Journal of Ovarian Research

RESEARCH

Open Access

Integrative metabolomics and proteomics reveal early cardiovascular risk signatures in PCOS female offspring

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Abstract

Background Polycystic ovary syndrome (PCOS) is associated with metabolism abnormalities and increased cardiovascular disease (CVD) risk. This study aimed to further investigate cardiovascular metabolism alterations specifically in the female offspring of PCOS patients using metabolomics and proteomics, and to identify potential early CVD-related biomarkers in this population.

Methods A total of 40 female offspring of PCOS patients and 40 female offspring of non-PCOS individuals (4–6 years old) were enrolled in this study. Cardiovascular risk features including height, weight, body mass index (BMI), blood pressure, glycemic and lipid parameters were assessed. Untargeted metabolomics and the Olink proteomics were applied to identify alterations in metabolites and CVD-related proteins. A further in-depth analysis of metabolomics and proteomics was conducted.

Results Lipid indicators, including Total Cholesterol（TC）, Low-Density Lipoprotein Cholesterol（LDL-C）, and high-density lipoprotein cholesterol (HDL-C), as well as γ-glutamyl transferase (GGT), a marker of liver function were significantly elevated in the female offspring of PCOS patients compared to the female offspring of non-PCOS women. Metabolomic profiling revealed significant alterations in lipid metabolites, particularly in glycerophospholipids and fatty acids, in the female offspring of PCOS patients. Additionally, elevated levels of CVD-related proteins, including proprotein convertase subtilisin/kexin type (PCSK9), tissue factor pathway inhibitor (TFPI), secretoglobin family 3A member 2 (SCGB3A2), junctional adhesion molecule A (JAM-A), interleukin-1 receptor-like 1 (ST2) and epidermal growth factor receptor (EGFR) were observed, with significant correlations between these proteins and the altered lipid metabolites. We conducted mediation analysis and identified several metabolites and proteins that mediate cardiovascular risk. A a combined metabolite and protein panel consisting of phospholipids (PIs), lysophosphatidylcholines (LysoPCs), and CVD-related proteins demonstrated an area under the curve (AUC) of

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0.93 based on receiver operating characteristic (ROC) curve analysis, effectively differentiating female offspring with hyperlipidemia.

Conclusions Female offspring of PCOS patients exhibit distinct lipid metabolic and protein profiles that suggest early cardiovascular-related alterations. These findings support the potential value of early metabolic monitoring in this population and highlight the need for longitudinal studies to determine whether these alterations translate to increased cardiovascular risk in adulthood. The identified metabolite and protein biomarkers may serve as tools for early risk assessment, pending prospective validation of their long-term predictive value.

Keywords PCOS, Female Offspring, Dislipidemia, Cardiovascular risk features, Proteomics, Metabolomics

Background

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder affecting 8–20% of reproductive-aged women worldwide [1, 2]. It is characterized by hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology [2]. Beyond reproductive abnormalities, PCOS is associated with a range of metabolic disturbances, including insulin resistance, obesity, dyslipidemia, and an increased risk of type 2 diabetes and cardiovascular diseases (CVD) [3–5]. Emerging evidence suggests that the metabolic abnormalities associated with PCOS may not be limited to affected women but may also extend to their offspring [6–13]. The intrauterine environment, genetic predispositions and epigenetic modifications may influence fetal reprogramming. The Barker hypothesis, also known as the Developmental Origins of Health and Disease (DOHaD) theory, suggests that adverse intrauterine environments can lead to long-term health consequences in offspring, including metabolic and reproductive disorders [9]. Previous studies indicate that the female offspring of PCOS patients may exhibit metabolic alterations that warrant monitoring, though the long-term implications for cardiovascular health remain to be determined through longitudinal studies [14, 15]. However, the specific early alterations in lipid metabolism and the underlying molecular mechanisms in the offspring of PCOS patients remain poorly understood.

Metabolomics and proteomics are powerful tools for investigating metabolic perturbations and identifying potential biomarkers associated with disease risk [16, 17]. Metabolomics enables the comprehensive analysis of small-molecule metabolites, providing insights into metabolic pathways and their dysregulation in disease states [18, 19]. Proteomics, on the other hand, allows for the large-scale study of proteins, their functions, and their interactions, facilitating the identification of disease-related protein markers [20, 21]. The integration of metabolomics and proteomics data can provide a more comprehensive understanding of the molecular mechanisms underlying disease pathogenesis and aid in the discovery of novel biomarkers for risk assessment and early intervention [21].

In this study, we aimed to investigate the alterations in CVD metabolism and risk specifically in the female offspring of PCOS patients compared to those of non-PCOS individuals using metabolomics and proteomics approaches. We hypothesized that the female offspring of PCOS patients would exhibit distinct metabolite and protein profiles, indicative of an increased risk of future CVD development. Furthermore, we sought to identify potential biomarkers that could serve as predictive tools for early cardiovascular risk assessment in this high-risk population.

Methods

Ethics

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Women's Hospital, School of Medicine, Zhejiang University (Approval No. IRB-20220331-R). Written informed consent was obtained from all participants and by a parent or guardian with parental responsibility on behalf of their children.

Study population

The maternal-child dyads were enrolled in this study in the reproductive center of Women's Hospital, School of Medicine, Zhejiang University based on an assisted reproductive technology (ART) cohort study. We included singletons born to PCOS mothers and followed up at 4–6 years in our ART Offspring Health Monitoring Center between January 2020 and October 2022.

PCOS diagnosis in the mothers was based on the Rotterdam criteria [22], which requires the presence of at least two out of three criteria: oligo- or anovulation, clinical and/or biochemical hyperandrogenism, and polycystic ovaries on ultrasound. Patients with other causes of hyperandrogenism or oligomenorrhea, such as thyroid disease, hyperprolactinemia and Cushing's syndrome, were excluded. The female offspring of non-PCOS females defined as the control group were recruited during the same period. They were matched 1:1 by maternal age at conception, paternal age at conception, maternal height, maternal preconception BMI, IVF or ICSI, fresh embryo transfer (fresh-ET) or frozen-embryo

transfer (FET), age at follow-up, and year of follow-up. Multi-omics analyses, including metabolomics and proteomics, were conducted using samples from final study population consisting of 40 female offspring of women diagnosed with PCOS (PCOS offspring) and 40 female offspring of non-PCOS women (non-PCOS offspring) in the control group. Exclusion criteria for both groups included maternal age > 40 years old, paternal age > 50 years old, donor gametes, birth defects, and a personal history of diabetes, CVD, or any other chronic diseases (including diabetes, hypertension and chronic kidney disease).

Anthropometric measurements

In the clinical cohort, anthropometric measurements were collected during clinical examinations. Weight and height were measured to calculate body mass index (BMI) as weight (kg) divided by height squared (m^2). Blood pressure was measured in a quiet room after the child had been seated and resting for at least 5 min. Measurements were taken in the morning, between 8:00 and 10:00 a.m., using an appropriately sized cuff on the right arm. Three consecutive readings were obtained at 1-min intervals. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded, with the mean values of at least three measurements used for analysis.

Biochemical analyses

Venous blood samples were collected after overnight fasting. Fasting biochemical measurements including in serum Total Cholesterol（TC）,Low-Density Lipoprotein Cholesterol（LDL-C）, high-density lipoprotein cholesterol (HDL-C), triglyceride (TG) [23], alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin [24], γ-glutamyl transferase (GGT), fasting glucose [25]. The triglyceride-glucose index (TyG index) was calculated using the formula ln [fasting triglycerides (mg/dL) × fasting glucose (mg/dL)/2], serving as a reliable surrogate marker for insulin resistance in which triglycerides (mmol/L) were converted to mg/dL using the formula mmol/L × 88.545, and HDL-C (mmol/L) using mmol/L × 38.67 for the calculation of the TyG index [26].

Serum sample collection

Fasting blood samples were collected from the children on the second morning after admission. The samples were transported in a temperature-controlled cooler by the research team to the laboratory of the Women's Hospital, School of Medicine, Zhejiang University. Upon arrival, the samples underwent centrifugation at 3,000 × g for 10 min, followed by a secondary centrifugation at 13,000 × g for 2 min. The resulting supernatant was carefully transferred into storage tubes and preserved at – 80 °C until further analysis.

