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Molecular characteristics of follicular fluid in advanced maternal age women with different ovarian reserves: a multi-omics study

Wenlong Qi¹, Zhicheng Jia², Mengyu Shi¹, Xinxin Yang¹, Xinwei Sun¹, Yongqian Li¹, Peixuan Wang¹ and Ying Guo^{1,3*}

Abstract

Background Age-related diminished ovarian reserve (DOR) exhibits marked significant individual variation. Some women of advanced age with normal ovarian reserve (NOR) achieve better reproductive outcomes than age-matched DOR patients.

Objective This study aimed to compare the metabolomic and proteomic profiles of follicular fluid (FF) from advanced maternal age (AMA) women with DOR and NOR, to reveal differences in the follicular microenvironment and identify potential biomarkers and related pathways.

Methods LC-MS/MS was employed to profile metabolites and proteins in FF samples from both groups. Differentially expressed metabolites (DEMs) and differentially expressed proteins (DEPs) were identified using multivariate and univariate statistical analyses. Functional annotation and pathway enrichment analyses were performed using the GO and KEGG databases. Metabolomic and proteomic data were analyzed using bioinformatic approaches to identify key molecules and potential biomarkers associated with DOR.

Results Untargeted metabolomic analysis identified a total of 89 DEMs, including 32 upregulated and 57 downregulated. Data-independent acquisition (DIA) quantitative proteomic analysis identified 335 DEPs, of which 40 were up-regulated and 295 were down-regulated. Multi-omics analysis revealed significant alterations in several pathways in DOR patients, including metabolic pathways, cholesterol metabolism, alanine, aspartate and glutamate metabolism, carbon metabolism, central carbon metabolism in cancer, and sulfur metabolism. Based on protein expression profiles associated with these dysregulated pathways, four candidate protein biomarkers were further identified.

Conclusions Based on proteomic and metabolomic analyses, this study systematically characterized the follicular microenvironment of AMA with different ovarian reserve. A series of key DEPs and DEMs were identified, along with potential biomarkers of clinical relevance. Among the altered pathways, alanine, aspartate and glutamate metabolism

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may play an important role in the development of DOR, providing new directions for further investigation into its molecular mechanisms.

Keywords Ovarian aging, Follicular fluid, Advanced maternal age, Metabolomics, Proteomics, Assisted reproductive technology

Introduction

Age is an independent risk factor for female reproductive potential, with female fertility showing a significant downward trend after the age of 30 years [1]. Women of childbearing age over 35 years old are defined as AMA [2]. With the increase of age, the ovarian reserve of women diminishes, oocyte number and quality decrease [3], and their response to exogenous gonadotropins weakens, which often makes AMA face the risk of increasing aneuploidy rate, decreasing live birth rate, and increasing abortion rate [4]. During in vitro fertilization/intracytoplasmic sperm injection-embryo transfer (IVF/ICSI-ET), AMA is often associated with a higher cycle cancellation rate, a reduction in the number of high-quality embryos, and a decreased cumulative pregnancy rate [5]. Currently, there are no effective interventions to combat age-related fertility decline, and even with assisted reproductive technology, the success rate in the AMA population is significantly lower [6]. The development of the social economy has led to the change of fertility concept, with the age of childbearing being continuously delayed and more women of advanced age having fertility needs. How to delay the fertility decline or improve the clinical fertility outcomes in AMA has become a hot issue in the field of reproductive medicine.

The quality and quantity of oocytes are key factors limiting the success rates of assisted reproductive technology in women of AMA. FF provides a specific microenvironment for oocyte development and maturation and is rich in various bioactive substances, including lipids, steroids, amino acids, and antioxidant enzymes, making it an ideal biological sample for studying female follicular development [7, 8]. At present, numerous studies have revealed the complex regulatory mechanisms underlying DOR; however, most of these investigations have focused on comparing differences in the follicular microenvironment between healthy women of reproductive age and older women with DOR [9, 10]. Notably, age-related DOR exhibits significant individual heterogeneity, and in clinical practice, some older women with NOR still achieve better reproductive outcomes than age-matched women with DOR. Therefore, an in-depth investigation of changes in the follicular microenvironment during the aging process may contribute to a deeper understanding of the impact of advanced age on female reproductive function and provide important directions for the development of new clinical intervention strategies and therapeutic approaches.

In recent years, the advancement of omics technology has made it possible to identify proteins and metabolites in biological fluids. This analytical method has emerged as an indispensable resource in the field of medical research, particularly for unraveling the complex mechanisms underlying disease development. Proteomics allows the study of intracellular protein composition, activity patterns, and protein interactions at a holistic level, thereby revealing the mechanisms and pathways of disease occurrence [11]. Zhang et al. found that RAC1 may be involved in the occurrence of premature ovarian insufficiency by modulating both cell proliferation and programmed cell death processes through the analysis of the granulosa cells proteomes of patients with premature ovarian insufficiency [12]. Metabolites are involved in the regulation of metabolic processes in organisms. Metabolomics can qualitatively and quantitatively characterize small molecule metabolites in organisms, comprehensively reflecting the body's metabolism changes under disease conditions [13]. Song et al. measured serum metabolites in women with NOR and DOR, revealing that nicotinic acid and nicotinamide metabolism pathways play key roles in DOR [14]. Zhang et al. measured the FF metabolites in women receiving assisted reproductive technology treatment. They found that the lipid components in FF changed with age, mainly manifested in the up-regulation of arachidonic acid [15]. However, most current studies on DOR rely on a single omics approach, which limits a systematic understanding of the initiation and progression of DOR.

Previously, we mapped the transcriptomic features of ovarian granulosa cells in AMA with different ovarian reserve and revealed the role of ferroptosis in AMA ovarian granulosa cells [16]. In this study, we employed proteomics and metabolomics to systematically analyze the molecular characteristics of FF from women of AMA with different ovarian reserve, to identify potential biomarkers at both the protein and metabolite levels, and to elucidate key pathways that may be altered in the follicular microenvironment of AMA women with DOR. This research aims to provide novel insights for alleviating age-related DOR.

Methods

Study samples

This study recruited 30 patients with DOR and 30 with NOR who received assisted reproductive technology treatment for the first time from January 2024 to

December 2024 at the Reproduction and Genetics Center of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine. All eligible patients with DOR and NOR who met the inclusion and exclusion criteria were consecutively recruited, and no eligible patients declined participation. The study was approved by the Reproductive Ethics Committee of Shandong University of Traditional Chinese Medicine (NO: SDTCM/E2408-24). All patients signed the informed consent form. The diagnostic criteria of this study were based on the POSEIDON criteria (Group 4) [17]. This standard was chosen to ensure that patient classification aligns with internationally recognized definitions, thereby enhancing the comparability and scientific rigor of our results. The inclusion criteria for the DOR group included: (a) age 35–42 years old; (b) antral follicle count (AFC) < 5, or anti-Müllerian hormone (AMH) < 1.2 ng/mL. The inclusion criteria for the NOR group included: (a) age 35–42 years old; (b) $5 \leq \text{AFC} < 12$ or $\text{AMH} \geq 1.2$ ng/mL. The exclusion criteria for both groups were as follows: (a) previous ovarian stimulation or ovulation induction therapy; (b) diagnosis of polycystic ovary syndrome, adenomyosis, endometriosis, or hyperprolactinemia; (c) history of ovarian/pelvic surgery or prior systemic radiotherapy/chemotherapy; (d) chromosomal abnormalities, autoimmune diseases, or unexplained infertility; (e) receipt of hormone therapy within the preceding three months; (f) current or past smoking, alcohol consumption, or exposure to toxic substances. All participants' body mass index (BMI, range 18–28) met the criteria and received in vitro fertilization / intracytoplasmic sperm injection treatment with GnRH-ant regimen.

