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Diabetes and Obesity Biomarkers in Association with Hyperandrogenism Reveal Key Networks in the Pathogenesis of Polycystic Ovary Syndrome: Study on a Cohort from the Southern Part of Kerala State

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Abstract

Polycystic Ovary Syndrome (PCOS) is a multisystemic disorder that is strongly associated with comorbid factors such as insulin resistance and obesity. PCOS women with high androgen levels are known to potently regulate insulin metabolism and increase the risk of type 2 Diabetes. Several diabetic biomarkers have been implicated, but how these biomarkers correlate with each other and the mechanistic pathways involved are poorly understood. Therefore, this study aims to comprehend the correlation and various pathways through which these key regulators interact with each other to regulate the PCOS-diabetes-obesity network. We conducted a retrospective cohort study, including 76 controls and 67 PCOS subjects. Plasma levels of 10 different diabetic and obesity biomarkers were measured by multiplex beads assay. Further, statistical correlation analysis and GOnet network prediction were done to understand the specific pathways altered in PCOS conditions. The levels of five biomarkers (ghrelin, C-peptide, insulin, and adipokines (leptin, and visfatin) were significantly changed in PCOS subjects. Regression and correlation analysis showed a significant correlation between ghrelin, C-peptide, insulin, leptin, visfatin, and high testosterone. Finally, GOnet network analysis revealed various processes and pathways including altered glucose metabolism, inflammatory response, cytokines production, regulation of stress, defective nitric oxide regulation, and impaired circadian rhythm. Our findings indicate intertwined metabolic-physiological-inflammatory complications in the pathogenesis of PCOS, highlighting the comorbidities between PCOS pathogenesis, diabetes, obesity, and inflammation.

Keywords: Diabetes, Hyperandrogenism, Multiplex, Obesity, Polycystic Ovary Syndrome

Abbreviations: PCOS (Polycystic ovary syndrome), WHO (World Health Organisation), IR (Insulin resistance), T2DM (type 2 Diabetes Mellitus), EDTA (Ethylenediaminetetraacetic acid), NaCl (Sodium chloride), RBC (Red blood corpuscles), ELISA (Enzyme-linked immunosorbent assay), LH (Luteinizing hormone), FSH (Follicle stimulating hormone), DHT (Dihydrotestosterone), C-peptide (Connecting peptide), GIP (Gastric inhibitory peptide), GLP- (1Glucagon-like peptide-1), PAI- 1 (Plasminogen activator inhibitor-1), RT (Room temperature), CI (Class interval), ROC (Receiver Operator Characteristics), BMI (Body mass index), SEM (Standard Error of Mean), OD (Ovulatory dysfunction), PCOM (Polycystic Ovarian Morphology), NAMPT (Nicotinamide phosphoribosyltransferase), NAD(P)H (Nicotinamide Adenine Dinucleotide Phosphate Hydrogen), NO (Nitric oxide), NOS (Nitric Oxide Synthase), non-REM (Non-rapid eye movement), MAPK (Mitogen-activated protein kinase), PK-B (Protein kinase-B), MD (Mediterranean diet), IL (Interleukin), CCL-5 (Chemokine (C-C motif) ligand 5), AUC (Area under curve), NREM (Non-rapid eye movement), TST (Total Sleep Time), TWT (Total Wake Time), WASO (Wakefulness After Sleep Onset), AHI (Apnea-hypopnea index).

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1. Introduction

Over the past few decades, PCOS, a complex endocrine/metabolic syndrome, has become an increasingly common cause of female infertility in reproductively aged women. According to the World Health Organisation (WHO) (https://www.who.int/news-room/fact-sheets/detail/polycystic-ovary-syndrome), 8–13% of women in their reproductive age have PCOS. Its prevalence varies depending on the diagnosis criteria. Irregular menstrual cycle, hyperandrogenism (HA), ultrasound-detected cystic ovaries, and abnormal ovulatory function are the hallmarks of PCOS but it varies from woman to woman. Due to the diverse range of signs and symptoms, diagnosing and treating PCOS became more challenging. It is associated with several comorbidities including insulin resistance (IR), type 2 Diabetes Mellitus (T2DM), cardiovascular diseases, visceral adiposity, and obesity that raise the risk of endocrine-metabolic complications in PCOS women[1]. Burghen *et al.* initially reported that PCOS has a major metabolic abnormality and is associated with hyperinsulinemia[2]. Subsequently, a thorough review by Dunaif provided extensive information indicating a more robust interaction between hyperandrogenism and IR[3]. Furthermore, a recent meta-analysis report by Ng *et al.* states that PCOS women have approximately a 4.4-fold risk of developing diabetes compared to normal women[4]. Additionally, it was stated that the incidence of T2DM in PCOS exhibits an early onset, beginning two decades earlier than in control women. Persson *et al.* also found that women with HA and PCOS have a 3.8-fold higher risk of developing T2DM than non-PCOS women[5]. Microarray analysis of granulosa cells from PCOS women also identified genes related to diabetes mellitus in PCOS women[6]. However, the cellular mechanisms of hyperandrogenism regulating insulin and causing T2DM are not well understood.

Moreover, the excessive intra-ovarian androgen levels also coexist with obesity in addition to raising the risk of T2DM onset. While obesity is not a necessary component of PCOS, it can exacerbate the syndrome's clinical symptoms in those who are already afflicted by increasing the degree of insulin resistance[7,8]. Obese PCOS women who are more likely to develop diabetes exhibit higher levels of abdominal obesity, which could be because visceral fat and insulin defects interact more strongly than subcutaneous fat does[8]. This increase in visceral adipocytes subsequently increases the production of several adipokines[9]. Various adipokines, along with androgen excess, aggravate PCOS pathogenesis[10]. However, the understanding of mechanisms and regulatory pathways via which hyperandrogenism, T2DM, and obesity interconnect with each other, leading to PCOS pathogenesis, is still a debate. Hence, a detailed analysis of major diabetic and obesity biomarkers in PCOS could lead to provide a better insight into the PCOS-diabetes-obesity nexus. Therefore, we conducted an extensive comparative analysis that included ten biomarkers in PCOS subjects.

