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Correlation between follicular fluid 25-hydroxyvitamin D levels and IVF embryo quality in women with diminished ovarian reserve: A metabolomic study

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Abstract

Introduction: Improving pregnancy outcomes for women with diminished ovarian reserve (DOR) is a key area of focus in assisted reproductive technology (ART).

Objective: This study aims to investigate the association between 25-hydroxyvitamin D (25-[OH]D) levels in follicular fluid (FF) of women with diminished ovarian reserve (DOR) and embryo quality during *in vitro* fertilization (IVF), and to perform a metabolomics analysis.

Methods: A total of 54 women with DOR and 62 women with normal ovarian reserve (control) were enrolled. Based on FF 25-(OH)D levels, both DOR and control cases were stratified into two subgroups: the vitamin D non-deficient (VDH) and vitamin D deficient (VDL). Baseline characteristics and IVF laboratory outcomes were compared among groups and subgroups. Untargeted metabolomic profiling of FF was then performed to identify differences between subgroups.

Results: FF vitamin D levels were significantly lower in the DOR group than in controls. In both cohorts, the normal fertilization rate was significantly higher in the VDH subgroup than in the VDL subgroup. Metabolites significantly upregulated in the VDH subgroup included phospholipids, hydroxychloroquine, and serinyl-tryptophan. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis revealed significant enrichment in glycerophospholipid metabolism in the VDH subgroup. In the DOR group, the DOR-VDH subgroup exhibited upregulation of steroid hormone biosynthesis and vitamin metabolism pathways, particularly those mediated by cytochrome P450, relative to the DOR-VDL subgroup.

Conclusion: Vitamin D may enhance the follicular microenvironment in DOR patients primarily by regulating lipid metabolism, thereby improving oocyte and embryo quality and developmental potential.

Keywords: Vitamin D; Diminished ovarian reserve; *In vitro* fertilization; Follicular fluid; Lipid metabolism

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1. Introduction

The ovary, the primary female reproductive organ, is responsible for oocyte production and the synthesis and secretion of estrogens, progesterone, and a small amounts of androgens. Normal ovarian function is essential for maintaining regular menstrual cycles and fertility. Diminished ovarian reserve (DOR), characterized by a decline in oocyte quantity and/or quality due to multiple causes, can lead to reduced fertility. This condition is typically associated with a decreased antral follicle count (AFC), elevated basal follicle-stimulating hormone (FSH) levels, and reduced anti-Müllerian hormone (AMH) levels.[1] DOR can be categorized into physiological and pathological forms. The former is primarily age-related, whereas the latter results from environmental, lifestyle, or iatrogenic factors independent of age. Given the increasing trend of delayed childbearing and the rising prevalence of pathological DOR, approximately 10% of women may experience a premature decline in ovarian reserve due to these factors.[2,3] Therefore, early diagnosis and management of DOR are crucial for preserving and enhancing female fertility.

Vitamin D (VD), a fat-soluble secosteroid prohormone, undergoes sequential hydroxylation: it is converted in the liver to 25-hydroxyvitamin D by 25-hydroxylases (primarily CYP2R1, with CYP27A1 also contributing) and subsequently in the kidney to the active metabolite 1,25-dihydroxyvitamin D (1,25-$[OH]_2$D) by CYP27B1.[4] Notably, 25-(OH)D, a key metabolic intermediate with a half-life of approximately three weeks, serves as a reliable biomarker for assessing VD status *in vivo*. VD deficiency has emerged as a global public health concern, particularly given the relatively high prevalence of VD deficiency and insufficiency among women of reproductive age, which may adversely affect reproductive health.[5]

Systematic reviews have demonstrated a complex association between VD status and AMH levels.[6] Moreover, VD deficiency has been linked to several reproductive disorders, including polycystic ovary syndrome (PCOS), endometriosis, and infertility.[7] Supplementation with VD has been shown to improve pregnancy and ovulation rates in women with PCOS, as well as to reduce serum androgens, FSH levels, and the risk of early miscarriage.[8] Currently, the influence of VD status on pregnancy outcomes following *in vitro* fertilization (IVF) and embryo transfer (ET) has become an area of growing interest in reproductive medicine.

Although several studies have explored the relationship between VD levels in serum and/or follicular fluid (FF) and assisted reproductive technology outcomes, the findings remain inconsistent. This study aims to investigate the association between FF 25-(OH)D levels and IVF outcomes in women with DOR. Furthermore, untargeted metabolomic analysis of FF was conducted to identify potential metabolic pathways associated with VD status and IVF success. These findings may provide novel insights into the pathophysiology of DOR and help identify potential protective factors that improve IVF outcomes in this patient population.