Controlled ovarian stimulation protocol and collection of follicular fluid and granulosa cells

To minimize the potential impact of variations in ovarian stimulation protocols on the follicular microenvironment, all participants in this study were subjected to a flexible GnRH antagonist regimen for controlled ovarian stimulation. Gonadotropin (Gn) administration was initiated on menstrual cycle days 2–3 at a starting dose of 150 IU/day via daily subcutaneous injections. After four days of stimulation, follicular development was assessed by transvaginal ultrasound, and serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E2) were measured. The Gn dosage was then individualized based on follicular number, diameter, and hormonal profile. GnRH antagonist (0.25 mg/day) was introduced when at least one follicle exceeded 12 mm in diameter or when serum E2 levels surpassed 500 pmol/L. When one dominant follicle reaches ≥ 18 mm in diameter, or three follicles reach ≥ 16 mm in diameter, administer human chorionic gonadotropin (hCG, Lizu Group, Zhuhai, China) at 5000–10,000 IU, or dual triggering with a

GnRH agonist (triptorelin hydrochloride, 0.1 mg, France) combined with low-dose hCG (3000 IU) to induce oocyte maturation [18]. FF aspiration was performed 34–36 h after trigger under transvaginal ultrasound guidance. After oocyte isolation, the FF obtained from dominant follicles of the same patient was pooled and collected in pre-cooled 15 mL sterile centrifuge tubes. The samples were then centrifuged at 4 °C at $3000 \times g$ for 10 min to remove cellular components and debris. Collect the supernatant without obvious blood contamination, transfer it to a 1.5 mL sterile EP tube, and immediately store it in an ultra-low temperature freezer at -80 °C for a long time to avoid repeated freezing and thawing until subsequent omics analysis. Concentrate the pellet into a round-bottomed test tube and resuspend thoroughly with 3 mL of PBS. Take a 15 mL centrifuge tube and add 5 mL of human lymphocyte isolation medium (Beyotime, Shanghai, China). Slowly add the cell suspension along the wall of the tube onto the upper layer of the isolation medium, taking care to avoid disturbing the interface. Subsequently centrifuge at 1500 r/min for 20 min. After centrifugation, the mixture was clearly separated into three layers. The flocculent granulosa cells in the intermediate layer were carefully and slowly aspirated using a pipette and transferred into a 15 mL centrifuge tube containing 5 mL PBS. After thorough mixing, the cells were centrifuged at 2000 r/min for 5 min. The supernatant was discarded, and the pellet was resuspended and transferred to a 1.5 mL centrifuge tube, followed by centrifugation at 1500 r/min for 2 min. Finally, the supernatant was gently removed, and the granulosa cell pellet was collected and stored at -80 °C for subsequent use. To control batch effects, all samples were thawed and subjected to omics testing during the same time period. During the experimental process, quality control samples (QC) were set up, prepared by mixing follicular fluid from 2 DOR patients and 2 NOR patients in equal volumes, to monitor the stability of LC-MS instrument operation and data reproducibility throughout the entire process, ensuring the reliability and repeatability of analysis results. This study referred to the commonly used sample sizes in previously published FF metabolomics and proteomics research [19–21]. Among the included 30 NOR and 30 DOR patients, 18 samples (9 per group) were first randomly selected for untargeted metabolomics analysis. Subsequently, another 10 samples (5 per group) were randomly selected from outside the metabolomics sample set for data-independent acquisition (DIA) proteomics analysis. There is no overlap between the metabolomics and proteomics samples. This independent sampling design was intended to reduce sample-dependent bias and to evaluate whether consistent biological alterations could be observed across independent cohorts, thereby

strengthening the robustness and reproducibility of the multi-omics analysis.

Follicular fluid metabolomic profiling and data processing

All specimens were gradually warmed to 4°C in a controlled environment. Twenty µL of samples were extracted with 120 µL of pre-cooled 50% methanol, vortexed for 60 s, and cultured at room temperature for 10 min. After overnight storage at -20°C, the extract was centrifuged at 4000 × g for 20 min, and the supernatant was collected for liquid chromatography-mass spectrometry analysis. Quality control (QC) samples were prepared by pooling equal volumes of FF from two DOR patients and two NOR patients. These were interspersed at fixed intervals throughout the testing process to monitor instrument stability and data reproducibility. This study employed no single compound or isotope-labelled internal standard; instead, data quality was controlled through combined QC sample assessment and a normalisation method based on global signal. Chromatographic separation was performed on a Vanquish ultra-high performance liquid chromatography system (Thermo Fisher Scientific) with a Waters ACQUITY UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 µm). The mobile phase A was an aqueous solution consisting of 25 mmol/L ammonium acetate and 25 mmol/L ammonia, while the mobile phase B consisted entirely of acetonitrile. The temperature of the column maintained a stable value of 4 degrees Celsius throughout the process. The Q-Exactive mass spectrometer, manufactured by Thermo Fisher Scientific, was employed to conduct high-resolution mass spectrometry analysis. By reversing the polarity of the ionized particles. The metabolic precursor ions were systematically analyzed within the mass range of 70 to 1050 m/z, employing a resolution setting of 70,000; the resolution of the secondary fragmentation scan was 17 500. The unprocessed data underwent transformation into mzXML format using ProteoWizard software. Then, the peaks were extracted, aligned, and integrated using an independently developed R package based on the XCMS algorithm and annotated by matching the self-constructed secondary mass spectrometry database (the algorithmic score threshold was set at 0.3). During the data preprocessing stage, missing values were uniformly handled: metabolites missing in a large proportion of samples were excluded, whilst low-proportion missing values were imputed using a method based on the minimum detectable signal. The identified metabolites were analyzed using a multivariate method. Metabolite data were normalized and first subjected to principal component analysis, followed by PLS-DA modeling (with principal component scores determined by 7-fold cross-validation) to evaluate the differences between groups. Given the exploratory nature of metabolomics profiling

and to avoid excessive false negatives caused by overly stringent correction, raw p values were used at the initial feature screening stage. DEMs were screened by t-test, and the screening criteria were $|\log_2FC| \geq 0.26$ ($FC \geq 1.2$ or ≤ 0.83), $VIP \geq 1$, and $p < 0.05$. Volcano maps were drawn using the ggplot2 package, and metabolic pathway enrichment analysis was performed in the KEGG database. The types of standardization for metabolomics data use PQN normalization.

Follicular fluid proteomics profiling and data processing

This study analyzed FF samples by data-independent acquisition quantitative proteomics. Samples were quality controlled, and trypsin digestion was performed after high abundance protein removal. Proteins were detected by Thermo Scientific UltiMate™ 3000 binary rapid separation system combined with Orbitrap Exploris™ 480 mass spectrometer (Thermo Fisher Scientific, San Jose, CA). PePSep C18 reversed-phase column (1.9 µm, 75 µm × 15 cm) was used for chromatographic separation. Bruker, Germany), with mobile phases A of 0.1% formic acid aqueous solution and B of 0.1% formic acid acetonitrile solution. The chromatographic column was balanced with 100% phase A for 10 min, and an automatic sampler loaded the sample. The gradient elution was performed at a flow rate of 300 nL / min for 60 min, with the following ratios of mobile phases B: 2% for 0 min, 2–22% for 45 min, 22–37% for 5 min, 37–80% for 5 min, and 80% for 5 min. Mass spectrometry acquisition parameters: primary mass spectrometry. The scanning range was 350–1500 m/z with a resolution of 120,000 and a normalized AGC target value of 300%; the data-independent acquisition mode divided 400–1200 m/z into 53 windows for acquisition. The high-energy collision dissociation energy is 32%, and the resolution of the secondary mass spectrometry was 30,000, with a normalized AGC target value of 200%. Raw data were subjected to database search and qualitative analysis using the Pulsar search engine of SpectroMine software (v4.2.230428.52329; Biognosys AG). Statistical analysis of proteomics data was mainly completed by R software (version 4.0). After a median normalized the raw intensity values of proteins, the metal package performed principal component analysis and difference analysis. During the data preprocessing stage, missing values were uniformly handled: proteins missing in the majority of samples were excluded, whilst low-proportion missing values were imputed using a method based on the minimum detectable signal. Considering the exploratory aim of identifying candidate proteins for downstream analyses, initial screening of DEPs was based on fold change and raw p values rather than FDR-adjusted thresholds, in order to avoid overly conservative filtering. DEPs were screened by t-test, and the screening criteria were $|\log_2FC| \geq 0.26$ ($FC \geq 1.2$ or

≤ 0.83) and $p < 0.05$. The heatmap package and function plotted hierarchical clustering heatmaps and KEGG pathway enrichment analyses were performed based on hypergeometric tests ($p < 0.05$). The types of standardization for proteomics data use median normalization.

Combined metabolomics and proteomics analysis

Proteins and metabolites represent different biological levels, with proteins mainly reflecting potential regulatory functions, while metabolites reflect the immediate phenotypic status of cells and tissues. The two cannot be directly compared at a quantitative scale. Therefore, this study adopts a pathway-level integration strategy based on consistency and correlation rather than a numerical fusion approach. We mapped DEPs and DEMs to pathways using the KEGG database and screened for common pathways with $p < 0.05$. The relationship between the DEMs and DEPs was investigated through a Pearson correlation analysis ($|r| > 0.6$, $p < 0.05$). Furthermore, a heatmap was constructed by hierarchical clustering to reveal the correlation between the two groups of molecules.