Metabolomics analysis

All metabolomics and proteomics samples were analyzed in duplicates to ensure the reliability and consistency of the data. Samples were thawed on ice, and metabolites were extracted using 50% methanol buffer. Briefly, 20 µL of each sample was mixed with 120 µL precooled 50% methanol, vortexed for 1 min, incubated at room temperature for 10 min, and stored overnight at – 20 °C. After centrifugation at 4,000 × g for 20 min, supernatants were transferred to 96-well plates and stored at – 80 °C for LC–MS analysis. Quality control (QC) samples were prepared by pooling 10 µL of each extraction mixture. All samples were analyzed using a Vanquish Flex UHPLC system (Thermo Fisher Scientific) with an ACQUITY UPLC T3 column (100 mm × 2.1 mm, 1.8 µm; Waters). Chromatographic separation was conducted at 35 °C with a flow rate of 0.4 mL/min. The mobile phase consisted of solvent A (water, 0.1% formic acid) and solvent B (acetonitrile, 0.1% formic acid), with a gradient elution of 0–0.5 min, 5% B; 0.5–7 min, 5–100% B; 7–8 min, 100% B; 8–8.1 min, 100–5% B; and 8.1–10 min, 5% B.

Metabolites were detected using a Q-Exactive mass spectrometer (Thermo Fisher Scientific) in positive and negative ion modes. Precursor spectra (m/z 70–1050) were acquired at 70,000 resolutions with an AGC target of 3×10^6 and a maximum injection time of 100 ms. DDA was used with a top 3 configuration, and fragment spectra were collected at 17,500 resolution with an AGC target of 1×10^5 and a maximum injection time of 80 ms.QC samples, analyzed every 10 runs, ensured system stability and data reliability.

Proteomics analysis

Proteins were quantified using the Olink Target 96 Cardiovascular Panel III (Olink Proteomics AB, Uppsala, Sweden) following the manufacturer's protocol. Briefly, pairs of oligonucleotide-labeled antibody probes specifically bind to their target proteins. When the probes are in close proximity, the oligonucleotides hybridize in a pairwise manner. The addition of a DNA polymerase triggers a proximity-dependent DNA polymerization event, generating a unique PCR target sequence. This DNA sequence is then detected and quantified using a microfluidic real-time PCR instrument (Signature Q100, LC-Biotechnology Co., Ltd., Hangzhou, China). Data were quality controlled and normalized using an internal extension control and an inter-plate control to account for intra- and inter-run variability. The final output is presented as Normalized Protein Expression (NPX) values, which are arbitrary units on a log2 scale, where higher values correspond to higher protein expression levels. Detailed assay validation data, including detection limits and intra- and inter-assay precision, are available on the manufacturer's website (www.olink.com) [27].

Defining cardiometabolic risk features

Dyslipidemia was defined as values above the 95th percentile according to pediatric guidelines, corresponding to TC ≥ 5.2 mmol/L, LDL-C ≥ 3.4 mmol/L, TG ≥ 1.1 mmol/L for 0–9 years or ≥ 1.5 mmol/L for 10–19 years or HDL-C < 1.0 mmol/L [28].

Statistical analysis

Clinical and biochemical characteristics were compared between the PCOS offspring and non-PCOS offspring groups using Mann–Whitney U test for continuous variables. For categorical variables, the chi-square test was used when all expected frequencies (T) were ≥ 5. When 1 ≤ T < 5, the Yate' continuity corrected chi-square test was applied.

Metabolomics data were log2-transformed and normalized protein expression values were produced as an arbitrary unit on a log2 scale prior to statistical analysis. Independent T test was used for statistical differential analysis. Metabolites with *P*-value < 0.05, fold change > 1.2 and variable importance for the projection (VIP) ≥ 1 were considered significantly altered. A cut of *P* -value < 0.05 and fold change > 1 was used to select statistically differential expressed proteins.

Pathway enrichment analysis was conducted using MetaboAnalyst 5.0 [29] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [30]. Spearman's rank correlation was used to assess the relationships between altered metabolites, proteins, and clinical parameters. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the predictive power of the identified biomarkers for hyperlipidemia risk. Statistical analyses were performed using R software (version 4.0.3), and *P*-value < 0.05 was considered statistically significant.

Mediation analysis

Mediation analysis was performed to explore the mediating role of PCOS-offspring-associated metabolites and proteins on cardiometabolic traits. The proportion of the effect mediated from PCOS exposure through the metabolites and proteins was determined by dividing its indirect effect by the total effect. We tested 80 paths identifying significant associations between PCOS exposure → metabolites and proteins change → cardiometabolic risk features at a significance level of *P*-value < 0.05.

Results

Study design and participant characteristics

In this study, a total of 80 female offspring (4–6 years old) were analyzed using untargeted metabolomics and olink proteomics. This included 40 female offspring born to mothers with PCOS and 40 born to mothers without PCOS (non-PCOS group). A schematic study design is illustrated in the accompanying diagram (Fig. 1).

No significant differences were observed in most parental parameters, including age at conception, BMI, ART parameters, and obstetric outcomes. However, PCOS mothers had lower basal follicle-stimulating hormone (FSH) level, higher basal luteinizing hormone (LH) level and elevated testosterone (T) level compared to non-PCOS mothers. The prevalence of gestational diabetes mellitus (GDM) was elevated in patients with PCOS, although this finding was not statistically significant (17.5% versus 2.5%) (Table 1). Additionally, there were no significant differences in early feeding patterns or residence between offspring of PCOS and non-PCOS groups (Table 1).

The cardiometabolic risk features of female offspring at a median age of 4.72 years of age revealed significantly elevated lipid parameters in PCOS female offspring, including TC (4.69 mmol/L versus 3.91 mmol/L), LDL-C (2.76 mmol/L versus 2.21 mmol/L) and HDL-C (1.59 mmol/L versus 1.40 mmol/L). The prevalence of dyslipidemia was increased (14.0% versus 6.0%) in PCOS female offspring compared to controls. Additionally, GGT, a marker of liver function, were higher (11.15 U/L versus 10.20 U/L). However, other cardiometabolic risk features including offspring BMI, as well as blood pressure, glycemic parameters including glucose and TyG index, and other liver enzymes including ALT, AST and bilirubin showed no significant differences between groups (Table 2).

Altered metabolomic profiles in PCOS female offspring

Untargeted metabolomic profiling revealed distinct patterns of metabolic alterations between female offspring of PCOS and non-PCOS groups. PCOS female offspring displayed a clearly distinguishable metabolic profile compared to those in the control group (Fig. 2A). We identified 74 metabolites as differential metabolites including 48 upregulated differential metabolites and 26 downregulated metabolites (Fig. 2B). These differential metabolites were characterized by substantial perturbations in the abundance and composition of lipid species, accounting for 62.2% of the total differential metabolites, notably fatty acids (FAs) and glycerophospholipids (GPs) (Fig. 2C-D). Pathway enrichment analysis elucidated significant dysregulation in key metabolic processes, particularly in choline metabolism, GP metabolism and biosynthesis of unsaturated FAs (Fig. 2E).

