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Original Article

Infertility

Increased serine synthesis in cumulus cells of young infertile women with diminished ovarian reserve

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ABSTRACT

STUDY QUESTION: What are the differences in gene expression of cumulus cells (CCs) between young women with diminished ovarian reserve (DOR) and those of similar age with normal ovarian reserve (NOR)?

SUMMARY ANSWER: Gene expression and metabolome profiling analysis demonstrate that the *de novo* serine synthesis pathway (SSP) is increased in the CCs of young women with DOR.

WHAT IS KNOWN ALREADY: The incidence of DOR has risen, tending to present at younger ages. Its mechanisms and aetiologies are still poorly understood. Abnormal metabolism is present in luteinized CCs of patients with DOR. Previous studies have revealed that mitochondrial dysfunction and impaired oxidative phosphorylation in CCs are related to DOR in women of advanced age. The pathogenic mechanisms likely differ between young women with DOR and cases associated with advanced maternal age. Several studies have examined amino acid metabolism in the follicle, with a focus on embryo development, but less information is available about CCs. The physiological significance of *de novo* serine synthesis in follicles and oocytes remains largely unknown.

STUDY DESIGN, SIZE, DURATION: CC samples were obtained from 107 young infertile women (age <38 years) undergoing ICSI, from July 2017 to June 2019, including 54 patients with DOR and 53 patients with NOR.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Oocyte development data were analysed retrospectively. Comprehensive genome-wide transcriptomics of CCs was performed. Differentially expressed genes (DEGs) were identified. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to categorize the functions of the DEGs and identify significantly enriched pathways. The transcript and protein levels of key enzymes involved in serine synthesis were verified in additional samples using quantitative real-time PCR (qRT-PCR) ($n = 10$) and capillary western blotting ($n = 36$). Targeted metabolomics of amino acids in CC extracts was performed by ultrahigh-performance liquid MS (UHPLC–MS/MS).

MAIN RESULTS AND THE ROLE OF CHANCE: The number of oocytes (2.4 ± 2.2 versus 12.1 ± 5.3) and metaphase II oocytes (2.1 ± 2.0 versus 9.9 ± 4.9) retrieved was significantly decreased in the DOR versus the NOR group, respectively ($P < 0.0001$). The rates of fertilization (80.7% versus 78.8%), viable embryos (73.7% versus 72.5%), and high-quality embryos (42.8% versus 49.0%) did not differ between the DOR and NOR groups, respectively ($P > 0.05$). A total of 95 DEGs were found by transcriptome sequencing. GO and KEGG analyses demonstrated that the DEGs were linked to amino acid metabolism and suggested significantly higher activity of the *de novo* SSP in the CCs of young women with DOR. Further qRT-PCR and capillary western blotting revealed that key enzymes (PHGDH, PSAT1, PSPH, and SHMT2) involved in *de novo* serine synthesis were upregulated, and UHPLC–MS/MS analysis showed increases in serine and glycine (a downstream product of serine) levels in the CCs of young patients with DOR. Our data clearly demonstrate that the *de novo* SSP, which diverts 3-phosphoglycerate from glycolysis to serine synthesis, was upregulated in young DOR CCs.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: Regarding the reproductive capacity of young patients DOR, the pregnancy outcomes were not analysed. The sample size was limited, and only women undergoing ICSI were examined since this was a prerequisite for the acquisition of CCs, which may cause selection bias. The exact mechanisms by which the SSP in CCs regulates ovarian reserve still require further study.

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WIDER IMPLICATIONS OF THE FINDINGS: Our research presents new evidence that alterations of the SSP in CCs of young infertile women are associated with DOR. We believe this is a significant contribution to the field, which should be key for understanding the cause and mechanisms of ovarian hypofunction in young women.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by grants from the Ministry of Science and Technology of China (2018YFC1005001) and National Natural Science Foundation of China (31601197). There were no competing interests.

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Introduction

Ovarian reserve is an important indicator of female fertility. Diminished ovarian reserve (DOR) refers to a decrease in the quantity or quality of follicles in the ovary. DOR manifests in the clinic as a high serum level of FSH, decreased level of anti-Müllerian hormone (AMH), and reduced antral follicle count (AFC). DOR is also characterized by poor fertility outcomes, and DOR accounts for 10% of cases of infertility (Cao *et al.*, 2018). In recent years, the incidence of DOR has risen, tending to present at younger ages. Although overcoming DOR is a focus of current ART, its mechanisms and aetiologies are still unclear and present a major challenge.

Oocyte development is coordinated with the concomitant growth of the corresponding follicle in the ovary. During folliculogenesis, cumulus cells (CCs) are an essential group of somatic cells surrounding the oocyte, connected to the oocyte by gap junctions, forming an entity called the cumulus–oocyte complex (COC). The bidirectional communication between CCs and oocytes is crucial for supporting oocyte maturation and follicular development because most of the metabolites in the oocyte, including amino acids, pyruvate, and intermediate metabolites, are provided by CCs through a gap junction formed by the COC (Gilchrist *et al.*, 2008; Dumesic *et al.*, 2015; Jiang *et al.*, 2021).

Previous studies have shown that amino acid metabolism supports oocyte growth and cytoplasmic maturation and may also be a predictor of oocyte developmental competence (Collado-Fernandez *et al.*, 2012). Despite their important roles, the uptake and metabolism of amino acids by follicles and oocytes, especially at the early stages of folliculogenesis, are not well understood (Collado-Fernandez *et al.*, 2012). A positive correlation between CC metabolites and follicle development was also reported (Richani *et al.*, 2021). It has also been suggested in mice that transporters in CCs mediate the uptake of L-serine into the COC, which is subsequently transferred to the growing and maturing enclosed oocyte (Zhang *et al.*, 2020). Serine is a precursor for many classes of biomolecules and a major donor of one-carbon (1C) units, which support methylation reactions and nucleotide synthesis. The *de novo* serine synthesis pathway (SSP) is upregulated in many cancers (Baksh *et al.*, 2020; Tajan *et al.*, 2021). Serine synthesis is a branching pathway from glycolysis that is initiated by the 3-phosphoglycerate dehydrogenase (PHGDH) enzyme. Similar to cancer cells, which have high proliferative capacity, CCs present high rates of aerobic glycolysis during COC maturation. However, the physiological significance of *de novo* serine synthesis in the COC remains largely unknown.