2. Materials and Methods

2.1 Subject Recruitment

This is a retrospective study conducted on 143 volunteers, 67 of whom were PCOS patients and the remaining 76 were healthy controls, between the ages of 18 and 40 years. Regular menstrual cycles, no history of metabolic/hormonal imbalance, and no fertility issues were used to select the healthy subjects. While, PCOS subjects were selected based on the Rotterdam Consensus criteria (The Rotterdam European Society for Human Reproduction and Embryology (ESHRE)/American Society for Reproductive Medicine (ASRM) - sponsored PCOS Consensus Workshop Group, 2003)[11]. Subjects with excess androgen disorders, autoimmune disorders, infections within 3 weeks, and aberrant Luteinizing Hormone (LH) levels were not included in our study.

2.2 Ethics Statement

This study has received the approval from Institute Ethical Review Board of RGCB (IHEC/01/2019/07) and KJK Hospital, located in the southern part of India (Kerala). Following the Declaration of Helsinki, blood samples were obtained from control and PCOS individuals only after receiving their informed consent.

2.3 Blood Sample Collection and Plasma Isolation

On days 2-5 of the follicular phase, 5 mL of peripheral blood was drawn from each subject in an evacuated blood collection tube coated with EDTA (CML Biotech (P) Ltd., Code VS-K3). Following centrifugation and 3% dextran-NaCl mediated RBC sedimentation during Peripheral Blood Mononuclear Cells preparation, plasma

was separated and stored in three aliquots at -80°C. One aliquot of each sample was thawed once before the experiment.

2.4 Hormone Profiling by ELISA

The plasma levels of LH, Follicle-Stimulating Hormone (FSH), progesterone, testosterone, Dihydrotestosterone (DHT), and Thyroid Stimulating Hormone (TSH) were estimated in 76 control and 67 PCOS subjects by using LH (human) ELISA kit (item no. 500720), FSH (human) (item no. 500710), progesterone (human) (item no. 582601), testosterone ELISA kit (Item no. 582701), Cayman chemicals, and DHT ELISA kit (MBS2000056, MyBioSource) and TSH ELISA kit (ELH-TSH-1).

2.5 Profiling of Diabetes-Associated Markers

Ten markers associated with diabetes were determined in the plasma of 76 PCOS and 67 control subjects by magnetic bead suspension assay using Bio-Plex Pro Human Diabetes 10-plex assay (Catalogue Number: 171A7001M, Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. The panel comprised of the following: Connecting peptide (C-peptide), insulin, ghrelin, leptin, visfatin, resistin, Gastric Inhibitory Peptide (GIP), glucagon, Glucagon-Like Peptide-1 (GLP-1), and Plasminogen Activator Inhibitor-1 (PAI-1) total. The assay was initiated by adding 50 µL of 1x beads to the 96-well plate followed by washing. Subsequently, 50 µL of blank, standards, and plasma samples were added to the corresponding wells, followed by 1-hour incubation on a shaker at 850±50 rpm at Room Temperature (RT). Following plate washing, samples were incubated with 25µL of detection antibodies for 30 min at room temperature. Next, it was incubated (10 min) with 50 µL of 1x fluorescent conjugate Streptavidin-Phycoerythrin. The plate was then resuspended with 125 µL of assay buffer. The intensity of the formed product was read and data was collected using a BioPlex 200 equipped with Bio-Plex Manager analysis software Version 6 (Bio-Rad Laboratories, Hercules, CA, USA). The BioPlex 200 instrument was configured with 50 beads per region, a flow rate of 60 µL/min, and a discriminator gate with a range of 5000 -25000. The standard curves of each analyte were generated with recombinant diabetic biomarkers included in the kit. The assay contained identical positive quality controls as well. The values denoted by <OOR were deemed to be zero, while values indicated by * represent machine-extrapolated values and were thus included in our study group.

2.6 Statistical Analysis

The concentration of each analyte was obtained in pg/mL, using a 5-parameter logistic regression fit by creating a standard curve with the standards of known concentration. Statistical analysis was performed through GraphPad Prism 9 software. Student's t-test was computed to determine the significance of differential expression. One-way ANOVA by Dunnett's multiple comparison tests was used in the phenotypic comparison of PCOS and control subjects. To identify the correlation between each analyte and hyperandrogenism, a linear regression-correlation analysis was performed using GraphPad Prism 9 software. Further, to understand the diagnostic potential a Receiver Operating Characteristics (ROC) Curve of significantly altered analytes was calculated along with the lower 95% Class Interval (CI) and upper 95% CI of altered analytes using MetaboAnalyst 6.0. Statistical significance in all the above analyses was considered with a $p<0.05$.

2.7 Gene Ontology and Network Analysis

Additionally, we also further analyzed the pathways in which the significantly altered biomarkers were involved, by using the GOnet network analysis tool. The inputs included gene annotation and the Log2 value of each analyte and the strongly interacting molecules in different biological processes were filtered out.

3. Results

3.1 Clinical and Biochemical Parameters of Control and PCOS Groups

The subjects included in our study were of similar age groups. In comparison to the control subjects, the PCOS subjects showed a significantly higher Body Mass Index (BMI) (Figure 1A). There was no difference in the mean FSH and progesterone levels between the two groups (Figures 1B and 1E). Significantly higher levels of LH were observed in PCOS subjects compared to control subjects ($p<0.0001$) (Figure 1C). The LH/FSH ratio was considerably higher in PCOS subjects compared to controls ($p<0.0001$) (Figure 1D). The total testosterone level ($p<0.001$) was increased to a significant level in

PCOS women (Figure 1F). We observed no considerable change in DHT levels between control and PCOS subjects (Figure 1G). The TSH level was significantly higher in PCOS subjects in comparison to controls ($p<0.0001$) (Figure 1H).

3.2 Phenotypic Classification of PCOS Subjects Included in the Study Group

The phenotypic classification of our PCOS group was done by the Androgen excess-PCOS phenotype Task Force based on NIH/NICHD Rotterdam criteria (1990 and 2003)[12]. The PCOS individuals (n=67) included in this study were classified into four main phenotypic subgroups, A, B, C, and D, based on the diagnostic criteria of Hyperandrogenism (HA), Ovulatory Dysfunction (OD) and Polycystic Ovarian Morphology (PCOM). Our study group comprised 46.3% phenotype A (HA+OD+PCOM) individuals (highest), while phenotype B (HA+OD) was the least subgroup with 4.5% cases. We observed that phenotype C (HA+PCOM) was the second highest phenotype with 37.3% cases, and phenotype D (OD+PCOM), with 11.9% cases in the cohort. There was no statistically significant difference in the levels of LH, FSH, LH/FSH, progesterone, DHT, and TSH among the four PCOS phenotypic populations of our study group. We observed a significantly higher level of testosterone in the population of phenotypes A, B, and C compared to phenotype D. Reproductive complications were observed in all four groups (Supplementary Table S1).