Western blot analysis

Protein expression levels of argininosuccinate synthase 1 (ASS1), glutamine-fructose-6-phosphate transaminase 2 (GFPT2), asparagine synthetase (ASNS), and glutamate dehydrogenase 1 (GLUD1) were validated by Western blot analysis. Samples were lysed in pre-chilled

RIPA lysis buffer (Cwbio, Jiangsu, China) and thoroughly homogenized, followed by incubation on ice for 30 min. The lysates were then centrifuged at 4 °C, and the supernatants were collected. Protein concentrations were determined using a BCA protein assay kit (Beyotime, Shanghai, China).

Equal amounts of protein were mixed with loading buffer, boiled for 10 min for denaturation, and centrifuged at 4 °C for 10 min. Proteins were separated by SDS-PAGE and subsequently transferred onto PVDF membranes (Merck Millipore, Ireland). After blocking with blocking buffer (Beyotime, Shanghai, China), the membranes were incubated with the corresponding primary antibodies at 4 °C. Following washing, the membranes were incubated with HRP-conjugated secondary antibodies. Visualisation was performed using ECL chemiluminescent reagents, with signals acquired in a chemiluminescence imaging system. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal reference protein. Band intensity values were quantified using ImageJ software (National Institutes of Health, USA).

Statistical analyses

A comprehensive statistical analysis was conducted employing the SPSS 25.0 (IBM, USA). The Kolmogorov-Smirnov test was employed to assess the normality of continuous variables, while data that met the normal distribution criteria were presented in terms of the mean and standard deviation. To evaluate the differences between the groups, a two-tailed t-test was employed as a statistical method for hypothesis testing. Non-normal distribution data were expressed as median (interquartile range), and the Mann-Whitney U test was used. Categorical variables were described by frequencies (percentages). The significance threshold was set at $p < 0.05$.

Result

Baseline characteristics and assisted reproductive technology outcomes of NOR and DOR groups

The clinical profiles of the patients are shown in Table 1. No statistically meaningful distinctions were observed between the NOR group ($n = 30$) and the DOR group ($n = 30$) in age, infertility years, BMI, basal estradiol, recombinant human follicle-stimulating hormone (rhFSH) stimulation duration, and total rhFSH ($p > 0.05$). However, the DOR group showed typical characteristics of decreased ovarian reserve function: a significant decrease in AFC ($p < 0.001$), lower AMH levels ($p < 0.001$), an increase in basal LH level ($p < 0.05$), and higher basal FSH levels ($p < 0.001$). Regarding assisted reproductive technology outcomes, the number of oocytes retrieved, 2PN fertilization rate, number of available embryos, number of blastocysts, and number of frozen embryos in the DOR group were significantly lower

Table 1 Baseline characteristics of patients

	NOR($n = 30$)	DOR($n = 30$)	<i>p</i> -value
Age(years)	37.83 ± 2.01	38.33 ± 2.29	0.373
Infertility duration(years)	3.13 ± 2.08	3.73 ± 2.36	0.300
BMI(kg/m ²)	23.57 ± 2.19	23.42 ± 2.73	0.815
Basal FSH level (IU/L)	5.91 ± 0.58	10.03 ± 1.44	< 0.001*
Basal LH level (IU/L)	3.57 ± 1.53	4.48 ± 1.36	0.017*
Basal E2 level (pg/ml)	40.21 ± 24.50	44.21 ± 18.80	0.481
AMH	2.91 ± 0.74	0.79 ± 0.25	< 0.001*
Antral follicle count	9.53 ± 2.50	4.8 ± 1.09	< 0.001*
Stimulation duration of rhFSH (day)	8.6 ± 1.35	8.9 ± 1.51	0.422
Total rhFSH (IU)	2117.91 ± 530.95	2235.41 ± 582.20	0.417
LH level on trigger day (IU/L)	2.55 ± 1.79	3.31 ± 2.15	0.144
Number of oocytes retrieved	10.5 ± 2.16	4.56 ± 1.10	< 0.001*
2PN Fertilization	6.76 ± 2.31	2.73 ± 1.46	< 0.001*
Number of available embryos	2.43 ± 1.30	1.7 ± 1.05	0.019*
Number of blastocyst	0.91 ± 1.59	0.2 ± 0.31	0.022*
Number of frozen embryos	1.8 ± 1.76	0.9 ± 1.15	0.023*

FSH Follicle-stimulating hormone, LH Luteinizing hormone, E2 Estradiol, rhFSH Recombinant human follicle-stimulating hormone

*Significant at $p < 0.05$

than those in the NOR group ($p < 0.05$). This suggests that AMA with NOR has better reproductive outcomes than AMA with DOR. Additionally, this study randomly selected 10 and 18 samples from the enrolled cohort for proteomics and metabolomics analysis. As shown in Supplementary Tables S1 and S2, the two subgroups undergoing omics analysis exhibited no significant differences in baseline parameters including age, duration of infertility, BMI, basal E2, rhFSH stimulation duration, or total rhFSH dosage ($p > 0.05$). The DOR subgroup further demonstrated significantly reduced antral follicle count ($p < 0.001$), reduced AMH levels ($p < 0.001$), and elevated baseline FSH levels ($p < 0.001$), indicating that the patient cohorts included in the omics analysis remained highly comparable between groups.

Metabolomics analysis of follicular fluid

In this study, Non-targeted metabolomics analysis was performed using liquid chromatography-mass spectrometry to identify differences in FF metabolism between the two groups of DOR and NOR patients. Through mass spectrometry 2, a total of 572 metabolites were identified between the two groups. (Supplementary Table S3). Metabolite classification analysis showed that lipid

and lipid-like molecules had the highest percentage (60.38%, Fig. 1A). Principal component analysis (PCA), an unsupervised method, revealed a partial separation trend between the DOR and NOR groups, although some overlap between the two groups was still observed (Supplementary Fig. S1A). Based on the PCA results, a supervised partial least squares discriminant analysis (PLS-DA) model was subsequently constructed as an exploratory approach to further characterize group-related metabolic differences. The PLS-DA score plot showed a separation trend between the DOR and NOR groups (Fig. 1B). Given the relatively small sample size and the inherent risk of overfitting associated with supervised models, the robustness of the PLS-DA model was evaluated using a permutation test with 200 iterations. The intercepts of R^2 and Q^2 were 0.882 and -0.616 , respectively, with a negative Q^2 intercept, suggesting that the model did not exhibit obvious overfitting under the current sample conditions (Fig. 1C). Nevertheless, considering the limited sample size, the PLS-DA results should be interpreted with caution and are primarily intended to support downstream feature selection rather than to provide definitive evidence of group separation. A total of 89 DEMs were identified, of which 32