Association of lipid metabolites with cardiometabolic risk features

Lipid metabolomic profiling revealed substantial alterations in the lipid metabolites of PCOS female offspring compared to controls, encompassing four major lipid speices: FA, GP, sphingolipid (SL) and sterol lipid (ST). 82% FAs (14 of 17), 77% GPs (20 of 26) and all STs

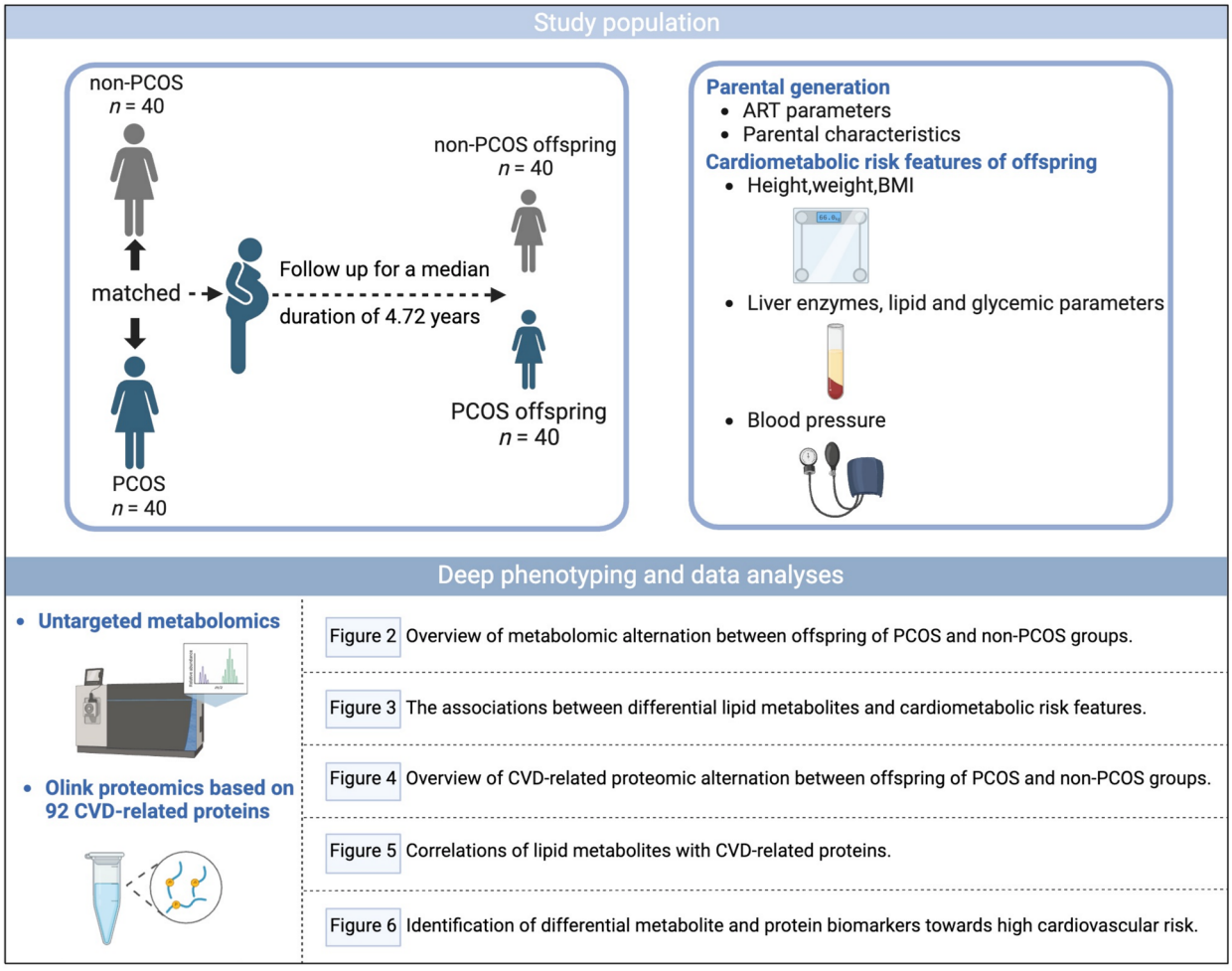

Fig. 1 Schematic study design. Non-targeted metabolomics and Olink proteomics were measured in the female offspring of 40 PCOS and 40 non-PCOS groups. We investigated the relationship between lipid metabolism dysregulation, cardiometabolic risk features and CVD-related proteins, as well as the ability of metabolites and proteins biomarkers to predict cardiovascular risk

exhibited the most pronounced upregulation while all SLs displayed downregulation. Further classification revealed that acylcarnitine (AC), unsaturated FA, phosphatidylinositol (PI), lysophosphatidylcholine (LysoPC) species and ursodeoxycholic acid (UDCA) exhibited pronounced upregulation, whereas plasmenyl-phosphatidylcholine (plasmenyl-PC), phosphatidylcholine (PC), and sphingomyelin (SM) species showed pronounced downregulation (Fig. 3A).

The integration of lipidomics and cardiometabolic risk features revealed that 50% ACs (3 of 6), 75% unsaturated FAs (3 of 4) and all LysoPCs showed positive associations with HDL-C while 87% PIs (13 of 15) and 80% LysoPCs (4 of 5) were positively associated with both LDL-C and TC. Additionally, 50% ACs (3 of 6) demonstrated positive associations with GGT (Fig. 3B and Supplementary Fig. 1). Linear regression analyses revealed the similar associations, displayed as all PIs and 80% LysoPCs (4 of 5) were positively associated with LDL-C while 93% PIs (14 of 15) and all LysoPCs were positively associated with TC. Additionally, 67% ACs (4 of 6) demonstrated positive associations with GGT (Fig. 3C and Supplementary Table 1–5). Logistic regression analyses for lipidomics and dyslipidemia revealed that three PIs identified as PI 36:3; PI (18:1/18:2), PI 34:2; PI(16:0/18:2) and PI 38:6; PI (16:0/22:6) as well as plasmenyl-PC 38:3; PC(P-16:0/22:3) were positively associated with an OR (95% CI) of 1.93 (1.06 ~ 3.53), 2.00 (1.05 ~ 3.83), 1.84 (1.01 ~ 3.34) and 2.78 (1.13 ~ 6.83), respectively (Fig. 3D-E and Supplementary Table 6).

Elevated CVD-Related proteins in PCOS female offspring

Proteomic analysis revealed distinct patterns between female offspring of PCOS and non-PCOS groups based

Table 1 Clinical characteristics of parents

	Non-PCOS (*n* = 40)	PCOS (*n* = 40)	*P*-value
Maternal age at conception (years)	29.4 ± 0.5	30.2 ± 0.4	0.106
Paternal age at conception (years)	31.7 ± 0.8	31.9 ± 0.8	0.909
Maternal height, cm	159.1 ± 0.8	158.5 ± 0.7	0.624
Maternal preconception weight, kg	56.2 ± 0.8	56.3 ± 1.1	0.836
Maternal preconception BMI (kg/m^2)	22.2 ± 0.3	22.4 ± 0.4	0.935
Feeding patterns			0.580
Breast feeding, n (%)	29(72.5%)	33(82.5%)	
Mixed feeding, n (%)	5(12.5%)	3(7.5%)	
Artificial feeding, n (%)	6(15.0%)	4(10%)	
Residence			0.644
Rural	16(40%)	24(60%)	
Urban	14(35%)	26(65%)	
ART parameters			
IVF	32(80.0%)	32(80.0%)	1.000
ICSI	8(20.0%)	8(20.0%)	
Frozen ET	28(70.0%)	30(75.0%)	0.617
Fresh ET	12(30.0%)	10(25.0%)	
Basal FSH	6.6 ± 0.2	5.7 ± 0.2	< 0.001
Basal LH	4.5 ± 0.3	8.2 ± 1.0	0.004
E2	114.6 ± 12.2	112.6 ± 9.5	0.715
T	0.9 ± 0.1	3.2 ± 2.1	0.047
Caesarean	28(70%)	27(67.5%)	0.809
Gestational age, weeks	39.2±0.2	38.9±0.3	0.847
Preterm delivery (< 37 weeks)	3(7.5%)	3(7.5%)	1.000
GDM	1(2.5%)	7(17.5%)	0.620
Hypertensive disease of pregnancy	2(5.0%)	1(2.5%)	1.000

Data are presented as mean ± SEM or n (%)

BMI Body mass index, *ART* Assisted reproductive technology, *IVF* In vitro fertilization, *ICSI* Intracytoplasmic sperm injection, *FET* Frozen-embryo transfer, *fresh-ET* fresh-embryo transfer, *FSH* Follicle-stimulating hormone, *LH* Luteinizing hormone, *E2* Estradiol, *T* Testosterone, *GDM* Gestational diabetes

P-value < 0.05 was considered to be a statistically significant difference

on 92 CVD-related proteins (Fig. 4A). 6 proteins that were significantly upregulated in PCOS female offspring compared to controls, including proprotein convertase subtilisin/kexin type (PCSK9), tissue factor pathway inhibitor (TFPI), secretoglobin family 3 A member 2 (SCGB3A2), junctional adhesion molecule A (JAM-A), interleukin-1 receptor-like 1 (ST2) and epidermal growth factor receptor (EGFR) (Fig. 4B-C). Linear regression analyses revealed significant interactions were detected in SCGB3A2, TFPI and TC, LDL-C while in EGFR and GGT (Fig. 4D and Supplementary Table 7–11). Logistic regression analyses for proteomics and dyslipidemia revealed that JAM-A was associated with an OR (95% CI) of 4.02 (1.13 ~ 14.23) (Fig. 4E and Supplementary Table 12).

