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Proteomic Dysregulations of Infertile PCOS vs. Non PCOS Females via Label-Free Quantitative Mass Spectrometry

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Key words
Infertility, Mass spectrometry, PCOS, Proteomics, Lipid metabolism, Clotting mechanism

ABSTRACT

Infertility presents as a complex and multifaceted challenge, characterized by inability to conceive, even after a year of engaging in regular and unprotected intercourse. The current study aims to investigate the underlying molecular mechanism of infertility related to PCOS and non-PCOS by analyzing the differential protein expression of the subgroups of infertile women compared to fertile healthy control (HC) group. Serum samples from 90 subjects were divided into three groups: infertile females with PCOS (n = 30), infertile females without PCOS (n = 30), and fertile healthy controls (n = 30). NanoLC-MS/MS analysis revealed significant differences in protein expression profiles between PCOS, non-PCOS and control groups. A total of 283 proteins were identified, with 51 significantly altered in PCOS and 62 in non-PCOS compared to controls. Thirty proteins showed similar expression patterns across subgroups, while 14 were unique to PCOS and 24 to non-PCOS. Key findings included the downregulation of CL-2 in both PCOS and non-PCOS, upregulation of CL-4 in both groups compared to controls, and unique expression patterns for CL-1, CL-3, and CL-5 of the heat map. Differentially expressed proteins included apolipoproteins, complement proteins, globins, protease inhibitors, and others. Pathway analysis indicated PCOS was associated with dysregulation of the alternative complement pathway and platelet activation, while non-PCOS showed variations in lipid metabolism and insulin-like growth factors. Comprehensive analysis revealed the significant molecular players and pathways implicated in lipid metabolism and clotting mechanisms. These findings offer valuable insights into the pathophysiology of infertility in PCOS and non-PCOS conditions. It may be used to systematically design molecular assays for understanding this disorder and improving its diagnosis and treatment in future.

INTRODUCTION

When a couple remains unable to conceive after a year of consistent unprotected intercourse, it is considered as infertility. It poses significant emotional, psychological, and financial burden on the individuals and families. According to recent global estimates, 8-12% of couples in their reproductive age range experience infertility, with specific regional variations, such as approximately 21.9% in Pakistan (Vander-Borght and Wyne, 2018; Ali *et al*., 2007). The most prevalent aetiologies of infertility including, ovulatory disorders, endometrial polyps and diminished ovarian reserve, constitute significantly to female infertility. Endometriosis can lead to anatomic distortions, such as development of adhesions that can impede the fallopian tubes, thereby hindering tubal function and patency, and is therefore classified as a significant contributor of female infertility (Carson and Kallen, 2021). A considerable number of couples experience infertility where exact cause is unknown or unidentifiable. This is termed as 'unexplained

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infertility, as female partner exhibits normal ovulation and the male partner demonstrates normal semen analysis (Wang *et al*., 2020).

Among ovulatory disorders, polycystic ovarian syndrome (PCOS) stands out as one of the most prevalent and clinically significant conditions, affecting approximately 70% of women with anovulation (irregular ovulation leading to the duration of cycle falling below 21 days or exceeding up to 35 days) (Carson and Kallen, 2021). In the South Asian region, particularly Pakistan, the prevalence rate is up to 52%, when compared to the white population, where it is about 20 - 25% (in the UK) (Azhar *et al*., 2020). First described by Stein *et al*. (1935), PCOS is considered a complicated hormonal disorder (Sagvekar *et al*., 2018) characterised by hirsutism, amenorrhea, and large polycystic ovaries (Lujan *et al*., 2018), predominantly affecting females at child bearing age. The Rotterdam criteria is used for the diagnosis of PCOS, which require the presence of two or three of the following conditions, such as oligo-anovulation, clinical and/or biochemical hyperandrogenism and polycystic ovaries (AFC ≥ 12 and/or ovarian volume >10 ml) (Lauritsen *et al*., 2014). PCOS is a multifactorial disease, demonstrating complex interplay between predisposition of genetic variants and environmental factors (Carmona-Ruiz *et al*., 2015), which results in diverse representation of its features across various age and ethnic groups, thereby limiting the true representation of the syndrome (Rashid *et al*., 2020). The major risk factors of PCOS, includes obesity, dyslipidemia, insulin resistance, hypertension, type 2 diabetes mellitus, and cardiovascular disease (CVD), further complicating its management (Group, 2003; Wilcock and taylor, 2018; Shi *et al*., 2021). There is no straight forward genetic test available that can be used to diagnose the condition and its underlying pathophysiological mechanisms (Rani *et al*., 2023).

Moreover, the proteomic approaches that are non-targeted have the benefit of determining location, abundance, protein-protein interactions and posttranslational modifications (Insenser *et al*., 2019). Any change in the expression of proteins depicts altered biological processes as proteins control the cellular functions (Khan *et al*., 2015). Therefore, in contrast to genome-based studies, advanced proteomics techniques are fairly beneficial as they interact first-hand with the functional molecules as compared to genes or mRNA (Abdallah *et al*., 2012). Moreover, the proteome of an organism undergoes posttranslational modifications and changes in relation to the environment cannot be predicted by the genetic coding (Abdallah *et al*., 2012). Thus, proteomics serves to further advance the understanding of pathophysiology of the disease (Pandeswari *et al*., 2019).

Despite the disease burden, molecular abnormalities and underlying pathophysiology of infertility caused by PCOS and non-PCOS have not yet comprehensively been explored. Currently, very limited studies have been conducted on the proteomics of PCOS in Pakistan. Hence, this study was designed to investigate the differential expression of proteins in infertile PCOS and non-PCOS groups as compared to fertile HC group by using nano LC-MS/MS mass-spectrometry. By exploring the shared differentially expressed proteins of PCOS and non-PCOS in comparison to HC, the study aims to elucidate common molecular pathways underlying infertility, providing valuable insights into the pathophysiology of PCOS-related infertility. Whereas unique differentially expressed proteins may offer clues to distinct mechanisms contributing to infertility in PCOS versus non-PCOS individuals, shedding light on the heterogeneity of infertility and potentially identifying novel diagnostic markers or therapeutic targets.

