.55571	Down
12S-HHTrE	1.76019	0.00415	0.52086	Down
15-deoxy-Δ12,14-PGJ_2	1.27131	0.03509	0.63420	Down
1a,1b-dihomo PGE_2	2.48940	0.013601	0.28674	Down
1a,1b-dihomo $PGF_{2\alpha}$	1.82901	0.0288	0.53937	Down
20-COOH-AA	1.75008	0.00401	0.67958	Down
20-HETE	1.11775	0.02031	0.29838	Down
8S,15S-DiHETE	1.38584	0.00450	0.70519	Down
PGA_2	2.32409	0.01828	0.13424	Down

PGD_2	1.08593	0.04879	0.36052	Down
PGE_1	2.01038	0.00705	0.41340	Down
$PGF_{1\alpha}$	1.33950	0.04522	0.54329	Down
$PGF_{2\alpha}$	2.18401	0.01426	0.34922	Down
PGJ_2	1.66952	0.03160	0.24083	Down

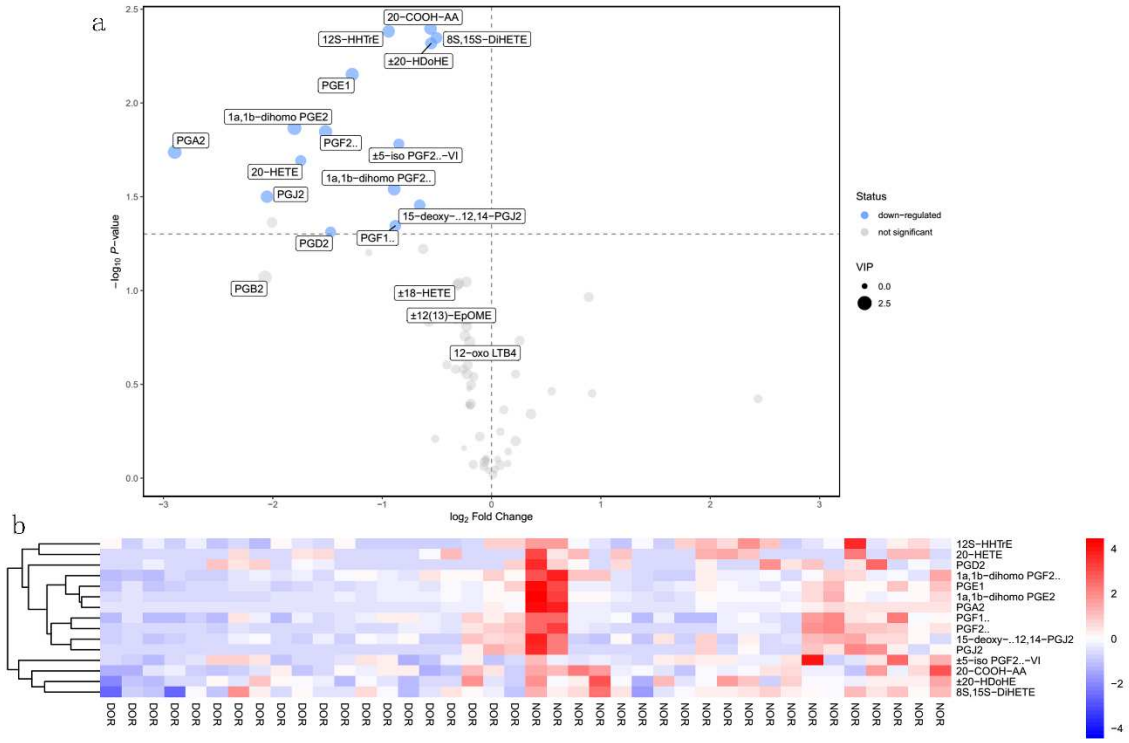


Fig. 2 Identification of the differential metabolomics profiles of FF between DOR and NOR based on a volcano plot and hierarchical clustering analysis. a Volcano plot, the down-regulated and up-regulated metabolites in DOR compared with NOR are marked in blue and red, respectively. X-axis: log_2 fold change of metabolites; Y-axis: fold change of –log10 *P* value determined by Student's t test. The dot size represents the variable importance in the projection (VIP) value. b Heatmap of the hierarchical clustering analysis. Fifteen differential metabolites are presented.

