DOI:10.31557/APJCC.2026.11.4.501

RESEARCH ARTICLE

Investigating the Roles of Follistatin and Sex Hormone-Binding Globulin in Polycystic Ovary Syndrome Pathogenesis: Implications for HOMA-IR and Hyperandrogenism

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Abstract

Introduction: Polycystic ovary syndrome (PCOS) is an endocrine disorder characterized by an imbalance of reproductive hormones that leads to anovulation and irregular menstruation. We aimed to evaluate serum levels of SHBG, follistatin and HSP-70, alongside HOMA-IR and hyperandrogenism markers, in PCOS vs. controls. **Materials and Methods:** Ninety women aged 15 to 45 years participated in this case-control study, with 45 diagnosed with polycystic ovary syndrome and 45 serving as controls. Blood samples were collected during the early follicular phase and analyzed using enzyme-linked immunosorbent assay (ELISA) to measure levels of SHBG, follistatin, HSP-70, anti-Müllerian hormone (AMH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), insulin, prolactin, and testosterone. **Results:** Results showed that women with polycystic ovary syndrome had significantly higher levels of follistatin (5.37 ± 0.45 ng/ml), HSP-70 (16.49±2.79), AMH (2386.14 ± 530.09 pg/dL), LH (3.33 ± 0.75 ng/dL), testosterone (2.73 ± 0.51 ng/ml), prolactin (64.21 ± 11.66 ng/ml), and free androgen index (773.23 ± 152.41), fasting insulin (15.21 ± 5.41 µIU/ml), glycated hemoglobin (HbA1c) (6.01 ± 0.24%), and HOMA-IR (3.72 ± 1.44), with reduced SHBG (0.35 ± 0.03 ng/dL) and FSH (2.61 ± 0.61 ng/ml) ($p \leq 0.01$ for all). **Conclusion:** These findings suggest that decreased SHBG and increased follistatin and HSP-70 contribute to the hormonal and metabolic disturbances observed in polycystic ovary syndrome. Therefore, SHBG, follistatin and HSP-70 serve as useful biomarkers for the early diagnosis and prognosis of this condition.

Keywords: Polycystic ovarian syndrome- follistatin- sex hormone binding globulin- HSP-70 HOMA-IR

Asian Pac J Cancer Care, **11 (4)**, 501-511 Submission Date: 03/23/2026 Acceptance Date: 05/07/2026

Introduction

Polycystic ovary syndrome (PCOS) is the most prevalent hormonal disorder affecting women of reproductive age. It is characterized by hyperandrogenism, irregular menstrual cycles, acne, depression, and hirsutism [1]. Insulin resistance is a critical element in the etiology of PCOS. It was shown to correlate with obesity and dyslipidemia; the pathophysiology of PCOS is diverse and various, involving environmental, inherited, and transgenerational factors. These sources explain the dysregulation of hypothalamus-pituitary-ovarian axis signaling, leading to ovarian and adrenal hyper-androgenism [2].

Follistatin, a glycoprotein a member of the superfamily of transforming growth factor-beta, decreases follicle-stimulating hormone (FSH) production, which can inhibit the growth of follicular cells and increase the ovarian production of androgenic hormones [3]. Follistatin may be a viable nonsurgical biomarker for PCOS [4]. PCOS may be associated with elevated follistatin gene expression. In transgenic mice, follistatin overexpression led to ovarian folliculogenesis arrest, either with or without a decrease in FSH serum concentrations [5]. Activin, a dimeric protein belonging to the transforming growth factor-beta (TGF-β) superfamily, plays a central role in ovarian physiology by stimulating follicle-stimulating hormone (FSH) synthesis and promoting granulosa

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cell proliferation. Its activity is tightly regulated by follistatin, a high-affinity binding protein that neutralizes activin bioavailability. The balance between activin and follistatin within granulosa cells is essential for normal folliculogenesis, as excess follistatin can inhibit activin signaling, leading to follicular arrest and impaired oocyte maturation. In women with polycystic ovary syndrome (PCOS), dysregulation of the activin–follistatin system has been reported, contributing to disrupted follicular development and altered steroidogenesis. Integrating this pathway into the current understanding of PCOS pathophysiology provides a more comprehensive mechanistic framework linking endocrine and metabolic dysfunctions [6]. SHBG is a homodimeric polypeptide protein with a molecular mass of 90–100 kDa, produced via the liver. It is the principal protein responsible for binding and transporting testosterone, estradiol, and other sex steroids in plasma with high affinity [7]. Studies consistently demonstrate reduced circulating SHBG levels and an essential component in many physiological and pathological settings in patients with PCOS compared to healthy individuals [8]. Heat shock proteins represent a vast family of proteins in eukaryotic and bacterial organisms, including hyperthermia, inflammation, infection, and food deficiency promotion of production in (HSPs). HSPs react to nearly all situations that induce physiological stress by folding peptides, unfolding and refolding misfolded proteins, destroying defective proteins, and transporting functional proteins [9, 10]. Compared to ovulatory controls of similar age and BMI, women with polycystic ovary syndrome have higher serum HSP-70 levels. These proatherogenic inflammatory markers are linked to ovarian androgenic hormone production, insulin sensitivity, obesity, and fasting lipid levels [11]. Certain investigations have indicated increased HSP70 levels in non-obese PCOS patients [12, 13]. Reduction of insulin capacity to perform the metabolic processes involved in glucose uptake, synthesis, and lipolysis is known as insulin resistance [14]. If pancreatic function is normal, this results in compensatory elevated insulin production both at baseline and during glucose intake. Nevertheless, the high level of insulin in polycystic ovarian syndrome is the source of hyperandrogenism, and it appears to be crucial to the development of the condition [15]. The objectives of study to estimating the level of SHBG, follistatin, and HSP-70 in PCOS patients and related with metabolic disorder.