Studies investigating amino acid metabolism have mainly focused on the embryo, while less information is available about CCs (Zhang *et al.*, 2020; Van Winkle, 2021). Accordingly, the study of CCs may help in understanding the reasons for DOR occurrence. Most previous studies are generally focused on the relations among ageing, ovarian function, and metabolism in human CCs or lack detailed age differentiation. In this study, we conducted research on the gene expression profile of CCs from mature follicles of young infertile women (age <38 years) with DOR or normal ovarian reserve (NOR) via transcriptome sequencing. Our study furthers the understanding of the metabolic and biological pathways that may be associated with DOR in young patients. Understanding these pathways appears to be important in elucidating the molecular mechanism of DOR and is helpful for early intervention and reducing the risk of female factor infertility, especially in the young population.

Materials and methods

Study population

The study population consisted of 107 young infertile women (age <38 years) undergoing ICSI treatment at the reproductive centre of Zhongshan Hospital affiliated with Fudan University, from July 2017 to June 2019. Participants were categorized into an NOR group (control group) and a DOR group. Written informed consent was obtained from all participants enrolled. This study protocol was approved by the ethics committee of Zhongshan Hospital, Fudan University (permit No. B2020110R). Repeated ICSI cycles for couples already included in the study were not considered.

Blood samples were collected from enrolled women on Days 2–4 of menstruation. AMH was measured with an ELISA (Ansh Labs, Webster, TX, USA). Basal FSH, LH, oestradiol (E2), and progesterone (P4) values in serum were determined by chemiluminescence immunoassays (Roche Diagnostics, Mannheim, Germany). Basal AFC was measured via transvaginal ultrasound.

The diagnosis of DOR was made based on the Bologna criteria (Ferraretti *et al.*, 2011). Any of the following three criteria were required to be present: basal FSH ≥ 10 mIU/ml; AFC < 5 in total (for both sides); or AMH < 0.5–1.1 ng/ml. Patients with NOR were enrolled as controls, and all of the following criteria were required to be present: normal basal hormone levels (FSH < 8 mIU/ml, LH < 6 mIU/ml, E2 < 60 pg/ml); AFC ≥ 5 per side; and AMH > 1.1 ng/ml. In this study, all enrolled patients had normal weight (18.5 kg/m^2 < BMI < 25 kg/m^2). Infertile women with PCOS, endometriosis, chromosomal abnormalities, a history of ovarian surgery, or other chronic diseases (e.g. endocrine, metabolic, or autoimmune diseases) were excluded.

Treatment protocol

Patients underwent ovulation induction using an antagonist programme with daily administration of recombinant FSH (rFSH, Gonal-F, Merck-Serono, Brazil). Follicular growth was monitored by vaginal ultrasound, and serum E2 concentrations were measured daily to tailor the dose of rFSH. GnRH antagonist (Cetrotide, Merck-Serono, Brazil) was administered along with rFSH when follicles reached 14 mm. Oocyte maturation was induced using HCG (Lizhu Pharmaceutical Trading Co., Zhuhai, China) (5000–10 000 IU) when at least three follicles ≥18 mm in diameter

were present. Approximately 36 h after HCG injection, oocyte retrieval was performed via ultrasound guidance.

Cumulus cell collection

CCs were collected as described previously (Liu *et al.*, 2015). Briefly, after oocyte retrieval, COCs were collected and washed in G-MOPSTM PLUS medium (Vitrolife, Göteborg, Sweden) to remove blood contaminants. After the COC was cultured for 2 h in G-IVF™ PLUS fertilization medium (Vitrolife, Göteborg, Sweden), the CCs were mechanically stripped from the oocytes by repeated gentle pipetting through a 180-μm diameter stripper pipette (Sunlight, Jacksonville, FL, USA) after brief hyaluronidase (Vitrolife, Göteborg, Sweden) (80 IU/ml) treatment. CCs were individually collected into cryogenic tubes and washed with centrifugation at 300*g* for 5 min in PBS. Each CC sample was derived from all the mature (Metaphase II: MII) oocytes retrieved per patient (i.e. one CC sample corresponds to one individual). The CC samples were then stored at −80°C until RNA extraction. A total of 107 CC samples from 107 participants were collected for further analysis.

RNA isolation

Total RNA was extracted from CCs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quantity was quantified using the NanoDrop ND-2000 (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was confirmed using RNA 6000 Nano Assay kits on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

cDNA library construction and sequencing

The extracted mRNA was used to construct RNA-seq libraries using rRNA-depleted RNAs with the NEBNext® Ultra™ II RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) following the manufacturer's protocol. To ensure that the sample quality requirements were met before testing, library quantification was performed using a Qubit 2.0 instrument, and library insert size was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The libraries were then pooled and sequenced on the HiSeqTM 2500 system (Illumina, San Diego, CA, USA). Gene expression levels were estimated according to the expected number of fragments per kilobase of transcript per million mapped fragments by HTSeq v0.9.1 (Anders *et al.*, 2015). Pairwise comparisons were performed, and clustering was implemented for all expressed genes using the Trinity v2.8.4 package (Grabherr *et al.*, 2011).

Differentially expressed genes

Differentially expressed genes (DEGs) were identified using DESeq2 (Love *et al.*, 2014). The *P* values were adjusted using multiple testing correction to control the false discovery rate (FDR). The DEGs were identified with at least a 2-fold change with adjusted $P < 0.05$ (FDR).