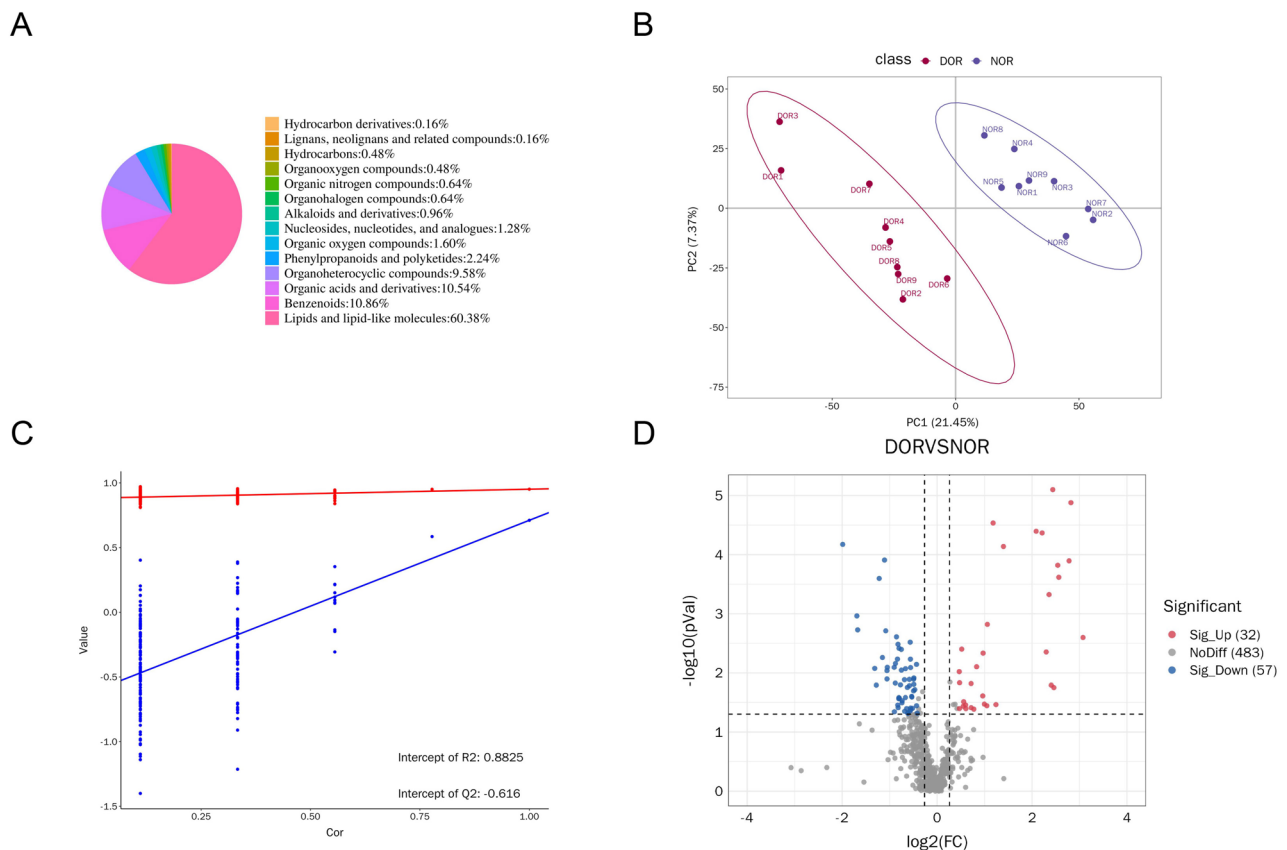


Fig. 1 **A** Proportion of identified metabolites by chemical classification. **B** PLS-DA score chart of metabolites in DOR and NOR. **C** The PLS-DA model's permutation test. **D** The volcano plot displays DEMs, The red and blue dots represent significantly up-regulated and down-regulated DEMs

were upregulated and 57 were down-regulated (screening criteria: $|\log_2FC| \geq 0.26$ ($FC \geq 1.2$ or ≤ 0.83), $VIP \geq 1$, $p < 0.05$). The volcano map (Fig. 1D) and the heat map of the top 30 DEMs (Fig. 2A) intuitively show the pattern of inter-group differences.

To explore the effect of metabolite changes on the pathological process of DOR, KEGG enrichment analysis of differential metabolites was performed ($p < 0.05$). The results showed that DEMs were significantly enriched in 105 metabolic pathways. According to the significance ranking, the top 20 pathways (Fig. 2B) were mainly involved: (1) lipid metabolism (glycerophospholipid metabolism, steroid hormone biosynthesis); (2) Amino acid metabolism (taurine and hypotaurine metabolism, alanine, aspartate, and glutamate metabolism); (3) Disease-related pathways (insulin resistance, choline metabolism in cancer, and autoimmune thyroid disease).

Proteomic analysis of follicular fluid

In this research, we used DIA quantitative proteomics technology to analyze the FF of DOR patients and NOR patients, and a total of 1068 proteins were quantified (Table S4). Structural domain analysis revealed that immunoglobulin-like folding and P-loop nucleoside triphosphate hydrolase were the most common (Supplementary Fig.S1B). Principal component analysis revealed that the protein expression patterns in the DOR group differed significantly from those in the NOR group, (Fig. 3A). A total of 335 DEPs were identified (screening criteria: $|\log_2FC| \geq 0.26$ ($FC \geq 1.2$ or ≤ 0.83), $p < 0.05$),

of which 40 proteins were upregulated and 295 proteins were down-regulated. The volcano map (Fig. 3B) and the hierarchical clustering heatmap of the top 100 DEPs (Fig. 3C) demonstrated the pattern of intergroup differences. The subcellular localization of 335 DEPs was analyzed, and the top three subcellular localizations were cytosol, nucleus, and extracellular (Supplementary Fig.S1C). To determine the characteristics of differential proteins, we performed GO and KEGG enrichment analyses on DEPs to characterize their functions.GO functional annotation divided DEPs into three major categories (Supplementary Fig.S1D): biological processes, cellular components, and molecular functions. Proteins in the biological processes classification were mainly involved in negative apoptosis regulation, positive DNA template transcription regulation, and innate immune response. In molecular functions classification, proteins were significantly enriched in RNA-binding, protein homologous binding, and ATP-binding pathways. For cellular components, proteins were mostly observed in the cytosol, nucleus, and mitochondria. The scatter plot of GO enrichment was shown (Fig. 4A). KEGG pathway analysis ($p < 0.05$) showed that DEPs were significantly enriched in the metabolic pathways of glycolysis/gluconeogenesis, oxidative phosphorylation and HIF-1 signaling pathway (Fig. 4B), indicating that glucose metabolism may change during ovarian aging.