2. Materials and methods

2.1. Study population

A total of 54 patients with DOR who underwent IVF at the Reproductive Medicine Center of the First Hospital of Lanzhou University between November 2023 and June 2024 were enrolled in this study. Additionally, 62 women with normal ovarian reserve undergoing IVF due to tubal factor infertility during the same period were recruited as controls for comparative analysis. The study protocol was approved by the Ethics Committee of Reproductive Medicine of the First Hospital of Lanzhou University (Approval No. LDYYSZLL2023-26), and written informed consent was obtained from all participants prior to enrollment.

The inclusion criteria were as follows:

(i) Age ≤40 years.
(ii) Diagnostic criteria for DOR required fulfillment of any two of the following conditions:

- AFC < 7 follicles.
- AMH < 1.1 ng/mL.
- Basal FSH ≥10 IU/L.

The exclusion criteria included: age > 40 years; uterine fibroids; endometriosis (stage III–IV); PCOS; premature ovarian failure; uterine malformations; thyroid dysfunction; chromosomal abnormalities; history of tuberculosis; autoimmune diseases; chronic systemic diseases (e.g., diabetes mellitus, renal insufficiency, and hypercholesterolemia); treatment involving preimplantation genetic testing cycles or oocyte-donation cycles; and male partners with moderate to severe oligoasthenoteratozoospermia.

2.2. *In vitro* fertilization cycle protocol

All patients were treated with a progestin-primed ovarian stimulation (PPOS) regimen. The initial dose of gonadotropins was determined according to patients' age, body mass index, AFC, and basal FSH levels. Follicular growth and endometrial thickness were monitored via transvaginal ultrasound. Gonadotropin administration was discontinued when the dominant follicles reached a diameter of ≥18 mm. Serum levels of estradiol (E_2),

luteinizing hormone (LH), and progesterone (P_4) were measured. On the same evening, 0.2 mg of gonadotropin-releasing hormone agonist was administered, along with 2,000–6,000 IU of human chorionic gonadotropin as a "double-trigger" approach. Oocyte retrieval was performed 35–37 hours later.

2.3. Collection of follicular fluid samples

On the day of oocyte retrieval, the first tube of clear, pooled FF was collected under transvaginal ultrasound guidance. Following centrifugation using a standardized protocol, the samples were stored at −80 °C for future analysis.

2.4. Outcome measures

The primary outcome of the study was the FF 25-(OH)D level. The following parameters were assessed on menstrual cycle days 2–3: FSH, LH, E_2, P_4, prolactin, AMH, thyroid-stimulating hormone, and basal AFC.

Laboratory outcomes during the IVF cycle included the following indices:

(i) Oocyte retrieval rate = $\frac{Number\ of\ retrieved\ oocytes}{Total\ number\ of\ punctured\ follicles} \times 100\%$

(ii) Metaphase II (MII) oocyte rate = $\frac{Number\ of\ MII\ oocytes}{Total\ number\ of\ retrieved\ oocytes} \times 100\%$

(iii) Normal fertilization rate = $\frac{Number\ of\ oocytes\ exhibiting\ two\ pronuclei\ and\ two\ polar\ bodies\ on\ the\ first\ day\ of\ IVF}{Total\ number\ of\ oocytes\ inseminated} \times 100\%$

(iv) Normal cleavage rate = $\frac{Number\ of\ cleaved\ embryos}{Number\ of\ normal\ fertilized\ oocytes} \times 100\%$

(v) Utilizable embryo rate on day 3 = $\frac{Number\ of\ morphologically\ acceptable\ embryos\ on\ day\ 3\ of\ IVF}{Number\ of\ normally\ fertilized\ oocytes} \times 100\%$

(vi) High-quality embryo rate on day 3 $\frac{Number\ of\ high-quality\ embryos\ on\ day\ 3\ of\ IVF}{Number\ of\ normally\ fertilized\ oocytes} \times 100\%$

2.5. Untargeted metabolomics

The criteria for defining VD nutritional status vary globally. In this study, based on the 2023 "Expert Consensus on the Evaluation and Improvement of Vitamin D Nutritional Status" in China,[9] 25(OH)D ≥12 ng/mL was considered non-deficient, and <12 ng/mL was considered deficient.

According to FF 25-(OH)D levels, participants in both the DOR and control groups were further stratified into two subgroups: VD non-deficient (VDH; ≥12 ng/mL) and VD deficient (VDL; <12 ng/mL). Untargeted metabolomics analysis was performed on 10 FF samples randomly selected from each subgroup.

