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Androgen Profile, Adiponectin, and Insulin Resistance Follow-Up Assessment for Combined Metformin and Vitamin D Treatment for Polycystic Ovary Syndrome

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ABSTRACT

Background & aim: Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age (15-44) years. Today, 50-80% of patients with PCOS exhibit insulin resistance (IR) syndrome, which is worsened by hyperandrogenism-related adipose tissue accumulation. Metformin at a dose of (1700-2000) mg/day, with and without lifestyle is associated with a beneficial improvement on BMI and menstrual cycles. This study investigated the role of androgen profile, insulin resistance and adiponectin to evaluate the benefits of combined vitamin D and metformin in the treatment of PCOS.

Methods: This cross-sectional study was included 50 infertile women who priory diagnosed with PCOS with age range of 18-40 year. They were classified into Group I: Twenty-five women who were treated with metformin 850 mg/twice daily and followed for 8 weeks and Group II: Twenty-five women who were treated with metformin 850 mg/twice daily plus vitamin D3 50.000 IU/wk., and followed for 8 weeks. Serum investigations included measurements of free testosterone, dihydrotestosterone, dehydroepiandrosterone-sulfate, sex-hormone binding globulin, HOMA-IR, 25-hydroxyvitamin D, and adiponectin by ELISA technique.

Results: This study revealed that the mean values of free testosterone (P<0.001), dihydrotestosterone (P<0.001) and waist circumference (P<0.021) were significantly improved (by lowering) after treatment with combined metformin plus vitamin D compared with metformin alone. Moreover, adiponectin mean value was significantly elevated after combined treatment compared with metformin alone (P<0.001).

Conclusion: Serum measurements of free testosterone, dihydrotestosterone and adiponectin are good indicators for measuring the efficacy of combined metformin plus vitamin D supplement in treatment of PCOS women.

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Introduction

As the most prevalent endocrine disorder, polycystic ovary syndrome (PCOS) is observed in females of childbearing age (1-2). According to the Rotterdam criteria as the most accepted, appropriate, and important diagnostic international criteria for PCOS, females are required to fulfill at least two of these criteria: clinical and/or biochemical evidence of hyperandrogenism, oligo and/or anovulation, and ultrasound-based polycystic ovaries (2-3).

PCOS is heterogeneous, and women may associate with several reproductive, endocrine, metabolic, and psycho-social symptoms (1, 3). Although the pathogenesis of PCOS has not been completely explained, recently conducted research has indicated that hyperandrogenism,

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inflammation, oxidative damage, as well as adipose tissue hypertrophy and dysfunction form a vicious cycle in this disease physiopathology (1, 4).

Insulin resistance is considered another frequent characteristic in patients involved with PCOS. This leads to hyperandrogenism and anovulation and has a significant role in its pathogenesis. It has been reported that insulin resistance syndrome is exhibited by 50%-80% of patients with PCOS. This condition is exacerbated by the accumulation of adipose tissue associated with hyperandrogenism contributing to the onset and progression of the disease (2, 4).

Metformin (1700-2000 mg/day), with and without lifestyle, is correlated with a beneficial improvement in body mass index (BMI) and menstrual cycles. The initiation of lower doses of metformin with gradual increases and the use of extended-release preparations can help reduce the side effects (5).

Women with PCOS require adequate vitamin D levels (≥30 ng/ml), and vitamin D deficiency may worsen hyperandrogenism and insulin resistance. As a relatively safe, inexpensive, and oral agent, vitamin D can help treat normal ovulatory dysfunction by enhancing follicular development and improving menstruation cycles; moreover, all women of childbearing age may use it during infertility (6-7). Accordingly, the supplementation of vitamin D for PCOS women, particularly those who are deficient in it, may be beneficial in the improvement of clinical characteristics and hormonal derangement of this disease. The objective of this study was to assess the role of androgen profile, insulin resistance, and adiponectin, to evaluate the benefits of combined vitamin D and metformin in the treatment of PCOS.

Materials and Methods

The present study was conducted based on observational research design at the Department of Biochemistry, College of Medicine, University of Baghdad, Baghdad, Iraq, and at Kamal Al-Samarraei Hospital for infertility management and IVF, from September 2022 to June 2023. The participants included 50 infertile previously diagnosed PCOS women (age range: 18-40 years) and without any infertility-related treatment for two months at least.