Table 2 Cardiometabolic risk features of offspring

	Non-PCOS (*n* = 40)	PCOS (*n* = 40)	*P*-value
Age, years	4.82 ± 0.05	4.84 ± 0.05	0.376
Height, cm	109.3 ± 0.7	107.8 ± 0.8	0.172
Weight, kg	17.7 ± 0.4	17.9 ± 0.5	0.799
BMI, (kg/m^2)	14.8 ± 0.2	14.3 ± 0.3	0.164
SBP, mmHg	99.2 ± 1.7	101.4 ± 1.6	0.218
DBP, mmHg	59.4 ± 1.2	57.8 ± 1.0	0.541
ALT, U/L	12.50 ± 4.16	12.72 ± 4.79	0.698
AST, U/L	29.47 ± 5.11	31.17 ± 5.96	0.147
Bilirubin, umol/L	9.09 ± 3.23	8.93 ± 2.83	0.996
GGT, U/L	10.20 ± 2.24	11.15 ± 2.26	0.022
TC, mmol/L	3.91 ± 0.07	4.69 ± 0.11	< 0.001
TG, mmol/L	0.76 ± 0.04	0.69 ± 0.04	0.264
LDL-C, mmol/L	2.21 ± 0.05	2.76 ± 0.08	< 0.001
HDL-C, mmol/L	1.40 ± 0.05	1.59 ± 0.05	0.003
Fasting glucose, mmol/L	4.62 ± 0.05	4.79 ± 0.09	0.136
Triglyceride-Glucose Index	7.88 ± 0.31	7.81 ± 0.40	0.879

Data are presented as mean ± SEM or n(%)

BMI Body mass index, *SBP* Systolic blood pressure, *DBP* Diastolic blood pressure, *ALT* Alanine transaminase, *AST* Aspartate transaminase, *GGT* γ-glutamyl transferase, *TC* Total cholesterol, *TG* Triglyceride, *LDL-C* Low-density lipoprotein cholesterol, *HDL-C* High-density lipoprotein cholesterol

P-value < 0.05 was considered to be a statistically significant difference

Correlations of lipid metabolites with CVD-related proteins

Correlation analysis between the lipid metabolites and CVD-related proteins revealed significant associations, with PCSK9 being the most enriched, associated with 13 lipid metabolites (Fig. 5A). Mainly, total 83% ACs (5 of 6) and all unsaturated FAs showed positive associations while all PIs and al LysoPCs showed positive correlation. Conversely, 67% plasmenyl-PC (2 of 3) showed negative correlation (Fig. 5B).

A comparison of the expression levels of bile acid metabolites revealed that UDCA had a significantly higher relative abundance in the female offspring of PCOS group (Fig. 5C). Additionally, correlation analysis showed that UDCA exhibited positive associations with LDL-C and PCSK9 (Fig. 5D and Supplementary Fig. 2).

Mediation effect of metabolites and proteins on cardiometabolic risk features

Lipid metabolic profile exhibits widespread associations with cardiometabolic risk features and CVD-related proteins (Fig. 6A). We conducted mediation analysis to explore the role of the 74 PCOS-offspring-differential metabolites and 6 PCOS-offspring-differential proteins on the development of cardiometabolic dysfunction in PCOS female offspring. The results revealed that PI and LysoPC species had positive mediation effects mainly, with mediation proportions ranging from 13% to 21% in PI species and 15% to 30% in LysoPC species (Fig. 6B and Supplementary Table 13).

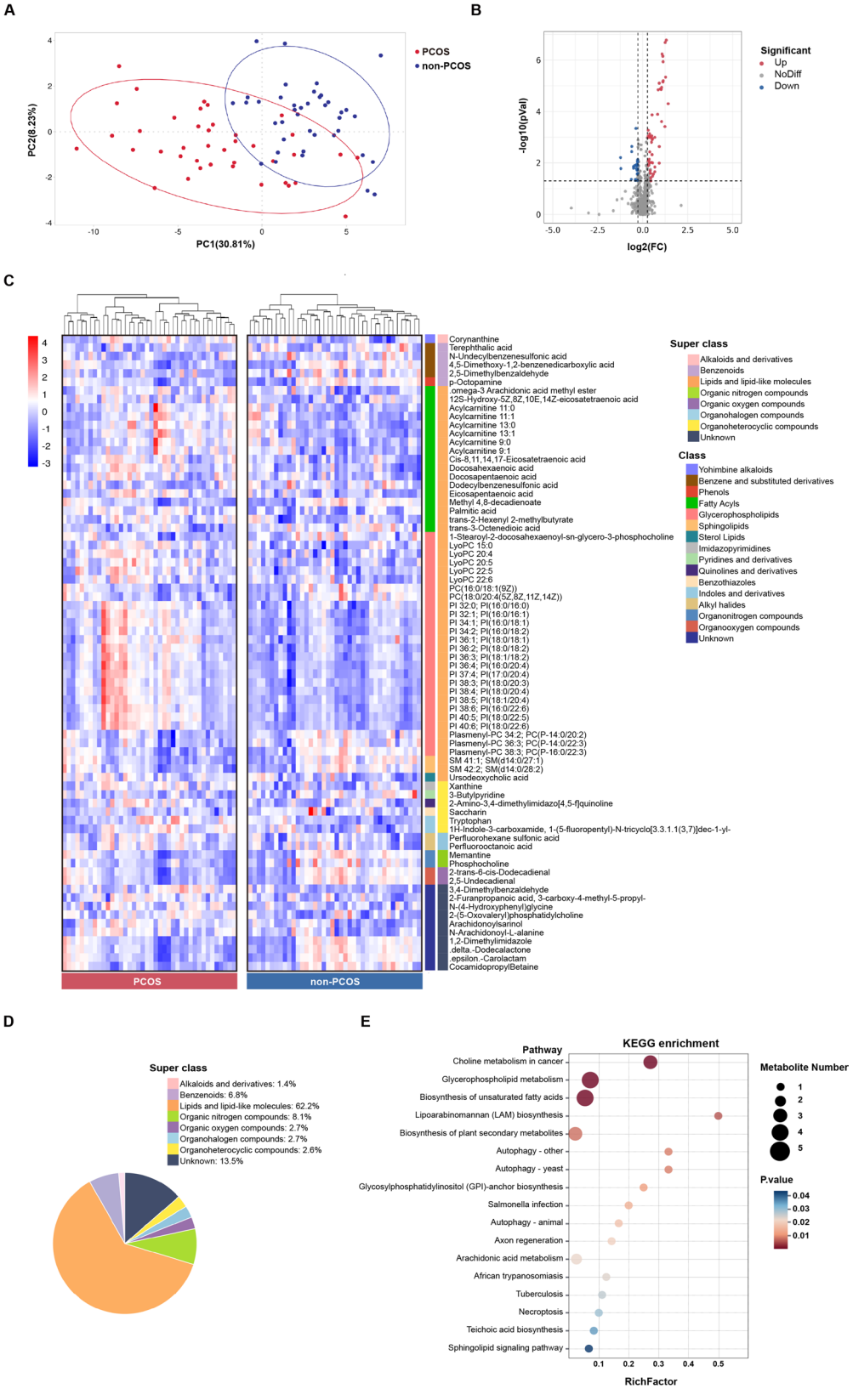

Fig. 2 (See legend on next page.)