Materials and Methods

Study design and participants

This case-control study was carried out to assess specific biomarkers linked to PCOS in females between the ages of 15 and 45. 90 women in all were enrolled, 45 of who had a PCOS diagnosis and 45 of who were controls. Diagnosis was based on clinical symptoms, ultrasound examination, and laboratory criteria according to Rotterdam guidelines. The data were collected and recorded from September 2024 to December 2024 at the hospital in the Fertility Unit in Karbala. In addition, information was collected for each group according to the questionnaire.

In accordance with form number IQ.UOK.CAMS.DCL.REC.2, the study protocol was approved by the institutional ethics committee, and all participants provided written informed permission before being included in the study, guaranteeing adherence to ethical guidelines for human research.

Inclusion and exclusion criteria

Eligible subjects were married women aged between (15-45) year. The excluded standards were a history of diabetes mellitus, smoking, hormonal contraceptive use, and previous ovarian surgery to minimize confounding factors affecting hormonal levels.

Sample collection and processing

Venous blood samples (5 mL) were collected from each participant on 2-3 days of menstrual cycle using standard phlebotomy techniques. Samples were divided into EDTA-coated tubes for whole blood and gel tubes for serum separation. Serum was separated by centrifugation at 3000 rpm for 10 minutes and stored under appropriate conditions until analysis.

Measurement of biochemical and hormonal markers

Levels of follistatin, sex hormone binding globulin (SHBG), HSP-70, anti-Müllerian hormone (AMH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), insulin, prolactin, and testosterone were quantified using enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's protocols. Optical density readings were measured at 450 nm and linear (chemical \Spanish) for fasting glucose. evaluation of insulin resistance was conducted using the homeostatic model HOMA-IR = fasting glucose (mg/dl) × fasting insulin (μU/ml) /405) [16]. It is important to note that this study was not specifically designed to diagnose metabolic syndrome; consequently, the data required to apply standard diagnostic criteria (such as NCEP-ATP III) are not available.

Statistical analysis

IBM SPSS software (version 23) was used to analyze the data. To summarize means and standard deviations, descriptive statistics were calculated. Levene's test analyzed variance homogeneity, and the Shapiro-Wilk test evaluated normality. When necessary, independent t-tests or Mann-Whitney U tests were used for group comparisons. The threshold for statistical significance was $p \leq 0.01$.

Sample Size Determination

Sample size was calculated for a two-sample (independent) t-test with equal group sizes using the normal approximation:

$$n = (2 (Z_{1-\alpha/2} + Z_{1-\beta})^2)/\delta^2$$

where n is the required number of subjects per group,

$Z_{(1-\alpha/2)}$ is the standard normal deviate for a two-tailed significance level ($\alpha = 0.05$; $Z_{(1-\alpha/2)}=1.96$, $Z_{(1-\beta)}$ corresponds to the desired power (80%; $Z_{(1-\beta)}$ $Z_{(1-\beta)}=0.842$, σ is the estimated pooled standard deviation, and δ is the expected mean difference between groups.

Based on pilot observations and literature values for follistatin and SHBG, the pooled standard deviation was assumed to be 1.2 ng/mL, and the minimum clinically significant difference between groups was set at 0.8 ng/mL. These values correspond to a standardized effect size (Cohen's d) of approximately 0.67, indicating a moderate effect. Substituting these values into the above equation yielded an estimated sample size of 35 participants per group to achieve 80% power at a 5% significance level (two-sided test).

To account for potential dropouts or unusable samples (approximately 10–20%), the target recruitment was increased to 40 subjects per group. Sample size calculations were performed and verified using G*Power version 3.1. A post-hoc power analysis, based on the observed mean differences and pooled standard deviations, was conducted after data collection and presented in the Results section.

As a result, 46 people per group was the estimated sample size. In order to provide sufficient statistical power and account for possible non-responses or exclusions, the final sample size was modified to include 45 PCOS patients and 45 healthy controls, for a total of 90 study participants.

Results

Comparison of fertility hormones between PCOS patients and control group

Hormonal assays revealed significant increases ($p \leq 0.005$) in luteinizing hormone (LH), prolactin, free androgen index, and testosterone levels in PCOS patients relative to controls, while (FSH) levels were significantly decreased in the patient group as show in Table 1.