2.5.1. Sample preparation

Follicular fluid (100 μL) was transferred into a 2 mL centrifuge tube, and 400 μL of acetonitrile/methanol extraction solution was added. The mixture was vortexed and then subjected to low-temperature ultrasonication at 5 °C for 30 min, followed by incubation at −20 °C to precipitate proteins. The mixture was centrifuged at 13,000 × g at 4 °C for 15 min, and the supernatant was collected and resuspended with 100 μL of acetonitrile/aqueous reagent. The samples were again extracted by low-temperature ultrasonication at 5 °C for 5 min and centrifuged at 13,000 × g for 10 min. The supernatant was collected and reconstituted with 100 μL of an acetonitrile/water mixture.

For quality control (QC), equal aliquots (20 μL) from each sample were pooled to create QC samples, which were injected at regular intervals throughout the analytical sequence to monitor instrument performance and reproducibility. Raw data were preprocessed, including missing value imputation and normalization, and the injection order of all study samples (excluding QC samples) was fully randomized.

2.5.2. Chromatographic and mass spectrometric conditions

The sample (10 μL) was separated on a HSS T3 column and then detected by mass spectrometry. The mobile phase A was water/acetonitrile solution containing 0.1% formic acid, and mobile phase B was acetonitrile/isopropanol/water solution containing 0.1% formic acid. The separation was performed using gradient elution at 0.40 mL/min with the column maintained at 40 °C, and data were acquired in both positive and negative ionization modes.

Mass spectrometry detection was carried out using a Thermo Fisher Exploris 240 UHPLC-FTMS system (Shanghai Meiji Biomedical Technology Co., Ltd., China) in both positive and negative ionization modes, with a scan

range of 70–1,050 m/z.

2.5.3. Data processing

Features with >80% non-zero values in at least one group were retained, whereas those with a relative standard deviation >30% in QC samples were excluded. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed using the ropls package in R (version 1.6.2, Etienne A. Thevenot France). The false discovery rate was controlled using the Benjamini–Hochberg procedure, with an adjusted p-value < 0.05 considered statistically significant.

To identify biologically relevant differential metabolites, a PLS-DA model was constructed and subjected to seven-fold cross-validation. Metabolites with a variable importance in projection (VIP) score >1, p < 0.05 (Student's t-test), and meeting the fold-change (FC) threshold were considered significant. These metabolites were subsequently subjected to pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database via the SciPy platform in Python (version 1.0.0, Travis Oliphant, USA), with all identified metabolites as the background.

Statistical significance was defined as false discovery rate <0.05. Metabolite classification and visualization were performed by matching against the KEGG compound database. All analyses were conducted using the Major BIOS cloud platform (Nanjing Baole Software Co., Ltd., China).

2.6. Statistical methods

Data were analyzed using SPSS version 25.0 (IBM, USA). Continuous variables were expressed as mean ± standard deviation. Comparisons between groups were performed using the Student's t-test for normally distributed data. Categorical variables were expressed as percentages (%) and compared using the $\chi 2$ test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Comparison of baseline characteristics between the diminished ovarian reserve and control groups

A total of 116 participants who met the inclusion and exclusion criteria were enrolled in this study, including 54 patients in the DOR group and 62 patients in the control group. No statistically significant differences were observed between the DOR and control groups with respect to age, body mass index, duration of infertility, or the rate of primary infertility ($p > 0.05$).

Regarding baseline hormonal parameters, the DOR group exhibited significantly higher basal FSH levels and significantly lower AMH levels and AFC compared with the control group. In contrast, basal LH and basal E2 levels did not differ significantly between the two groups ($p > 0.05$). Furthermore, FF VD levels were significantly lower in the DOR group than in the control group ($p < 0.05$) (Table 1).

Table 1. Comparison of baseline characteristics between the diminished ovarian reserve group and the control group

Baseline characteristic	DOR (n = 54)	Control (n = 62)	p-value
Age (year)	35.69 ± 3.75	34.87 ± 4.26	0.285
Body mass index (kg/m^2)	22.88 ± 3.08	22.81 ± 3.21	0.901
Duration of infertility (year)	4.28 ± 4.04	3.93 ± 3.29	0.603
Primary infertility rate (n [%])	46.30% (25/54)	50% (31/62)	0.690
Baseline hormones			
FSH (mIU/ml)	12.69 ± 7.96	7.11 ± 1.73	<0.01
LH (mIU/ml)	7.34 ± 10.93	5.37 ± 2.65	0.244
E_2 (pg /ml)	44.03 ± 24.15	43.01 ± 22.37	0.855
AMH (ng/mL)	0.45 ± 0.32	2.58 ± 1.31	<0.01
AFC	3.83 ± 1.84	12.26 ± 4.26	<0.01
VD (ng/mL)	7.23 ± 5.62	9.71 ± 6.92	0.038

Abbreviations: AFC: Antral follicle count; AMH: Anti-Müllerian hormone; DOR: Diminished ovarian reserve; E_2: Estradiol; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; VD: Vitamin D.