These 50 women with PCOS were diagnosed by a Specialist Gynecologist after proper physical, biochemical, and gynecological examinations and confirmed by ultrasound. Rotterdam consensus defines PCOS by fulfilling at least two of the following criteria: hyperandrogenism, oligo and/or anovulation, and polycystic ovaries (≥12 follicles, 2-9 mm in diameter, and/or an ovarian volume more than 10 mL in at least one ovary) (8). Under the guidance of a Gynecologist, at Kamal Al-Samarraei Hospital for Infertility Management and In Vitro Fertilization, Baghdad, Iraq, the included 50 PCOS participants were classified according to their designed treatment and followed precisely for 8 weeks. They were then divided into two sub-groups. The participants in Group I (n=25) were subjected to metformin (850 mg/twice daily) treatment and followed for 8 weeks, and the women in Group II (n=25) were under treatment using metformin (850 mg/twice daily) plus vitamin D3 50.000 IU/wk., and also followed for 8 weeks. Sample size of groups was accepted according to proportionate stratified sampling equation and the power of sample size was ≥ 80% to identify medium-to-large effect.

This study excluded women with any type of cancer, acute and chronic illness, diabetes mellitus, chronic liver disease, pregnant women, smokers, endocrine disorders, and chronic renal failure. The first author collected all the data from PCOS study group and collected blood samples. All the collected samples assessed in the same laboratory. After 10-12 hours of overnight fasting state, blood samples were obtained from each included woman at studied groups in the follicular phase between the 2nd and 7th day of the menstrual cycle, before starting their designed treatment, between 08:00 and 10:00 A.M. from an antecubital vein after 5 minutes rest in the supine position. The blood samples were then separated by centrifugation at 3000 rpm for 5 minutes to obtain serum after remaining to be clotted at room temperature for 10-15 minutes. The resulting serum samples were aliquot, frozen, and maintained at -20°C for two months till the day of measurement of fasting serum glucose, insulin, 25 hydroxyvitamin D, adiponectin, free testosterone, dihydrotestosterone, dehydroepiandrosterone-sulfate (DHEA-S) and

sex-hormone binding globulin (SHBG) by the quantitative sandwich and competitive enzyme immunoassay technique for the in vitro determination of human serum and plasma.

HOMA-IR=[insulin (mU/L) * glucose (mg/dl)]/405 equation was utilized to measure the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) (9). Furthermore, BMI (Kg/m^2)=Weight (Kg)/height (m^2) equation was employed to measure weight and height, as well as calculate the BMI (10). Following that, a 6.5 MHz transducer was used to perform the ovaries transvaginal ultrasound scan and identify the total number of early antral follicles at Kamal Al-Samarraei Hospital for infertility management and IVF.

SPSS software (version 23.0) and Microsoft Office (version 2010) were utilized to analyze the obtained data. The analysis revealed a normal distribution of the data. The descriptive statistics (i.e., mean±SD) were measured to describe the data. Following that, a paired sample t-test (Paired t-test of a single variable with two periods in one group) was applied to compare the groups. Moreover, the degree of association between continuous variables was measured using the Pearson's correlation coefficient (r). It is worth mentioning that a P-value equal to or less than 0.05 was considered statistically significant.

Results

According to the findings obtained from the follow-up of PCOS women, out of 25 females, who selected for treatment in each group, 18 patients completed their course for metformin group and 19 females completed their course for combined group.

Table 1 depicts no significant difference between metformin and combined groups (P>0.05) regarding the mean±SD values of age, BMI, waist circumference, adiponectin, and 25 hydroxyvitamin before treatment. However, the combined group shows higher mean±SD value of HOMA-IR of (7.12 ± 3.21, P<0.001), compared to the metformin-treated group (4.37 ± 1.19).