Our study identified 15 differentially expressed oxidative lipids that could diagnosis of ovarian reserve function. The diagnostic efficacy of 15 differentially expressed oxidative lipids was evaluated by the ROC curve between the DOR patients and the NOR controls, and the values of AUC, 95%CI, specificity, and

sensitivity were obtained (Table 3). These differences in the 15 metabolites between the two groups are displayed with Software of R (Version 3.6.3). Among the differential metabolites in FF, the levels of 15 different metabolites in DOR group were all lower than in NOR control group (Fig.3).

Table 3. The ROC reports between DOR and NOR group

Compounds	AUC	95%CI	Cut-off	Sens	Spec	Sens+Spec
20-HDoHE	0.782	0.632-0.933	0.461	0.900	0.600	1.500
±5-iso $PGF_{2\alpha}$-VI	0.740	0.579-0.901	0.171	0.900	0.650	1.550
12S-HHTrE	0.762	0.612-0.913	0.138	0.800	0.650	1.450
15-deoxy-Δ12,14-PGJ_2	0.667	0.492-0.843	0.045	0.600	0.850	1.450
1a,1b-dihomo PGE_2	0.667	0.492-0.843	0.045	0.600	0.850	1.450
1a,1b-dihomo $PGF_{2\alpha}$	0.680	0.512-0.848	0.372	0.650	0.650	1.300
20-COOH-AA	0.758	0.606-0.909	0.469	0.900	0.550	1.450
20-HETE	0.665	0.516-0.814	0.229	0.450	0.950	1.450
8S,15S-DiHETE	0.800	0.655-0.945	0.060	0.900	0.650	1.550
PGA_2	0.850	0.729-0.971	0.232	0.800	0.800	1.600
PGD_2	0.657	0.498-0.817	0.045	0.550	0.800	1.350
PGE_1	0.818	0.687-0.948	0.170	0.800	0.700	1.500
$PGF_{1\alpha}$	0.644	0.470-0.818	0.057	0.750	0.500	1.250
$PGF_{2\alpha}$	0.735	0.576-0.894	0.660	0.650	0.800	1.450
PGJ_2	0.675	0.535-0.815	0.127	0.500	0.850	1.350

AUC: area under the curve; 95%CI: 95% confidence interval, Sens: Sensitivity; Spec: Specificity; Sens+Spec: Sensitivity+Specificity.