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Fig. 2 Overview of metabolomic alternation between offspring of PCOS and non-PCOS groups. **A** PLS-DA illustrated the separation of samples from offspring of PCOS and non-PCOS groups based on metabolomic profiles. **B** The volcano plot showed differential metabolites between offspring of PCOS and non-PCOS groups. Significantly upregulated and downregulated metabolites were highlighted as red and blue based on thresholds of |FC|> 1.2 and *P*-value < 0.05. **C** The heatmap showed 74 differential metabolites between offspring of PCOS and non-PCOS groups. **D** The pie chart showed the proportion of super class of metabolites in the total differential metabolites. **E** The KEGG pathway enrichment scatter plot depicted significantly enriched pathways based on metabolomic profiles. PLS-DA, the partial least-squares discrimination analysis; FC, fold change; KEGG, Kyoto Encyclopedia of Genes and Genomes

Predictive performance of metabolites and proteins to detect hyperlipidemia

We evaluated the predictive performance of the identified metabolites positively correlated with cardiometabolic risk features and proteins for detecting hyperlipidemia in PCOS female offspring. We identified a combined metabolite and protein panel comprising PI, LysoPC species, and CVD-related proteins, demonstrating the area under the curve (AUC) of 0.93(95% CI 0.88–0.98) while metabolites and proteins demonstrated the AUC of 0.84 (95% CI 0.76–0.95) and 0.78 (95% CI 0.67–0.89) respectively (Fig. 6C and Supplementary Fig. 3).

Discussion

This study provides novel insights into the alterations in lipid metabolism and CVD risk in the female offspring of PCOS patients using metabolomics and proteomics approaches. Our findings reveal distinct lipid metabolite and protein profiles in PCOS female offspring compared to non-PCOS female offspring, characterized by elevated levels of glycerophospholipids and sterol lipids and CVD-related proteins as well as decreased level of sphingolipids. These alterations were significantly associated with cardiovascular risk features, and an increased prevalence of hyperlipidemia in the PCOS female offspring group.

Our findings confirm a significant increase in TC, LDL-C, and HDL-C in PCOS offspring. Elevated TC and LDL-C are positively correlated with CVD risk in later life [31, 32]. An intriguing finding is the simultaneous elevation of HDL-C alongside LDL-C and TC in PCOS female offspring, which appears contradictory given HDL-C's protective role. This pattern likely reflects overall lipid metabolic dysregulation rather than isolated protective changes. The concurrent elevation of all lipid fractions suggests fundamental perturbations in lipid homeostasis, potentially involving altered hepatic synthesis or lipoprotein clearance mechanisms [33]. Moreover, HDL-C functionality may be compromised in the pro-inflammatory environment indicated by our proteomic findings, as dysfunctional HDL-C particles can lose their anti-atherogenic properties despite elevated levels [34]. This paradoxical lipid pattern emphasizes that cardiovascular risk assessment in PCOS offspring cannot rely solely on traditional lipid levels but requires comprehensive evaluation of both lipid functionality and inflammatory markers.

Consist with our study, several research have found early metabolic derangements in female offspring of PCOS [14, 35]. It seems that the alterations in lipid metabolism can be detected in PCOS offspring as early as birth [15]. A Dutch study observed subtle, but distinct, cardiovascular and metabolic abnormalities in prepubertal PCOS offspring, comprising early arterial stiffness, left ventricular dilation, and higher TG, LDL-C and carotid intima thickness [36]. It has been proposed that a combination of environmental and genetic factors determines the health of the offspring [37]. According to the Barker hypothesis, early genetic adaptation to less-than-ideal conditions in utero may impact fetal programming and result in poor health in later life [9]. Increasing evidence showed that PCOS may modify the endocrine and metabolic functions of offspring, which may be attributed to a combination of genetic inheritance and intrauterine environment, epigenetic programming, and hormonal influences [38]. It is noteworthy that the incidence of GDM was higher among patients with PCOS, although the difference was not statistically significant, possibly due to the limited sample size. Previous studies have indicated that women with PCOS have a higher risk of developing GDM [39], which may influence the lipid metabolism of their offspring through intrauterine environmental effects [40].

To date, rather than single biomarkers, simultaneous evaluation of numerous biomarkers using novel technology may have potential to enhance prediction of CVD risk at an early age. In this study, we combined Olink proteomics [41, 42], which involved 92 cardiovascular risk protein markers, and untargeted metabolomics to comprehensively analyze the alternations related to CVD.

The observed elevation of PI and LysoPC species in PCOS female offspring, positively correlated with lipid indicators, suggests dysregulation of glycerophospholipid metabolism. PIs, beyond structural roles, are central to signaling pathways like PI3K/Akt, which regulate endothelial nitric oxide production and vascular tone [43, 44]. Disrupted PI metabolism impairs this pathway, contributing to endothelial dysfunction, vascular inflammation, and hypertension [44]. LysoPCs are potent bioactive lipids involved in oxidative stress and inflammation. They enhance ROS production via NADPH oxidase and activate pro-inflammatory signaling through receptors such

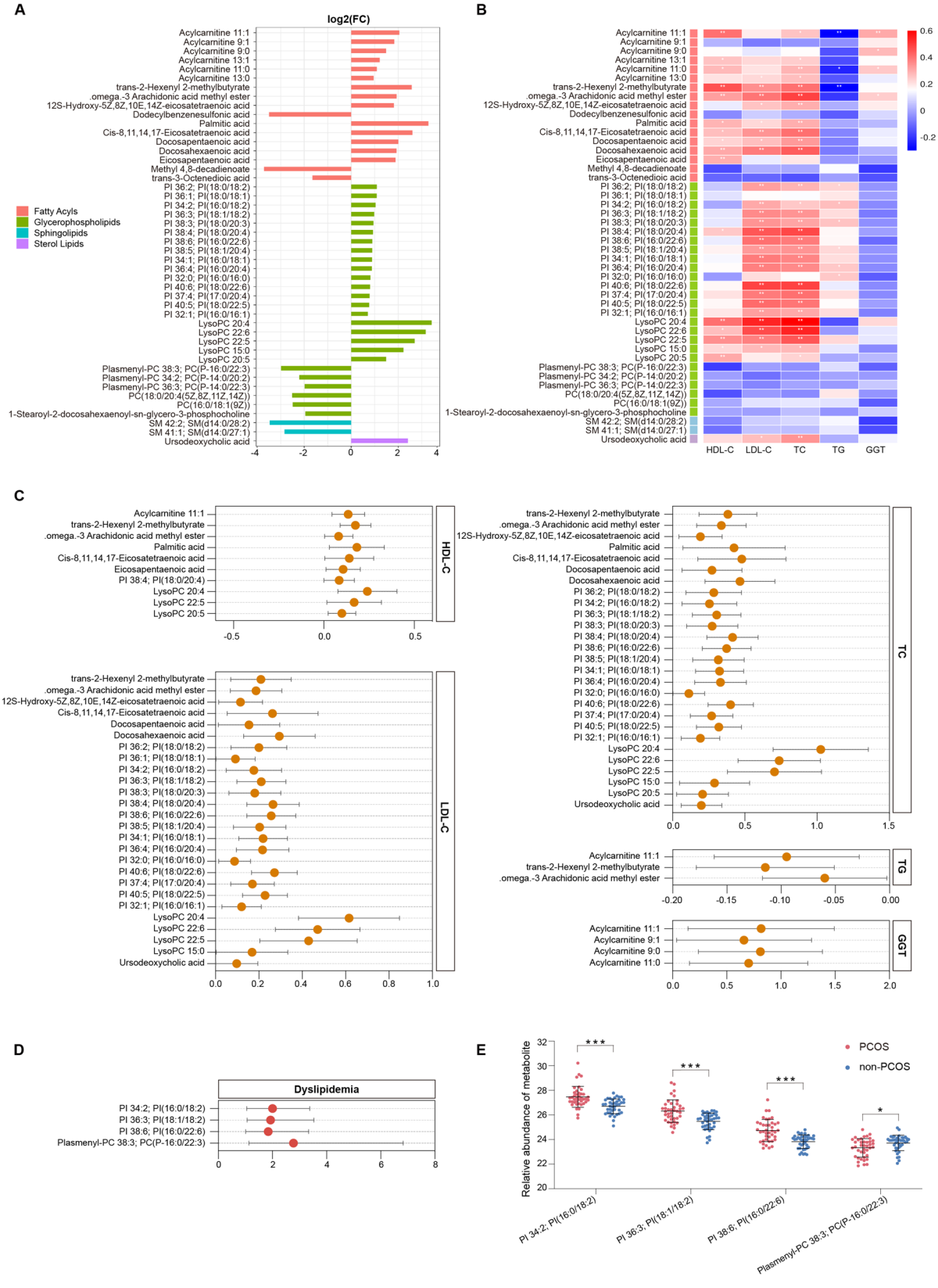

Fig. 3 (See legend on next page.)