3.2. Comparison of oocyte and embryo quality among subgroups stratified by follicular fluid 25-hydroxyvitamin D levels

Based on FF 25-(OH)D levels, both the DOR and control groups were divided into two subgroups: VDH and VDL. In the DOR group, the normal fertilization rate was significantly higher in the DOR-VDH subgroup than in the DOR-VDL subgroup ($p < 0.05$). However, no statistically significant differences were observed between the two subgroups in terms of oocyte retrieval rate, MII oocyte ratio, normal cleavage rate, utilizable embryo rate on day 3, or high-quality embryo rate on day 3 ($p > 0.05$). Within the control group, the normal fertilization rate was significantly higher in the VDH subgroup than in the VDL subgroup ($p = 0.046$), whereas other oocyte and embryo quality parameters did not differ significantly ($p > 0.05$; Table 2).

3.3. Untargeted metabolomics analysis

A total of 14,878 metabolites were detected using liquid chromatography–mass spectrometry, including 6,334 metabolites in positive ion mode and 8,544 metabolites in negative ion mode. The relative standard deviation for QC samples was <30%, confirming satisfactory analytical repeatability and instrumental stability throughout the analytical sequence (Figure S1).

3.3.1. Metabolite analysis of the two subgroups of FF 25-(OH)D in the DOR group and the control group

A PLS-DA model was constructed to evaluate the metabolic differences between subgroups. The DOR-VDH and DOR-VDL subgroups exhibited clear separation along the PC1 axis (Figures 1A and S2). A total of 2,753 metabolites were detected in the DOR-VDH subgroup and 2,512 in the DOR-VDL subgroup, with 2,464 metabolites shared between the two subgroups (Figure 1B).

Similarly, in the control group, the PLS-DA model revealed distinct separation between the control-VDH and control-VDL subgroups along PC1. Samples within each subgroup were tightly clustered in the PCA plots, indicating high experimental repeatability and suggesting differences in the metabolic profiles between subgroups (Figures 1C and S3). The control-VDH and control-VDL subgroups contained 2,548 and 2,515 metabolites, respectively, with 2,467 shared metabolites (Figure 1D).

3.3.2. Differential metabolite and pathway analysis between VD subgroups in the DOR and control groups

Comparative analysis identified 231 significantly altered metabolites between the DOR-VDH and DOR-

Table 2. Comparison of oocyte and embryo quality in different subgroups of FF 25-(OH)D between the DOR and control groups

Oocyte and embryo quality	DOR				Control			
	VD ≥12 ng/mL *n* = 17	VD <12 ng/mL *n* = 37	χ^2	*p*	VD ≥12 ng/mL *n* = 24	VD <12 ng/mL *n* = 38	χ^2	*p*
Oocyte retrieval rate (%)	75.34% (55/73)	72.30% (107/148)	0.232	0.63	93.06% (228/245)	93.34% (477/511)	0.21[a]	0.884
MII oocyte rate (%)	89.09% (49/55)	88.79% (95/107)	0.003[a]	0.953	89.47% (204/228)	90.57% (432/477)	0.208[a]	0.648
Normal fertilization rate	75.51% (37/49)	58.95% (56/95)	3.877	0.049	75.98% (155/204)	68.29% (295/432)	3.963[a]	0.046
Normal cleavage rate	100% (37/37)	98.21% (55/56)	0.668[a]	0.414	99.35% (154/155)	97.62% (288/295)	1.737[a]	0.188
Utilizable embryo rate on day 3	72.97% (27/37)	75.00% (42/56)	0.048	0.827	76.13% (118/155)	75.59% (223/295)	0.13[a]	0.909
High-quality embryo rate on day 3	21.62% (8/37)	23.21% (13/56)	0.032	0.857	25.16% (39/155)	24.41% (72/295)	0.19[a]	0.889

Note: [a]indicates that none of the cells in the statistical table had an expected count less than 5 (0.0%).
Abbreviations: DOR: Diminished ovarian reserve; MII: Metaphase II; VD: Vitamin D.