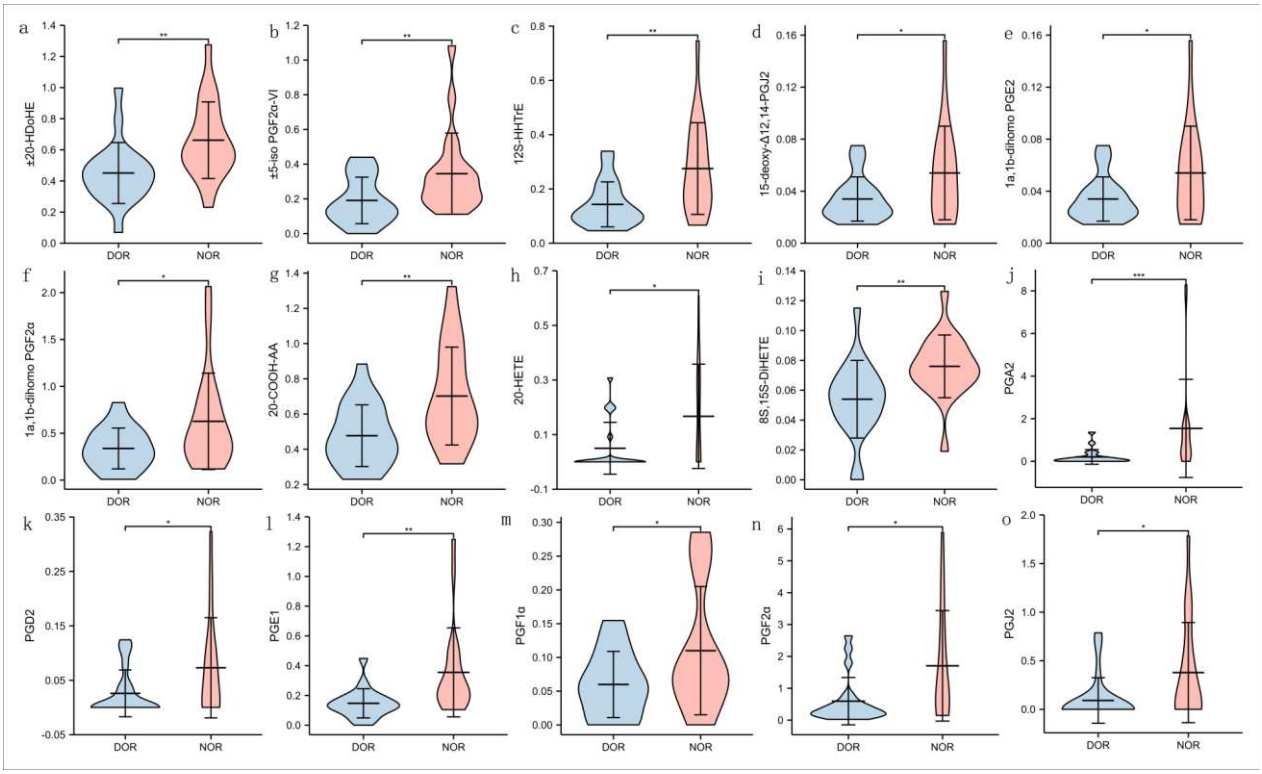
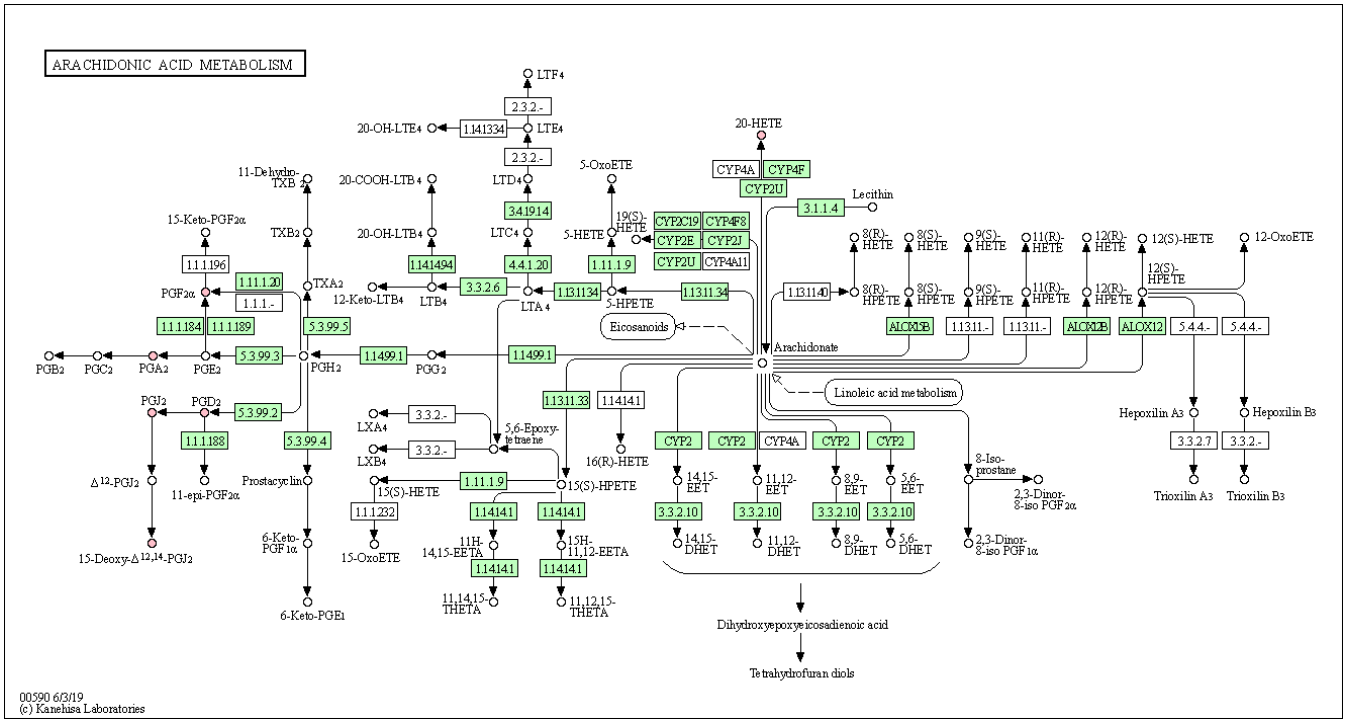


Fig. 3 a-o The violin diagram showing 15 differential metabolite profiles concentration in the quantitative analysis of DOR and NOR groups. The y-axis represents for mass spectral concentration integration data. The x-axis represents for each component group. *P <0.05, **P <0.01,***P < 0.001.