3.3 Diabetic Biomarkers are Altered in PCOS Conditions

Out of the ten analytes measured, five showed statistically significant differential levels in PCOS subjects compared to the control (Figures 2A - 2E and Table 1). The mean level of ghrelin significantly decreased in PCOS women compared to healthy subjects ($p<0.001$) (Figure 2A). PCOS subjects showed significantly lower levels of C-peptide ($p<0.001$) and insulin ($p<0.05$) as compared to controls (Figures 2B, C). The levels of leptin ($p<0.05$) and visfatin ($p<0.05$) were significantly high in PCOS conditions (Figures 2D, E). There was no difference in the plasma levels of GIP, GLP-1, glucagon, PAI-1, and resistin between healthy and PCOS subjects (Table 1). Additionally, the relative differential expression of five significantly altered analytes was analyzed in the four PCOS phenotypes to understand the phenotypic variation of these analytes. Our comparative analysis revealed a significant diminution in the levels of ghrelin and C-peptide in PCOS phenotype A and phenotype C groups compared to the control group (Figures 3A, B and Supplementary Table S1). Significant differences were not observed for insulin, leptin, and visfatin between the four PCOS phenotypes.

3.4 Obesity and Diabetic Indicators are Correlated with High Testosterone in PCOS Women

To determine the relationship between all the five altered analytes with each other and with hyperandrogenism

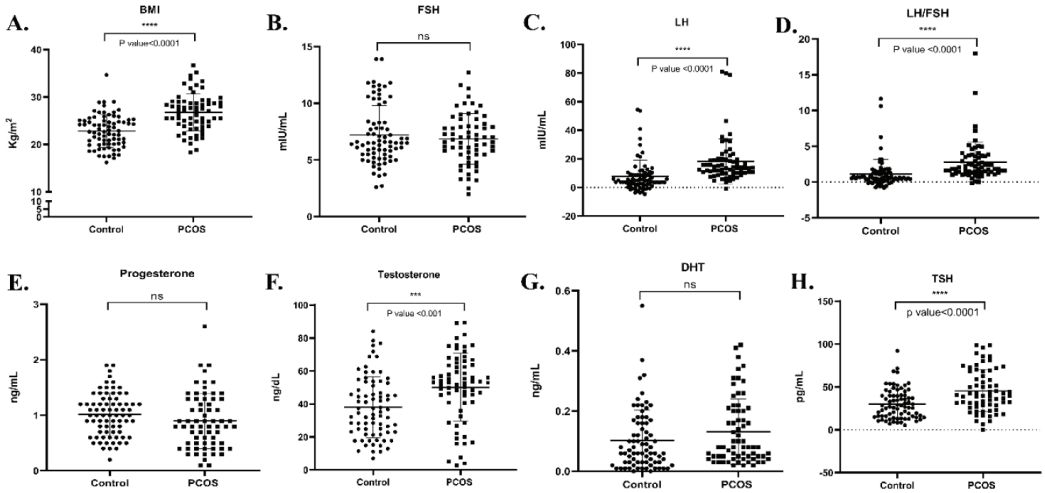


Figure 1. Comparative analysis of clinical and biochemical parameters in control and PCOS groups.
Physiological and hormonal analysis in control (n=76) and PCOS (n=67) groups. A-H: scatter dot plot represents BMI, FSH, LH, FSH/LH, progesterone, testosterone, DHT, and TSH levels respectively. Values shown are mean ± SEM. Every dot denotes an individual subject, and a p-value<0.05 is considered statistically significant.

Table S1: Clinical characteristics and plasma biomarker levels with respect to the four different PCOS phenotypes.

Characteristics/ Cytokines	Phenotype A (46.3%)	Phenotype B (4.5%)	Phenotype C (37.3%)	Phenotype D (11.9%)
Age (years)	26.9 ± 0.6	23.7 ± 1.3	27.5 ± 0.8	25.5 ± 1.3
BMI (kg/m^2)	27.1 ± 0.7	24.2 ± 2.4	26.7 ± 0.8	26.6 ± 1.8
LH (mIU/mL)	17.9 ± 3.3	9.7 ± 5.3	20.2 ± 3.0	16.4 ± 2.6
FSH (mIU/mL)	7.1 ± 0.4	6.9 ± 0.5	7.8 ± 1.1	6.0 ± 1.0
LH/FSH	2.5 ± 0.4	1.3 ± 0.7	3.2 ± 0.7	2.9 ± 0.8
Progesterone (ng/mL)	1.0 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	1.1 ± 0.1
Testosterone (ng/dL)	53.3 ± 2.4	59.5 ± 4.2	57.6 ± 3.7	10.9 ± 2.4
DHT (ng/mL)	0.11 ± 0.01	0.07 ± 0.01	0.17 ± 0.03	0.12 ± 0.04
TSH (pg/mL)	45.47 ± 4.37	23.92 ± 4	44.95 ± 4.65	54.08 ± 10.84
Ovary size (cm^3)	11.7 ± 0.8	8.1 ± 3.3	11.6 ± 0.7	9.6 ± 1.5
Ghrelin (pg/mL)	124.6 ± 29.4	40.2 ± 39.7	69.4 ± 21.3	313.2 ± 123.6
C-peptide	73.5 ± 14.8	83.3 ± 72.2	65.7 ± 16.7	141.5 ± 35.9
Insulin	218.4 ± 44.5	74.0 ± 42.1	164.7 ± 56.4	221.0 ± 75.7
Leptin	2921.3 ± 399.5	2114.6 ± 1110.8	2445.3 ± 580.6	3119.2 ± 573.0
Visfatin	3991.9 ± 1715.5	1294.1 ± 647.5	3592.3 ±1656.1	5355.7 ± 2044.3

Clinical characteristics and plasma biomarker levels in individuals with four different PCOS phenotypes. All values were presented as mean ± SEM. Each PCOS phenotype is shown with the percentage of the population (in our study group).