Comparison of the level of SHBG, follistatin, and AMH in the PCOS patients as compared to the control group

The results show a highly significant decrease ($p \leq 0.0062$) in SHBG and elevated follistatin, AMH and HSP-70 in the PCOS patients compared to healthy group. Mean values for SHBG 0.35±0.03 (ng\dl) in PCOS patients versus 1.05±0.16 (ng\dl) and mean values for follistatin 1.96±0.36 (ng\ml) in PCOS patients versus 5.37±0.45 (ng\ml) in controls, and for AMH, 2386.14 ± 530.09 pg/dL in patients versus 895.01 ± 198.18 pg/dL in controls, also for HSP-70 16.49±2.79 (ng\ml) versus 4.79±0.56 (ng\ml) in control as show in Table 2.

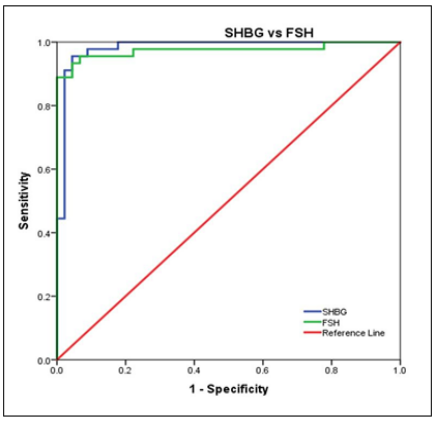

Figure 1. Receiver Operation Characteristic Curve for SHBG and FSH Biomarker in PCOS Patients

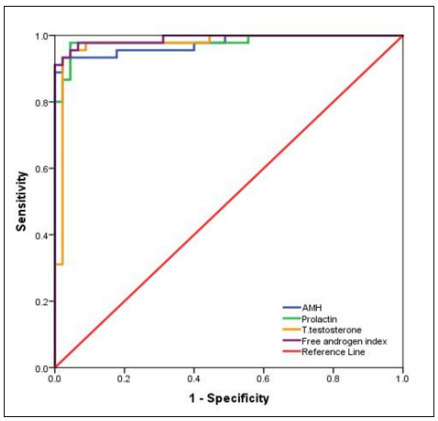

Figure 2. Receiver Operation Characteristic Curve for AMH, Testosterone, Prolactin, and Free Androgen Index Biomarker in PCOS Patient

Table 1. The Level of Different Fertility Hormones in PCOS Patients Compared to Controls

Parameters	Groups	Mean	Std. Deviation	P-value
T.testosterone (ng\mL)	Control	1.08	0.05	0.00002
	Patient	2.73	0.51	
Prolactin (ng\mL)	Control	20.74	4.33	0.0001
	Patient	64.21	11.66	
FSH (ng\mL)	Control	5.72	0.7	0.0004
	Patient	2.61	0.61	
LH (ng\dL)	Control	0.51	0.16	0.0008
	Patient	3.33	0.75	
Free androgen index	Control	104.59	13.55	0.0007
	Patient	773.23	152.41	

Bonferroni-adjusted significance level set at 0.005, LH= Lutinizing Hormone, FSH= Follicle-Stimulation Hormone

Table 2. Comparison of the Level of SHBG, Follistatin, and AMH in the PCOS Patients as Compared to the Control Group

Parameters	groups	Mean	Std. Deviation	P-value
SHBG (ng\dL)	Control	1.05	0.16	0.0007
	Patient	0.35	0.03	
Follistatin (ng\mL)	Control	1.96	0.36	0.0007
	Patient	5.37	0.45	
AMH (pg\dL)	Control	895.01	198.18	0.0001
	Patient	2386.14	530.09	
HSP-70 (ng\mL)	Control	4.79	0.56	0.0006
	Patients	16.49	2.79	

Bonferroni-adjusted significance level set at 0.0062, SHBG= Sex Hormone Binding Globulin, AMH= Anti-mullerian Hormone, HSP-70= Heat Shock Protein-70.

Table 3. Estimation of SHBG in PCOS Patients According to Different Demographic Criteria

Parameter	Classification	Groups	Control		Patient		P-value
			Mean	Std. Deviation	Mean	Std. Deviation	
SHBG (ng\dL)	Last date of delivery	None	1.023	0.042	0.355	0.031	0.00001
		One Year	1.09	0.119	0.367	0.049	0.0155
		Two Year	0.978	0.115	0.356	0.069	0.00009
		More than two years	1.07	0.173	0.353	0.023	0.00002
	Type of delivery	Cesarean	1.003	0.075	0.353	0.036	0.0001
		Natural	1.065	0.173	0.356	0.028	0.0003
	Address	Rural	1.08	0.192	0.357	0.028	0.00002
		Urban	1.009	0.072	0.354	0.032	0.00001
	Age (yr)	Less than 30	1.008	0.095	0.352	0.034	0.00004
		Greater than 30	1.086	0.189	0.363	0.015	0.00003
	Nature of food	Healthy	1.023	0.082	0.345	0.025	0.00001
		Unhealthy	1.084	0.212	0.359	0.033	0.00002
	BMI kg\m²	Normal	1.004	0.107	0.354	0.046	0.00001
		Overweight	1.048	0.16	0.354	0.034	0.00005
		Obese	1.137	0.201	0.359	0.01`9	0.00004

Bonferroni-adjusted significance level set at 0.0033, SHBG=Sex Hormone Binding globulin, BMI=Body Mass Index.