MATERIALS AND METHODS

Subject selection

A case control study was designed following the approval from Ethical Review Committee (ERC AKU 2022-7509-22501) of Aga Khan University, Karachi. Participants were recruited from the Endocrinology Clinic of the same hospital, where after obtaining an informed consent, socio-demographic data including age, weight, height, and brief medical history were recorded in order to classify fertile and infertile subjects. The subjects were categorized into three groups: healthy fertile females as healthy controls, and primary infertile females with and without PCOS as cases. The control group consisted of females matched in age, gender, and BMI with the cases, having a child aged less than 2-3 years, showing no signs of disease, and not using oral contraceptives. For the case groups, primary infertile females aged 20-40 years diagnosed with PCOS using the Rotterdam Criteria were included, regardless of ethnic background. Each group comprised 30 subjects. Exclusion criteria applied to those with secondary infertility, recent hormonal therapy, BMI < 18 or > 30 Kg/m², or diagnosed with other relevant conditions.

Sample size calculation

$$n = \frac{Z^2_{\alpha/2}\, pq}{e^2}$$

N= Sample size; α = Confidence Interval = 95%; p= Prevalence= 22% (Sami and Ali, 2012); q= 1 – p; e= Error Margin= 5% = 0.05

Overall sample

$$n = \frac{(1.96)^2\,(0.22)(0.78)}{(0.05)^2} = 264$$

Proportionate into fertile and infertile groups: Infertility 21.9% = 22%; $P_{inf\,(pco)}$ = 52% (Amera-Tariq *et al*., 2021); $n_{inf} = n * P_{inf} = 264 * 22\% = 58 \approx 60$; $n_{fer} = n - n_{inf} = 264 - 60 = 204$; $n_{inf\,(pco)} = n * P_{inf\,(pco)} = 60 * 52\% = 31 \approx 30$; $n_{fer\,(nonpco)} = n_{inf} - n_{inf\,(pco)} = 60 - 30 = 30$

Calculated sample size for control group came out to be 204, while for both the cases it came out be 30 each. However, due to time constraint and limitation of resources, the sample number of control group was reduced to 30 as well.

Peripheral blood collection

Blood samples (4 mL) from each study subject were collected in gel-coated vacutainers tubes, on the 2nd day of menstrual cycle. All the samples were centrifuged at 4000 rpm, and sera were transferred into the separate sterile 1.5 mL Eppendorf tubes and stored at -80°C for proteomic analysis (Gayosso-Gómez *et al*., 2014).

Clinical and biochemical data

Radioimmunoassay was performed using an automated chemiluminescence analyzer (Abbott, USA) to evaluate the hormonal profile, encompassing follicle stimulating hormone (FSH), luteinizing hormone (LH), anti mullerian hormone (AMH), prolactin (PRL), and estradiol (E2) levels.

Mass spectrometric based proteomics

For proteomic analysis, serum samples (10 µL) from each participant were pooled based on their respective case and control groups into three separate tubes labelled as Control, PCOS, and Non-PCOS. Protein estimation of each pooled sample was measured using the Invitrogen Qubit™ Protein Assay Kit and Qubit™ 2.0 Fluorometer (Nowak *et al*., 2021). For tryptic digestion, previously optimized protocol was followed with some modifications (Zafar *et al*., 2021). 200 µg of protein from each sample group was mixed with 160µL of 1M NH_4HCO_3. Following denaturation with 100 µL of 45 mM DT and alkylation with 100 µL of 100 mM iodoacetamide,10 µg of trypsin was introduced for protein digestion. Desalting was performed using custom-made tips containing C18-resin. Peptides were eluted sequentially into Protein LoBind tubes by increasing the concentration of ACN from 30% to 70% along with 0.1% TFA. Peptide quantification was performed by Qubit™ Protein Assay Kit (Nowak *et al*., 2021). The peptide mixture (20 µg) was applied to nano-LC–MS/MS using an Orbitrap Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific, USA) which was connected to an EASY-LC 1000 system, as previously described in protocol (Beltran-Camacho *et al*., 2020).

Preprocessing of proteomics data

For identification of proteins, raw files generated fromOrbitrap Q-Exactive HF-X were analyzed on MaxQuant (version 2.3.2, Matrix Science, UK) in conjunction with the Andromeda search engine (Cox *et al*., 2011). False discovery rate (FDR) was set at 1% and spectra were matched against the proteins of Homo sapiens present in UniProt/Swiss-Prot database (Zafar *et al*., 2021). The quantitative proteomic data analysis was conducted using Perseus (v.1.6.10.50). Multiple filtration steps present in Perseus, were applied, and significant proteins were obtained using student's t test. Benjamini and Hochberg method were used for correction of p value for t-test testing with 10% FDR cutoff (Green *et al*., 2007). Multi scatter plot, profile plot, volcano plot and heat map were obtained in order to visualize the variability of protein expression of all groups.

Functional analysis of proteomics data

The PANTHER (protein analysis through evolutionary relationships) database Version 17.0 (Enli *et al*., 2013; Thomas *et al*., 2022) was used to identify classes of common proteins and their proportions which was visualized using a pie chart. For pathway overrepresentation analysis, Gene Ontology database ShinyGO 0.76 (sdstate.edu) was used (Ashburner *et al*., 2000; Consortium *et al*., 2023). The *p*-value of at least 0.05 was considered as significant with FDR correction. String version 12.0 was used to elucidate protein–protein interactions and critical analysis of physical and functional association (http://string-db.org/) (Szklarczyk *et al*., 2023). The KEGG (Kyoto encyclopedia of genes and genomes) pathway maps were obtained at 0.05 FDR cut off for up-regulated and down-regulated proteins in both subgroups groups in order to gain insight into the role of protein in the biological system (Ge *et al*., 2019; Kanehisa *et al*., 2020; Luo *et al*., 2013).

Statistical analysis

For all the continuous variables, data were presented as mean ± standard deviation. ANOVA was performed using SPSS (IBM SPSS 21) statistical software. *P*-value ≤ 0.05 considered as significant.