Table 1. Mean±SD values of age, BMI, waist circumference, HOMA-IR, adiponectin, and 25 hydroxyvitamin between the two groups before the treatment

Variable	Metformin group (N=25)	Combined group (N=25)	P-Value
Age (years)	26.32 ± 6.11	24.92 ± 4.70	0.546 •
BMI (kg/m^2)	30.76 ± 6.40	30.58 ± 5.43	0.382•
Waist circumference (cm)	99.76 ± 11.68	105.48 ± 17.45	0.101•
HOMA-IR	4.37 ± 1.19	7.12 ± 3.21	< 0.001••
Adiponectin (ng/ml)	2.49 ± 0.29	2.53 ± 0.28	0.873•
25 hydroxyvitamin D (ng/ml)	12.35 ± 1.65	12.04 ± 2.27	0.836•

T-test revealed, ● non-significant differences between two groups,
●● Significant difference in HOMA-IR between two groups (p<0.001)
BMI: Body mass index, DHEA-S: Dehydroepiandrosterone-sulfate;
SHBG: Sex hormone binding globulin

The results also reveal that there is no significant difference between the metformin and combined groups in terms of the mean±SD values of dihydrotestosterone, DHEA-S, and SHBG before treatment (P>0.05, Table 2). However, the combined group obtained significantly higher value of free testosterone (4.48 ± 0.29 pg/ml, P=0.028), compared to the metformin-treated group (4.24 ± 0.28 pg/ml).

The mean±SD values of comparison of demographic, biochemical, and hormonal parameters before and after metformin treatment are presented in Table 3. Accordingly, after metformin treatment, PCOS women showed significantly lower mean±SD values of BMI (27.56 ± 5.30 kg/m^2, P<0.001), waist circumference (93.22 ± 10.00 cm, P=0.003), HOMA-IR (2.84 ± 1.25, P<0.001), free testosterone (3.22 ± 0.42 pg/ml, P<0.001), and dihydrotestosterone (450 ± 33.25 pg/ml, P<0.001), compared to values before treatment (BMI: 30.76 ± 6.40 kg/m^2, WC: 99.76 ± 11.68 cm, HOMA-IR: 4.37 ± 1.19, free testosterone; 4.24 ± 0.28 pg/ml and dihydrotestosterone: 496 ± 15.88 pg/ml).

Table 2. Mean±SD values of Androgen profile between the two groups before the treatment

Androgen profile	Metformin group (N=25)	Combined group (N=25)	P-Value
Free testosterone (pg/ml)	4.24 ± 0.28	4.48 ± 0.29	0.028 •
Dihydrotestosterone (pg/ml)	496 ± 15.88	503 ± 13.25	0.198 ••
DHEA-S (ng/ml)	315 ± 7.99	316 ± 7.73	0.842 ••
SHBG (ng/ml)	14.68 ± 4.67	14.17 ± 5.37	0.260••

T-test revealed, ● significant difference in free testosterone between two groups (p<0.028), ●● non-significant differences in (Dihydrotestosterone, DHEA-S, and SHBG) between two groups. DHEA-S: Dehydroepiandrosterone-sulfate; SHBG: Sex hormone binding globulin

Table 3 also reveals significantly higher mean±SD values of adiponectin (4.25 ± 0.39 ng/ml, P<0.001), 25 hydroxyvitamin D (13.63 ± 1.64 ng/ml, P=0.01), and SHBG levels (17.64± 4.66 ng/ml, P<0.001) after treatment, compared to values before treatment (adiponectin: 2.49 ± 0.29 ng/ml; 25 hydroxyvitamin D: 12.35 ± 1.65 ng/ml; and SHBG: 14.68 ± 4.67 ng/ml). However, no remarkable differences were noted in the DHEA-S levels after metformin treatment (P>0.05).

Table 3. Mean±SD values of demographic, biochemical, and hormonal variables before and after the treatment in metformin Group

Variable	Before metformin treatment (N=25)	After metformin treatment (N=18)	P-Value
BMI (kg/m^2)	30.76 ± 6.40	27.56 ± 5.30	< 0.001•
Waist circumference (cm)	99.76 ± 11.68	93.22 ± 10.00	0.003 ••
HOMA-IR	4.37 ± 1.19	2.84 ± 1.25	< 0.001•
Adiponectin (ng/ml)	2.49 ± 0.29	4.25 ± 0.39	< 0.001•
25 hydroxyvitamin D (ng/ml)	12.35 ± 1.65	13.63 ± 1.64	0.01 •••
Free testosterone (pg/ml)	4.24 ± 0.28	3.22 ± 0.42	< 0.001 •
Dihydrotestosterone (pg/ml)	496 ± 15.88	450 ± 33.25	< 0.001 •
DHEA-S (ng/ml)	315 ± 7.99	316 ± 7.35	0.122••••
SHBG (ng/ml)	14.68 ± 4.67	17.64 ± 4.66	< 0.001•