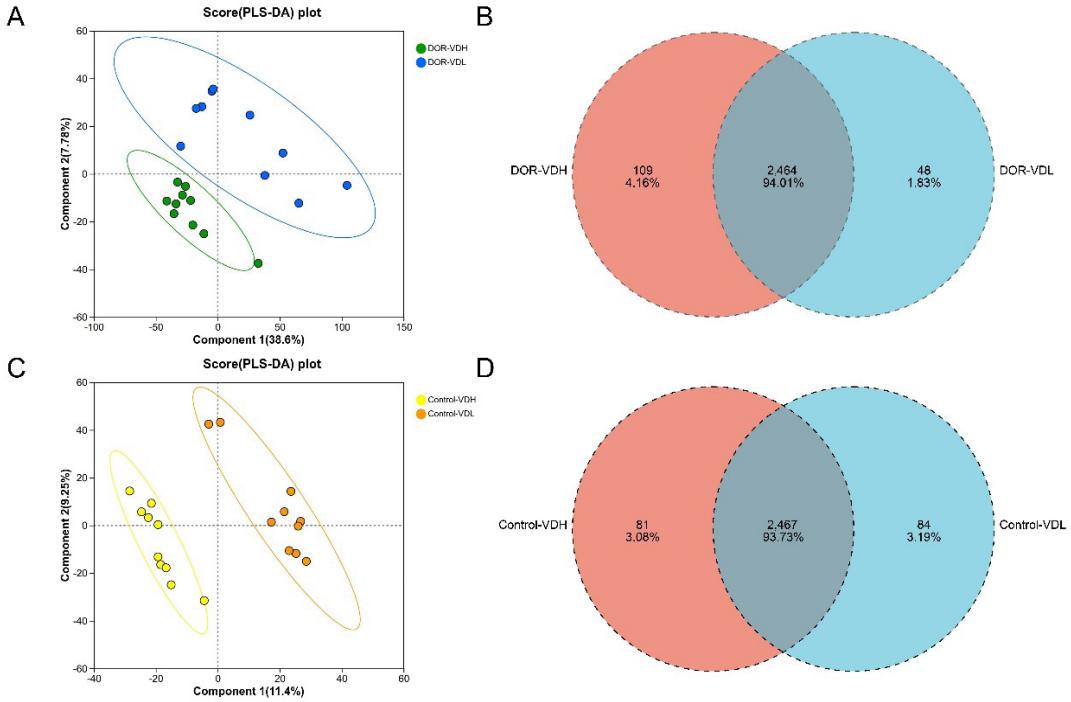


Figure 1. Differential follicular fluid metabolites between VDH and VDL subgroups in the DOR and control groups. (A) PLS-DA score plot of the DOR subgroups. (B) Venn diagram illustrating metabolite overlap between the DOR-VDH and DOR-VDL subgroups. (C) PLS-DA score plot of the control subgroups. (D) Venn diagram illustrating metabolite overlap between the control-VDH and control-VDL subgroups.
Abbreviations: DOR: Diminished ovarian reserve; PLS-DA: Partial least squares discriminant analysis; VDH: Vitamin D non-deficient; VDL: Vitamin D deficient.

VDL subgroups, together with 2,190 metabolites that were not significantly altered. Among the significantly altered metabolites, 203 were upregulated and 28 were downregulated (Figure 2A, Table S1).

Key upregulated metabolites included glycerol 3-phosphate; estriol 3-glucuronide; L-proline; acetamide; 3-hydroxydodecanedioic acid; alfentanil; kyotorphin; isoleucyl–isoleucyl–valine; cysteinyl-lysine; lucidenic acid D2; hydroxyprolinyl-proline; hydroxyprolyl-proline; hydroxychloroquine; and serinyl-tryptophan.

In contrast, prominent downregulated metabolites comprised hymenoxon; menthyl ethylene glycol carbonate; fluticasone; acrylamide; O-acetylcyclopicolidine A; taxine B; vidarabine; guanine; glucuronolactone; cresopyrine; cinobufagin; idebenone; 22-hydroxydocosenoate; and 1-piperazinylethanol.

Between the control-VDH and control-VDL subgroups, 256 metabolites were significantly altered, including 110 upregulated and 146 downregulated, while 2,165 metabolites showed no significant changes (Figure 2C, Table S2). Notable downregulated metabolites included phosphoramidite; altanserin; glutamylglutamic acid; hydroxychloroquine; 4,6-dihydroxy-2-quinolinecarboxylic acid; benzonitrile; octopine; glycolithocholic acid; L-proline; 6-hydroxyhexanoic acid; 3-chlorotyrosine; and serinyltryptophan. Major upregulated metabolites included bialaphos; 10,16-dihydroxyhexadecanoic acid; hexosylsphingosine; menthyl ethylene glycol carbonate; leucyl–phenylalanine; taxine B; 4-carbamimidoyl-L-phenylalanine; and donepezilin.