RESULTS

Demographics and biochemical analysis

Overall work flow of the study has been shown in Figure 1. Initially, anthropometric parameters and

biochemical profile of the subjects were compared among subgroups of infertility in comparison to healthy control fertile group. The age and BMI of these females and the hormonal profile including FSH, LH, prolactin, estradiol and AMH were also recorded in all groups as shown in Table I. The results showed that neither age nor BMI was statistically significant in comparison of infertile cases to healthy fertile control group. The hormonal profiles showed significant variation among the subgroups of infertility. In comparison of PCOS *vs* HC, FSH levels showed no significant difference, whereas LH hormone levels were notably elevated, and significant reductions were observed in the mean levels of prolactin and estradiol. Conversely, when comparing the non-PCOS subgroup to the HC group, there were distinct mean reductions in the levels of LH, prolactin, and estradiol. Moreover, a comparison between the PCOS and non-PCOS subgroups revealed distinct mean elevation in LH and reductions in prolactin and estradiol levels, as delineated in Table I. AMH levels did not show any significant difference among the groups.

Table I. Bio demographical characteristics, Biochemical profile of fertile and infertile female subgroups (PCOS and non-PCOS).

Variable	HC (N=30)	PCOS (N=30)	Non-PCOS (N=30)	p value
Age	32±3.63	32.13±4.23	31.7±5.12	0.92
BMI	23.3±1.27	22.78±2.15	22.74±2.09	0.40
FSH (unit)	9.2±11.10	9.5±2.86	6.8±2.20$$	$$
LH (IU/L)	9.2±8.98	10.9±13.08*##	5.1±1.99$$	*##$$
Prolactin (µg/L)	19.9±15.2	14.7±9.25*#	13.1±7.30$$	*#$$
Estradiol (pcmol/L)	189.5±131.29	71.7±45.04*##	77.5±64.77$$	*##$$
AMH (ng/ml)	2.8±2.38	3.3±3.28	2.2±1.28	-

FSH, follicular stimulating hormone; LH, luteinizing hormone; AMH, anti mullerian hormone; HC, healthy control; PCOS, polycystic ovary syndrome; BMI, body mass index; */**, showing significant difference between PCOS and HC group. $/$$ showing significant difference between non-PCOS and HC group. #/## showing significant difference between PCOS and non-PCOS group.

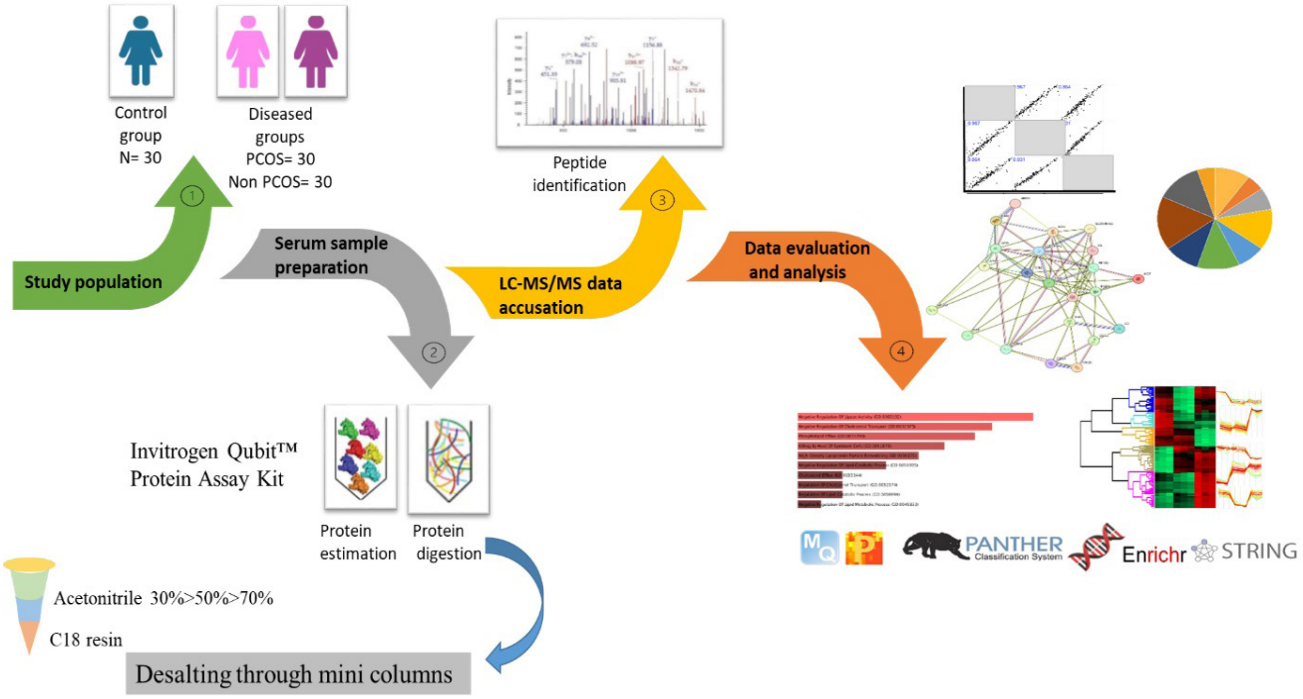

Fig. 1. Diagrammatic representation of the study.

Identification and qualitative assessment of proteins

A total number of 283 proteins were identified using MaxQuant (Fig. 2A). Complete list of identified proteins is provided in Electronic supplementary file Supplementary Table SI. Multiple filtration steps were applied in Perseus (v.1.6.10.50) including, only identified by site, reversed and, removing contaminants to identify the most authentic proteins of interest (Fig. 2A). The total number of proteins in HC group was 168, whereas 176 total proteins were present in each PCOS and Non-PCOS groups. Among these 176 proteins, 10 proteins were exclusively present in infertile PCOS and non-PCOS subgroups (Electronic Supplementary Table SII), while 2 proteins were exclusive in HC. A total of 164 proteins were common in all infertility (PCOS and non-PCOS) and HC group (Fig. 2B). The variation between the infertile (PCOS and non-PCOS) and HC groups was evaluated by using multi-scatter plot analysis correlation coefficient (Fig. 2C), while profile plot showed an overall quantitative trend of proteins, with each line representing a unique protein (Fig. 2D).