Additionally, a KEGG pathway analysis was performed to explore the enriched metabolic pathways associated with ovarian reserve function, the pathway enrichment analysis of the above 15 different oxidative lipids were conducted by overexpression analysis method to reveal the biological functional background of FF metabolic disorders in infertile patients with DOR. The KEGG Mapper Search results showed that different Oxylipins metabolites were involved in metabolic pathways including arachidonic acid (AA) metabolism, metabolic pathways, serotonergic synapse, neuroactive ligand-receptor interaction, asthma, phosphoolipase D signaling pathway, vascular smooth muscle contraction, oxytocin signaling pathway, FC epsilon RI signaling pathway, african trypanosomiasis, bile secretion, ovarian steroidogenesis. Furthermore, we input 15 differential metabolites into the MetaboAnalyst 5.0 database for metabolic pathway enrichment analysis. Based on the Holm *P*

value, FDR (false discovery rate) value and Impact value, only one important metabolic pathway of arachidonic acid metabolic was found. Therefore, combined with the results of KEGG database analysis, it is suggested that arachidonic acid metabolic pathway is closely related to DOR(Fig.4).



Discussion

Oxylipins, also known as lipid mediators, are a series of oxidative metabolites produced by the automatic oxidation of polyunsaturated fatty acids (arachidonic acid, linoleic acid, alpha-linolenic acid, DHA, EPA, etc.) or by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450(CYP) enzymes. Oxylipins are signal transduction molecules that participate in almost all physiological activities of the body and play a very important regulatory role in life activities of the organism, including inflammatory response, immune defense, endocrine regulation and oxidative stress, etc [19]. It has been reported that it is closely related to the occurrence and development of a variety of diseases, such as tumor [20], cardiovascular disease [21], diabetes [22], lung disease [23] and Alzheimer disease [24]. However, the relationship between oxylipins and the occurrence and development of DOR has not been reported. Therefore, we performed UHPLC-MS-MS analysis of FF oxylipins metabolites

composition from patients with DOR and NOR. Our study revealed the changes of oxylipins metabolites in FF of infertile patients with DOR, and reflected the changes of ovarian reserve function in infertile patients with DOR. We identified 15 differentially expressed oxylipins metabolites that were associated with DOR, including±20-HDoHE, ±5-iso PGF2α-VI, 12S-HHTrE, 15-deoxy-Δ12,14-PGJ2, 1a,1b-dihomo PGE_2, 1a,1b-dihomo $PGF_{2\alpha}$, 20-COOH-AA, 20-HETE, 8S,15S-DiHETE, PGA_2, PGD_2, PGE_1, $PGF_{1\alpha}$, $PGF_{2\alpha}$ and PGJ_2. The contents of these 15 different oxylipins metabolites in FF of DOR patients were all lower than those of NOR group, and the differences were statistically significant. Based on the KEGG and Metaboanalyst database, the results of pathway enrichment analysis showed that the differential oxylipins metabolites were closely related to arachidonic acid metabolic pathway. These differential oxylipins metabolites and arachidonic acid metabolic pathway may play a non-negligible role in DOR infertility. Dysfunctions in the metabolism of oxylipins in FF of DOR patients related to follicular development may provide possible detection and therapeutic targets for promoting oocyte maturation.

Polyunsaturated fatty acids (PUFAs) are long chain fatty acids containing two or more double bonds, of which the n-3 and n-6 series fatty acids have important biological significance. Imbalanced intake of n-6 to n-3 PUFAs in the diet has been linked to cardiovascular and cerebrovascular diseases, cancer, inflammation and autoimmune diseases [25]. Wathes et al. [26] showed that dietary n-6 and n-3PUFAs may influence reproductive processes by providing precursors for prostaglandin synthesis and regulating the expression patterns of many key enzymes involved in prostaglandin and steroid metabolism. AA is an essential fatty acid of n-6 PUFAs, cleaved from phospholipids by phospholipase A2 (PLA2) phosphatidyl inositol specific phospholipase C and the fatty acylglycerol lipase. Then, AA could metabolize to eicosanoids via COX, LOX, or P450-dependent cyclooxygenase pathways [27]. A study demonstrates that elevated concentrations of AA derivatives in human FF at the time of oocyte retrieval significantly decrease the ability of oocytes to form pronuclei after