Metabolites with VIP scores > 1.0 in the first component of the PLS-DA model were considered key contributors to subgroup discrimination and were therefore selected for further analysis (Figure 2B and 2D). Subsequent KEGG pathway enrichment analysis demonstrated that the differential metabolites identified between the DOR-VDH and DOR-VDL subgroups were significantly enriched in pathways related to lipid, nucleotide, and carbohydrate metabolism. Specifically, pathways involved in lipid metabolism (including steroid hormone biosynthesis and glycerophospholipid metabolism), cofactor and vitamin metabolism, and drug metabolism (primarily mediated by cytochrome P450 enzymes), were significantly upregulated. In contrast, metabolites mapping to carbohydrate and nucleotide pathways were less represented (Figure 3A).

For the control-VDH and control-VDL subgroups, differential metabolites were significantly enriched in

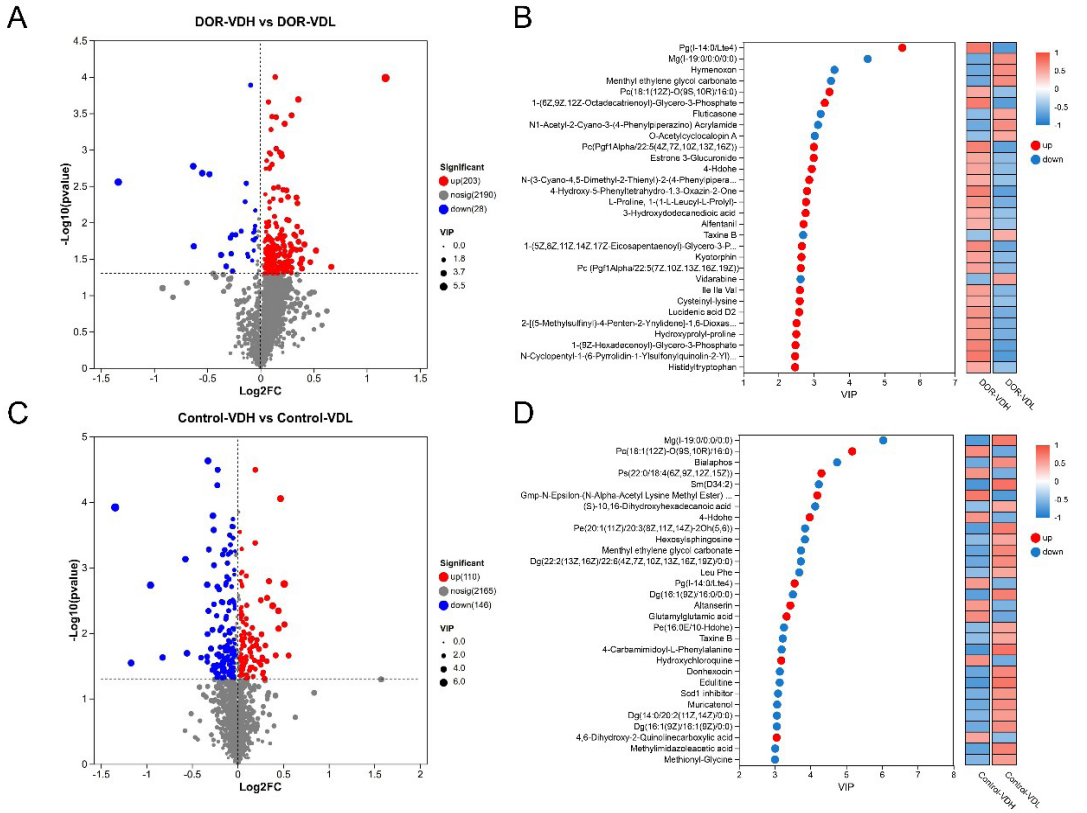


Figure 2. Differential metabolites between VDH and VDL subgroups in follicular fluid from the DOR and control groups. (A) Principal component analysis score plot of the DOR subgroups. (B) VIP scores derived from the partial least squares discriminant analysis model for the DOR subgroups. (C) Principal component analysis score plot of the control subgroups. (D) VIP scores derived from the partial least squares discriminant analysis model in the control subgroups.
Abbreviations: DOR: Diminished ovarian reserve; PLS-DA: Partial least squares discriminant analysis; VDH: Vitamin D non-deficient; VDL: Vitamin D deficient; VIP: Variable importance in projection.