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Fig. 3 The associations of differential lipid metabolites with cardiometabolic risk features. **A** The bar chart showed the log2(FC) of 46 differential lipid metabolites between offspring of PCOS and non-PCOS groups. **B** The correlation heatmap of 46 differential lipid metabolites related with cardiometabolic risk features, including HDL-C, LDL-C, TC, TG and GGT. Two-sided spearman correlations are shown. * *P*-value < 0.05, ** *P*-value < 0.001. **C** The forest plot showed the β-coefficients (95% CI) of differential lipid metabolites with cardiovascular risk feature (*P*-value < 0.05). Linear regression analysis was performed while the measure of centre for the error bars presented the β-coefficients of differential lipid metabolites, the solid line demonstrated the 95% CI of β-coefficients. **D** The forest plot showed the ORs (95% CI) of differential lipid metabolites with dyslipidemia (*P*-value < 0.05). Logistic regression analysis was performed. **E** Relative abundance of specific metabolites in figure D. Data were presented as median values ± SD. * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001. FC, fold change; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; GGT, γ-glutamyl transferase; OR, odds ratio; CI, confidence interval; SD, standard deviation

as G2A and Toll-like receptors. This leads to endothelial activation, cytokine release, and monocyte recruitment, key events in early atherogenesis [45]. Elevated LysoPCs thus indicate a shift toward a pro-inflammatory vascular environment in PCOS offspring.

The observed reduction in SM levels is consistent with trends seen in obese children, and SM depletion has been correlated with inflammation [46, 47]. Chronic low-grade inflammation is a major risk factor for cardiovascular diseases, as it damages endothelial cells and promotes atherosclerosis, increasing the risk of cardiovascular events [48].

A significant increase in ursodeoxycholic acid level was found in the female offspring of PCOS group along with changes in other bile acids, indicating a disruption in bile acid metabolism. Disrupted bile acid metabolism is linked to dyslipidemia associated with increased cardiovascular risk [49]. Bile acids regulate lipid metabolism through nuclear receptors like farnesoid x receptor. A disturbance in this process may impair lipid homeostasis [50]. Together, these metabolic alterations suggest an early imbalance in lipid signaling pathways that may predispose PCOS offspring to cardiovascular dysfunction.

The proteomic analysis revealed several elevated CVD-related proteins in PCOS female offspring, with PCSK9 emerging as a particularly significant finding. As a key regulator of LDL-C metabolism, PCSK9's upregulation likely contributes to the observed elevations in LDL-C levels and increased hyperlipidemia prevalence in these children [51]. This aligns with previous research showing elevated PCSK9 levels in women with PCOS, where it correlates positively with androgen levels and insulin resistance [52]. Recent genetic studies have further strengthened this connection, demonstrating that decreased PCSK9 levels are associated with lower PCOS risk, and genetic variants affecting PCSK9 correlate with reduced PCOS susceptibility [53].

The study also identified elevated levels of TFPI, ST2, JAM-A, EGFR, and SCGB3A2, providing additional evidence of cardiovascular risk. TFPI plays a crucial role in regulating coagulation by inhibiting both FXa and the tissue factor-FVIIa complex [54], while ST2 has emerged as a validated biomarker in protein-based models for predicting cardiovascular events [42]. JAM-A's involvement in endothelial barrier function and leukocyte transmigration suggests its potential role in early atherosclerosis development [55, 56]. EGFR signaling affects vascular smooth muscle cell behavior and subsequent atherosclerotic progression [57], while SCGB3A2 modulates lipid metabolism through phospholipase A2 inhibition [58]. Together, these proteins point to dysregulation across multiple cardiovascular pathways including coagulation, inflammation, and vascular remodeling in PCOS female offspring.

The strong predictive power of a combined metabolite and protein panel comprising PI, LysoPC species and proteins, highlights the potential utility of these markers for early identification of PCOS female offspring at high risk of developing hyperlipidemia and future CVD. Specifically, the significant association between the altered phospholipid metabolism, CVD-related proteins and hyperlipidemia further support the potential role of these molecular markers in the pathogenesis of CVD risk in PCOS female offspring. Early identification of at-risk individuals is crucial for implementing targeted interventions and lifestyle modifications to prevent the development of overt cardiovascular complications [59, 60].

The strengths of this study include the comprehensive metabolomics and proteomics approach, which allowed for the simultaneous investigation of a wide range of metabolites and proteins associated with lipid metabolism and CVD risk. The integration of these omics data with clinical parameters provided a more holistic understanding of the molecular mechanisms underlying the increased CVD risk in PCOS female offspring. Additionally, the inclusion of a well-characterized cohort of PCOS female offspring and age-matched controls enabled the identification of specific alterations associated with PCOS. Consistent with previous studies reporting metabolic abnormalities before the onset of hyperandrogenism in girls with precocious puberty, our findings suggest that the observed metabolic changes and cardiovascular risk may serve as early indicators of PCOS, emphasizing the need for early monitoring [14, 61].

However, several limitations of this study should be acknowledged. First, the cross-sectional design precludes the establishment of causal relationships between the observed molecular alterations and CVD risk. Longitudinal studies are needed to evaluate the temporal dynamics of these alterations and their long-term impact on cardiovascular health. Second, the cross-sectional design