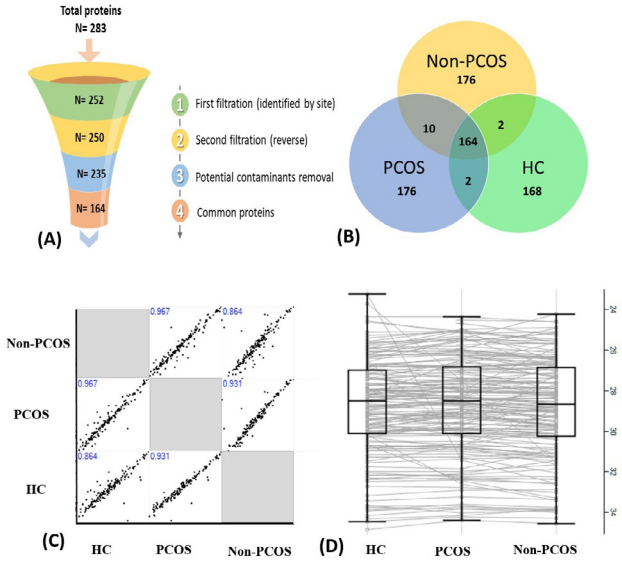

Fig. 2. Graphic representation of qualitative proteomic data analysis. (A) Filtration procedure implemented on the identified and quantified proteins. (B) Venn diagram showing proteins distribution in each group. Healthy control (HC), polycystic ovary syndrome (PCOS) and Non-PCOS. (C) Analysis of variability of proteins between the groups as shown by multi scatter plot. (D) Profile plot of proteomic data between study groups.

Quantitative proteomic data analysis

From the 283 identified proteins, 51 proteins were found to be significantly differentially expressed proteins between PCOS *vs* HC group. Among these, 18 proteins exhibited up-regulation, while 33 proteins showed down-regulation. Conversely, in the non-PCOS *vs* HC group, we observed 62 significantly differentially expressed proteins, with 15 proteins displaying up-regulation and 47 proteins showing down-regulation. The pattern of protein expression in infertile groups was found to be consistent for about 30 proteins. Specifically, 13 proteins exhibited down-regulation and 17 showed up-regulation in both the infertility subgroups, as depicted in (Fig. 3A) and detailed in (Supplementary Table SIII). Additionally, 24 and 14 proteins were found to be exclusive identified in the non-PCOS *vs* HC group and PCOS *vs* HC group, respectively. To further understand the expression profiles, a heat map was generated based on *z*-score values of LFQ intensities, illustrating differential expression in both infertility subgroups (Fig. 3B). Proteins present in each cluster of heat map are listed in (Supplementary Table SIV). Clustering analysis identified five main clusters of differentially expressed proteins. Specifically, CL-1 was exclusively down-regulated in PCOS as compared to HC group, CL-2 was downregulated in both PCOS and non-PCOS group when compared to HC group, CL-3 was exclusively and significantly down-regulated in non-PCOS as compared to HC group, CL-4 displayed up-regulation in both the PCOS and non-PCOS when compared to HC group, and CL-5 was exclusively and significantly up-regulated in non-PCOS when compared to HC (Fig. 3C). The protein categories of differentially expressed proteins of PCOS *vs* HC group are apolipoproteins, complement component, globins, protease inhibitor, protein-binding activity modulator, serine protease and transfer/carrier proteins (Fig. 3D). The protein categories of differentially expressed proteins of non-PCOS *vs* HC group comprise of apolipoproteins, complement component, globins, protease inhibitor, protein-binding activity modulator, serine protease and transfer/carrier proteins and proteases (Fig. 3E). Overall both the groups revealed similar protein classes with different percentages except proteases that were only observed in non- PCOS group.

Functional analysis

To elucidate the roles of the significantly differentially expressed proteins common in PCOS vs non PCOS group, GO functional enrichment analysis was performed for upregulated and downregulated proteins respectively. Complement activation and immunoglobulin mediated immune response were the enriched biological processes by upregulated proteins while downregulated proteins were involved in negative regulation of very-low-density lipoprotein particle clearance, chylomicron remnant clearance, negative regulation of cholesterol transport and lipoprotein lipase activity, nitric oxide transport, negative