Estimation of SHBG in PCOS patients according to different demographic criteria

Significant decreased (p≤0.0033) of SHBG in PCOS patients were observed across different demographic criteria, including address (rural vs. urban), age groups (less than 30 and greater than 30 years), type of food (healthy vs. unhealthy), type of delivery (cesarean vs. natural), and BMI categories (normal, overweight, obese), in contrast to the comparable control groups, as show in Table 3.

Estimation of Follistatin in PCOS patients according to different demographic criteria

Significant elevations (p≤0.0033) of follistatin in PCOS patients were observed across different demographic criteria, including address (rural vs. urban), age groups (less than 30 and greater than 30 years), type of food (healthy vs. unhealthy), type of delivery (cesarean vs. natural), and BMI categories (normal, overweight, obese), compared to their respective control groups as show in Table 4.

Estimation of HSP-70 in PCOS patients according to different demographic criteria

Significant elevations (p≤0.0045) of HSP-70 in PCOS patients were observed across different demographic criteria, including address (rural vs. urban), age groups (less than 30 and greater than 30 years), type of food (healthy vs. unhealthy), type of delivery (cesarean vs. natural), and BMI categories (normal, overweight, obese), compared to their respective control groups as show in Table 5.

Level of SHBG based on clinical features and fertility indicators in the PCOS patients compared to the control

Serum SHBG concentrations were significantly decreased (p≤0.0041) in PCOS patients regardless of clinical symptoms such as hirsutism and acne, as well as fertility parameters including type of infertility (primary and secondary) and parity status (nulliparous vs. parous), when compared to controls, as show in Table 6.

Table 4. Estimation of Follistatin in PCOS Patients According to Different Demographic Criteria

Parameters	Classification	Groups	Control		Patient		P-value
			Mean	Std. Deviation	Mean	Std. Deviation	
Follistatin (ng\mL)		None	1.803	0.092	5.325	0.439	0.00001
	Last date of delivery	One Year	2.182	0.067	5.173	0.605	0.0201
		Two Year	1946	0.394	5.941	0.456	0.00007
		More than two years	1.958	0.38	5.365	0.425	0.00004
		Cesarean	2.038	0.46	5.543	0.564	0.0002
	Type of delivery	Natural	1.932	0.332	5.31	0.343	0.0003
		Rural	1.953	0.41	5.321	0.489	0.00007
	Address	Urban	1.96	0.285	1.274	0.434	0.00004
		Less than 30	1.987	0.315	5.351	0.476	0.00001
	Age	Greater than 30	1.931	0.4	5.446	0.38	0.00003
	Nature of food	Healthy	1.901	0.296	5.331	0.487	0.00002
		Unhealthy	2.01	0.425	5.392	0.445	0.00004
		Normal	1.923	0.326	5.375	0.618	0.00001
	BMI (km\m²)	Overweight	1.996	0.36	5.401	0.382	0.00005
		Obese	1.886	0.447	5.346	0.465	0.00002

Bonferroni-adjusted significance level set at 0.0033

Table 5. Estimation of HSP-70 in PCOS Patients According to Different Demographic Criteria in PCOS and Control Groups

Parameters	Classification	Groups	Control		Patient		P-value
			Mean	Std. Deviation	Mean	Std. Deviation	
	Address	Rural	4.842	0.552	17.176	3.707	0.00002
		Urban	4.724	0.582	16.216	2.328	0.00003
		Less than 30	4.919	0.539	16.409	3.143	0.00001
HSP-70 (ng\mL)	Age	Greater than 30	4.696	0.568	16.754	1.199	0.00001
		Healthy	4.738	0.55	16.916	3.211	0.00002
	Nature of food	Unhealthy	4.86	0.579	16.322	2.63	0.00005
		Ceserian	4.795	0.614	17.443	2.916	0.0002
	Type of delivery	Natural	4.795	0.554	16.371	1.274	0.0001
		Normal	4.869	0.723	16.235	3.591	0.00003
		Overweight	4.791	0.434	16.4	2.918	0.00004
	BMI kg\m²	Obese	4.688	0.662	16.235	2.318	0.00006

Bonferroni-adjusted significance level set at 0.0045

Level of follistatin based on clinical features and fertility indicators in the PCOS patients compared to the control

Serum follistatin concentrations were significantly higher ($p \leq 0.0041$) in PCOS patients regardless of clinical symptoms such as hirsutism and acne, as well as fertility parameters including type of infertility (primary and secondary) and parity status (nulliparous vs. parous), when compared to controls, as shown in Table 7.