Gene ontology and pathway enrichment analysis

Gene interactions play key roles in specific biological functions. GO (http://www.geneontology.org) annotates genes according to three categories: biological process, molecular function, and cellular component. KEGG analysis was also performed to identify the metabolic pathways that were significantly enriched among the DEGs.

Validation of real-time quantitative PCR

Quantitative real-time PCR (qRT-PCR) assays were carried out to validate the transcriptomic analysis results. Four genes that were deemed biologically significant participants in key serine metabolism gene pathways were chosen from the list of DEGs. To ensure the validity of the results for these selected genes, total RNA was extracted from CC samples from 20 additional patients (10 with NOR and 10 with DOR) with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and 1 μg of total RNA was transcribed to cDNA with a Reverse Transcription Kit (Takara, Tokyo, Japan) following the manufacturer's instructions. qRT-PCR was performed using an ABI Prism 7500 system (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master Mix (Toyobo, Osaka, Japan). Each RNA sample was analysed in triplicate. GAPDH was chosen as a reference gene. Primer sequences are provided in Supplementary Table S1. Relative gene expression level changes in young patients in the NOR and DOR groups were analysed. The relative fold change ($2^{-\Delta\Delta Ct}$) was used to quantify gene expression.

Capillary western blot analysis

Protein content analysis was performed using Simple Western™ Automated Western Blot Systems (Protein Simple, San Jose, CA, USA) using a 12–230 kDa separation module (SM-W004) and anti-rabbit detection module (DM-001) according to the manufacturer's instructions (Mishra *et al.*, 2017; Castle *et al.*, 2019). Briefly, CCs were lysed with lysis buffer, and the protein concentration was determined using the BCA protein assay (Thermo Scientific, Waltham, MA, USA). Lysate was loaded into designated wells in a 25-well plate followed by electrophoresis. The primary antibodies used were mouse anti-actin (Servicebio, Wuhan, China), 1:3000; rabbit anti-PHGDH (Servicebio, Wuhan, China), 1:750; rabbit anti-PSAT1 (Abcam, Cambridge, MA, USA), 1:1000; rabbit anti-PSPH (ABclonal, Wuhan, China), 1:750; and rabbit anti-SHMT2 (Servicebio, Wuhan, China), 1:750. The relative expression of the protein was corrected according to that of actin as the internal reference. The digital image was analysed using Compass software (ProteinSimple, San Jose, CA, USA), and the quantified data of the detected protein were reported as molecular weight and signal/peak intensity.

HPLC–MS/MS analysis

Metabolite extraction from the CC samples, ultrahigh-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS), and bioinformatics analysis were performed as previously described (Hishinuma *et al.*, 2021; Read *et al.*, 2021). Briefly, UHPLC (1290 Infinity LC, Agilent Technologies, Palo Alto, CA, USA) was used to separate samples in the automatic sampler at 4°C with the column temperature constant at 40°C. The mobile phase contained A = 25 mM CH_3COONH_4 + 0.08% formic acid in water and B = 0.1% formic acid in acetonitrile. The gradients were applied at a flow rate of 250 μl/min, and a 2 μl aliquot of each sample was injected. The quality control sample was set up to test and evaluate the stability and repeatability of this system, and a standard mixture of amino acid metabolites was tested to correct the chromatographic retention time.

After sample separation, a 5500 QTRAP mass spectrometer (AB SCIEX, Framingham, MA, USA) was used for mass spectrometric analysis (MRM) in ESI positive mode. The conditions were set as follows: source temperature, 500°C; ion source gas 1 (Gas 1), 40; ion source gas 2 (Gas 2), 40; curtain gas (CUR), 30; ion spray voltage floating, 5500 V; MRM detection mode, ion pair.

For data processing, peak areas and retention times were extracted using MultiQuant software (AB SCIEX, Framingham,

MA, USA). Retention times were corrected using standards of amino acids and their derivatives for metabolite identification.

Statistical analysis

To analyse the clinical and laboratory data, between-group differences were tested using the two-tailed unpaired Student's *t* test or Mann–Whitney *U* test if the data were not normally distributed, and frequency data were analysed with the chi-square test. The statistical analyses were performed using SPSS 17.0 (SPSS 17.0, Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference. The data are presented as the mean ± SD. Comparisons between two sample groups were performed by calculating the fold changes and *P* values obtained by Student's *t* test. GO terms and KEGG pathways were considered significantly enriched if they contained at least a 2-fold change with adjusted $P < 0.05$ (FDR). To test correlation between RNA and protein expression levels in the CCs, Pearson's correlation coefficient was calculated. Metabolites with *P* values < 0.05 and fold changes >1.5 were marked as significantly changed metabolites between sample groups.

Results

Clinical and laboratory parameters of the study population

A total of 107 cycles from 107 young infertile women (age <38 years) undergoing ICSI cycles participated in this study. NOR (n = 54) and DOR (n = 53) subjects were classified according to the Bologna criteria (Ferraretti *et al.*, 2011). Age was defined as the woman's age on the day of oocyte retrieval.

Table 1 shows the baseline characteristics and laboratory results of the patients in the NOR and DOR groups. All participants were under 38 years old, and the mean ages of the NOR and DOR groups, respectively, were similar (31.7 ± 2.9 years versus 32.6 ± 3.0 years) ($P > 0.05$). Patients with DOR had significantly higher FSH (11.3 ± 4.6 versus 6.7 ± 1.2), lower AMH (0.6 ± 0.3 versus 3.4 ± 1.3), and lower AFC (3.8 ± 1.6 versus 14.5 ± 4.6), as expected, since these values were the criteria used for classifying the groups. Basal serum E2, LH, and P4 were not significantly different between the two groups. The BMI values of the women in both groups were within the normal range (18.5 kg/m^2 < BMI < 25 kg/m^2) ($P > 0.05$). The causes of infertility were not significantly different ($P > 0.05$). The duration of infertility in the DOR group was significantly longer ($P < 0.05$).