Table 1. Plasma level differential expression of biomarkers in control and PCOS subjects

Analytes	Control (n=76) Mean± SEM	PCOS (n=67) Mean± SEM	p-value
Ghrelin	137.1 ± 12.3	79.1 ± 10.7	<0.001
C-peptide	289.5 ± 38.2	122.7 ± 22.9	<0.001
Insulin	301.5 ± 42.5	192.2 ± 30.7	<0.05
Leptin	2039.4 ± 163	2731.2 ± 293.9	<0.05
Visfatin	1711.3 ± 381	3884.8 ± 1026.3	<0.05
GIP	299.4 ± 98.2	115.9 ± 19.7	> 0.05
GLP-1	5.9 ± 3.5	0.44 ± 0.4	> 0.05
Glucagon	258.3 ± 17.4	285.9 ± 26.5	>0.05
PAI-1	2341.5 ± 108.7	2445.9 ± 179.1	>0.05
Resistin	1402.9 ± 135.5	1600.6 ± 372.4	>0.05

Plasma levels of various diabetic biomarkers in control (n=76) and PCOS (n=67) subjects. All values are represented as Mean ± SEM. The concentration of analytes is shown as pg/mL. p<0.05 are considered statistically significant.

(since high testosterone is the hallmark of PCOS), we carried out a linear regression analysis for every possible pairwise combination of these analytes. We observed a substantial correlation between ghrelin and C-peptide (p<0.0001) as well as with leptin (p<0.05) (Figures 4A, B). However, in PCOS patients, both C-peptide and leptin were significantly reliant on insulin levels (p<0.01 and p<0.05, respectively; Figures 4C, D). Additionally, a significant relationship (p<0.05) was found between leptin and visfatin (Nicotinamide phosphoribosyltransferase, NAMPT) (Figure 4F). Furthermore, increased testosterone in PCOS patients showed a stronger correlation with C-peptide (Figure 4F), suggesting the formation of an interconnecting loop involving hyperandrogenism, obesity, and diabetes.

3.5 Obesity and Diabetic Indicators Show a Higher Diagnostic Potential for PCOS Diagnosis

Several studies have reported alteration in the five significantly altered analytes viz., ghrelin, insulin, C-peptide, leptin, and visfatin, in PCOS condition. To predict the diagnostic potential of these altered obesity and diabetic indicators for PCOS diagnosis, we performed an ROC curve analysis of the altered analytes. The Area Under Curve (AUC) values of ghrelin (0.721), C-peptide (0.736), insulin (0.619), leptin (0.565), and visfatin (0.616) showed significant changes (p<0.05) indicating their diagnostic value in PCOS condition

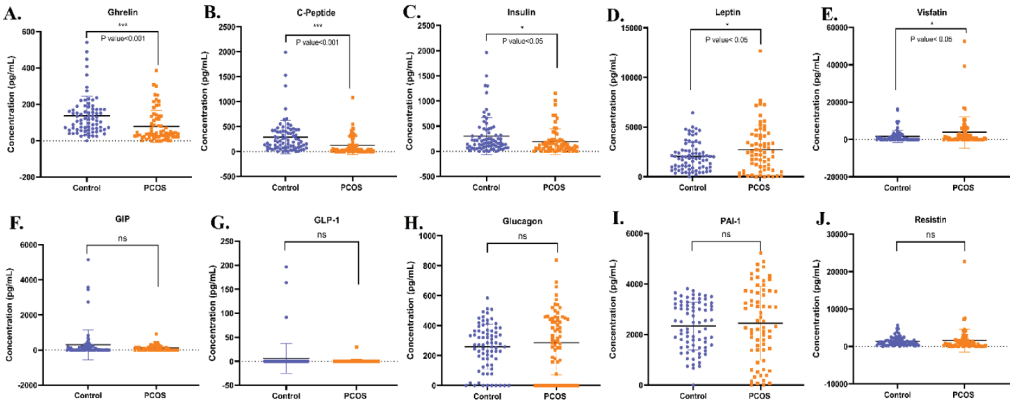


Figure 2. Estimation of biomarkers in the plasma of control and PCOS groups.

Plasma levels of diabetic bio-makers in control (n=76) and PCOS (n=67) subjects. Values expressed are mean ± SEM. A-E: scatter dot plot representation for ghrelin, C-peptide, insulin, leptin, and visfatin in control and PCOS groups. F-J: scatter plot representation for GIP, GLP-1, glucagon, PAI-1, and resistin. Each dot represents a single subject. Values shown are mean ± SEM. p-value <0.05 is considered statistically significant.

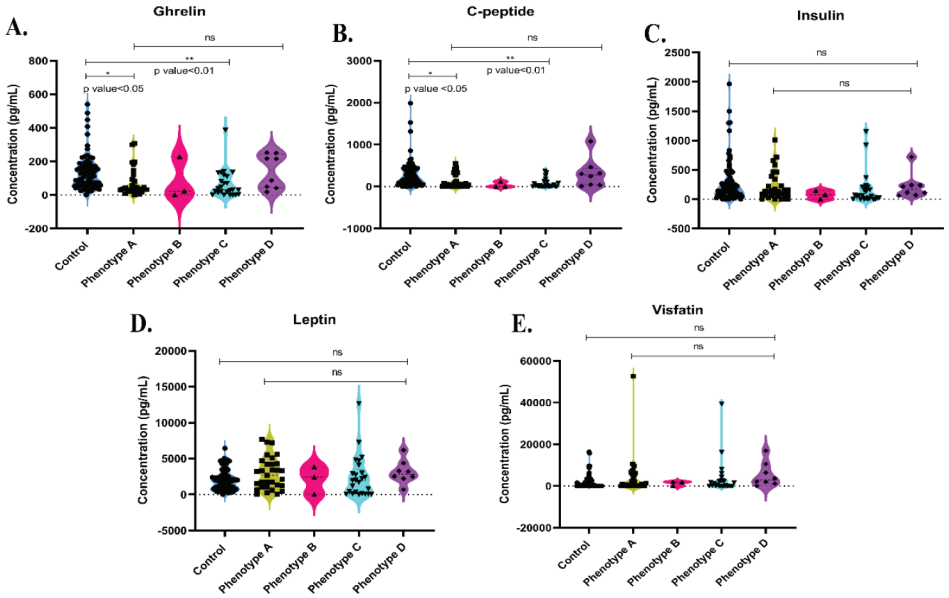


Figure 3. Comparison of plasma level changes in control and the four PCOS phenotypes.