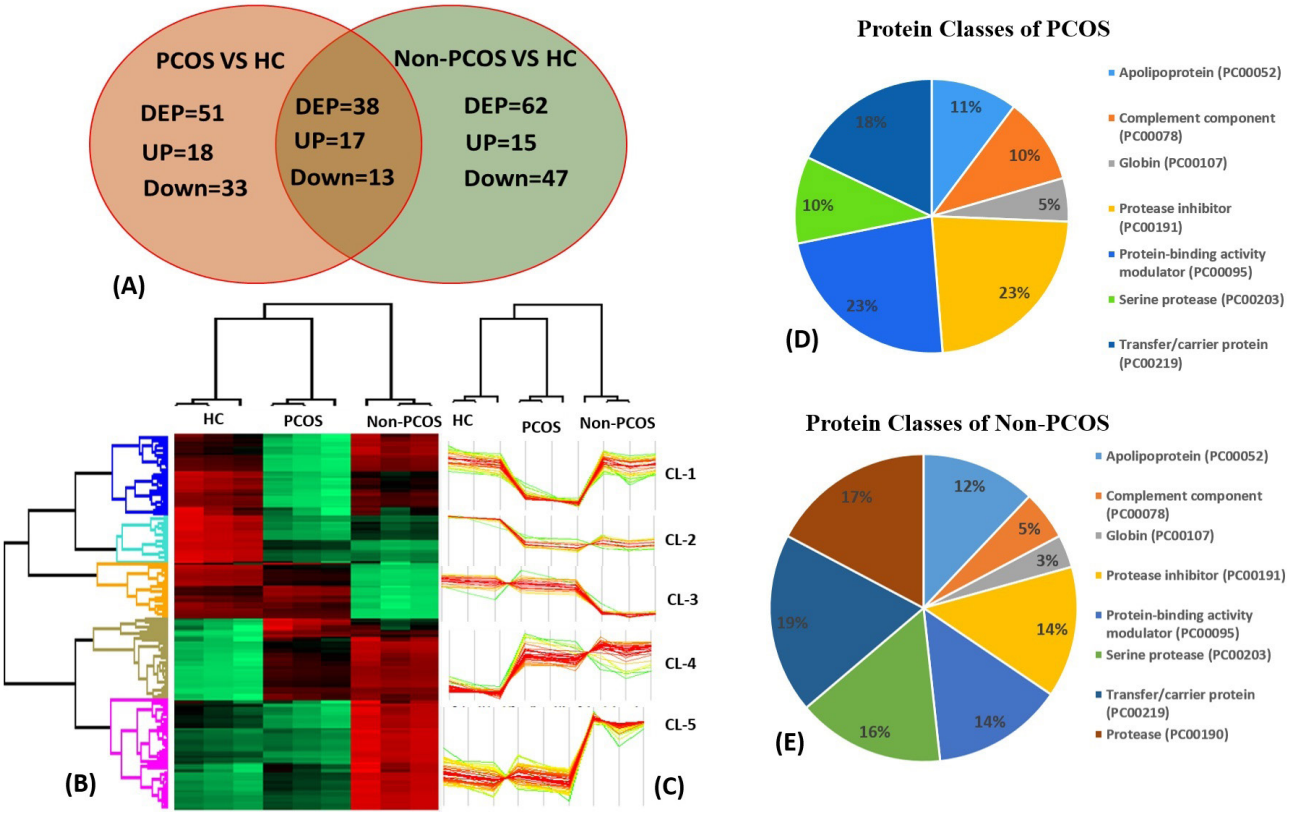

Fig. 3. Quantitative analysis of proteins in subgroups (PCOS, and non-PCOS) of infertility compared to HC. (A) Statistical data analysis revealing the differentially expressed proteins (DEPs), significantly up-regulated and down-regulated in both subgroups of infertility as well as in individual groups. (B) Heat map analysis of significant proteins in all groups. (C) Cluster analysis of significant proteins in all groups. (D) Protein classes of differentially expressed proteins of PCOS (E) Protein classes of differentially expressed proteins of non-PCOS.

GO enriched biological process by upregulated proteins (p value <0.001)
Complement activation
Immunoglobulin mediated immune response
GO enriched biological process by downregulated proteins (p value <0.001)
Negative regulation of very-low-density lipoprotein particle clearance
Chylomicron remnant clearance
Negative regulation of cholesterol transport
Negative regulation of lipoprotein lipase activity
Nitric oxide transport
Negative regulation of triglyceride catabolic process
High-density lipoprotein particle clearance
Positive regulation of apoptotic cell clearance
Reverse cholesterol transport

(A)

GO enriched biological process by upregulated proteins (p value <0.001)
Nitric oxide transport
Complement activation, classical pathway
Humoral immune response mediated by circulating immunoglobulin
GO enriched biological process by downregulated proteins (p value <0.001)
Negative regulation of complement activation, lectin pathway
Negative regulation of very-low-density lipoprotein particle clearance
Chylomicron remnant clearance
Regulation of very-low-density lipoprotein particle remodelling
High-density lipoprotein particle clearance
Regulation of phospholipid catabolic process
Phospholipid efflux
Fibrinolysis

(B)

GO enriched biological process by upregulated proteins (p value <0.001)
Regulation of high-density lipoprotein particle clearance
Negative regulation of very-low-density lipoprotein particle clearance
Nitric oxide transport
Negative regulation of triglyceride catabolic process
Chylomicron remnant clearance
Regulation of very-low-density lipoprotein particle remodelling
GO enriched biological process by downregulated proteins (p value <0.001)
Complement activation
Positive regulation of phagocytosis

(C)

Fig. 4. Biological process enrichment of differentially expressed proteins common in (A) PCOS/Non PCOS group, (B) PCOS/HC group and (C) Non PCOS/HC group. Upregulated (blue) and downregulated (pink) gene ontology annotations. PCOS, polycystic ovary syndrome; HC, healthy control.

regulation of triglyceride catabolic process, high-density lipoprotein particle clearance, and reverse cholestrol transport (Fig. 4A)

Moreover, GO enriched biological functions of PCOS vs HC upregulated proteins revealed that these proteins were linked to nitric oxide transport, complement activation (classical pathway) and humoral immune response mediated by circulating immunoglobulin. The proteins that showed a decreased differential expression were involved in negative regulation of complement activation and negative regulation of very-low-density lipoprotein particle clearance, chylomicron remnant clearance, regulation of very-low-density lipoprotein particle remodelling, high-density lipoprotein particle clearance, regulation of phospholipid catabolic process, phospholipid efflux and fibrinolysis (Fig. 4B).

Conversely, GO enriched biological functions of non PCOS vs HC showed that the upregulated proteins were involved in regulation of high-density lipoprotein particle clearance, negative regulation of very-low-density lipoprotein particle clearance, nitric oxide transport, negative regulation of triglyceride catabolic process, chylomicron remnant clearance and regulation of very-low-density lipoprotein particle remodelling while the downregulated proteins were involved in complement activation and positive regulation of phagocytosis (Fig. 4C).

Pathway over representation analysis was also conducted on both common and unique proteins associated with infertility, aiming to uncover common and unique disrupted pathways in both study groups (PCOS and non-PCOS). Shared DEPs unveiled significant dysregulation in the coagulation pathways, alongside disturbances in the classical complement system as displayed in (Fig. 5A). Protein-protein interaction (PPI) analysis conducted through STRING also demonstrated a notable association among all common proteins. Subsequently, K-means clustering was employed to classify the proteins into distinct groups. This analysis revealed that 18 out of 30 common proteins were found to be associated with the complement and coagulation pathway (red nodes). The second cluster predominantly exhibited connections to lipid metabolism (dysregulated apolipoproteins) (green nodes), while the third cluster differentiated into two proteins associated with steroid hormone binding proteins (blue nodes) (Fig. 5B).