T-test revealed, ● significant difference in BMI, HOMA-IR, adiponectin, free testosterone, dihydrotestosterone, and SHBG between two groups (for all, P<0.001),
●● significant difference in waist circumference (P< 0.003), ●●● significant difference in 25 hydroxyvitamin D (P< 0.01), ●●●● non-significant difference in DHEA-S BETWEEN TWO GROUPS. BMI: Body mass index, DHEA-S: Dehydroepiandrosterone-sulfate; SHBG: Sex hormone-binding globulin

As can be observed in Table 4, the mean±SD values of demographic, biochemical, and hormonal parameters are compared before and after combined treatment (metformin plus vitamin D). The mean values of BMI (30.58 ± 5.43 kg/m^2 vs. 29.09 ± 5.24; P=0.001), waist circumference (105.48 ± 17.45 cm vs. 102.37 ± 17.68; P=0.003), free testosterone (4.48 ± 0.29 pg/ml vs. 2.27 ± 0.31; P<0.001), and dihydrotestosterone (503 ± 13.25 pg/ml vs. 415 ± 7.64; P<0.001) were significantly decreased after combined treatment, compared to those before treatment.

Table 4 also reveals that the mean±SD values of adiponectin (5.22 ± 0.49 ng/ml vs. 2.53 ± 0.28; P<0.001), 25 hydroxyvitamin D levels (22.32 ± 1.75 ng/ml vs. 12.04 ± 2.27; P<0.001), and SHBG (17.52 ± 5.34 ng/ml vs. 14.17 ± 5.37; P<0.001) were significantly elevated after combined treatment, compared to those before. However, the same table shows no remarkable differences in the HOMA-IR (P=0.012) and DHEA-S levels (P=0.441).

A significant decrease can be observed in the levels of free testosterone (P<0.001) and dihydrotestosterone (P<0.001) after treatment (metformin plus vitamin D), compared to those after treatment with metformin alone (Table 5). Moreover, the mean value of adiponectin was significantly increased after combined

treatment, compared to that after metformin alone (P<0.001). However, the mean values of waist circumference (P<0.021) and HOMA-IR (P<0.001) decreased significantly in the group treated with metformin in comparison with those of the combined group regarding this fact that the mean values of these two later parameters (i.e., WC & HOMA-IR) were markedly high in the combined group before starting treatment, compared to those of the metformin alone.

Table 4. Mean±SD values of demographic, biochemical, and hormonal variables before and after the treatment in combined Group

Variablee	Before combined treatment (N=25)	After combined treatment (N=19)	P-Value
BMI (kg/m^2)	30.58 ± 5.43	29.09 ± 5.24	< 0.001•
Waist circumference (cm)	105.48 ± 17.45	102.37 ± 17.68	0.003 ••
HOMA-IR	7.12 ± 3.21	5.53 ± 3.24	0.012 •••
Adiponectin (ng/ml)	2.53 ± 0.28	5.22 ± 0.49	< 0.001•
25 hydroxyvitamin D (ng/ml)	12.04 ± 2.27	22.32 ± 1.75	< 0.001•
Free testosterone (pg/ml)	4.48 ± 0.29	2.27 ± 0.31	< 0.001 •
Dihydrotestosterone (pg/ml)	503 ± 13.25	415 ± 7.64	< 0.001 •
DHEA-S (ng/ml)	316 ± 7.73	317 ± 6.05	0.441 ••••
SHBG (ng/ml)	14.17 ± 5.37	17.52 ± 5.34	< 0.001•

T-test revealed, ● significant difference in BMI, adiponectin, 25 hydroxyvitamin D, free testosterone, dihydrotestosterone, and SHBG between two groups (for all, P<0.001),
●● significant difference in waist circumference (P< 0.003), ●●● significant difference in HOMA-IR (P< 0.012), ●●●● non-significant difference in DHEA-S between two groups. BMI: Body mass index, DHEA-S: Dehydroepiandrosterone-sulfate; SHBG: Sex hormone binding globulin.