A–E depict violin plots for the mean plasma level changes between the control and the four distinct PCOS phenotypes. Values shown are mean ± SEM. p<0.05 is considered statistically significant.

(Figures 5A-E) in conjunction with the other classical criteria.

3.6 GOnet Network Analysis of Altered Pathways in PCOS Condition

To comprehend the networks and biological processes in which these obesity and diabetic indicators are involved, we carried out a GOnet network biological process analysis (Figure 6). Figure 6A shows the colour matrix ranging from blue to red colour. Analytes with lower fold change are represented in shades of blue colour, and analytes with higher fold change are represented in shades of red. Enrichment analysis with a p≤0.05 was considered for our network selection and unconnected or poorly connected networks were removed from our analysis. The intricate relationships between ghrelin, insulin, leptin, and visfatin were revealed by the GOnet network (Figure 6B). Several important biological networks and processes that are affected in PCOS condition were identified by this interaction, including impaired glucose metabolism

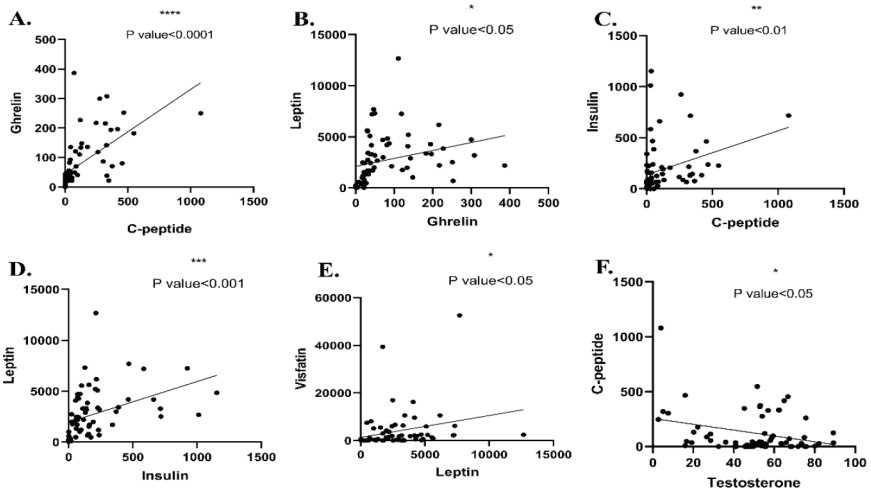


Figure 4. Linear regression - correlation analysis between diabetes and obesity indicators as well as high testosterone in PCOS women.

Linear regression - correlation analysis between (A) ghrelin and C-peptide, (B) ghrelin and leptin, (C) C-peptide and insulin, (D) leptin and insulin, (E) leptin and visfatin, and (F) C-peptide and testosterone. p<0.05 are considered statistically significant.

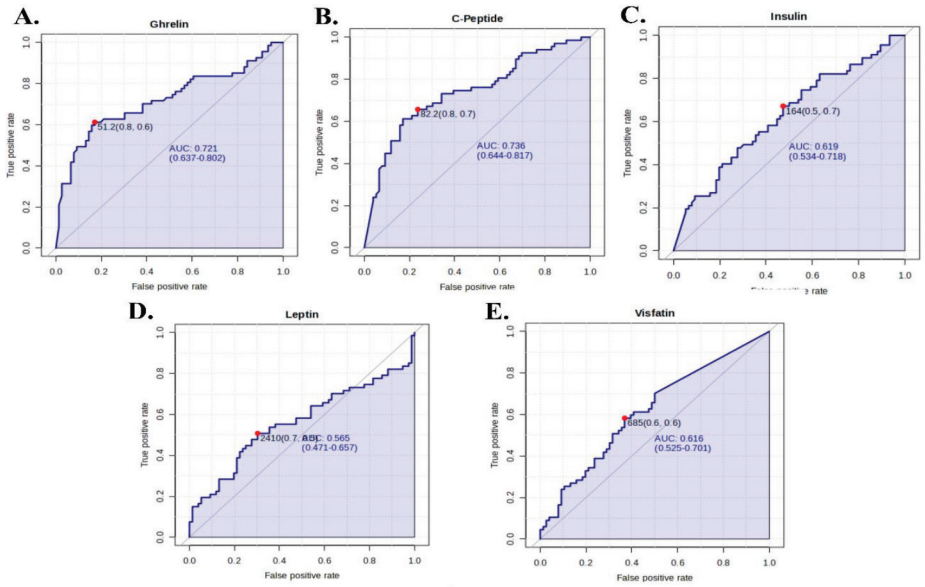


Figure 5. ROC curve analysis for the diagnostic potential of significantly changed analytes for PCOS diagnosis.

ROC curves showing the AUC as well as lower and upper 95% CI of significantly altered analytes: ghrelin, C-peptide, insulin, leptin, and visfatin. p<0.05 are considered statistically significant.

affecting gluconeogenesis, glucose import, and negative regulation of glucagon production as well as adult feeding behaviour and appetite regulation. This analysis also revealed impaired immune signaling like cytokine production, T cell activation, inflammatory response, positive regulation to interferon beta and tumour necrosis factor superfamily, positive regulation to interleukin-12 (IL-12), IL-8, IL-6, and chemokine (C-C motif) ligand 5 (CCL-5) secretion. It also impacts the stress mechanisms that include negative regulation of respiratory burst in the inflammatory response, peroxisome organization, response to stress, regulation to reactive oxygen species, monooxygenase activity, Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NAD(P)H) activity, and nitric oxide synthase activity. Furthermore, it revealed many important pathways associated with obesity as well as steroid hormone regulation, such as fat cell differentiation, lipid transport, prostaglandin,