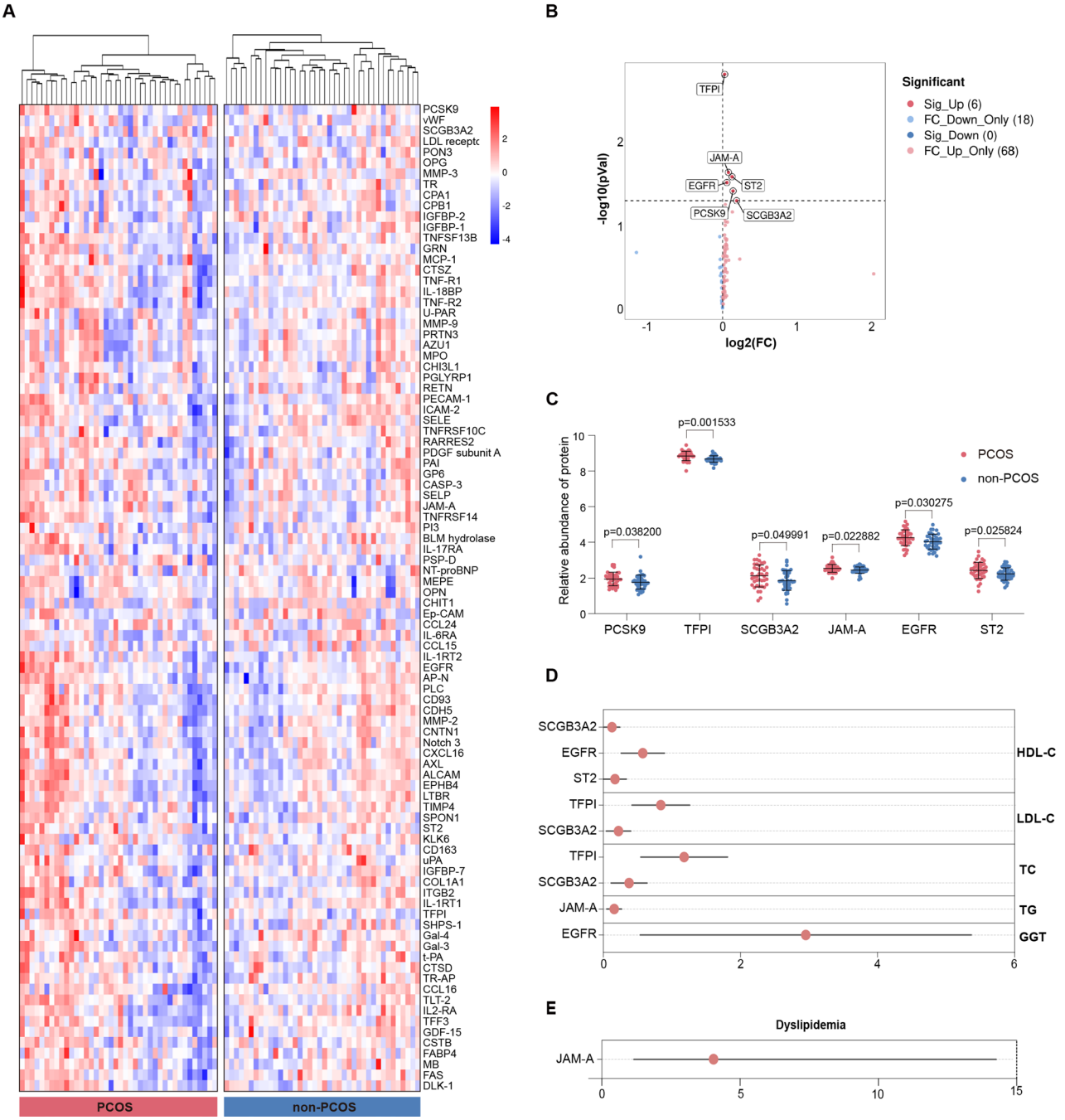

Fig. 4 Overview of CVD-related proteomic alternation between offspring of PCOS and non-PCOS groups. **A** The heatmap of proteins in Olink proteomics between the offspring of the PCOS and non-PCOS groups. **B** The volcano plot showed differential proteins marked with their name between offspring of PCOS and non-PCOS groups. Upregulated and downregulated proteins were highlighted as red and blue based on thresholds of FC = 1. Proteins with *P*-value < 0.05 are shown in deeper colors. **C** Relative abundance of differential proteins shown in figure B. Data were presented as median values ± SD. **D** The forest plot showed the β-coefficients (95% CI) of differential proteins with cardiovascular risk feature (*P*-value < 0.05). Linear regression analysis was performed. **E** The forest plot showed the ORs (95% CI) of differential proteins with dyslipidemia (*P*-value < 0.05). Logistic regression analysis was performed. CVD, cardiovascular disease; FC, fold change; SD, standard deviation; OR, odds ratio; CI, confidence interval

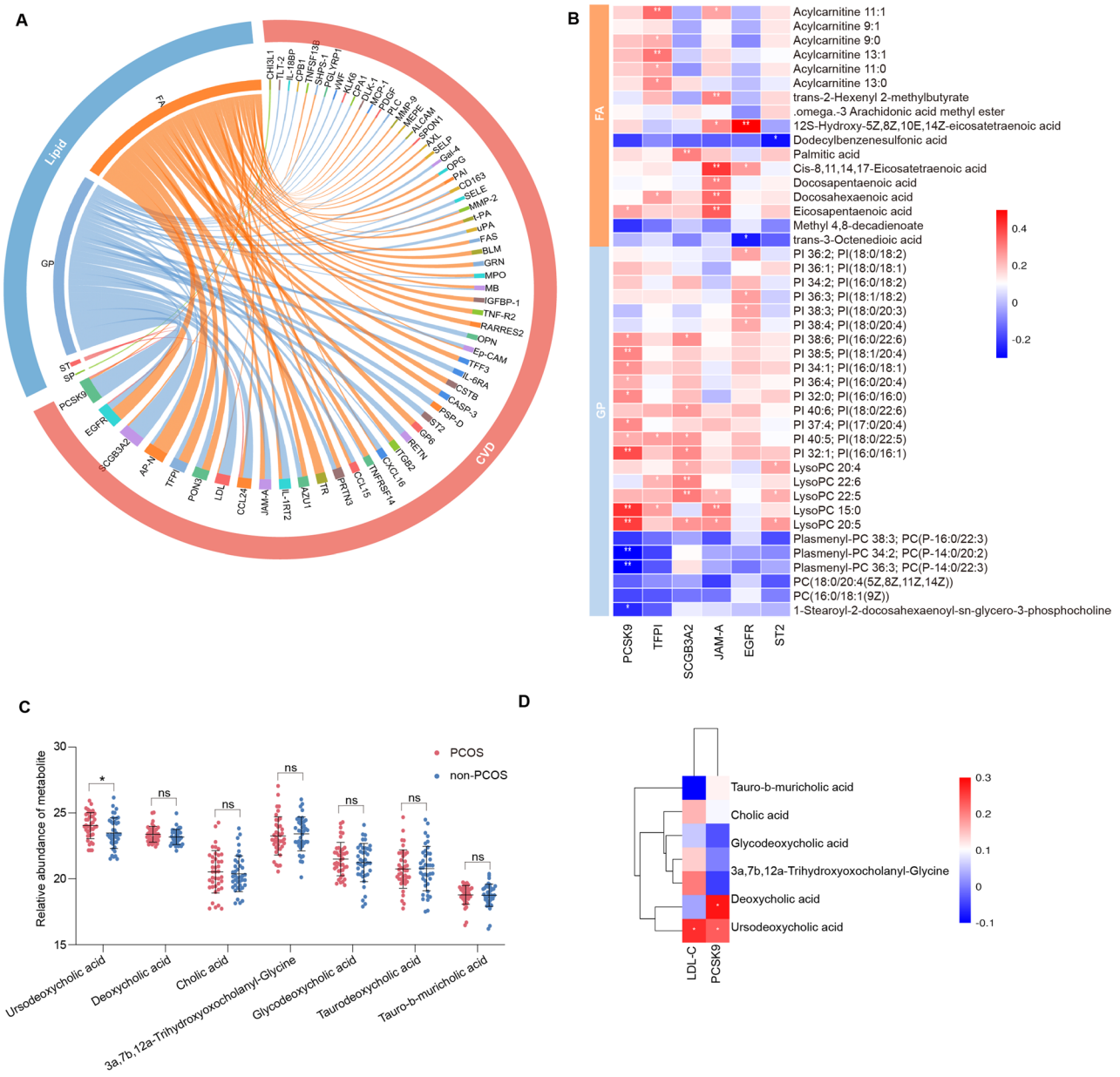

Fig. 5 Correlations of lipid metabolites with CVD-related proteins.A.The circular chord diagram illustrated the associations between lipid metabolites and CVD-related proteins. Each connection represents significant correlation between specific lipid metabolites and CVD-related proteins (P-value <0.05). Two-sided spearman correlations were conducted.B.The correlation heatmap displayed the relationships between differential lipid metabolites classfied as GP and FA species and 6 differential CVD-related protein. * P-value < 0.05, ** P-value < 0.001.C.Relative abundance of bile acids in the offspring of PCOS and non-PCOS groups. Data were presented as median values ± SD. * P-value < 0.05, ** P-value < 0.01, *** P-value < 0.001, ns, not significant.D.Heatmap illustrated the correlation between bile acids and CVD markers (LDL-C, PCSK9). Two-sided spearman correlations were conducted. * P-value < 0.05, ** P-value < 0.001.CVD, cardiovascular disease; GP, glycerophospholipid; FA, fatty acid; SD, standard deviation, LDL-C, low-density lipoprotein cholesterol; PCSK9, proprotein convertase subtilisin/kexin type

and the relatively small sample size precluded a detailed subgroup analysis to determine which specific maternal PCOS phenotype is most strongly associated with the observed early cardiometabolic alterations in the offspring. Future, larger-scale, longitudinal studies are warranted to address this critical question and to guide phenotype-specific screening in the offspring population. Third, despite comparable residence and feeding patterns between groups, the potential influence of unmeasured confounders such as diet, physical activity, and parental socioeconomic status on metabolic and proteomic outcomes cannot be excluded.