HSP-70 levels based on clinical features and fertility indicators

Serum HSP-70 concentrations were significantly higher ($p \leq 0.0041$) in PCOS patients regardless of clinical symptoms such as hirsutism and acne, as well as fertility parameters including type of infertility (primary and secondary) and parity status (nulliparous vs. parous), when compared to controls as show in Table 8.

Estimation of different glucose tests and HOMA-IR in the PCOS patients compared to the control group

The results in Table 7 demonstrate a significant increase ($p \leq 0.0062$) in f. insulin, HbA1c, and HOMA-IR found in the PCOS patients (15.21±5.41), (6.01±0.24) and (3.72±1.44) compared to the control group (6.65±1.46), (5.00±0.19) and (1.57±0.33), respectively. As show in Table 9.

Tables 10-12 present the comparison of serum Heat Shock Protein (HSP), Sex Hormone-Binding Globulin (SHBG), and Follistatin levels between patients and controls across different BMI categories (normal, overweight, and obese). The aim was to determine whether BMI modifies the relationship between disease status and these biomarkers.

The mean HSP levels were markedly higher in patients compared to controls across all BMI groups. In the normal-weight group, the mean HSP level in patients (17.298 ± 3.591 ng/mL) was substantially elevated

Table 6. Level of SHBG based on Clinical Features and Fertility Indicators in the PCOS Patients Compared

Parameters	Classification	Groups	Mean	Std. Deviation	P-value
SHBG (ng\dL)	Type of infertility	Primary	0.355	0.031	0.0002
		Secondary	0.354	0.032	
		Control	1.051	0.158	
	Hairsitsm	No	0.346	0.014	0.0002
		Yes	0.357	0.034	
		Control	1.051	0.158	
	Acne	No	0.348	0.028	0.0003
		Yes	0.361	0.033	
		Control	1.051	0.158	
	Nuliparus\Parus	Nuliparus	0.355	0.031	0.0003
		parus	0.354	0.032	
		Control	1.051	0.158	

Bonferroni-adjusted significance level set at 0.0041, SHBG=sex hormone binding globulin.

Table 7. Follistatin Based on Clinical Features and Fertility Indicators in the PCOS Patients Compared to the Control

Parameters	Classification	Level	Mean	Std. Deviation	P-value
follistatin (ng\mL)	Type of infertility	Primary	5.325	0.439	0.0003
		Secondary	5.426	0.471	
		Control	1.956	0.361	
	Hairstism	No	5.197	0.472	0.0001
		Yes	5.419	0.361	
		Control	1.956	0.227	
	Acne	No	5.366	5.366	0.0004
		Yes	5.382	5.382	
		Control	1.956	1.956	
	Nuliparus\parus	NuliParus	5.325	0.439	0.0003
		Parus	5.426	0.471	
		Control	1.956	0.361	

Bonferroni-adjusted significance level set at 0.0041.

compared to controls (4.869 ± 0.723 ng/mL). A similar pattern was observed among overweight (16.4 ± 2.918 ng/mL vs. 4.791 ± 0.434 ng/mL) and obese individuals (16.235 ± 2.318 ng/mL vs. 4.688 ± 0.662 ng/mL). Despite the consistent trend of higher HSP levels in patients, the interaction between status and BMI group was not statistically significant (p = 0.723). This indicates that BMI did not influence the relationship between disease status and HSP levels the increase in HSP among patients was consistent across BMI categories. These findings suggest that HSP elevation is primarily associated with disease status rather than body mass. HSPs are known to respond to cellular stress and inflammation, so their upregulation in patients may reflect a systemic stress response independent of BMI.

In all BMI categories, SHBG levels were significantly lower in patients compared to controls. For instance, among normal-weight individuals, the mean SHBG concentration was 1.004 ± 0.107 in controls versus 0.354 ± 0.046 in patients. Similar reductions were observed in overweight and obese groups. However, the interaction between status and BMI was not significant (p = 0.152), indicating that BMI did not significantly modify the effect of disease status on SHBG levels. The consistently lower SHBG levels in patients may suggest metabolic or endocrine alterations associated with the disease, possibly linked to insulin resistance, inflammation, or hormonal imbalance. The lack of BMI effect implies that

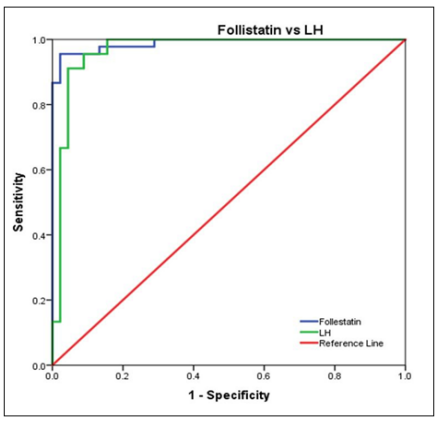

Figure 3. Receiver Operating Characteristic Curve for Follistatin and LH Biomarker in PCOS Patient

Table 8. Comparison of Serum Levels of HSP-70 According to Psychological and Fertility Indicators in PCOS Patients and the Control Groups

Parameters	Groups	Groups	Mean	Std. Deviation	P-value
HSP-70 (ng\mL)		No	15.228	2.695	0.0001
	Hirsutism	Yes	16.81	2.753	
		Control	4.795	0.561	
		No	16.392	2.116	0.0001
	Acne	Yes	16.582	3.306	
		Control	4.795	0.561	
		nuliparus	16.098	3.208	0.0006
	nuliparus\paruslady	Parus lady	16.907	2.263	
		Control	4.795	0.561	
		Primary	16.098	3.208	0.0005
	Type of infertility	Secondary	16.907	2.263	
		Control	4.795	0.561	

Bonferroni-adjusted significance level set at 0.0041.