intracytoplasmic sperm injection (ICSI) [28]. As cell signaling intermediates, AA and its derivatives are responsible for regulating cAMP activation, Ca^{2+} influx, Ca^{2+}/CaM, PKC, MAPK and PI3K/ Akt, which results in regulation of cellar growth, proliferation and differentiation [29-30]. A study has shown that AA is one of the most abundant polyunsaturated fatty acids [31]. In bovine ovaries, the concentration of AA in FF accounts for about 2.5% of total fatty acids, while the concentration in plasma is 1.2% [32]. It has been reported that follicular AA in both human [33] and non-human mammals [34] affects cumulus granulosa cells. Zhang N et al. [35] demonstrated that AA can regulate the survival, gene expression, lipid formation, steroid production and intracellular signaling pathways of bovine granulosa cells. Support for the hypothesis that AA in FF can directly affects the function of granulosa cells, thereby affecting follicular development and ovulation. Khajeh M et al. [36] investigated the biological effects of AA in human cumulus granulosa cells (CGCs) after exposure to acetylsalicylic acid (ASA), ASA treatment reduced E2 production, Cyp19a1 expression, glutathione peroxidase (GPx) activity, and estradiol receptor expression in CGCs. The addition of AA could prevented ASA-induced E2 reduction and expression of Cyp19a1, increased the antioxidant capacity of CGCs exposed to ASA by promoting GPx activity, triggered the synthesis and secretion of PGE2 and increased the expression of the estrogen receptor. Li S et al. [37] studied the levels of AA and its metabolites in the FF of non-obese PCOS patients, found that the levels of AA and its metabolites in the FF of PCOS patients increased, and insulin played a key role in the increase of AA metabolites generated by cyclooxygenase-2(COX-2), which may be another new molecular pathophysiological mechanism of PCOS. In this study, we found that the levels of AA metabolites in FF of DOR patients were lower than those of DOR control group, which was consistent with the results of Li S et al.

Under the influence of cytochrome P450 (CYP) enzymes, AA is transformed into 20-hydroxyeicosatetraenoic acid (20-HETE) [38]. A study showed that the serum concentration of 20-HETE in obese women is higher

than that in normal weight women. Strong adipogenic 20-HETE activity has also been observed [39]. Another study has demonstrated that the concentration of 20-HETE in urine and plasma of patients with metabolic syndrome was higher than that of control [40]. In addition, the level of 20-HETE in blood is significantly correlated with BMI, where the higher the BMI, the higher the level of 20-HETE [41]. A study found that the level of 20-HETE increased in peripheral circulation and adipose tissue of mice fed with high-fat diet. In addition, a correlation with damaged insulin signaling was demonstrated. It is concluded that 20-HETE has a significant impact on obesity induced by a high-fat diet, impaired insulin signaling and the occurrence of insulin resistance (IR) [42]. The levels of 20-HETE in the FF of non-obese PCOS patients that underwent IVF was higher than in non-PCOS group, but there was no significant difference [37]. We found that the levels of 20-HETE in FF of DOR patients were lower than those of NOR control group in our study. However, the correlation and mechanism between 20-HETE and DOR has not been reported.

AA is a well-known precursor of prostaglandins (PGs), which are synthesized PGs by prostaglandin-endoperoxide synthase S1 (PTGS1) or PTGS-2 (previously identified as cyclooxygenase enzymes COX1 and COX2) [43-44]. In the ovary, PTGS-2(COX2) is the critical prostaglandin-endoperoxide synthase, and PTGS-2(COX2) deficient female mice exhibit multiple failures in female reproductive processes, including ovulation, fertilization, implantation and decidualization [45-46]. In previous studies, prostaglandins, especially PGE_2 and $PGF_{2\alpha}$, have been shown to regulate oocyte maturation, ovulation and cumulus expansion [47-48]. In addition, it has been found that PGE_2 plays an important role in protecting oocytes against oxidative stress, PGE_2 could represent a novel autocrine/paracrine player in the mechanisms that can facilitate successful oocyte maturation and oocyte survival in the cow [49]. Although $PGF_{2\alpha}$ is also synthesized in response to LH surge, its function during the ovulatory process remains uncertain [50]. Some studies suggested both $PGF_{2\alpha}$ and PGE_2 to be important to the ovulatory process [51-52]. However,