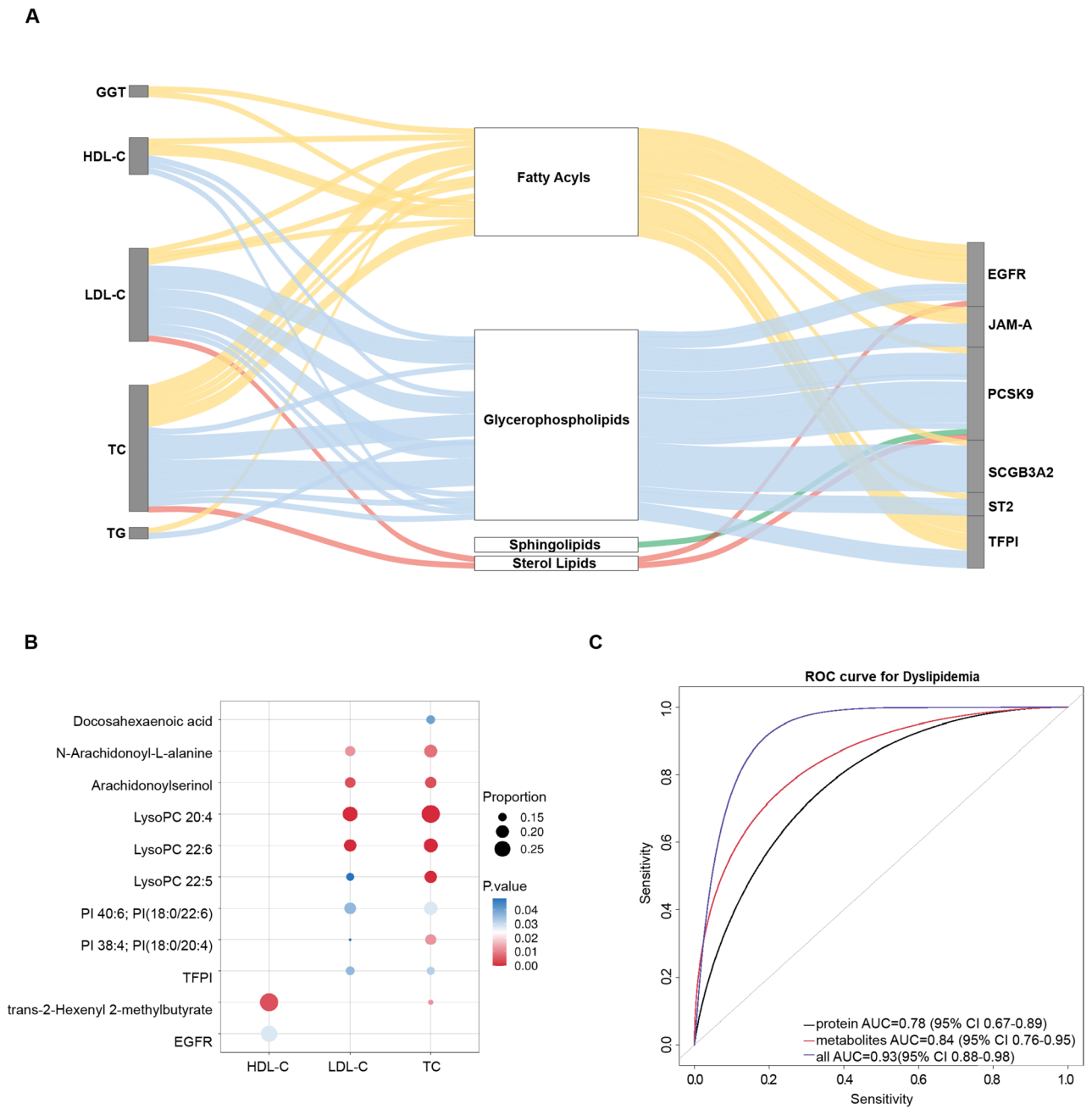

Fig. 6 Identification of differential metabolite and protein biomarkers towards high cardiovascular risk. **A** The sankey diagram depicted the relationships between lipid classes and cardiometabolic risk features and CVD-related proteins. The colors of curved lines represent different lipid classes of metabolites. **B** Mediation analysis was performed. Each dot represents a significant indirect effect (*P*-value < 0.05), with dot size indicating the mediation proportion. **C** ROC curves for logistic regression model used a combined metabolite and protein panel consisting of PI, LysoPC species, and CVD-related proteins to predict hyperlipidemia. The mean AUC values with their respective 95% CI were also provided for each ROC curve. ROC, receiver operating characteristic; PI, phosphatidylinositol; LysoPC, lysophosphatidylcholine; AUC, area under the curve

Conclusion

This study reveals significant alterations in lipid metabolism and CVD risk in the female offspring of PCOS patients, as evidenced by the distinct lipid metabolite and protein profiles. These findings suggest the potential value of early metabolic monitoring in PCOS female offspring and highlight the need for longitudinal studies to determine whether these early alterations translate to increased cardiovascular risk in adulthood. While early intervention strategies targeting lipid metabolism pathways may be beneficial, their necessity and efficacy require further investigation through prospective studies.

Abbreviations

AC	Acylcarnitine
ALT	Alanine Aminotransferase
ART	Assisted Reproductive Technology
AST	Aspartate Aminotransferase
AUC	Area Under the Curve
BMI	Body Mass Index
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
DOHaD	Developmental Origins of Health and Disease
EGFR	Epidermal Growth Factor Receptor
FA	Fatty Acid
FET	Frozen-Embryo Transfer
FSH	Follicle-Stimulating Hormone
FVIIa	Factor VIIa
FXa	Factor Xa
GDM	Gestational Diabetes Mellitus
GGT	γ-Glutamyl Transferase
GP	Glycerophospholipid
HDL-C	High-Density Lipoprotein Cholesterol
ICSI	Intracytoplasmic Sperm Injection
IRB	Institutional Review Board
IVF	In Vitro Fertilization
JAM-A	Junctional Adhesion Molecule A
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC–MS	Liquid Chromatography-Mass Spectrometry
LDL-C	Low-Density Lipoprotein Cholesterol
LH	Luteinizing Hormone
LysoPC	Lysophosphatidylcholine
NPX	Normalized Protein Expression
OR	Odds Ratio
PC	Phosphatidylcholine
PCOS	Polycystic Ovary Syndrome
PCSK9	Proprotein Convertase Subtilisin/Kexin Type 9
PI	Phosphatidylinositol
PI3K/Akt	Phosphoinositide 3-Kinase/Protein Kinase B
QC	Quality Control
ROC	Receiver Operating Characteristic
SBP	Systolic Blood Pressure
SCGB3A2	Secretoglobin Family 3A Member 2
SL	Sphingolipid
SM	Sphingomyelin
ST	Sterol Lipid
ST2	Interleukin-1 Receptor-Like 1 (also known as IL1RL1)
T	Testosterone
TC	Total Cholesterol
TFPI	Tissue Factor Pathway Inhibitor
TG	Triglyceride
TyG Index	Triglyceride-Glucose Index
UDCA	Ursodeoxycholic Acid
UHPLC	Ultra-High Performance Liquid Chromatography
VIP	Variable Importance for the Projection

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13048-026-02019-8.

Supplementary Material 1.

Supplementary Material 2.

Acknowledgements
Not applicable.

Authors' contributions
Xinyue Wu: Methodology, Resources, Formal analysis, Software, Data curation, Writing-Original Draft. Xiangning Deng: Conceptualization, Formal analysis, Writing-Original Draft, Validation, Funding acquisition. Shuangying Liu: Software, Data Curation, Validation. Haining Wang: Investigation, Writing-Review & Editing. Yingzhi Yang: Data Curation, Methodology. Aqiong, Xie: Data Curation, Methodology. Yimin Zhu: Conceptualization, Project administration, Writing-Review & Editing, Supervision, Funding acquisition, Minyue Tang: Conceptualization, Project administration, Data analysis, Writing-Original Dra ft and Editing, Supervision.

Funding
This work was supported by the Key research and development Program of Zhejiang Province (2025C02116), Chinese Cardiovascular Association-Natural lipid-lowering drugs fund (2023-CCA-NLD-563).

Data availability
Data could be accessed on reasonable request to the corresponding author. A proposal with detailed description of study objectives and statistical analysis plan will be needed for evaluation of the reasonability of requests.

Declarations

Ethics approval and consent to participate
All sample collection procedures were approved by the Institutional Review Board of Women's Hospital, School of Medicine, Zhejiang University (Approval No. IRB-20220331-R) and written informed consent was obtained from all participants and by a parent or guardian with parental responsibility on behalf of their children.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 2 November 2025 / Accepted: 2 February 2026
Published online: 10 February 2026

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