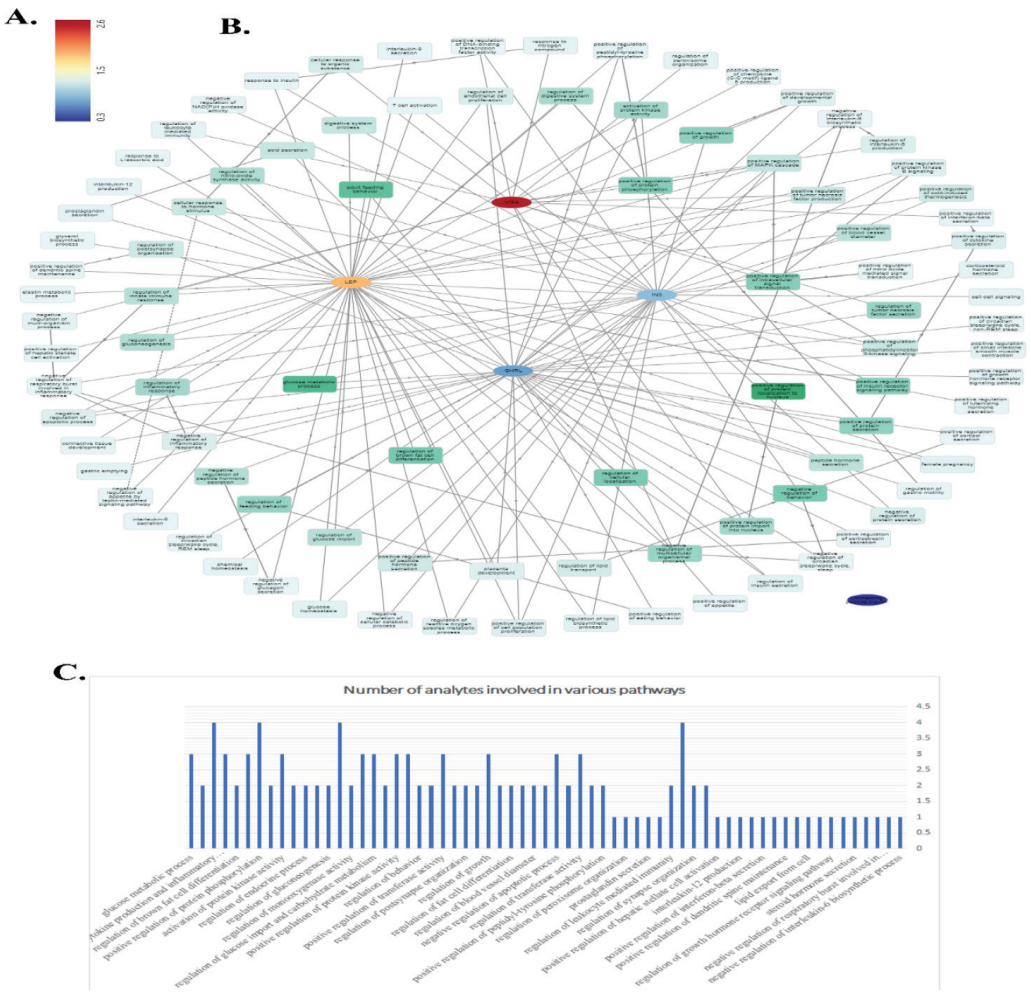


Figure 6. GOnet network analysis of altered obesity and diabetic markers.

GOnet network analysis of altered analytes displaying every biological process and pathway. A: Colour matrix ranging from blue to red. Blue colour represents a lower fold change and red color represents a higher fold change. B: GOnet network of five significantly altered analytes. Strongly involved analytes with a higher fold change value are represented as red colour shades and weakly involved analytes with a lower fold change value are in blue colour shades (oval shape). Similarly, the strongly involved networks are shown in shades of dark green (square shape). C: Bar graph showing the number of analytes involved in the selected key pathways from the GOnet network analysis.

corticosteroid hormone secretion, and positive regulation of LH secretion. Remarkably, we discovered that PCOS patients had poorer control over non-rapid eye movement (non-REM) sleep, the circadian sleep/wake cycle, and the regulation of blood vessel diameter. In addition to these, our investigation also revealed a significant contribution of impaired regulation of protein phosphorylation, Mitogen-Activated Protein Kinase (MAPK) cascade, PI-3 kinase, protein kinase-B (PK-B), and transferase activity to the PCOS pathogenesis.

4. Discussion

PCOS is a multisystemic disorder that affects the female body's entire metabolic, hormonal, physiological, and immune networks. The arrested immature follicles in the PCOS ovary show a follicotoxic effect, leading to an elevation of androgen in the ovary. As a result, the underdeveloped follicles are unable to ovulate from the ovary. This aberrant condition not only affects the ovarian microenvironment but also exhibits an extremely wide range of effects on the entire body by hindering the pituitary-ovarian hormonal nexus, hormonal-metabolic synchrony, metabolic-chronobiology interactions, as well as immune mechanisms. Several studies have already shown an association between hyperandrogenism with type 2 diabetes mellitus. Zhao *et al.* reported that the risk of T2DM is seven-fold higher in PCOS women with hyperandrogenism when compared to PCOS women without hyperandrogenism[13]. Here, we have assessed plasma levels of 10 diabetic biomarkers in PCOS and control

subjects. Our results showed five biomarkers as significantly altered in the PCOS group. Interestingly, we found a significant decrease in the level of ghrelin in PCOS subjects. Schofl *et al.* reported a decreased ghrelin level in PCOS women with insulin resistance[14]. Further, a significantly low level of ghrelin was reported in obese PCOS subjects, and after following the Mediterranean Diet (MD)-based diet and weight loss program for 12 weeks, ghrelin levels increased in PCOS women[15]. In contrast, Daghestani *et al.* found no difference in the level of ghrelin between PCOS and control Saudi women[16,17]. Daghestani *et al.* observed a significant difference in ghrelin levels in obese PCOS women compared to lean PCOS, suggesting a significant role of ghrelin in causing obesity in PCOS women[16]. Thus, our report of lowered ghrelin in the South Indian (Kerala) population is in agreement with the other previous reports.

In several studies, the plasma levels of insulin were significantly elevated in PCOS conditions. Maliqueo *et al.* reported a significant increase in pro-insulin and C-peptide levels in PCOS women compared to normal, thus reflecting a condition of insulin resistance[18]. However, their data showed a wide range, and thus the significance level was not clear. PCOS women exhibit significantly higher C-peptide concentration as compared to normal women and the levels are already reported in T2DM and CVD predictors. Thus, this analyte could also be used as a biomarker for PCOS[19]. Surprisingly, we found significantly lower levels of both circulating C-peptide and insulin in the plasma of PCOS women when compared to healthy women. Thus, our results indicate a potential defect either in the conversion of pro-insulin to active insulin and C-peptide or a decrease in pro-insulin secretion in our study group. Therefore, not all women with PCOS are insulin resistant; some women with high BMI also possess less insulin, which increases their risk of Type 2 diabetes.