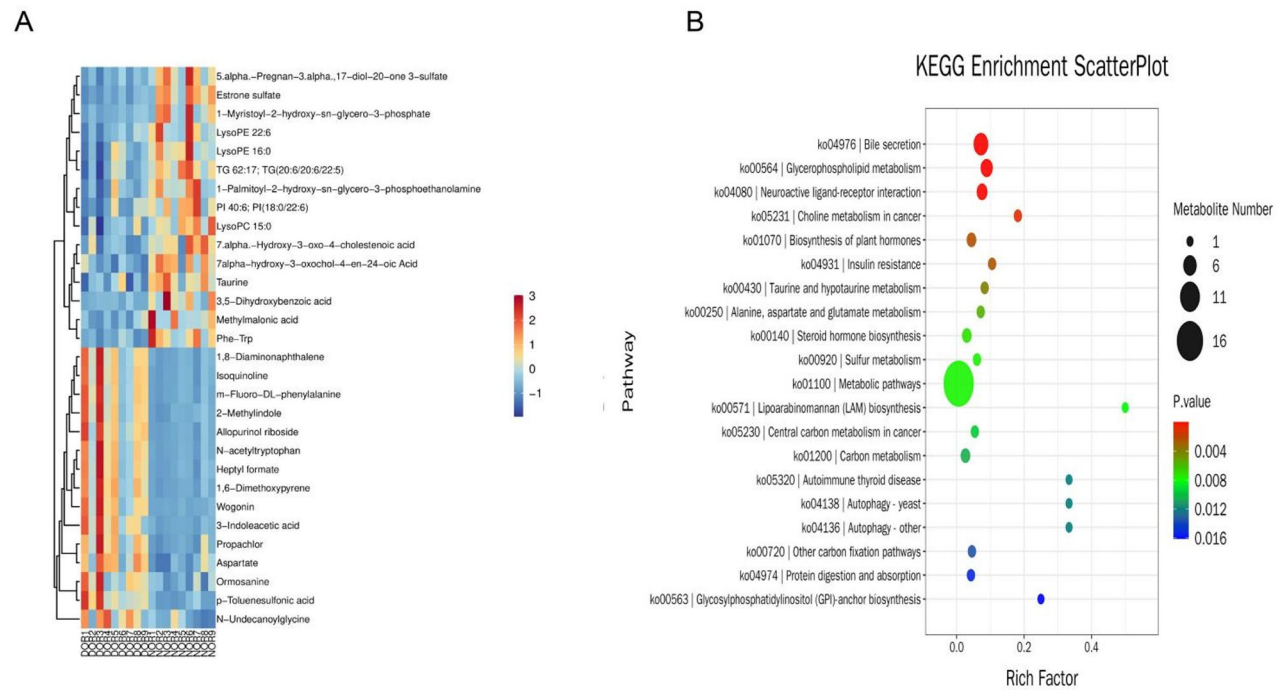


Fig. 2 A Heatmap of DEMs. B Bubble map of the KEGG enrichment analysis of DEMs

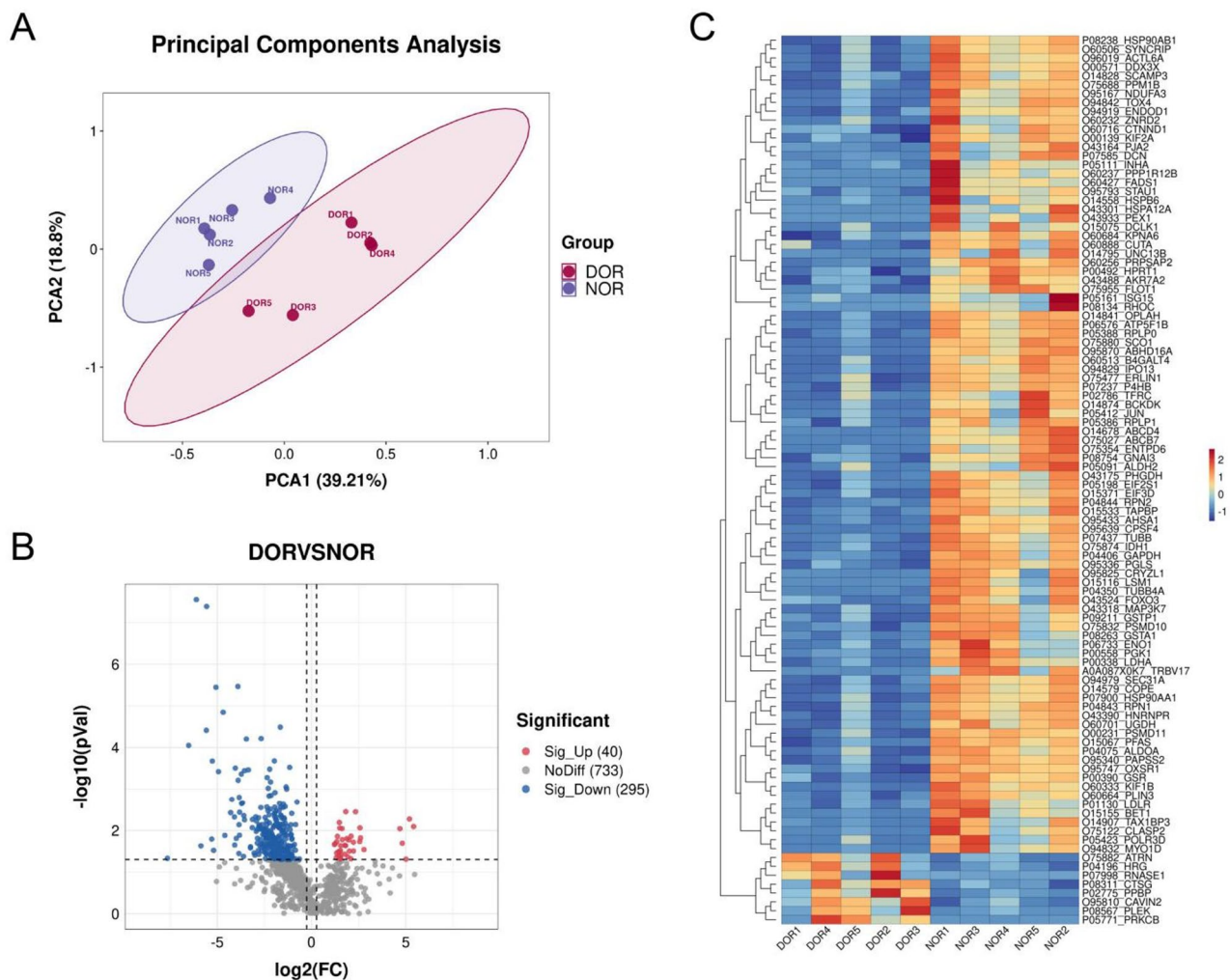


Fig. 3 **A** PCA plot of protein expression profiles in DOR and NOR groups. **B** The volcano plot displays DEPs, The red and blue dots represent significantly up-regulated and down-regulated DEPs. **C** Heatmap of DEPs

Combined analysis of metabolomics and proteomics of follicular fluid

To avoid bias introduced by subjective weighting between heterogeneous omics layers, we integrated proteomic and metabolomic data at the functional pathway level. Our analysis focused specifically on pathways that exhibited significant disturbances in both layers, thereby enhancing the biological coherence and interpretability of the multi-omics integration. Pearson correlation analysis ($|r| > 0.6$, $p < 0.05$) was performed on DEPs and DEMs based on the order of significance (p -value top 30) (Fig. 5A). The DEPs and DEMs were mapped to the KEGG database at the same time. A total of 62 common pathways were identified, of which six were significantly enriched ($p < 0.05$), including metabolic pathways, cholesterol metabolism, alanine, aspartate and glutamate metabolism, carbon metabolism, central carbon metabolism in cancer, and sulfur metabolism. Amino acids, as the fundamental building blocks of proteins, play critical roles in follicular

development, oocyte maturation, and early embryonic development. Within the alanine, aspartate, and glutamate metabolism pathway, we observed a significant increase in aspartate levels in the FF of patients with DOR, accompanied by marked downregulation of key metabolic enzymes, including ASS1, GFPT2, GLUD1, and ASNS. These findings indicate that dysregulation of alanine, aspartate, and glutamate metabolism may be involved in the pathological state of DOR. Accordingly, among the significantly enriched pathways, this metabolic pathway was selected for further focused analysis.

Correlation analysis between differential metabolites and clinical indicators

To investigate the association between differential substances and DOR in FF, Spearman rank correlation analysis was conducted to evaluate the relationship between DEPs and DEMs in the co-enrichment pathway of proteomics and metabolomics and clinical parameters

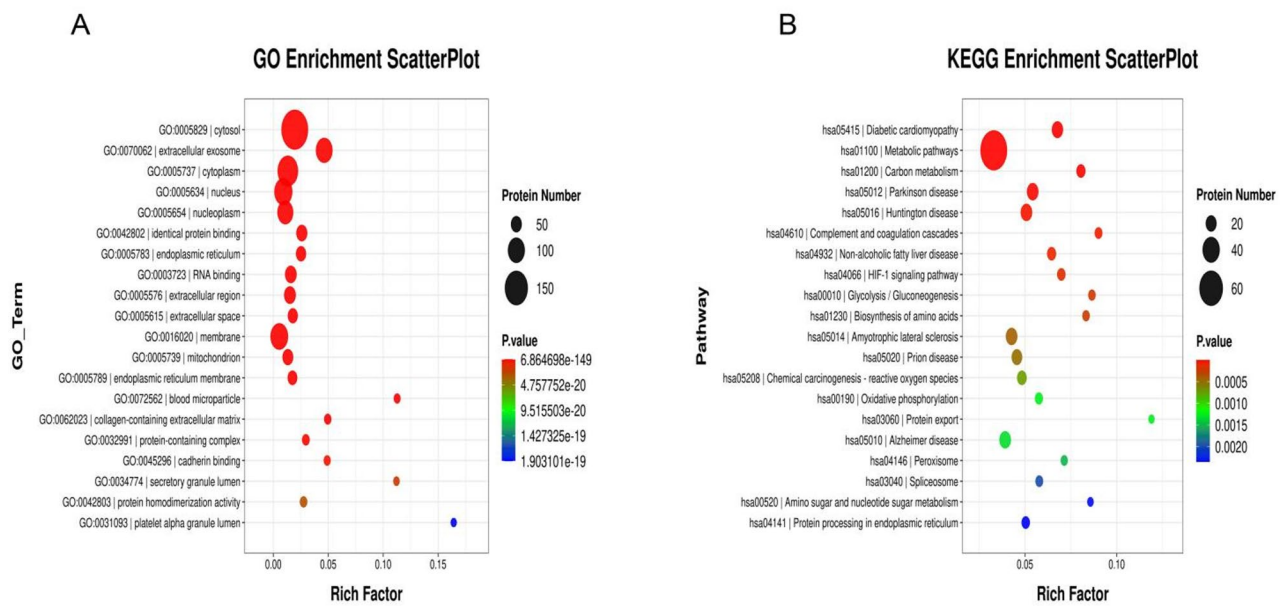


Fig. 4 **A** GO enrichment analysis bubble plot of DEPs. **B** Bubble map of the KEGG enrichment analysis of DEPs