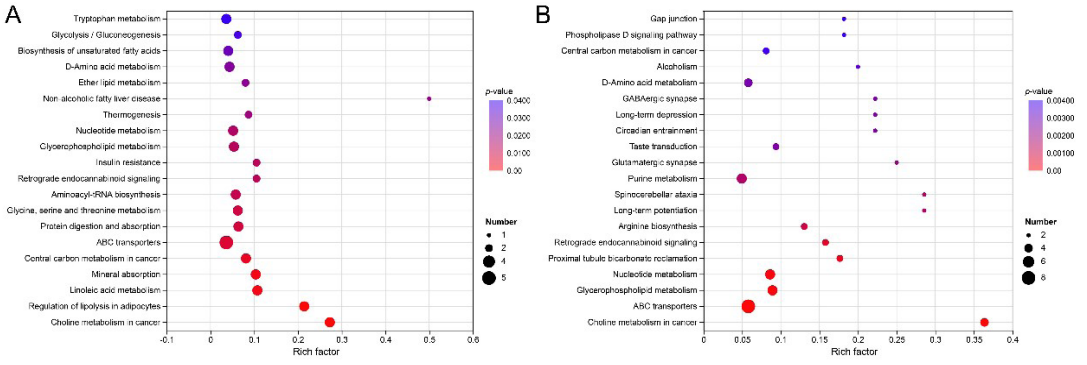


Figure 3. Kyoto Encyclopedia of Genes and Genomes pathways enrichment analysis of differentially expressed metabolites. (A) Comparison between the DOR-VDH and DOR-VDL subgroups. (B) Comparison between the control-VDH and control-VDL subgroups.
Abbreviations: DOR: Diminished ovarian reserve; VDH: Vitamin D non-deficient; VDL: Vitamin D deficient.

lipid metabolism, amino acid metabolism, and membrane transport, including ATP-binding cassette transporters. Pathways related to lipid metabolism (glycerophospholipid metabolism), amino acid metabolism (glycine, serine, and threonine metabolism), signal transduction, and arachidonic acid metabolism were upregulated, whereas phosphate and phosphite metabolism were downregulated (Figure 3B).

3.3.3. Comparison of results between two pairs of subgroups: DOR-VDH vs. DOR-VDL and control-VDH vs. control-VDL

Comparative analysis of the two subgroup pairs (DOR-VDH vs. DOR-VDL and control-VDH vs. control-VDL) revealed shared upregulated metabolites, including phospholipids, hydroxychloroquine, and serinyl-tryptophan. KEGG pathway analysis demonstrated common significant enrichment in glycerophospholipid metabolism. Furthermore, within the DOR group, the VDH subgroup showed distinct upregulation of steroid hormone biosynthesis and vitamin metabolism pathways, particularly those mediated by cytochrome P450 enzymes, relative to the VDL subgroup.

4. Discussion

Follicular growth is tightly regulated by the intrafollicular microenvironment, which mediates somatic cell–oocyte communication and provides essential support for oocyte development. FF, a complex mixture of hormones, enzymes, antioxidants, and metabolites, serves as a key medium for nutrient exchange and signaling within antral follicles. Alterations in FF composition reflect follicular status and are associated with oocyte developmental competence, fertilization potential, and early embryo outcomes.[10] VD exerts a broad spectrum of physiological functions and plays a critical role in maintaining calcium, magnesium, and phosphate homeostasis. Additionally, it functions as an immunomodulator and possesses anti-proliferative properties.[11] Specifically, VD modulates immune responses by suppressing pro-inflammatory cytokines and promoting the expression of anti-inflammatory cytokines. Previous studies have demonstrated a significant correlation between VD levels and follicular development, supporting the notion that VD is essential for normal ovarian function and female fertility.[12] Accordingly, we hypothesized that VD may significantly influence the IVF outcomes of patients with DOR.

In this study, we measured VD levels in the FF of women undergoing IVF and observed significantly lower levels in those with DOR compared with controls. Both groups were further stratified into high-VD (VDH; ≥12 ng/mL) and low-VD (VDL, <12 ng/mL) subgroups based on FF VD levels. Although the normal fertilization rate was significantly higher in VDH subgroups of both the DOR and control groups, no significant differences were observed in the MII oocyte rate, utilizable embryo rate on day 3, or high-quality embryo rate on day 3 between the VDH and VDL subgroups. These findings suggest that VD deficiency is associated with DOR and may adversely affect normal fertilization during IVF cycles.

Our results align with prior reports indicating that severe VD deficiency is associated with reduced ovarian reserve[13] and that VD supplementation may improve ovarian markers in DOR patients.[12] Some studies have reported that adequate VD levels are associated with higher IVF success rates among women undergoing treatment;[14] however, VD does not appear to enhance indices related to oocyte quality, such as oocyte yield and oocyte maturation rate.[15] Nevertheless, the criteria and methods used to define VD nutritional status vary across different countries and regions, leading to different conclusions.

For instance, Antunes *et al.*[16] reported no significant differences in the number of retrieved follicles, oocytes, and mature oocytes between VD-deficient and VD-sufficient patients. Skowrońska *et al.*[17] conducted a prospective intracytoplasmic sperm injection study and found no correlation between VD concentration in FF and fertilization success rate.