PCOS metformin-treated participants showed a significant negative correlation between HOMA-IR and SHBG (r= -0.593, P=0.009), as well as SHBG and BMI (r= -0.880, P<0.001). On the other hand, there was a significant positive association between HOMA-IR and BMI (r= 0.631, P=0.003). Moreover, a remarkable negative association was noted between SHBG and BMI (r= -0.914, P<0.001) among PCOS women treated with metformin plus vitamin D_3.

Table 5. Mean±SD values of demographic, biochemical, and hormonal variables between the two groups after the treatment

Variable	After metformin treatment (N=18)	After combined treatment (N=19)	P-Value
BMI (kg/m^2)	27.56 ± 5.30	29.09 ± 5.24	< 0.054•
Waist circumference (cm)	93.22 ± 10.00	102.37 ± 17.68	< 0.021••
HOMA-IR	2.84 ± 1.25	5.53 ± 3.24	< 0.001•••
Adiponectin (ng/ml)	4.25 ± 0.39	5.22 ± 0.49	< 0.001•••
25 hydroxyvitamin D (ng/ml)	13.63 ± 1.64	22.32 ± 1.75	< 0.001•••
Free testosterone (pg/ml)	3.22 ± 0.42	2.27 ± 0.31	< 0.001•••
Dihydrotestosterone (pg/ml)	450 ± 33.25	415 ± 7.64	< 0.001•••
DHEAS (ng/ml)	316 ± 7.35	317 ± 6.05	0.869••••
SHBG (ng/ml)	17.64 ± 4.66	17.52 ± 5.34	0.255••••

T-test revealed, ● significant difference in BMI (P< 0.054), ●● significant difference in waist circumference (P<0.021), ●●● significant differences in HOMA-IR, adiponectin, 25 hydroxyvitamin D, free testosterone, and dihydrotestosterone (for all, P< 0.001),●●●● non-significant differences in DHEAS and SHBG between two groups. BMI: Body mass index, DHEA-S: Dehydroepiandrosterone-sulfate; SHBG: Sex hormone binding globulin

Discussion

The findings of the present study demonstrated that the mean values of free testosterone, dihydrotestosterone and waist circumference were significantly improved (by lowering) after treatment with combined metformin plus vitamin D compared with metformin alone. Moreover, adiponectin mean value was

significantly elevated after combined treatment compared with metformin alone.. Saleh BO et al. (2015) demonstrated high levels of BMI and free testosterone among the PCOS groups (11). Alawad (2018) revealed that there were mostly deficient or insufficient levels of 25 hydroxyvitamin in PCOS women (12), which was in line with the results of this study. Qasim MN et al. (2022) mentioned that the mean values of BMI were remarkably more significant in the PCOS group, while the serum level of vitamin D_3 decreased significantly in the PCOS group. They also revealed that females who suffer from PCOS were more susceptible to lack of vitamin D levels than those without PCOS, resulting in obesity contributing to vitamin D deficiency risk (13). Teede H. et al. (2019) demonstrated that participants across all BMI categories had statistically remarkable enhancements with metformin for fasting insulin, management of weight, and metabolic outcomes (reduction of BMI and HOMA-IR) (14), which was in line with the results of this study. Furthermore, Duan X. et al. (2021) demonstrated a correlation between metformin treatment and remarkably increased serum adiponectin concentrations (15), which was concordant with the findings of this study.

Teede H. et al. also (2019) mentioned the advantages of metformin alone for PCOS women in terms of the management of hormonal status across all BMI categories (14). Lashen H. et al. (2010) demonstrated several effects related to metformin (therapeutic intervention with metformin) in PCOS patients, including the restoration of ovulation and improvement of ovarian function (16). Their results were also consistent with the findings of this study.

Williams A. et al. (2020) confirmed that metformin can help reduce the hyperandrogenic symptoms and signs of patients with PCOS by minimizing the androgen levels (2). In addition, Teede H et al. (2019) revealed a statistically significant difference post-treatment with metformin for testosterone for all participants (14). Moreover, Lashen H. et al. (2010) mentioned several effects related to metformin therapeutic intervention in PCOS women, such as a reduction in circulating androgen levels with confirmed increased serum levels of SHBG (16). Upon these findings, their results were consistent with the results of this study. PCOS women treated with metformin showed a remarkable positive correlation between HOMA-IR and BMI which might be due to the correlation between the level of IR and glucose transporter 4 (GLUT4) expressions. Accordingly, the increment in the utilization of peripheral glucose after treatment with metformin is most probably caused by the GLUT4 expression induction and the increase in the translocation to the plasma membrane (17).