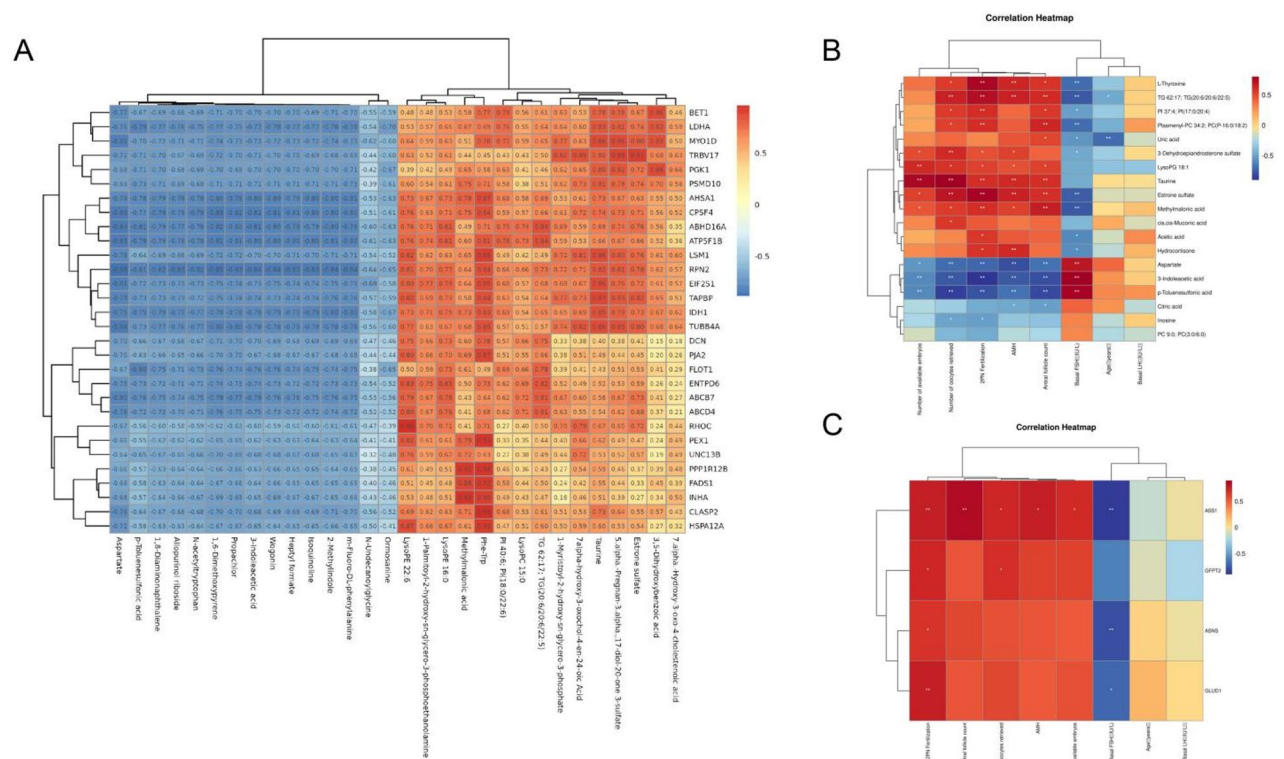


Fig. 5 **A** Correlation analysis of the differential proteins and metabolites. In the figure, red is positively correlated and blue is negatively correlated. **B** Spearman correlation analysis between candidate metabolites and clinical parameters in FF. **C** Spearman correlation analysis between candidate proteins and clinical parameters in FF

(Fig. 5B and C). A threshold of significant correlation was set at $|\rho| > 0.5$ and $p < 0.05$. Lipid metabolites (LysoPG 18:1, PI 37:4, TG 62:17, etc.) were found to be significantly and positively associated with oocyte retrieval

rate, 2PN fertilization rate, and AFC; estrone sulfate and taurine were positively correlated with the number of available embryos, oocyte retrieval rate, 2PN fertilization, AMH, and AFC, whereas estrone sulfate also negatively

correlated with basal FSH. Aspartate levels were inversely related with the number of available embryos, oocyte retrieval rate, 2PN fertilization rate, AMH, and AFC but positively correlated with basal FSH. Compared with NOR patients, the expression levels of ASS1、GFPT2、ASNS、and GLUD1 in the metabolic pathways of alanine, aspartate, and glutamate metabolism in DOR patients were significantly decreased (Fig. 6A). Similarly, the results of Western blot also showed a similar trend (Fig. 6B and C). ASS1, GFPT2, ASNS and GLUD1 were all positively correlated with 2PN fertilization rate. Among them, ASS1, ASNS, and GLUD1 were negatively correlated with basal FSH, while ASS1 significantly correlated with the number of available embryos, oocyte retrieval rate, AMH, and AFC.

Discussion

In this study, we performed proteomic and metabolomic analyses of FF from women of AMA with DOR and NOR, identifying 335 DEPs and 89 DEMs, respectively. Importantly, as shown in Supplementary Tables S1 and S2, the subgroups selected for omics analyses were well balanced in baseline clinical characteristics, including age, body mass index, duration of infertility, basal hormone levels, and ovarian stimulation parameters, thereby minimizing potential selection bias and enhancing the robustness of subsequent multi-omics comparisons. Pathway enrichment analysis of the two omics datasets revealed that alanine, aspartate, and glutamate metabolism was one of the

key metabolic pathways significantly enriched at both the proteomic and metabolomic levels. Within this pathway, several core enzymes, including ASS1, GFPT2, GLUD1, and ASNS, were significantly downregulated in the FF of advanced-age DOR patients. These findings suggest that dysregulation of alanine, aspartate, and glutamate metabolism is closely associated with ovarian functional decline, and that the associated enzymes may serve as potential biomarkers and intervention targets for age-related DOR.

Proteomic characteristics of the follicular microenvironment in DOR

FF provides a specific microenvironment for the development and maturation of oocytes. In-depth investigation of the protein composition of FF can help reveal the signaling mechanisms within the follicle and the microenvironmental changes that occur during reproductive aging. Previous studies have preliminarily mapped the proteome of human FF [22], and more recent research has employed proteomics to analyze the dynamic changes in the FF microenvironment during follicle maturation, as well as to explore mechanisms underlying disease-induced infertility [23, 24]. Bai et al. conducted a proteomic analysis of naturally aging mouse ovaries and revealed that ovarian aging is closely associated with biological processes such as chronic inflammation, oxidative stress, and dysregulated lipid metabolism [25]. However, due to species differences, these findings cannot be

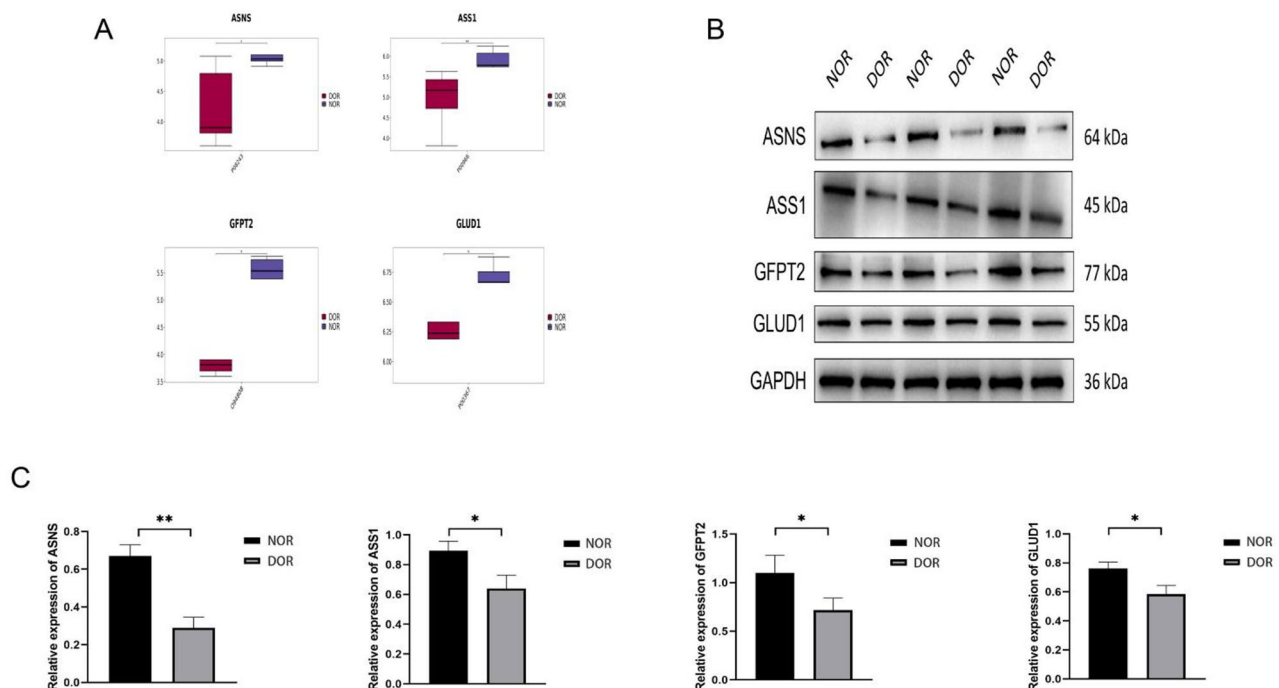


Fig. 6 **A** DEPs levels in the metabolic pathways of alanine, aspartate, and glutamate. **B** Representative protein bands of ASNS、ASS1、GFPT2 and GLUD1. **C** Verification of the expression levels of DEPs by western blot. * $p < 0.05$, ** $p < 0.01$, DOR vs. NOR

directly extrapolated to humans and require validation in clinical samples. Liu et al. profiled the proteome of exosomes derived from human FF and found that DEPs were significantly enriched in pathways related to carbon metabolism, tyrosine metabolism, and amino acid biosynthesis [26]. Notably, the expression levels of these metabolism-associated proteins were significantly lower in older women compared to younger women, suggesting that reduced metabolic capacity in aged females may affect reproductive potential via exosome-mediated protein regulation. To date, no study has reported the proteomic characteristics of FF in AMA with different ovarian reserve.