Pereira de Moraes F et al. [53] concluded that $PGF_{2\alpha}$ alone does not induce ovulation in cattle. Sharma AK et al. [54] investigated PGE_2 treated follicles revealed a reduction in the attributes of apoptosis in granulosa cells while $PGF_{2\alpha}$ showed an increased in the apoptotic characteristics. Kemiläinen H et al. [55] found a broad expression of HSD17B12 enzyme in both human and mouse ovaries, indicated a role for HSD17B12 in the synthesis of PGD_2, PGE_2, $PGF_{2\alpha}$, and TXB2, namely prostaglandins that are well-known regulators of ovarian function.15-deoxy-Δ12,14-PGJ_2 is also a product of AA cyclooxygenase pathway. It is a specific endogenous ligand for a peroxisome proliferator-activated receptor γ (PPARγ) in vivo [56]. PPARγ was detected in primary and secondary follicles, being more expressed in the large follicles, may be responsible for regulating the expression of genes involved with growth, development, and/or differentiation of the follicle. Treatment of granulosa cells with 15-deoxy-Δ12,14-PGJ_2 stimulated basal progesterone secretion, whereas had no significant effect on FSH-stimulated steroid production, which suggested that PPARγ may regulate genes involved with follicular differentiation and the decline in PPARγ in response to LH is important for ovulation and/or luteinization [57]. The levels of PGE_2, PGD_2, PGJ_2, 15-deoxy-Δ12,14-PGJ_2 and $PGF_{2\alpha}$ in FF of PCOS patients that underwent IVF were significantly higher than those of non-PCOS group [37]. In our study, the levels of PGE_2, PGD_2, PGJ_2, 15-deoxy-Δ12,14-PGJ_2 and $PGF_{2\alpha}$ in FF of DOR patients were lower than in NOR control group.

Conclusions

Based on UHPLC-MS/MS, we investigated oxylipins metabolites changes in the FF of the DOR and NOR patients. Metabonomic analysis of FF showed that 15 differentially expressed oxylipins metabolites that were associated with DOR, and all the differentially oxylipins metabolites in FF of DOR patients were lower than those of NOR group. The pathway enrichment analysis showed that the oxylipins metabolites of these differences were mainly concentrated in the arachidonic acid (AA) metabolic pathway, while AA is involved in the regulation

of follicular development and oocyte maturation, and its complex changes are closely related to follicular development. These differential oxylipins metabolites and arachidonic acid metabolic pathway may play a non-negligible role in DOR infertility. Dysfunctions in the metabolism of oxylipins in FF of DOR patients related to follicular development may provide possible detection and therapeutic targets for promoting oocyte maturation, and provide scientific basis for understanding the microenvironment of oocyte development and improving oocyte quality in patients with DOR.

Abbreviations

DOR: Diminished ovarian reserve, POF: Premature ovarian failure, POR: Poor ovarian responder, NOR: Normal ovarian reserve, IVF: In vitro fertilization, ICSI-ET: Intraepithelial sperm injection-embryo transfer, OS: Oxidative stress, ROS: Reactive oxygen species, MDA: Malondialdehyde, FF: Follicular fluid, AGE: Advanced glycation end-products, AMH: Anti-Mullerian hormone, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, bAFC: basic antral follicle count, E2: Estrogen, P: Progesterone, SWATH: Sequential window acquisition of all theoretical fragment-ion spectra, MRM: Multiple reaction monitor, PCA: Principal component analysis, OPLS-DA: Orthogonal projections to latent structure discriminant analysis, QC: Quality control, AA: Arachidonic acid, FDR: False discovery rate, COX: Cyclooxygenase, LOX: Lipoxygenase, CYP: Cytochrome P450, PUFAs: Polyunsaturated fatty acids, PLA2: Phospholipase A2, CGCs: Cumulus granulosa cells, ASA: Acetylsalicylic acid, GPx: Glutathione peroxidase, COX-2: Cyclooxygenase-2, 20-HETE: 20-hydroxyeicosatetraenoic acid, IR: Insulin resistance, PGs: Prostaglandins, PTGS1: Prostaglandin-endoperoxide synthase S1, PTGS2: Prostaglandin-endoperoxide synthase S2, PPARγ: Peroxisome proliferator-activated receptor γ.

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Authors' contributions

LCC performed the literature search, data analysis and interpretation, and wrote the manuscript. ZXL and HH constructed the tables and figures. LCC and ZXX performed the sample collection and data analysis. ZQH, QC and FYL contributed to the design of the research study and provided critical review of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Research Ethics Committees of Shuguang hospital affiliated to Shanghai university of traditional Chinese medicine (No.2020-833-40-01). All participants signed an informed consent form prior to the study.

Conflict of interest

The authors declare that they have no conflict of interest.

Consent for publication

Not applicable.

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