Additionally, we observed a significant increase in the levels of leptin and visfatin in the plasma of PCOS subjects compared to healthy women, which is in agreement with an earlier report[20]. Leptin and visfatin are adipokines secreted from the adipose tissue. Adipose tissue, apart from its role in energy storage, also functions as a potent endocrine organ. In this study, we report higher plasma leptin levels in PCOS and this is comparable with the levels reported by Mohiti *et al.*[21]. A meta-analysis by *Caprio et al.* indicated that leptin has a negative effect on ovarian steroid regulation in an *in-vitro* model of granulosa and thecal cells[22]. Leptin inhibits the secretion of insulin-induced 17β-estradiol and progesterone from bovine granulosa cells[23], and androstenedione and progesterone secretion by bovine thecal cells[24]. Further, incubation of fertile women's granulosa cells with higher concentrations of leptin considerably reduces FSH and estradiol secretion[25]. This signifies that increased levels of leptin may prevent the dominant follicle from developing and, hence, leave the ovary with an anovulatory response. Chan *et al.* reported a significantly high plasma visfatin level in PCOS women[26] and our results are in agreement with this study. Visfatin is a prominent adipokine that is secreted mainly from visceral adipose tissue. It has been found that visfatin has an insulin-like activity that promotes the uptake of glucose into adipocytes and prevents the release of glucose from hepatocytes[27]. Other than regulating glucose metabolism, visfatin also actively participates in inflammation. Enhanced visfatin production might be an attribution of higher macrophages in obese adipose tissue and, thus, might be considered a proinflammatory marker[28]. Apart from this, visfatin synthesis is also negatively regulated by the anti-inflammatory myokine, "IL-6"[29]. Fulghesu *et al.* reported that PCOS women with Insulin Resistance (IR) have significantly higher IL-6 serum levels and increased IL-6 secretion upon lipopolysaccharide-mediated activation when compared with PCOS women without IR (NIR) and normal women[30], suggesting that inflammatory stimuli lead to an altered immune response in IR-PCOS subjects. As visfatin is negatively regulated by IL-6, our findings strongly demonstrate the absence of insulin resistance and high visfatin level, thus suggesting that the PCOS group we recruited might have a low IL-6 level. These results also corroborate with a previous report, wherein it was shown that PCOS women without IR possess low IL-6.

The relationship between PCOS and the five analytes included in this study has been already reported as described earlier; however, the intricacies of their regulation and function with each other leading to "PCOS" was our focus. Further, little information is available on the pathways that are affected in this entire loop. To understand this further, we performed a regression-correlation analysis to check how these five analytes are correlating with each other in PCOS conditions. Interestingly, our linear regression-correlation analysis revealed a significant correlation between hyperandrogenism with these five metabolites. Our analysis showed a significant correlation between ghrelin

and C-peptide, ghrelin with leptin, C-peptide with insulin, leptin with insulin as well as visfatin, and C-peptide with high testosterone (Figure 4). This demonstrates a strong interaction between diabetes, obesity, and PCOS.

Furthermore, we attempted to analyze all the crucial processes and pathways that are getting affected as a result of altered ghrelin, C-peptide, insulin, leptin, and visfatin through GOnet network analysis. The GOnet network analysis showed a stronger interaction between ghrelin, insulin, leptin, and visfatin, affecting various pathways such as regulation of glucose metabolism, impaired immune signaling, T cell activation and several cytokine regulations including (IL-6, IL-8, IL-12, CCL-5, IFN-beta, and TNF superfamily), regulation to stress and reactive oxygen species, monooxygenase activity, regulation to Nitric Oxide (NO) and Nitric Oxide Synthase (NOS) activity (Figure 6). Our analysis revealed that insulin and leptin regulate the nitric oxide synthase activity and NO level, which supports our previous finding of reduced plasma NOx as well as iNOS/NOS2, and eNOS/NOS3 expression in PCOS women[31]. This network also showed that altered ghrelin, insulin, and leptin affect the MAPK cascade, PI3 kinase, protein kinase activity, and apoptosis which in turn alter many cellular signals and this is in agreement with our report of overactivated MAPK pathways in PCOS[32]. Leptin and ghrelin affect prostaglandin and corticosteroid secretion as reported in this study. PEG2 prostaglandins are shown to be severely elevated in PCOS patients' serum[33] and the media of cultured granulosa cells[34] when compared to healthy individuals. Our finding of elevated leptin might be responsible for prostanoid elevation in PCOS. It is notable that hypercortisolism in Cushing syndrome patients and exogenous treatment with prednisolone lead to reduced plasma ghrelin levels[35]. Higher hair cortisol levels in PCOS patients point to hypothalamic-pituitary-adrenal axis hyperactivation[36]. Thus, our network analysis suggests the importance of studying the impact of ghrelin on steroid biosynthesis.

Our GOnet network analysis indicated that PCOS patients had scantier management of non-rapid eye movement (NREM) sleep and the circadian sleep/wake cycle. This finding gains support from the findings of de Sousa *et al.* wherein PCOS girls significantly differed from the controls with regard to the percentage of Stage 1 of NREM sleep, sleep efficiency, Total Sleep Time (TST), Total Wake Time (***TWT***), Wakefulness After Sleep Onset (WASO), and REM sleep percentage in polysomnography analysis[37]. Incidentally, these authors did not find a significant correlation between insulin resistance and the apnoea–hypopnoea index, and this goes in the sink with our findings of lack of insulin resistance in our study group. Further support comes from the finding of an increased Apnea-Hypopnea Index (AHI) notably in the non-rapid eye movement period (AHI(NREM))[38] suggesting that PCOS women encounter obstructive respiratory stress during the NREM stage. The GOnet network analysis showed that ghrelin positively regulates the circadian sleep/wake cycle and, thus, ghrelin may increase NREM sleep[39]. This indicates that low ghrelin in PCOS patients severely impairs the latters' circadian sleep/wake cycle by impairing NREM. Increased plasma leptin and visfatin are thought to be a consequence of the reduction in sleep and REM sleep[40], and our PCOS subjects exhibited increased leptin and visfatin which corroborates with our previous findings that PCOS patients have disturbed chronology and poor circadian rhythm[41].