In this study, we performed proteomic analysis on FF from AMA women with DOR and those with NOR, identifying a total of 335 DEPs, of which 40 were upregulated and 295 downregulated. KEGG pathway enrichment analysis indicated that these DEPs were primarily involved in key metabolic pathways, including glycolysis/gluconeogenesis, OXPHOS, and the HIF-1 signaling pathway. Glycometabolism, as a central process for cellular energy supply, comprises multiple stages including glycolysis, gluconeogenesis, and OXPHOS. Studies have shown that with increasing age, oocytes and GCs exhibit abnormal glycometabolism characterized by a shift from OXPHOS to glycolysis, which impairs oocyte maturation, fertilization, and early embryonic development [27]. Concurrently, the antioxidant defense system declines with age, leading to elevated reactive oxygen species (ROS) production and exacerbated oxidative stress in the ovary [28]. HIF-1, as a key transcription factor mediating hypoxic responses, can induce the expression of glycolytic genes to adaptively regulate cellular responses to hypoxia, favoring glycolysis over OXPHOS to meet energy demands, thereby reducing ROS generation and mitigating oxidative damage [29, 30]. This metabolic shift may represent a compensatory protective mechanism aimed at alleviating ovarian oxidative stress by limiting excessive ROS accumulation. In summary, the proteomic findings of this study are consistent with previous reports, highlighting that metabolic dysregulation plays a crucial role in reproductive aging of advanced-age women.

Metabolomic characteristics of the follicular microenvironment in DOR

Women with ovarian aging are prone to metabolic disturbances, particularly abnormalities in lipid and glucose metabolism [31]. Metabolomics enables the systematic analysis of dynamic changes in endogenous metabolites and has been widely applied in recent years to investigate the metabolic characteristics of FF, providing a powerful tool for elucidating the mechanisms of female reproductive aging and identifying potential biomarkers.

For example, Liang et al. reported significant alterations in arachidonic acid metabolism in the FF of patients with DOR, which were associated with reduced fertility [32]. Similarly, de la Barca et al. demonstrated that levels of polyunsaturated choline plasmalogens and arginine methyltransferase activity were markedly decreased in the FF of DOR patients, accompanied by a reduced dimethylarginine/arginine ratio, suggesting that metabolic abnormalities in FF represent an important molecular feature of DOR [33]. Based on these findings, the present study performed untargeted metabolomic profiling of FF from AMA with different ovarian reserve, identifying a total of 89 DEMs, including 32 upregulated and 57 downregulated metabolites. KEGG pathway enrichment analysis revealed that these DEMs were mainly enriched in glycerophospholipid metabolism, steroid hormone biosynthesis, and taurine and hypotaurine metabolism pathways, indicating that lipid- and hormone-related metabolic pathways are disrupted during ovarian aging.

Glycerophospholipids are essential structural components of cellular membranes and play critical roles in cell signaling, immune regulation, and protein functional modulation [34]. Wu et al. reported that GPD1L expression and GPD1L-mediated glycerophospholipid metabolic function were significantly downregulated in the FF of DOR patients, accompanied by decreased levels of phosphatidylcholine (PC), phosphatidic acid (PA), lysophosphatidylethanolamine (LysoPE), and lysophosphatidylcholine (LysoPC), ultimately impairing oocyte quality [35]. Consistent with these findings, our study also observed decreased levels of glycerophospholipid-related metabolites, including LysoPG 18:1, PI 37:4 [PI(17:0/20:4)], and TG 62:17 [TG(20:6/20:6/22:5)], further confirming that glycerophospholipid metabolic dysfunction is a key metabolic characteristic of female ovarian aging. Steroid hormones play a crucial regulatory role in follicular development and the maintenance of intrafollicular microenvironmental homeostasis, directly influencing cellular proliferation, apoptosis, and angiogenesis within the follicle [36]. Bildik et al. proposed that defects in steroid hormone biosynthesis are commonly present in patients with poor ovarian response (POR) [37]. In addition, Zeng et al. used metabolomic analysis to demonstrate a significant reduction in serum testosterone sulfate levels in women with ovarian aging [38]. In line with these observations, our study revealed marked dysregulation of the steroid hormone biosynthesis pathway in the FF of DOR patients, with significant decreases in key metabolites such as estrone sulfate and dehydroepiandrosterone sulfate. These metabolites may serve as specific metabolic markers of physiological ovarian aging, providing a basis for early clinical identification and precision intervention. Moreover, taurine,

an important sulfur-containing amino acid, exhibits potent antioxidant and anti-apoptotic properties and can improve ovarian function by alleviating oxidative stress and apoptosis [39]. Previous studies have shown that dietary taurine deficiency may lead to female reproductive dysfunction, including estrogen imbalance and embryonic loss [40]. Our results further demonstrated that taurine levels in the FF of DOR patients were significantly lower than those in NOR patients, suggesting that taurine deficiency may be an important metabolic feature of an impaired follicular microenvironment. Targeted taurine supplementation may therefore represent a potential intervention strategy to improve reproductive outcomes. In summary, the metabolomic findings of this study are highly consistent with previous studies, further validating the critical roles of lipid metabolism and steroid hormone biosynthesis in ovarian aging from the perspective of the FF microenvironment.

Multi-omics analysis reveals a critical role of alanine, aspartate and glutamate metabolism in DOR

Based on proteomic and metabolomic data, we jointly identified six significantly altered biological pathways at both the protein and metabolite levels, including metabolic pathways, cholesterol metabolism, alanine, aspartate and glutamate metabolism, carbon metabolism, central carbon metabolism in cancer, and sulfur metabolism. These findings provide clues for exploring whether protein-level alterations during ovarian aging influence metabolite levels through enzymatic reactions, thereby participating in the functional regulation of the follicular microenvironment. Amino acids, as the fundamental building blocks of proteins, play essential roles in follicular development, oocyte maturation, and early embryonic development. Therefore, among the significantly enriched pathways, we focused primarily on the alanine, aspartate, and glutamate metabolism. Proteomic analysis revealed that, compared with AMA with NOR, multiple key enzymes involved in this pathway, including ASS1, GFPT2, GLUD1, and ASNS, were significantly downregulated in the FF of AMA with DOR. Consistently, metabolomic analysis demonstrated a marked elevation of aspartate levels in the FF of DOR patients, suggesting that downregulation of these enzymes may attenuate metabolic flux through this pathway, leading to abnormal substrate accumulation.

ASS1 is the rate-limiting enzyme of the mammalian urea cycle, catalyzing the condensation of citrulline and aspartate to form argininosuccinate, which is subsequently converted to arginine by argininosuccinate lyase (ASL). Studies have shown that arginine plays a crucial role in female reproduction. Herring et al. reported that arginine promotes placental blood flow, stimulates placental angiogenesis, and enhances maternal–fetal

nutrient transport [41]. During early pregnancy, arginine depletion can induce reversible developmental arrest of blastocysts by inhibiting trophoblast cell growth [42]. Therefore, downregulation of ASS1 may limit intracellular arginine biosynthesis, thereby adversely affecting embryo implantation and early embryonic development by disrupting nitric oxide-mediated signaling and energy metabolism [43]. ASNS is the only enzyme catalyzing the biosynthesis of asparagine (Asn), catalyzing the ATP-dependent conversion of aspartate and glutamine into Asn and glutamate [44]. ASNS deficiency has been recognized as a molecular hallmark of cellular and tissue aging; its downregulation can induce premature cellular senescence by promoting the secretion of senescence-associated secretory phenotype (SASP) factors, such as IL-1 β and IL-6, while concurrently suppressing cell proliferation [45]. In addition, ASNS is involved in glucose transport and energy production and plays an important role in maintaining cellular redox homeostasis. ASNS knockdown has been shown to reduce glutathione levels, increase malondialdehyde content, and upregulate HIF-1 α expression [46]. Downregulation of ASNS in the FF of DOR patients may exacerbate oxidative damage within the follicular microenvironment, directly impacting oocyte quality. These findings further support our earlier conclusion that the FF microenvironment of DOR patients is characterized by significant disturbances in glucose metabolism and oxidative stress. GLUD1 is highly expressed in steroidogenic tissues, including the ovary, testis, and adrenal glands. Its main function is to catalyze the deamination of glutamate to produce α -ketoglutarate (α -KG), thereby linking amino acid metabolism to the tricarboxylic acid cycle. GLUD1 also plays an essential role in epigenetic regulation and normal embryonic development [47]. Cheng et al. demonstrated that GLUD1 knockdown reduces α -KG levels, resulting in delayed embryonic development, decreased blastocyst formation rates, and increased apoptosis [48]. GFPT2 is the key rate-limiting enzyme of the hexosamine biosynthesis pathway and is highly expressed in human embryonic stem cells [49]; however, its role in female reproductive aging has not yet been reported. Furthermore, we validated these findings at the granulosa cells level. Western blot analysis demonstrated that the expression levels of four key proteins involved in the alanine, aspartate, and glutamate metabolism pathway were significantly decreased in granulosa cells from DOR patients, consistent with the FF proteomic results. This cellular-level validation further strengthens the reliability and biological credibility of our conclusions.