Likewise, patients with DOR had significantly fewer retrieved oocytes (2.4 ± 2.2 versus 12.1 ± 5.3) and MII oocytes (2.1 ± 2.0 versus 9.9 ± 4.9) ($P < 0.0001$). There was no significant difference between the NOR and DOR groups, respectively, for the rates of MII oocytes (82.1% versus 86.8%), fertilization (80.7% versus 78.8%), viable embryos (73.7% versus 72.5%), and high-quality embryos (42.8% versus 49.0%) ($P > 0.05$). These data indicate that DOR is associated with decreases in the number of oocytes retrieved and the number of MII oocytes but has little influence on embryo quality.

Table 1. Patient characteristics and laboratory results in young women with diminished or normal ovarian reserve.

Characteristic	NOR group	DOR group	P value
No. of patients	53	54	–
Age (years)	31.7 ± 2.9	32.6 ± 3.0	0.10
Duration of infertility (years)	3.5 ± 2.8	6.0 ± 4.7	<0.05
Infertility type (n, %)	–	–	–
Primary	36 (67.9)	31 (57.4)	0.261
Secondary	17 (32.1)	23 (42.6)	0.261
Cause of infertility (n, %)	–	–	–
Female factors	40 (75.5)	45 (83.3)	0.314
Male factors	11 (20.8)	5 (7.4)	0.095
Mixed factors	2 (3.8)	4 (7.4)	0.414
Unknown factors	0	0	–
BMI (kg/m^2)	21.9 ± 2.2	21.8 ± 2.2	0.905
AFC (n)	14.5 ± 4.6	3.8 ± 1.6	<0.0001
Basal serum FSH (mIU/ml)	6.7 ± 1.2	11.3 ± 4.6	<0.0001
Basal serum LH (mIU/ml)	5.7 ± 2.0	5.6 ± 3.5	0.782
Basal serum E2 (pg/ml)	46.4 ± 16.4	47.0 ± 20.0	0.865
Basal serum P4 (ng/ml)	0.2 ± 0.1	0.2 ± 0.1	0.191
Basal serum AMH (ng/ml)	3.4 ± 1.3	0.6 ± 0.3	<0.0001
Oocytes retrieved (n)	12.1 ± 5.3	2.4 ± 2.2	<0.0001
MII oocyte number (n)	9.9 ± 4.9	2.1 ± 2.0	<0.0001
MII oocyte rate (n, %)	505 (82.1)	66 (86.8)	0.305
Normal fertilization rate (n, %)	411 (80.7)	52 (78.8)	0.705
Effective embryo rate (n, %)	303 (73.7)	37 (72.5)	0.858
High-quality embryo rate (n, %)	176 (42.8)	25 (49.0)	0.400

Data are presented as the mean ± SD or %.
Between-group differences were tested using the two-tailed unpaired Student's *t*-test or Mann–Whitney *U* test if the data were not normally distributed, and frequency data were analysed with the chi-square test.
$P < 0.05$ is considered statistically significant.
NOR, normal ovarian reserve; DOR, diminished ovarian reserve; AFC, antral follicle count; AMH, anti-Müllerian hormone; E2, oestradiol; P4, progesterone; MII, metaphase II.
Female factors include ovulation disorders, blocked fallopian tubes, uterus issues, endometriosis, and other female diagnoses. Male factors include low sperm count, poor sperm quality, and abnormal sperm function. Mixed factors include problems that affect both partners. The unknown factor classification is applied when a precise reason for infertility cannot be identified, even with extensive examinations of both individuals involved.

Identification of DEGs in CCs between the DOR and NOR groups

To identify the genes and pathways altered in CCs in young patients with DOR, RNA was extracted from the CCs, and a comprehensive genome-wide transcriptomic analysis was performed. Genes with $\log_2$(fold change) >1 and adjusted *P* value <0.05 were considered significant DEGs. Hierarchal clustering of the DEGs was performed. Heatmap results showed that DOR and NOR samples were partitioned into two distinct clusters, suggesting high reproducibility of the sequencing data. Furthermore, heatmap results revealed that the gene expression pattern was different between the DOR and NOR groups (Fig. 1A). Overall, 95 genes were differentially expressed between the NOR and DOR groups. Among these DEGs, 62 genes were upregulated, while 33 genes were downregulated, in the DOR group compared with the NOR group (Fig. 1B).

Functional enrichment analysis of DEGs

GO enrichment analysis was conducted to characterize the distribution of young DOR-related DEGs among different biological functions. The results showed that 30 GO terms were significantly enriched among the DEGs, of which 4, 6, and 20 were cellular component, molecular function, and biological process terms, respectively (FDR < 0.05) (Fig. 2A). In the cellular component category, the most significantly enriched GO terms were platelet dense tubular network membrane and sarcoplasmic reticulum. In the molecular function category, the most significantly enriched GO terms were inositol-1,4,5-trisphosphate binding and oxidoreductase activity. For biological processes, 20 GO terms were significantly enriched, including carboxylic acid biosynthetic process and L-serine biosynthetic process (Fig. 2A). Moreover, we performed KEGG analysis to explore the related signalling pathways. We found that several important pathways, including biosynthesis of amino acids, carbon metabolism, cellular senescence, and glycine, serine and threonine metabolism, were increased in the DOR group (FDR < 0.05) (Fig. 2B; Supplementary