Table 9. Estimation of Different Glucose Tests and HOMA-IR in the PCOS Patients Compared to the Control Group

Parameters	groups	Mean	Std. Deviation	P-value
Fasting glucose (mg\d)	Control	95.71	4.66	0.0328
	Patient	98.4	6.87	
Fasting insulin (MIU\ ml)	Control	6.65	1.46	0.0006
	Patient	15.21	5.41	
HbA1c %	Control	5	0.19	0.0007
	Patient	6.01	0.24	
HOMA-IR	Control	1.57	0.33	0.0004
	Patient	3.72	1.44	

Bonferroni-adjusted significance level set at 0.0062, HOMA-IR= Homeostatic Model Assessment for Insulin Resistance

these mechanisms are disease-related rather than weight-dependent.

Follistatin levels were substantially higher in patients compared to controls in all BMI groups. For example, in the normal BMI group, patients exhibited a mean of 5.375 ± 0.618 ng/mL, while controls averaged 1.923 ± 0.326 ng/mL. Similar differences persisted in the overweight and obese groups. The interaction between status and BMI group was not significant (p = 0.962), suggesting that BMI did not influence the pattern of Follistatin expression between groups. This pattern supports the finding from the two-way ANOVA that disease status is the dominant factor affecting Follistatin levels. Follistatin is known to regulate inflammatory and metabolic pathways, and its consistent elevation across BMI groups highlights its potential as a robust disease-related biomarker. 3.13. Diagnostic performance of biomarkers using ROC curve analysis.

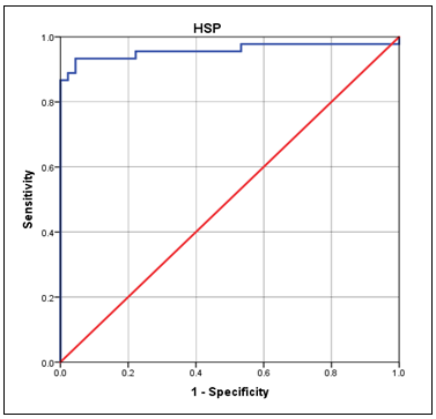

Figure 4. Receiver Operating Characteristic Curve for HSP-70 Biomarker in PCOS Patient

Analysis of the receiver operating characteristic (ROC) curve showed that the detected biomarkers had a good diagnostic accuracy., including SHBG, follistatin, HSP-70, AMH, testosterone, prolactin, FSH, and LH, with areas under the curve (AUC) ranging from approximately 95.852% to 98.86%, reflecting excellent sensitivity and specificity for differentiating PCOS patients from controls as show in Table 13, (Figure 1-4).

Receiver Operating Characteristic (ROC) analysis was performed to evaluate the diagnostic performance of each biomarker for discriminating PCOS patients from controls.

The Area Under the Curve (AUC) was computed using the non-parametric DeLong method [17] with 95% confidence intervals (CI). Standard error, sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) were also calculated for each parameter at the optimal cut-off point determined by Youden's index (J = sensitivity + specificity – 1).

To compare diagnostic accuracy among biomarkers, pairwise AUC comparisons were conducted using DeLong's test for two correlated ROC curves. This test

Table 10. Comparison of HSP Levels between Patients and Controls Across Different BMI Categories

BMI Group	Status	Mean	Std. Deviation	95% CI	P. value (Status * BMI)
Normal	Control	4.869	0.723	3.745 - 5.992	0.72311
	Patient	17.298	3.591	15.866 - 18.730	
Overweight	Control	4.791	0.434	3.964 - 5.617	
	Patient	16.4	2.918	15.470 - 17.329	
Obese	Control	4.688	0.662	3.256 - 6.120	
	Patient	16.235	2.318	15.280 - 17.189	

Table 11. Comparison of SHGB Levels between Patients and Controls Across Different BMI Categories

BMI Group	Status	Mean	Std. Deviation	95% CI	P. value (Status * BMI)
Normal	Control	1.004	0.107	0.942 - 1.066	0.15212
	Patient	0.354	0.046	0.275 - 0.433	
Overweight	Control	1.048	0.16	1.003 - 1.094	
	Patient	0.354	0.034	0.303 - 0.405	
Obese	Control	1.137	0.201	1.059 - 1.216	
	Patient	0.356	0.019	0.304 - 0.408	

Table 12. Comparison of Follestatin Levels between Patients and Controls Across Different BMI Categories