Enhancements in insulin sensitivity that are mediated by metformin might be correlated with some mechanisms, such as enhanced glycogen synthesis, higher activity of insulin receptor tyrosine kinase, and finally, increased recruitment and activity of GLUT4 (17). Additionally, adenosine 50-monophosphate-activated protein kinase (AMPK), a so-called cellular energy sensor, can be activated by metformin. It can also stimulate glucagonlike-peptide-1 release, and as a result, improve the secretion of insulin and lower the levels of plasma glucose (17).

Zhuang L. et al. (2019) revealed significant lower levels of BMI and fasting insulin after combined treatment, compared to those before treatment which is concordant with the findings of this study (7). Irani M. et al. (2014) and Firouzabadi R.D. et al. (2012) mentioned a positive effect of vitamin D supplementation on infertile females suffering from PCOS (18, 19), which was also consistent with the findings of the current study. Kadoura S. et al. (2019) clarified improvements in the irregularity of the menstrual cycle in 58.8% of those in the metformin-supplementation group which is in line with the findings of the present study (20). As well, Irani M. et al. (2014) and Firouzabadi R.D. et al. (2012) found a positive effect of vitamin D on follicular maturation and menstrual regularity in infertile females with PCOS which is similar to the results of this study (18, 19). The presence of vitamin D receptors (VDRs) in the human and animal granulosa cells and the cumulus oophorus cells approves the vital role of vitamin D supplementation in the precise female reproductive cycle regulation (6).

On the other hand, Kadoura S. et al. (2019), Irani M. et al. (2015), and Selimoglu H. et al. (2010) confirmed that they could not find any superior effects on enhancing hormonal levels in

the supplementation group after treatment. That was not consistent with the results of this study (20-22). The difference in findings reported in this study with those in the literature may be related to the different diagnostic criteria of PCOS or various geographic locations. Zhuang L. et al. (2019) demonstrated that after combined treatment, the serum level of testosterone was significantly lower than before (7). VDR and traces of vitamin D metabolizing enzyme were found in syncytioblast procured from human culturing. VDR was observed both in decidua and placenta. Several studies have associated the increased level of androgen with a decrease in the precursors of vitamin D (25-hydroxyvitamin) plasma levels (6). Therefore, vitamin D plays an important role in the enhancement of metabolic, endocrine, and reproductive disorders or dysfunctions in females suffering from PCOS (23-24).

The limitation of this study was that the sample size of the participants was relatively small, which is due to difficulties in the follow-up of women who were taken treatment and/or supplements leading to ignoring of those who did not return to the hospital center.

The strength of this study was that it opens sights about the possibility of using vitamin D supplementation, as an adjuvant treatment in the correction of one of the most complaining characteristics of PCOS, which is obesity represented by BMI and WC, as well as the improvement of the most major factor of PCOS, which is hyperandrogenemia as found by a significant decrease in androgen profile.

Future research and clinical practice are recommended to screen, diagnose, and treat PCOS women to decrease the incidence of irregular menstruation, and the decrease in serum level of vitamin D. This study revealed that vitamin D supplementation had a significant effect on metabolic and hormonal profiles. Accordingly, it is suggested to advise patients with PCOS to take vitamin D supplementation for its beneficial effects.

Conclusion

According to the study findings, we concluded that Serum measurements of free testosterone, dihydrotestosterone, and adiponectin could be good indicators for measuring the efficacy of combined metformin plus vitamin D supplement in the treatment of PCOS women. Combined treatment was superior to that of metformin alone in the treatment of PCOS.

Declarations

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Conflicts of interest

The authors declared no conflicts of interest.

Ethical Considerations

Verbal (oral) consent was attained from each included adult woman. Regarding the ethical considerations, the Scientific Committee of the Department of Biochemistry, College of Medicine, University of Baghdad, Baghdad, Iraq, and the Ministry of Health, Iraq, approved the research ethically.

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Authors' contribution

ZN collected data from PCOS study group, collected blood samples, conducted practical measurements, and contributed to the writing of introduction, methods, and results sections. BS performed statistical analysis, designing the study, and contributed to the discussion and conclusion sections. Both authors read and approved the final manuscript and agreed to be accountable for all aspects of the work.

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