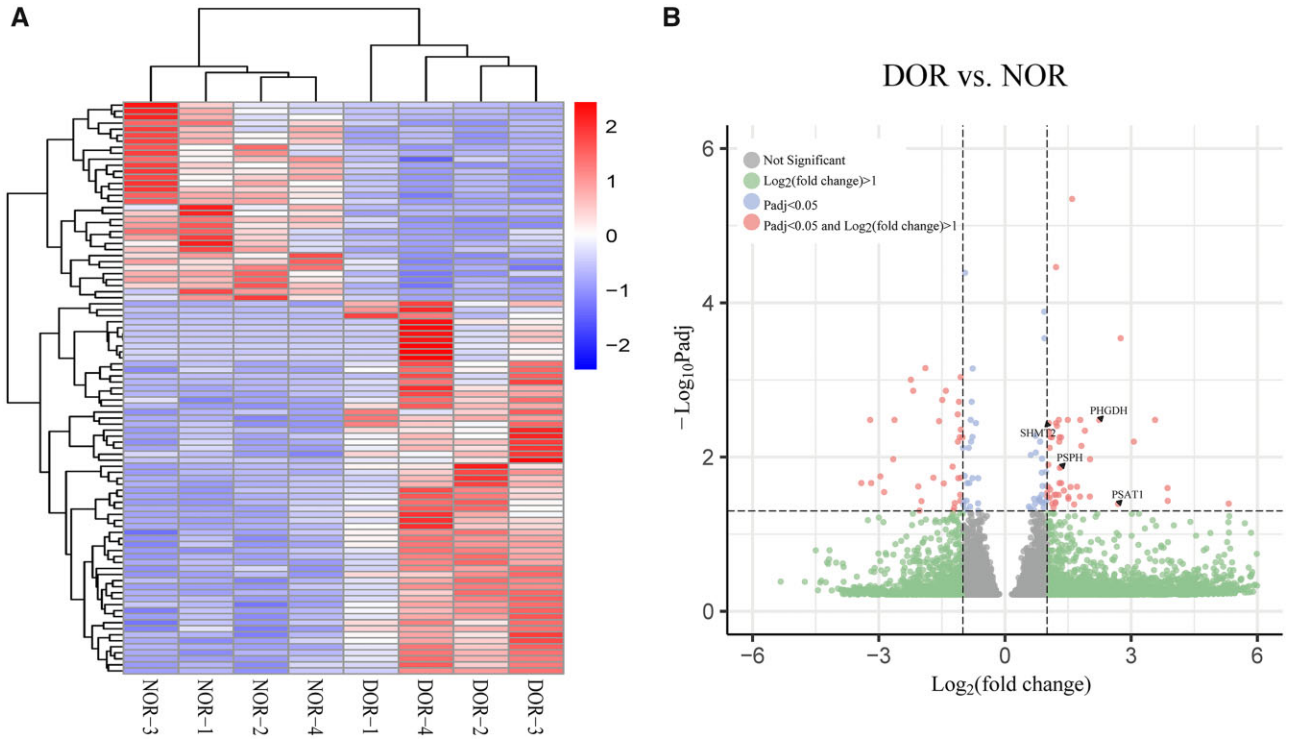

Figure 1. Hierarchical heatmap and volcano plot analysis of differentially expressed genes in cumulus cells between young patients with diminished ovarian reserve and those with normal ovarian reserve. (**A**) Heatmap of significant differentially expressed genes (DEGs) in cumulus cells (CCs) between young patients with diminished ovarian reserve (DOR) and those with normal ovarian reserve (NOR) (n = 4). Red and blue on the y-axis represent upregulated and downregulated DEGs, respectively. (**B**) Volcano plot for DEGs in CCs between young patients with DOR or NOR, showing the distribution of significance ($-\log_{10}$Padj) and fold change ($\log_2$(fold change)) for all genes. Genes in pink are significant DEGs (Padj < 0.05 and $\log_2$(fold change) >1, n = 4).

Figs S1 and S2). Figure 2B also shows that genes such as PHGDH, phosphoserine aminotransferase 1 (PSAT1), phosphoserine phosphatase (PSPH), and serine hydroxymethyltransferase-2 (SHMT2) were involved in biosynthesis of amino acids, carbon metabolism, and glycine, serine and threonine metabolism.

The serine biosynthesis pathway was upregulated in CCs in patients with DOR

To ensure the validity of the transcriptomic analysis results, 20 individual CCs (10 from patients with NOR and 10 from patients with DOR) were tested using qRT–PCR. The results revealed that three genes (PHGDH, PSAT1, and PSPH), encoding metabolic enzymes involved in the SSP, were upregulated in young DOR CCs, validating the RNA-seq data (Figs 1B and 3). Of these, PHGDH was upregulated over 3.8-fold (Fig. 3A).

Then, to investigate the protein levels of serine synthesis enzymes and the correlation between mRNA and protein levels, CCs from 36 patients were collected (24 from NOR and 12 from DOR). The CC samples collected from each patient were divided into two parts; one for qRT-PCR and the other for capillary western blotting. The results from one patient sample of the DOR group were discarded because no significant band was found for the reference. Results showed that PHGDH, PSAT1, and PSPH protein levels were higher in the CCs of young patients with DOR (Fig. 3B; Supplementary Fig. S3). The correlation analysis results indicated that the levels of mRNA and protein related to serine synthesis genes in each CC sample were highly correlated, with Pearson's correlation coefficients ranging from 0.61 to 0.80 (Supplementary Fig. S4).

SHMT2 is a mitochondrial enzyme that transfers a methyl group from serine to tetrahydrofolate (THF), producing glycine, and methylene-THF. Therefore, serine provides one-carbon units for carbon metabolism and is involved in a variety of methylation reactions. qRT-PCR and capillary western blot results showed that SHMT2 was upregulated in the CCs of young patients with DOR (Fig. 3A and B).

Guided by the finding of serine synthesis metabolic enzyme changes, the relative contents of serine and glycine in CCs were analysed by UPLC–MS/MS. The results showed that the relative contents of serine and glycine were upregulated in CCs from the young DOR group (Fig. 3C). Figure 3D shows a schematic of serine and one-carbon metabolism in CCs. Although L-serine is available from the extracellular environment, it can be synthesized from the glycolytic intermediate 3-phosphoglycerate (3-PG). PHGDH, PSAT1, and PSPH have been reported as key enzymes in the SSP. Among them, PHGDH, which catalyses the first step, is the rate-limiting enzyme in the pathway. Serine feeds into the folate and methionine cycles via SHMT2, transferring one carbon to THF, which eventually provides one-carbon units for methylation reactions (Fig. 3D).