BMI Group	Status	Mean	Std. Deviation	95% CI	P. value (Status * BMI)
Normal	Control	1.923	0.326	1.693 - 2.154	0.96205
	Patient	5.375	0.618	5.081 - 5.668	
Overweight	Control	1.996	0.36	1.827 - 2.166	
	Patient	5.401	0.382	5.211 - 5.592	
Obese	Control	1.886	0.447	1.593 - 2.180	
	Patient	5.346	0.465	5.150 - 5.541	

Table 13. Area Under the ROC Curve (AUC) Analysis for Research Parameters

Metrics		T.testosterone	Prolactin	FSH	LH	SHBG	Follistatin	AMH	FAI	HSP-70
Std. Error		0.018	0.013	0.018	0.021	0.013	0.008	0.015	0.021	0.025
Asymptotic Sig.		0.001	0.003	0.009	0.002	0.003	0.001	0.008	0.003	0.006
Asymptotic 95% Confidence Interval	Lower Bound	0.938	0.955	0.939	0.925	0.956	0.973	0.946	0.946	0.946
	Upper Bound	1	1	1	1	1	1	1	1	1
Cutoff Point		1.198	35.143	4.329	2.455	0.589	3.773	1465.212	143.96	8.151
Area Under Curve (AUC)		97.33%	98.12%	97.43%	96.64%	98.17%	98.86%	97.53%	96.64%	95.85%
Sensitivity		95.56%	97.78%	95.45%	91.11%	95.56%	95.56%	93.43%	95.56%	93.33%
Specificity		95.36%	95.46%	93.33%	95.54%	95.55%	97.78%	97.78%	95.43%	95.56%
Accuracy		95.56%	96.67%	94.44%	93.33%	95.56%	96.67%	95.56%	96.67%	94.44%
Positive Predictive Value		95.46%	95.65%	93.48%	95.35%	95.66%	97.73%	97.67%	95.65%	95.46%
Negative Predictive Value		93.48%	97.73%	95.46%	91.49%	95.55%	95.65%	93.62%	97.73%	93.48%

accounts for the correlation arising from using the same set of subjects and provides a p-value for whether the difference in AUCs between two markers is statistically significant.

When multiple AUC comparisons were made (e.g., between Follistatin vs. SHBG, Follistatin vs. AMH, and SHBG vs. HSP-70), p-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) procedure to control for Type I error inflation. Statistical significance was defined as adjusted $p < 0.05$ (Table 14).

ROC analysis revealed excellent diagnostic accuracy for all tested parameters (AUC > 0.95). Among them, Follistatin exhibited the highest AUC (0.9886, 95% CI: 0.973–1.000), followed by SHBG (0.9817, 95% CI: 0.956–1.000) and AMH (0.9753, 95% CI: 0.946–1.000).

Pairwise AUC comparisons using DeLong's test showed that Follistatin performed significantly better than AMH ($p = 0.009$, $q = 0.027$) and HSP-70 ($p = 0.00002$,

Table 14. Statistical Comparison of Diagnostic Performance (ΔAUC) between Studied Biomarkers

Comparison	ΔAUC	Standard Error	Z-value	p-value	Adjusted q-value (FDR)	Interpretation
Follistatin vs SHBG	0.007	0.004	1.75	0.08	0.12	Not significant
Follistatin vs AMH	0.013	0.005	2.6	0.009	0.027	Significant
Follistatin vs HSP-70	0.03	0.007	4.28	0.00002	0.00006	Highly significant
SHBG vs AMH	0.006	0.004	1.45	0.147	0.196	Not significant
AMH vs HSP-70	0.017	0.006	2.83	0.005	0.015	Significant

q = 0.00006), but not significantly better than SHBG (p = 0.080).

These findings suggest that Follistatin and SHBG are strong and comparable diagnostic markers for PCOS, with Follistatin showing the numerically highest discriminative power.