Visfatin increase is associated with atherosclerosis inflammation in acute myocardial infarction patients and correlates with elevated and early onset of major adverse cardiovascular events[42,43]. Heightened visfatin levels correlate with abridged brachial artery flow-mediated vasodilatation in PCOS suggesting weakened endothelial function[44]. Our finding of increased visfatin complements the findings of its correlation with cardiovascular disorder in PCOS. Interestingly, similar to no correlation of visfatin with high sensitivity C-reactive protein in PCOS, there is an inverse correlation seen between visfatin and C-peptide, the two inflammatory markers, suggesting that visfatin may have a central role in PCOS pathogenesis. Further, increased leptin in PCOS patients along with high visfatin may increase cardiovascular risk since leptin has a role in cardiovascular disease[45]. Decreased ghrelin in PCOS subjects could exacerbate the cardiovascular risk as ghrelin is known to impart cardiovascular protective effects[46] and is also proposed as a treatment for cardiovascular diseases[47]. On the other hand, leptin exhibits pulsatility and has a diurnal rhythm[48]. Leptin is proposed to up-regulate LH secretion[49] and thus the high LH secretion in PCOS could be a consequence of increased leptin levels.

Additionally, we confirmed the diagnostic potential of the five analytes for PCOS via ROC curve analysis. We found significantly high AUC values for all the five analytes in the PCOS condition (although not predominantly, but significant), which emphasizes their diagnostic potential towards PCOS. Altogether, this study

revealed the various crucial pathways altered in PCOS conditions as a result of aberrant production of insulin, ghrelin, leptin, and visfatin. We also demonstrated a vast network of complicated interconnections between hyperandrogenism, diabetes, and obesity. Analysis of these factors in a larger Indian population either in isolated regions or in the general population will provide further evidence for the prevalence of insulin resistance in PCOS patients. It will also provide a better understanding of their role in follicular arrest, abnormal hormone regulation and impaired pituitary-ovarian hormonal nexus in PCOS pathogenesis.

5. Conclusion

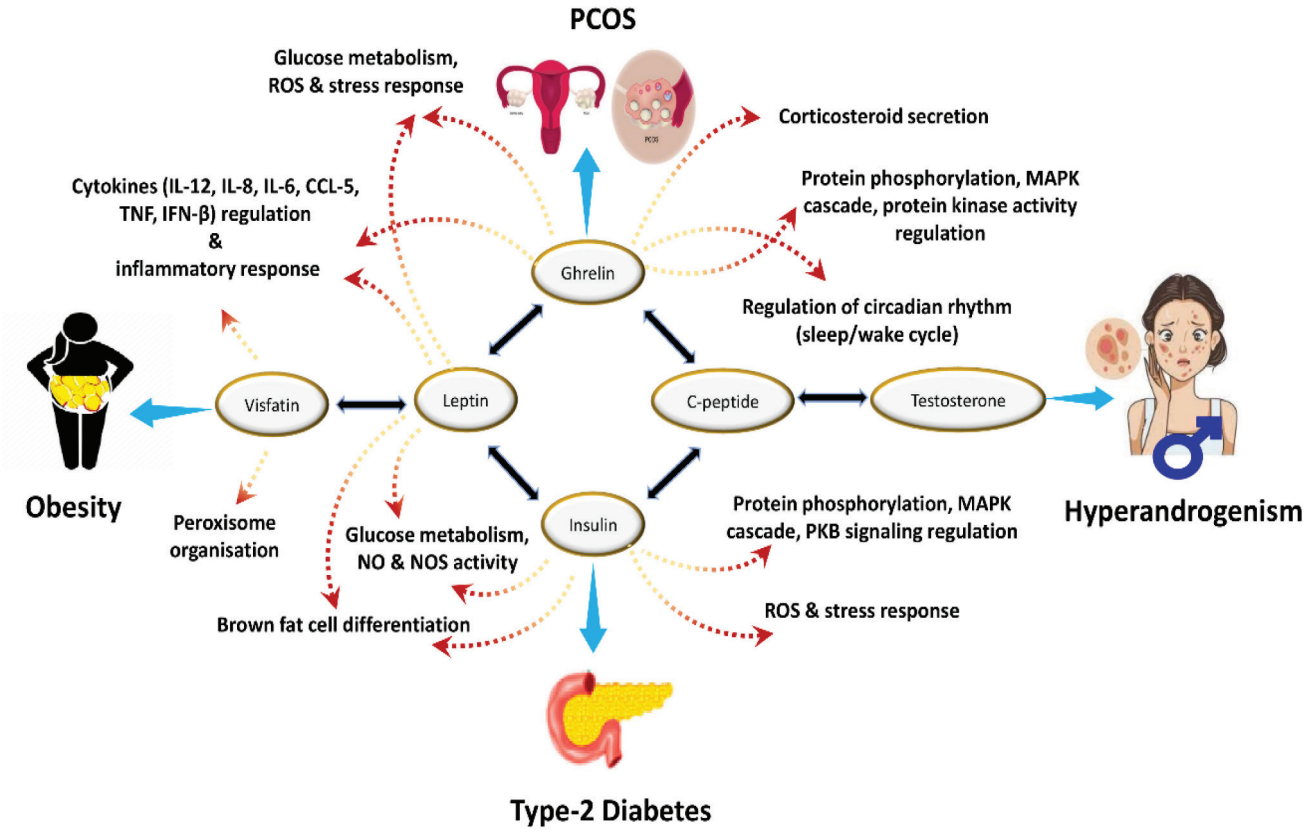
In conclusion, our data showed that not all PCOS women show insulin resistance. PCOS women of Indian ethnicity (derived from Kerala state) show less circulating plasma insulin and C-peptide levels. We found marked alteration of plasma ghrelin and the adipokines "leptin and visfatin" in the PCOS subjects. An aberrant metabolism altered immune mechanisms and cytokine production, oxidative stress, impaired circadian and affected phosphorylation mechanisms, which indicates intertwined metabolic-physiological-inflammatory complications in PCOS pathogenesis (summary figure). The strength of our study is derived from our detailed correlation and pathways analysis, wherein it is shown how PCOS pathogenesis correlates with comorbidities like diabetes, obesity, cardiovascular diseases, and inflammation.

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8. Author contribution

ML contributed to project conception, study design, experiment supervision, data interpretation, and manuscript review. LPP executed the experiments, analyzed, and interpreted all the data for Figures 1-7 and all supplementary data. LPP wrote the manuscript. The diagnosis and recruitment of PCOS patients were done by KJK. All authors were involved in the discussion of the results.

9. Conflict of Interest

There is no Conflict of Interest between authors.

10. References

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