Discussion

In this study, we retrospectively analysed oocyte development data after ICSI from young infertile women with DOR. Our studies have demonstrated that DOR in young women is associated with decreases in the number of oocytes acquired and the number of MII oocytes but has little influence on embryo quality. This result is consistent with those of previous studies (Thum *et al.*, 2008; Chang *et al.*, 2018; Morin *et al.*, 2018). However, several studies have also suggested that patients with evidence of follicular depletion also exhibit reduced oocyte developmental competence

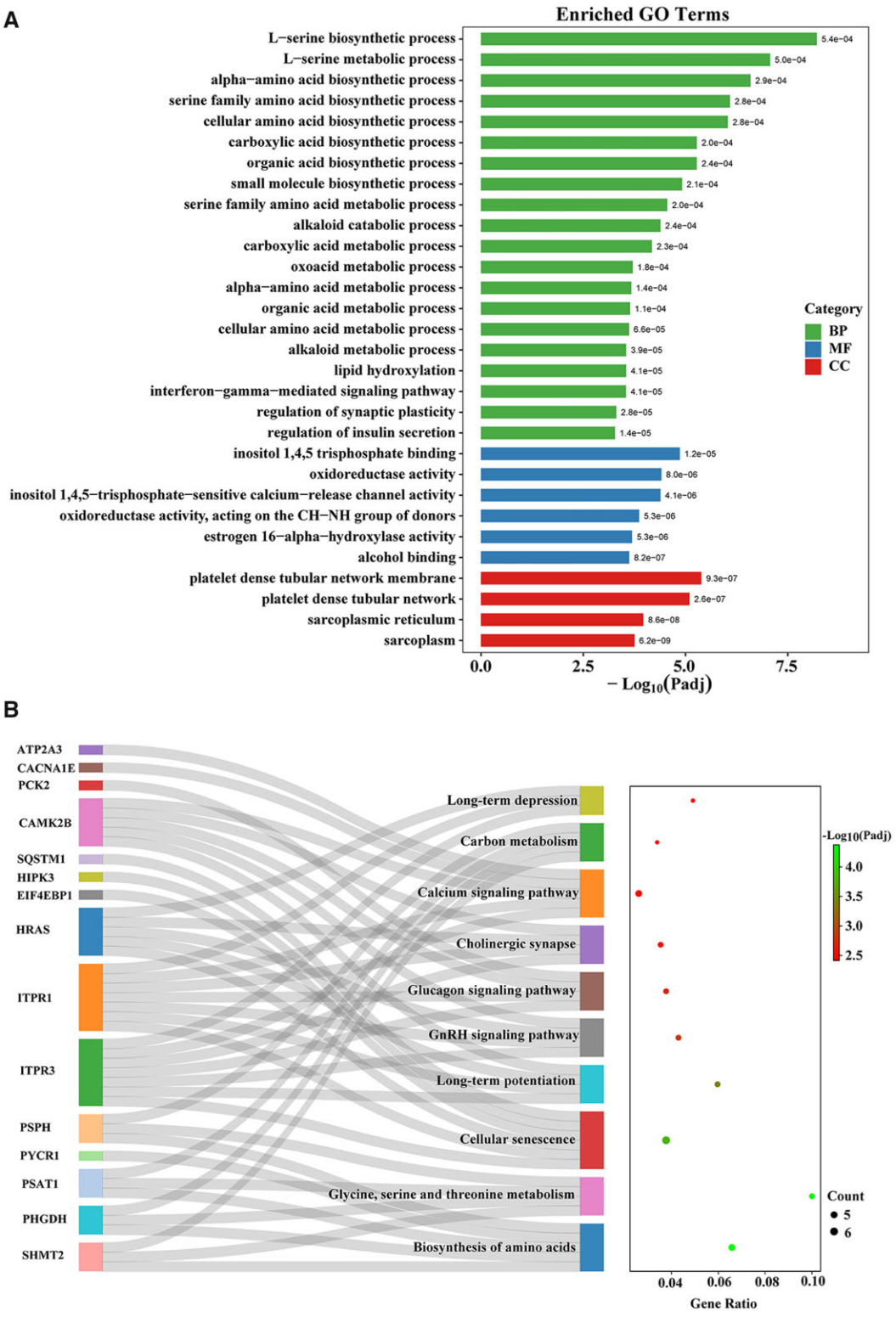

Figure 2. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses of differentially expressed genes. (**A**) Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs). Thirty GO terms were significantly enriched among the DEGs, of which 20, 6, and 4 were biological process (BP), molecular function (MF), and cellular component (CC) terms, respectively (false discovery rate < 0.05). (**B**) Sankey plot showing the relationships of DEGs with the pathways enriched in cumulus cells (CCs). The dot plot shows the top 10 identified KEGG pathways enriched in CCs from young patients with diminished ovarian reserve (DOR). Green and red indicate high and low *P* values, respectively.

(Haadsma *et al.*, 2010; Cohen *et al.*, 2017; Tarasconi *et al.*, 2017). A major limitation of the current literature characterizing oocyte quality in the context of DOR is that the studies rarely untangle physiological ageing (DOR in older patients) from premature ageing in ovarian function (DOR in younger patients), even though depletion of the follicular pool accompanies reproductive ageing (Bowen *et al.*, 2007; Sun *et al.*, 2008; Laisk *et al.*, 2019). All these studies lack detailed age differentiation. In this study, we