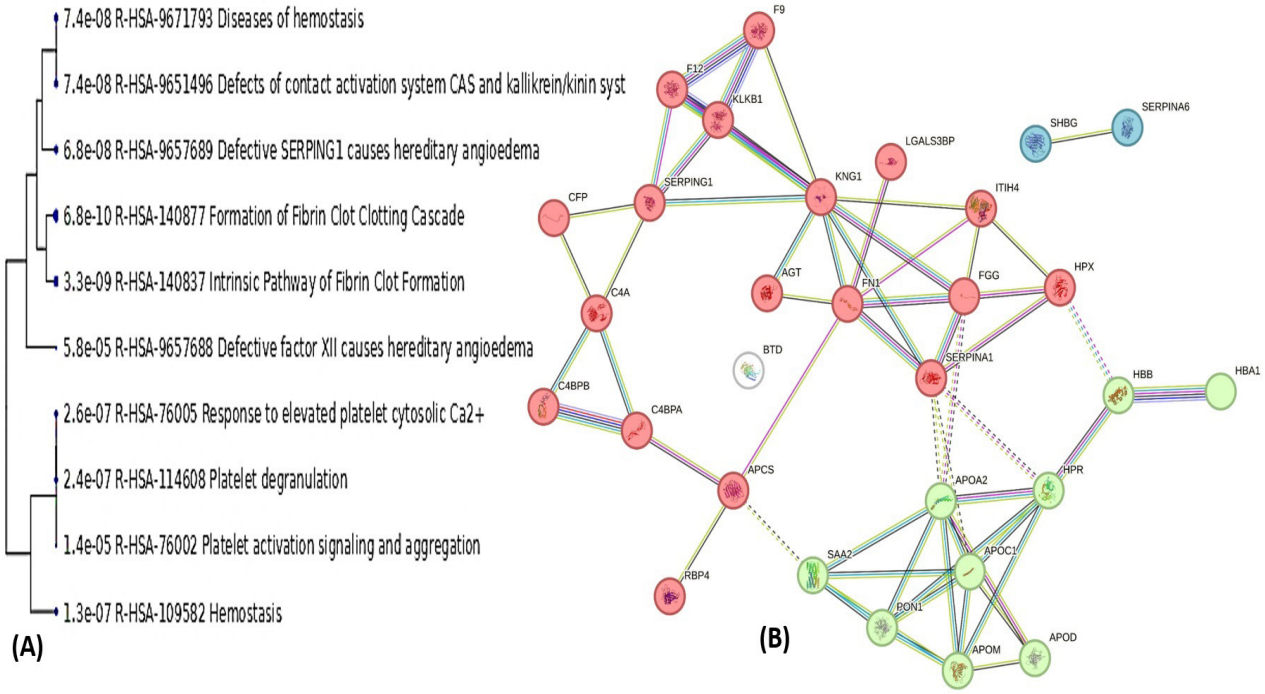

Fig. 5. Functional analysis of common proteins of infertility. Showing similar trend of gene expression in both subgroups of infertility (PCOS, and non-PCOS). (A) Pathway analysis of shared DEPs in both subgroups (PCOS, and non-PCOS) of infertility. (B) PPI network analysis of shared DEPs along with K-means clustering. Colours representation: red nodes= complement and coagulation pathway; green nodes= lipid metabolism; blue nodes= steroid hormone binding proteins.