In summary, the reduced expression of ASS1, ASNS, and GLUD1 may synergistically impair female reproductive potential through distinct biological processes, including arginine biosynthesis, asparagine metabolic

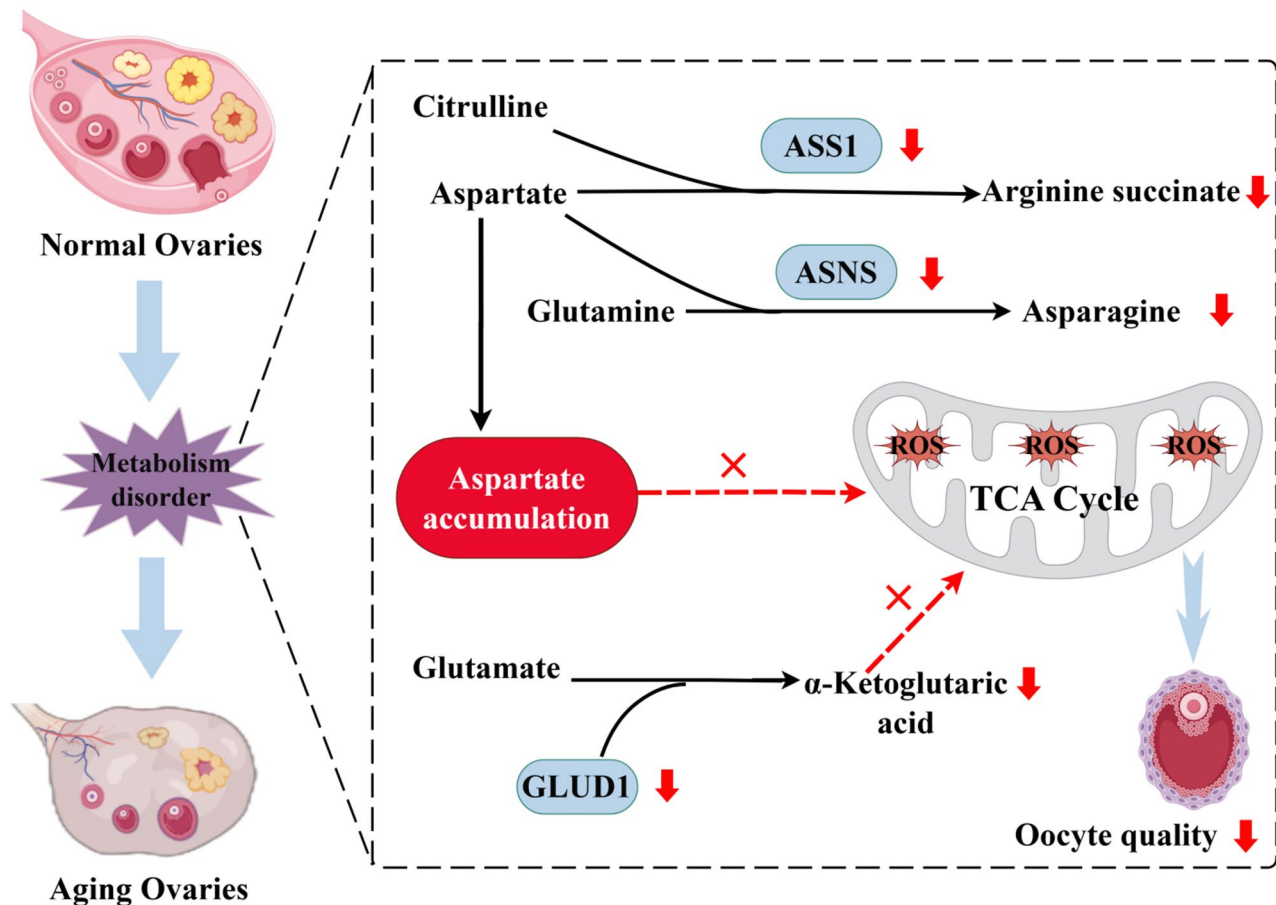


Fig. 7 Schematic illustration showing how differential expression of key enzymes regulates amino acid metabolism in follicular fluid during ovarian aging. In the FF of DOR patients, ASS1, ASNS, and GLUD1 are significantly downregulated, leading to impaired conversion of aspartic acid and glutamate, accumulation of aspartic acid, and interruption of the tricarboxylic acid cycle. These changes can lead to energy metabolism disorders, oxidative stress, and mitochondrial dysfunction, ultimately reducing the quality of oocytes. (Note: Drawn by figdraw)

homeostasis, and energy metabolism. Previous studies have shown that aspartate levels are positively correlated with aging, with serum concentrations gradually increasing with advancing age [50]. Li et al. also reported significantly elevated aspartate levels in the FF of DOR patients [51], which is highly consistent with our findings. A plausible mechanism is that downregulation of ASS1, ASNS, and GLUD1 reduces aspartate consumption across multiple metabolic pathways, leading to its abnormal accumulation within the FF microenvironment (Fig. 7). However, direct evidence elucidating how aberrantly elevated aspartate in FF accelerates ovarian aging by affecting oocyte energy metabolism, granulosa cells function, and follicular microenvironment homeostasis remains lacking, and the precise molecular mechanisms underlying this process warrant further investigation in future studies. Importantly, although oxidative stress, mitochondrial dysfunction, and reduced oocyte developmental competence have been implicated in ovarian aging, the present study does not provide direct evidence linking elevated aspartate levels in FF to these processes. Therefore, the

proposed associations should be interpreted as hypothesis-generating rather than causal. Further functional studies are required to clarify how disturbances in alanine, aspartate, and glutamate metabolism within the FF microenvironment may influence oocyte quality and developmental potential.

Limitations

One major limitation of the present study is the relatively small sample size used for the proteomic and metabolomic analyses. Due to the difficulty in obtaining follicular fluid samples and limitations in research funding and experimental conditions, this study only selected a portion of the overall sample for proteomic and metabolomic analysis. The relatively small sample sizes for both omics analyses may increase statistical variance and raise the risk of model overfitting. In addition, this study is exploratory in nature. Although efforts were made to minimize potential confounding effects through the use of a uniform ovarian stimulation protocol and standardized FF collection procedures, the influence of

stimulation-related clinical variables on the study outcomes cannot be completely excluded. Future research should expand sample sizes and incorporate multi-centre data to enhance statistical power and improve result reproducibility, and identify additional metabolites and proteins exhibiting biologically significant differences. Another important limitation of this study is the lack of targeted validation for key differential metabolites identified by untargeted metabolomics, particularly aspartate. Although protein-level validation was performed using Western blot analysis, the absence of targeted metabolomics or validation in an independent cohort limits the strength of the findings as definitive biomarkers. Therefore, the metabolomic results should be interpreted as exploratory. In the future, we will carry out targeted studies on key differential metabolites and proteins to provide a more reliable theoretical basis for studying pathological mechanisms and clinical intervention of DOR. Last but not least, this study was not designed or powered to evaluate key clinical outcomes such as clinical pregnancy, ongoing pregnancy, or live birth. Therefore, the conclusions of this study are limited to differences in the follicular microenvironment rather than clinical outcome prediction.

Conclusions

In conclusion, this study performed proteomic and metabolomic analyses of FF from AMA with different ovarian reserves, identifying a series of biologically meaningful DEPs, DEMs, and significantly enriched key metabolic pathways. Among these, dysregulation of the alanine, aspartate, and glutamate metabolism pathway may play an important role in the initiation and progression of ovarian aging, providing new insights and clues for elucidating the underlying mechanisms of ovarian aging. However, the precise role and mechanistic involvement of this metabolic pathway in ovarian aging remain to be further clarified through additional animal and cell-based studies in the future.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12905-026-04393-3>.

Supplementary Material 1.

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Authors' contributions

QWL and JZC were jointly responsible for the experimental design; QWL completed the first draft of the paper; LYQ and WPX were responsible for the collection of clinical data and biological samples; SMY and YXX were involved in data analysis and results collation; QWL, JZC and SXW were jointly involved in the revision of the paper and the discussion of key academic issues; GY

was responsible for the design of the research program and provided financial support. All authors contributed to the article and approved the version submitted.

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Data availability

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

Declarations

Ethics approval and consent to participate

This study follows the Helsinki Declarations. The study was approved by the Reproductive Ethics Committee of Shandong University of Traditional Chinese Medicine (NO: SDTCM/E2408-24). All participants provided written informed consent before sample collection.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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