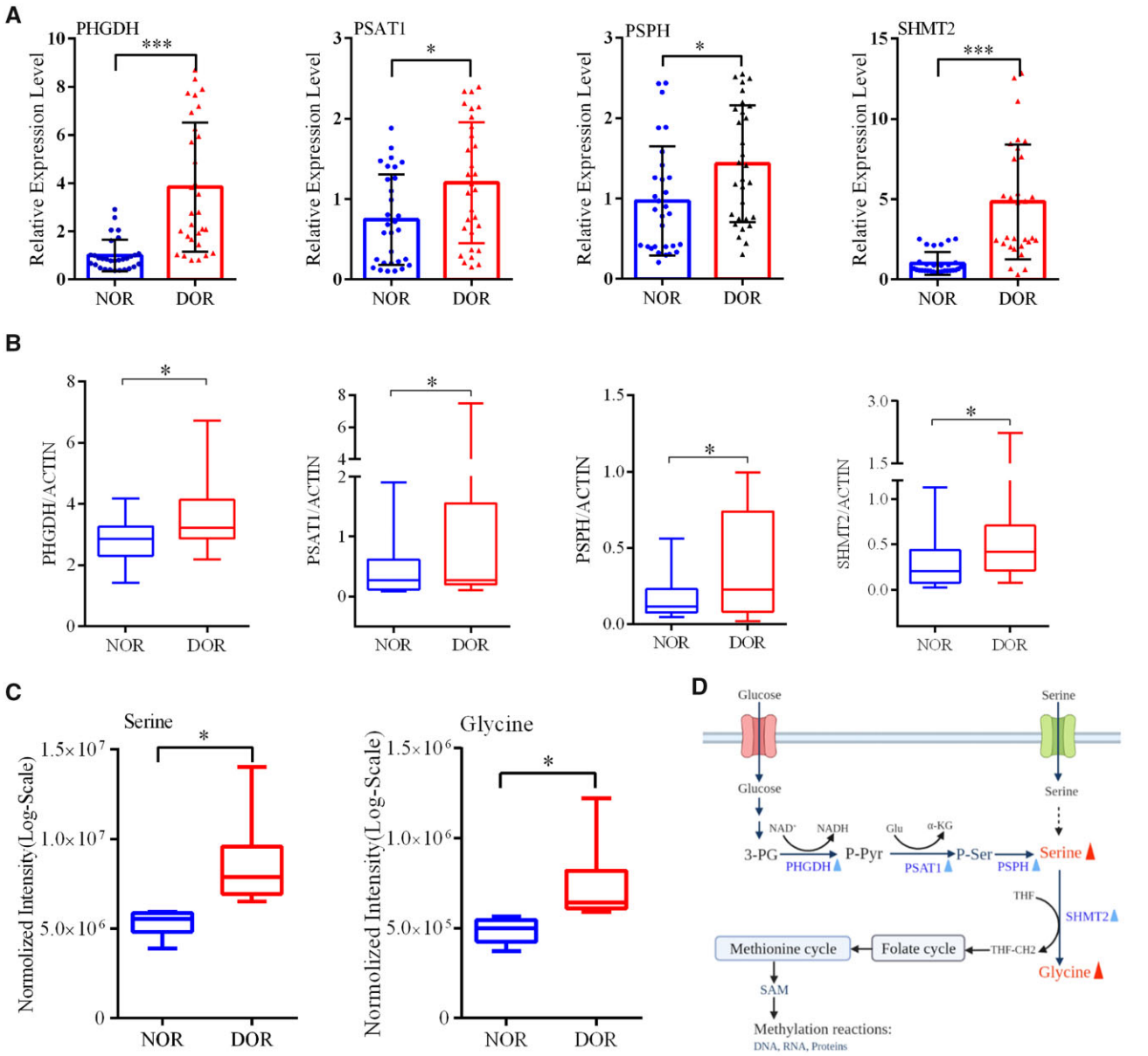

Figure 3. Serine biosynthesis pathway activity was markedly increased in cumulus cells from young patients with diminished ovarian reserve. (**A**) Transcription of serine synthesis genes (phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), phosphoserine phosphatase (PSPH) and serine hydroxymethyltransferase-2 (SHMT2) were verified by quantitative real-time PCR (n = 10). (**B**) Quantification of serine synthesis genes (PHGDH, PSAT1, and PSPH) and SHMT2 protein expression in cumulus cells from 35 patients (24 from normal ovarian reserve (NOR) and 11 from diminished ovarian reserve (DOR)) analysed by capillary western blot. (**C**) The relative contents of serine and glycine in cumulus cells (CCs) were analysed by ultrahigh-performance liquid chromatography-tandem mass spectrometry (n = 6). (**D**) Serine and one-carbon metabolism (the folate cycle and the methionine cycle) link the SSP and DNA methylation reactions. Serine and glycine levels, indicated by red-filled triangles, were increased in CCs from young patients with DOR; metabolic enzymes upregulated in CCs from young patients with DOR are denoted by blue-filled triangles. The values are the means ± SDs. The asterisks of the bar chart indicate statistically significant differences (*$P < 0.05$; ***$P < 0.001$).

studied only patients <38 years of age to separate out the impacts of age-related changes in oocyte quality, and the inclusion criteria were strictly followed.

Although no decline in embryo quality was found in this study, the extensive literature reports poor pregnancy outcomes, a high spontaneous abortion rate, and a lower live birth rate (LBR) in young patients with DOR, as beginning with fewer oocytes leads to a lower probability of acquiring transplantable embryos in each round of ART (Morin *et al.*, 2018; Bunnewell *et al.*, 2020). Moreover, young women with DOR are at risk for accelerated premature ovarian insufficiency, resulting in infertility, but the mechanism regulating this process is unclear.