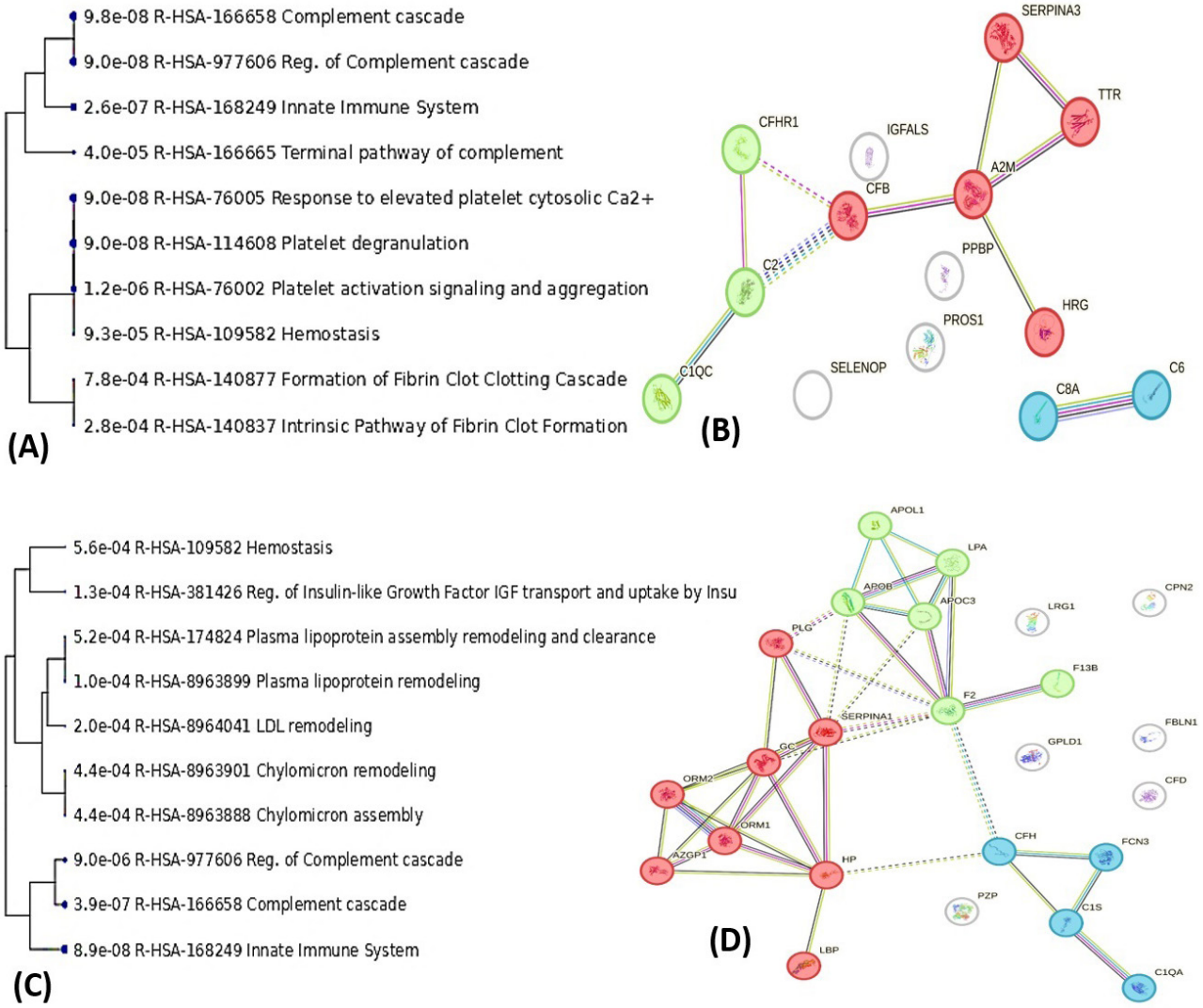

Fig. 6. Functional analysis of unique proteins of infertility. (A) Pathway analysis of exclusively DEPs of PCOS *vs* HC. (B) PPI network analysis of exclusively DEPs of PCOS along with K-means clustering. Colours representation: red nodes= complement activation; green nodes= cytolysis; blue nodes= blood micro particle proteins. (C) Pathway analysis of exclusively DEPs of non-PCOS vs HC. (D) PPI network analysis of exclusively DEPs along with K-means clustering. Colours representation: red nodes= acute phase response; blue nodes= immune response; green nodes=lipid metabolism proteins.

The pathway analysis of unique DEPs associated with PCOS indicated dysregulation in the whole complement cascade, and disruptions in platelet aggregation and degranulation (Fig. 6A). Additionally, the PPI network analysis highlighted strong connections of most of the DEPs as shown in (Fig. 6B), while clustering analysis categorized these proteins into functional groups, such as complement activation (red nodes), cytolysis (green nodes), and anti-inflammatory (blue nodes) proteins. The pathway analysis of unique DEPs associated with non-PCOS condition showed more pronounced disturbances in apolipoproteins associated with the dysregulation of lipid metabolism (Fig. 6C), while PPI network analysis highlighted strong connections of the DEPs, acute phase response (red node), immune response (blue node) lipid metabolism (green node) and proteins (Fig. 6D).

The most significant pathway of both subgroups of infertility are complement and coagulation pathway, KEGG map of up-, and down-regulated proteins in both subgroups highlighted in green and red colours as shown in (Supplementary Fig. 1).

DISCUSSION

Infertility is one of the most prevalent chronic health disorders involving young adults all around the globe (Smith *et al*., 2003). In pursuit of a comprehensive understanding of the pathology, clinical and experimental studies have been performed associated with the female reproductive health, environment and lifestyle (Bala *et al*., 2021). Infertility associated with PCOS and non-PCOS is considered to be the complex disease rather than merely a gynaecological problem. Hence, in order to identify the molecular mechanism linked to pathophysiology of this disease, a comprehensive proteomic profile of the infertile female patients (PCOS and non-PCOS) in comparison with fertile females was performed using Nano-LC MS/MS.

Demographic results showed no significant difference in age and BMI as our samples were matched, however, the biochemical profile showed a significant difference in the levels of various hormones routinely tested for the diagnosis of the infertility. The levels of LH were significantly higher in PCOS as compared to the fertile group which is consistent with the previous findings (Yang and Chen, 2024; Mahmud *et al*., 2022) and represents a neuroendocrine disturbance in the subjects (Pratama *et al*., 2024). Similarly, in the non-PCOS group, both the FSH and LH levels were significantly reduced as compared to the fertile group showing hormonal disturbances. Furthermore, Estradiol and Prolactin levels were significantly decreased in both PCOS and Non-PCOS infertile groups in contrast to the fertile group. Reduced Estradiol concentration is considered to be the result of endogenous inhibitors like 5 alpha-androstane-3, 17-dione within PCOS follicular fluid, which hampers the synthesis of estradiol (Agarwal *et al*., 1996). Contrary to our findings, prolactin has been reported to be increased in cases of PCOS and hyperprolactinemia is considered as a well-established cause of anovulatory infertility (Falaschi *et al*., 1977; Bernard *et al*., 2015; Melmed *et al*., 2011). Though, some of the findings reported lower levels of prolactin in PCOS women as well, which is negatively correlated with LH and positively associated with Estradiol levels (Yang *et al*., 2020; Glintborg *et al*., 2014). Lastly, AMH levels did not show any significant difference between healthy, infertile PCOS and infertile non-PCOS groups though it is considered to be higher in PCOS females and serves as an established biomarker for assessing female reproductive capacity (Rudnicka *et al*., 2021; Ran *et al*., 2021).

Followed by biochemical analysis, proteomic analysis was performed which revealed 30 common proteins among different subgroups of infertility that showed uniform pattern of gene expression, however, among these, 8 proteins revealed differential expression in both infertility subgroups. Among exclusively identified proteins in PCOS group, 14 proteins showed significant differential expression and non-PCOS group showed differential expression of 24 proteins.

The common proteins significantly altered in both diseased groups, were involved in complement and coagulation cascade pathways. These proteins include fibrinogen, coagulation factor 9, complement factor B, complement factor H, C2 and C4A. Fibrinogen exhibited the most statistically significant log-fold change within both infertility groups with an increase of up to 1.3 times in PCOS and up to 1.4 times in non-PCOS group. This finding is consistent with the previous findings of PCOS in younger and obese individuals (Ji *et al*., 2015). Fibrinogen is associated with an elevated risk for cardiovascular disease which is a long term complication of PCOS (Wang *et al*., 2017) and non-PCOS infertile women as well (Mu *et al*., 2016; Okoth *et al*., 2020). Moreover, it has also been suggested as a potential laboratory screening biomarker for PCOS, however, other research has questioned its status as such (Ji *et al*., 2015; Ozgokce *et al*., 2020).