Conversely, Ciepiela *et al.*[18] demonstrated that FF 25-(OH)D levels were negatively correlated with oocyte fertilization ability and subsequent pre-implantation embryo development ability; however, lower concentrations were associated with higher chances of obtaining high-quality embryos, as well as higher pregnancy and delivery rates. Moreover, a meta-analysis by Karimi *et al.*[19] found no significant association between VD levels and ovarian reserve markers in adolescent and adult women. Collectively, these findings indicate the impact of FF VD levels on oocyte and embryo quality remains inconclusive and warrants validation in studies with larger sample sizes and rigorous methodological design.

Based on the clinical findings, we performed untargeted metabolomic profiling of FF from 10 samples per subgroup in the DOR and control cohorts. Comparative analysis between DOR-VDH vs. DOR-VDL and control-VDH vs. control-VDL revealed consistent upregulation of phospholipids, hydroxychloroquine, and serinyl-tryptophan in the VDH subgroups. KEGG pathway enrichment analysis highlighted significant upregulation of glycerophospholipid metabolism in both comparisons. Specifically, within the DOR group, the VDH subgroup exhibited marked upregulation of steroid hormone biosynthesis, vitamin metabolism, and cytochrome

P450-mediated metabolic pathways compared with the VDL subgroup.

A decline in oocyte quality is a critical factor influencing the outcome of IVF–ET. Oocyte developmental competence is closely associated with its metabolic microenvironment.[20] Lipids are essential for oocyte energy metabolism, supporting growth, maturation, and subsequent embryonic development. The balance between glucose and lipid metabolism within oocytes critically influences female fertility.[20,21] Impaired lipid metabolism has been linked to reduced oocyte quality in obese women.[22,23] *In vivo* studies have demonstrated that maternal VD deficiency disrupts fatty acid metabolism, increases maternal inflammation,[24,25] impairs placental function and offspring brain angiogenesis,[25] and induces oxidative stress along with eicosanoid imbalance, ultimately contributing to adverse pregnancy outcomes.[26,27]

Glycerophospholipids are key structural and functional components of cell membranes.[28] Previous studies have established that impaired glycerophospholipid metabolism is associated with a variety of diseases, including diabetes, autoimmune diseases, neurological disorders, and cancer.[29] In a related context, Wu *et al.*[29] reported that the glycerophospholipid metabolism pathway was downregulated in patients with DOR and advanced age. VD metabolites, synthesized via cytochrome P450 (CYP) enzymes, play crucial roles in human physiological processes, primarily mediating cellular signaling pathways through the activated VD receptor/retinoid X receptor heterodimer complex.[30] Notably, 1α,25-(OH)2VD can suppress NLR family pyrin domain-containing 3 inflammasome activation and improve lipid metabolism in hepatic models,[31] while also influencing mechanistic target of rapamycin signaling[32] and amino acid catabolism[33] in immune and breast cells.

As a nutritional supplement, VD exerts moderate effects on inflammation, plasma fatty acid levels, and physical function in overweight and obese patients with low-grade inflammation.[34] Hydroxychloroquine is a substrate of P450s 2C8, 2D6, and 3A enzymes *in vivo*.[35] Similarly, it has been shown to promote lipid metabolism, modulate adipose tissue inflammation, and inhibit cytokine release.[36] In our study, higher hydroxychloroquine levels in VDH subgroups suggest that VD may help modulate oxidative stress and inflammatory responses within the follicle.

5. Conclusion

As a steroid hormone, VD likely regulates physiological functions through multiple pathways. In patients with DOR, reduced VD levels may compromise oocyte quality and embryo developmental potential. Our findings suggest that, in the DOR population, VD may predominantly improve the follicular microenvironment by regulating lipid metabolism.

All subjects in this study were IVF patients treated with the PPOS ovulation induction protocol, without fresh embryo transfer. Consequently, associations between FF 25-(OH)D levels and pregnancy outcomes could not be evaluated. Future studies are warranted to further evaluate the impact and mechanisms of VD on reproductive function and pregnancy outcomes. In summary, appropriate VD supplementation may improve IVF outcomes and promote reproductive health.

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Conflict of interest

The authors declare they have no competing interests.

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Ethics approval and consent to participate

Ethical approval for this study was obtained from the Ethics Committee of the First Hospital of Lanzhou University (approval number: LDYYSZLL2023-26). All participants were provided with information about the study and gave written informed consent before participating in the study.

Consent for publication

Informed consent was obtained from the patient for the publication of their data and/or images in this manuscript. The consent was documented in writing through a signed informed consent form.

Availability of data

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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