In the ovarian follicle, CCs form a direct barrier between the oocyte and external environment. Owing to their bidirectional communication, CCs have a direct impact on the maturation and development of the oocyte (Bulgurcuoglu *et al.*, 2022; Tian *et al.*, 2023). In this study, we performed transcriptome sequencing to obtain expression data for CCs from mature follicles of young women (<38 years) undergoing ovarian stimulation. A distinct difference in CC gene expression was found between young women with NOR and those with DOR. GO and KEGG analyses revealed major differences in the L-serine biosynthetic process. All three key enzymes (PHGDH, PSAT1, and PSPH) involved in *de novo* serine synthesis were upregulated in CCs of young women

with DOR. This observation was confirmed by qRT-PCR testing of an additional 20 samples from patients with NOR/DOR. In addition, the protein levels of all three key enzymes (PHGDH, PSAT1, and PSPH) were observed to be upregulated in CCs of young women with DOR. This observation was further confirmed by testing a set of 36 patient samples (including both NOR and DOR groups) using capillary western blot analysis. Furthermore, a significant correlation was found between the expression levels of mRNA and protein content associated with serine synthesis genes across each CC sample. In addition, MS analysis indicated that the relative contents of serine and glycine were upregulated in DOR CCs. Our data clearly demonstrate that the *de novo* SSP, which diverts 3-phosphoglycerate from glycolysis to serine synthesis, was upregulated in CCs from young patients with DOR. Consequently, we speculated that the decreased ovarian function in young patients with DOR is associated with increased serine synthesis in CCs, providing insight into the possible causes of DOR among young women.

Serine, as the principal source of one-carbon groups, can be converted to glycine by serine hydroxymethyltransferases (SHMT1 and SHMT2) to provide a one-carbon unit for one-carbon metabolism (Parker and Metallo, 2016; Pan *et al.*, 2021). In our study, we found that the SHMT2 protein and serine and glycine contents were significantly upregulated in CCs from young patients with DOR. This suggests that serine–glycine one-carbon metabolism was upregulated, a feature that is also associated with the rapid proliferation of cancer cells (Amelio *et al.*, 2014). The proliferation of granulosa cells is crucial for the development of follicles, and CC cell proliferation *in vivo* is inhibited after exposure to the HCG trigger (Amelio *et al.*, 2014). A previous study showed a higher rate of cell proliferation in the granulosa cells of patients with DOR (Woo *et al.*, 2018). This is considered to be abnormal because granulosa cells should have begun differentiation, luteinization, and reduced cell proliferation after encountering the HCG trigger. It is well known that DNA methylation processes are strongly correlated with one-carbon metabolism. The main metabolic product of one-carbon metabolism, S-adenosyl-methionine, is a major methyl donor required for DNA methylation. Meanwhile, abnormal DNA methylation patterns at CpG islands have been observed in mural granulosa cells from women with DOR (Olsen *et al.*, 2020; Bernabeu *et al.*, 2021; Olsen *et al.*, 2021). Our data showed that serine and glycine were upregulated in CCs from young women with DOR, which leads us to suggest that serine synthetic pathway alterations may contribute to aberrant DNA methylation patterns.

DOR is a severe disorder with a complex multifactorial pathophysiological mechanism that causes infertility in females. An increasing number of DOR cases have been diagnosed in young patients in recent years. Several studies revealed that abnormal metabolism is present in luteinized granulosa cells of patients with DOR (Chen *et al.*, 2022). Yang *et al.* (2022) and Bildik *et al.* (2022) revealed that decreased cholesterol metabolism in granulosa cells might be a possible mechanism for DOR. Our study investigated the aetiology of DOR from the perspective of amino acid metabolism in CCs. Few previous studies have explored the relation between follicular development and amino acid metabolism. Hemmings *et al.* (2013) reported that the developmental competence of oocytes is associated with amino acid turnover capacity. Akamine *et al.* (2021) confirmed that fertilization, embryo quality, and clinical pregnancy could be affected by one-carbon metabolism in follicular fluid. Moslehi *et al.* (2021) revealed that serum concentrations of 29 metabolites, including serine, were positively correlated with the annual rate of AMH decline. These studies demonstrated that ovarian ageing is associated with amino acid metabolism. Our research provides new evidence that the metabolism of certain amino acids is also clearly altered in the CCs of young women with DOR.

Most studies lack detailed age differentiation in their analysis. Indeed, age is considered an important contributor to DOR. The pathogenic mechanisms likely differ between young women with DOR and cases associated with advanced maternal age. Advanced maternal age is associated with a reduction in not only oocyte quantity but also oocyte quality. In contrast, oocyte quality and embryo developmental competence seem to be unaffected in young patients. Previous studies found that in older women, DOR was associated with decreased oocyte developmental potential, abnormal mitochondrial function, and impaired oxidative phosphorylation in CCs (Cecchino *et al.*, 2021; Lu *et al.*, 2022). However, in this study, we confirmed that ovarian reserve was affected through different physiological mechanisms in a young population; rather, we found that the SSP is altered and that this alteration may be an important contributor to the diminished ovarian function in the young population. Activating the SSP has been assumed to be important not only for serine synthesis but also for α-ketoglutarate generation and redox maintenance in cells. The exact mechanisms by which the SSP in CCs regulates DOR still require further study.

One limitation of our study is the small sample size. To reduce the potential for confounding, stringent exclusion and inclusion criteria were employed. In this study, CCs were pooled from each patient, meaning that our findings could not be extrapolated to embryonic potential at the individual oocyte level. The other limitation of this study is that pregnancy outcomes were not analysed. In addition, only women undergoing ICSI were examined, since this was a prerequisite for the acquisition of CCs in this study. ICSI is generally indicated owing to associated unexplained infertility, male factor infertility, or unexplained fertilization failure in a prior IVF cycle, which may cause selection bias.

In conclusion, we reported here an obviously decreased number of oocytes retrieved and MII oocytes obtained in young patients with DOR. We present new evidence that alterations of the SSP in CCs make important contributions to the cause of DOR in the young population. Gene expression and metabolome profiling might open up new possibilities for the study of follicle, oocyte, and embryo metabolism at many levels and with broad scope, which should be key for understanding the mechanisms of ovarian hypofunction in young women.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

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

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Authors' roles

H.S. and Y.L. contributed to the current study design. X.L. performed the CCs isolations and CCs-related experiments. Y.L. did the statistical analyses and interpretations. X.L. contributed to the capillary western blot and data analysis. X.D. and S.L. contributed to patient recruitment and oocyte retrievals. Y.L. and X.W. performed data collection and register. X.L. and Y.L. contributed to the drafting of the manuscript. H.S. and B.T. revised the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

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