Discussion

PCOS is a multifactorial heterogeneous disorder associated with a variety of etiologies, outcomes, and clinical manifestations. The result of the present study found that PCOS patients have a decrease in SHBG due to insulin resistance. Hyperinsulinemia inhibiting production of SHBG from liver, raises fatty liver, abnormalities in the metabolism of lipid and glucose, and low estrogen levels. This process impedes production of SHBG from the liver, making inactive androgen free and leading to a condition called hyperandrogenism [18]. This finding is in agreement with studies that found that a decrease in SHBG is caused by high insulin [19]. In the current study, elevated follistatin levels were observed in women with polycystic ovary syndrome aged over 30, which may be associated with obesity, hyperinsulinemia, and fatty liver; these factors can impair follicular development and contribute to increased ovarian androgen production, Serum follistatin demonstrated exceptional diagnostic performance for PCOS in our cohort. ROCanalysis revealed an AUC of 0.973 (p = 0.001), with a cutoff of 3.773 ng/mL yielding a sensitivity of 98.86% and specificity of 95.56%. These findings suggest that follistatin is a highly reliable biomarker, reflecting both activin–follistatin imbalance and associated endocrine-metabolic disturbances. Clinically, this threshold could aid in diagnosing PCOS in ambiguous cases and serve as a longitudinal marker for monitoring therapeutic response to metformin or combined oral contraceptives. Future multicenter and prospective studies are needed to validate this cutoff, assess dynamic changes during treatment, and standardize assay methods to ensure reproducibility across populations. Until then, the 3.8 ng/mL cutoff should be considered hypothesis-generating [6]. This is in line with a study that found that an increase in follistatin levels can induce adipose tissue, insulin resistance, and thus could increase the risk for type 2 diabetes [20]. Elevated serum follistatin levels were particularly evident in obese PCOS phenotypes, reflecting a phenotype-specific metabolic alteration linked to insulin resistance and adipokine imbalance [21]. This heterogeneity emphasizes the need for individualized management strategies. Therapeutic agents such as GLP-1 receptor agonists have shown promise in obese PCOS patients by improving insulin sensitivity, reducing body weight, and potentially modulating follistatin-related pathways [22, 23]. These findings highlight the potential of integrating metabolic-targeted therapies into phenotype-based treatment approaches for PCOS.

Additionally, the study discovered that PCOS patients' ovarian tissue contained noticeably more HSP70 than the healthy group. Ovarian tissue contains higher levels of the most conserved protein, HSP70, which has been connected to a decrease in ovarian follicular cell death. Because HSP70 may affect apoptosis by preventing the Bcl-2 family protein from moving from the cytosol to the mitochondria, women with PCOS may also experience apoptosis in their ovarian tissues as a result of this increase in HSP70 [24], which also agrees with [14][25].

HOMA-IR, glycated hemoglobin (HbA1c), and fasting insulin levels were found to be elevated in patients with PCOS, reflecting the close linked between insulin resistance and various metabolic disturbances, including increased aromatase activity and androgen production, pancreatic beta-cell dysfunction, and reduced progesterone synthesis in granulosa cells. These findings are consistent with previous studies indicating that elevated fasting insulin and HOMA-IR result from pancreatic beta-cell impairment [26]. That agreement with study found elevated insulin levels in women with PCOS align with evidence that hyperinsulinemia can enhance aromatase activity in adipocytes, increasing local estrogen production. This mechanism may contribute to the hormonal imbalances observed in our cohort, linking metabolic disturbances directly to ovarian dysfunction and supporting the importance of targeting insulin resistance in PCOS management [27].

The analysis of data showed a decrease in FSH and an increase in LH, prolactin and t. testosterone due to the disturbed hypothalamic-pituitary-ovarian (HPO) axis, which is associated with low FSH secretion. Increased LH frequency increases theca cell production of androgens (total testosterone) due to obesity. Impairment estrogen feedback mechanism and insulin resistance work together to stimulate the production of testosterone in the ovaries and adrenal glands, leading to hyperandrogenism. Meanwhile, the decreasing FSH levels impair follicle development and, consequently, anovulation [28], which agrees with [29]. In the current study, an increase in AMH was found due to the enhanced production and release of AMH by the pre-antral and tiny antral follicles. AMH levels rise proportionately to the antral follicle count

(AFC) at a steady 0.2 ng/ml for each follicle. Furthermore, granulosa cells in PCOS patients' follicles have been shown to generate 75 times as much AMH as normal cells. It can be used as an indicator of ovarian activity [30], which agrees with [31].

Previous studies documented results comparable to the results of the current study, showing that Prolactin AUC, sensitivity, and specificity were (83.2%, 77%, and 88%), respectively [32]. Testosterone AUC, sensitivity, and specificity were (88.9%, 92.6%, 85.4%) [33-35] demonstrated that LH AUC, sensitivity, and specificity were (93.2%, 86.3%; 95%). FSH sensitivity of 65.00% and specificity of 87.80% [36]. AMH has a specificity and sensitivity of 95.52%,93.46% [36-38].

This study's inability to determine the prevalence of metabolic syndrome using accepted clinical criteria since all required data is missing is one of its limitations. To allow for a methodical examination of the connection between metabolic syndrome and other biomarkers, we advise that future research incorporate the complete set of data (such as waist circumference, triglycerides, etc.). We sincerely hope that our response meets your needs, and we sincerely appreciate your time and hard work in reviewing this work.

SHBG decrease in women with PCOS leads to an increase in free androgens like testosterone, follistatin and HSP-70 contributing to the insulin resistance. They play a critical role in the development of polycystic ovarian syndrome. They can be used as diagnostic markers for PCOS.

Acknowledgements

We express our gratitude to the patients who took part in the research.

Authors' contributions

All authors made substantial contributions to data collection, analysis, the preparation of the results and write the manuscript.

Data availability

You can obtain the datasets used and/or analyzed in this study from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Ethical approval and informed consent

In accordance with form number IQ.UOK.CAMS. DCL.REC.2, the study protocol was approved by the institutional ethics committee, and all participants provided written informed permission before being included in the study, guaranteeing adherence to ethical guidelines for human research.

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