Another protein, Coagulation factor 9, was found to be significantly up-regulated in both the infertile groups. This protein is linked to greater risk of venous thromboembolisms (Lagrange *et al*., 2019). Moreover, blood coagulation is crucial in the interaction of embryo and endometrium (Guo *et al*., 2021), and coagulation defects result in impaired implantation (Gerotziafas *et al*., 2017). However, increased coagulability was documented to be less likely in these non-obese PCOS women (Moin *et al*., 2023). Hence, we suggest that this factor could also be associated with the cause of infertility in PCOS and non-PCOS subjects. The complement system has three alternative routes which merge to a common pathway. It is one of the main component of innate immunity which is involved in inflammatory and immune response. The classical pathway is activated through C4 (Carroll, 2004) which was found to be up-regulated in both the infertile PCOS and non-PCOS groups. As opposed to classical pathway, the lectin pathway activates through C2 (Carroll, 2004) which exhibited down-regulation, as low as 0.98 times log-fold change, exclusively in the PCOS group. Complement activation plays a role in various disease processes, such as diabetes (Engström *et al*., 2005) and cardiovascular disease (Bjerre *et al*., 2008), both of which are linked to PCOS (Torchen, 2017; Arffman *et al*., 2019) as well as non-PCOS related infertility (Gleason *et al*., 2019; Tobias *et al*., 2015). Moreover, mounting evidence suggests that PCOS is associated with an elevated risk of infection and its consequences (Kyrou *et al*., 2020). It has also been reported that one of the classical pathways

leads to infertility by the formation of membrane attack complex (MAC) that perforates the spermatozoa leading to its destruction (Msc and Makki, 2010). Though we did not take account for antisperm antibodies which activate this pathway. Another complement protein, factor H was significantly up-regulated exclusively in non-PCOS infertile group, which is an inhibitor of complement pathway (Parente *et al*., 2017).

Among the lipid metabolism proteins, several apolipoproteins were significantly differentially expressed. ApoA-II was found to be down-regulated in both of the infertile groups. Koike *et al*. (2021) proved the atheroprotective attribute of apoA-II in rabbits, which was substantiated by the augmentation of cholesterol efflux activity and the anti-inflammatory capabilities of HDL particles. Moreover, the risk of coronary artery disease was also found to be inversely associated with APOA-II levels in human subjects (Florea *et al*., 2022). Hence down-regulation of ApoA-II demonstrates the increased cardiovascular risk of both infertile groups. Furthermore, Apo D and Apo M proteins were significantly up-regulated in both the infertile groups while Apo B was exclusively up-regulated in non-PCOS group, by up to 1.01 times log-fold change. Apo B and ApoM have been reported to be potential contributors to the atherosclerosis (Hahn *et al*., 1995), diabetes and coronary artery disease (Butler *et al*., 2023; Zannis *et al*., 2015). Though, a cohort study revealed that the levels of Apo M were significantly reduced in the PCOS group (Butler *et al*., 2023), which is in contrast to our findings.

Among binding proteins, sex hormone binding globulin (SHBG) was found to be down-regulated in both the infertile groups, as low as 0.97 log-fold change. Alterations in the SHBG and sex hormone levels have been linked with hypo/hyperthyroidism and in turn thyroid disorders have been linked to reduced fertility (Krassas *et al*., 2010). Reduced SHBG levels cause an increase in androgen bioavailability, which causes anovulation and the clinical traits of PCOS to progress (Qu and Donnelly, 2020). However, evidence shows that liraglutide can reverse these symptoms (Nylander *et al*., 2017). Another protein, S100A9 was observed to exhibit lower expression levels, as low as 0.95 log-fold change in the infertile PCOS group while higher expression, as much as 1.01 times log-fold change in the non-PCOS infertile group. This protein has not been reported in association with PCOS before, but it plays a crucial role in embryo implantation by binding with calcium ions and have been regarded as potential candidate marker for assessing endometrial receptivity (Sadigh *et al*., 2019). The impact of this protein on adverse pregnancy outcomes is also being investigated (Verma *et al*., 2018).

Among serine protease inhibitors, alpha1-antitrypsin (SERPINA1) was found to be up-regulated in both the infertile groups which is consistent with the previous findings in peritoneal fluid of infertile patients (Ferrero *et al*., 2009). This protein has been suggested to potentially alleviate PCOS symptoms by inhibiting pro-inflammatory factors through direct interactions (Pan *et al*., 2022). Moreover, SERPINA3 was down-regulated exclusively in the PCOS group as low as 0.96 times log-fold change. This protein has also not been associated with PCOS in previous findings but has been known to limit coagulation and inflammation by inhibiting Cathepsin G (Chelbi *et al*., 2012), hence, the downregulation of this protein could likely be the reason of pronounced inflammation in PCOS patients.

In future, ELISA experiments can be carried out for further validation of these proteins. The limitations of our study included smaller sample size which could compromise the statistical significance of the study and restrict the generalizability. Moreover, it was not possible to match the lifestyle of case and control groups adding to confounders in the study.

To conclude, this study revealed significant changes in protein expression in both the infertile PCOS and non-PCOS groups utilizing the mass spectrometry technique in comparison with healthy controls. The shared and exclusive DEPs among different subgroups of infertility shed light on the diverse biological process, including lipid metabolism, complement and coagulation cascade, inflammation and reproductive functions.. The knowledge acquired through this research has the potential advantage to lay the foundation for personalized medicine strategies. Tailored treatment designed to address the precise molecular anomalies identified in each patient, may result in more effective and personalised therapeutic approaches.

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Compliance with ethical standards

All procedures performed in this study involving human participants were approved by Ethical Review Committee (ERC AKU 2022-7509-22501**)** of Aga Khan University, Karachi.

Consent to participate

Participants were recruited from the Endocrinology Clinic of the same hospital, after obtaining an informed consent from each participant.

Consent for publication

The author confirms that the work described has not been published before (except in the form of thesis) and it is not under consideration for publication elsewhere.

Availability of data and material

Data is available upon request.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20250625050207

Statement of conflict of interest

The authors have declared no conflict of interest.

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