BIOMARKER ASSESSMENT OF SERUM INTERLEUKIN-18 TOGETHER WITH HOMOCYSTEINE FOR POLYCYSTIC OVARIAN SYNDROME

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ABSTRACT

Background

Polycystic ovary syndrome (PCOS) a complex endocrine disorder associated with reproductive disorders and metabolic dysfunctions, insulin resistance with compensatory hyperinsulinemia, obesity, endometrial carcinoma, and cardiovascular diseases.

Objectives

This study aims to evaluate the levels of IL-18 and homocysteine in serum as a possible biomarker for cardiovascular disease in 150 cases positive with PCOS and in 150 negative control females.

Patients and Method

This study was a case-control study and serum samples randomly taken from 300 individuals (150 samples from patients with PCOS and 150 samples from healthy controls). Five milliliters of venous blood has been taken from each individual and the samples were analyzed for interleukin-18 and homocysteine by using enzyme-linked immunosorbent assay, hormones profile include LH, FSH, LH/FSH ratio, TSH, PRL, and Testosterone.

Results

We found that the average level of IL-18 and homocysteine in serum were 378.3 ±181.21 pg/ml and 10.36 ± 5.98 nmol/ml respectively in PCOS patients, while in the control group the values were 224.98± 131.885 pg/ml and 5.17± 5.24 nmol/ml respectively.

Conclusions

The results show a highly significant difference (p-value < 0.001) and high serum concentration of IL-18 and homocysteine in PCOS as compared to the control group. Therefore, elevation of IL-18 combined with homocysteine is a selective indicator for higher risk of PCOS, which is closely related to cardiovascular abnormality as we have gated for this category of PCOS patients.

Keywords: *Polycystic Ovarian Syndrome, Interleukin-18, Homocysteine, Cardiovascular Disease, Biomarker*

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INTRODUCTION

Polycystic ovary syndrome is the most common endocrine disease in women throughout the child-bearing ages. The prevalence ranges in the United States are from 9% when the NIH (National Institutes of Health) criteria are used to 18% when using the guidelines of the Rotterdam consensus [1-3]. PCOS is a complex disorder influenced by both environmental and genetic factors [4-6]. It is characterized by hyperandrogenism, menstrual irregularity, polycystic ovarian morphology (PCOM), insulin resistance with compensatory hyperinsulinemia, obesity and increase the risk for developing type 2 diabetes, endometrial carcinoma and cardiovascular disease [7].

Clinical hyperandrogenism is defined by the presence of hirsutism, acne, or androgenic alopecia and biochemical hyperandrogenism includes elevated circulating androgen levels [8], while serum levels of free testosterone are more frequently elevated in women with PCOS. Therefore, it is considered the most sensitive biochemical marker for the diagnosis of PCOS [9].

Menstrual irregularity is found in about two-thirds of adolescents with PCOS. These girls may present with oligomenorrhea (means menstrual bleeding that occurs at intervals over 40 days or fewer than 9 periods yearly), primary amenorrhea (the absence of menarche by 16 years of age), secondary amenorrhea (the absence of menses for at least 3 months), dysfunctional uterine bleeding (excessive and irregular vaginal bleeding). Furthermore, monthly menstrual cycles may still be anovulatory, which is suggested when there is a paucity of menstrual cramps, absence of premenstrual molimina (breast tenderness, lower abdominal bloating, or moodiness), and menorrhagia (excessive menstrual bleeding) [10].

The diagnosis of PCOS can be established when at least two of the three following criteria are present, with the exclusion of other etiologies: clinical and/or biochemical signs of hyperandrogenism, oligo- and anovulation and polycystic ovaries (PCO) observed in an ultrasound examination, which is determined by the presence of ≥12 follicles within the ovary with a diameter of 2–9 mm and/or ovarian volume ≥10 cm, Such an ultrasound image in one gonad only is sufficient to define polycystic ovaries [11].

Hyperinsulinemia resulting from insulin resistance has an important role in the pathogenesis of PCOS at several levels [12]. Firstly, insulin stimulates the production of androgens by ovarian thecal cells because insulin has a direct synergistic effect with LH in enhancing androgen production. Secondly, high insulin levels increase LH secretion from the pituitary, elevating the LH/FSH ratio, and further contributing to anovulation [13]. Thirdly, hyperinsulinemia further decreases of sex hormone-binding globulin (SHBG) levels, elevating free testosterone level. Fourthly, Insulin also appears to potentiate basal and adrenocorticotropic hormone (ACTH), stimulated adrenal androgen production [14]. Another hypothesis suggests that PCOS is attributable to androgen excess, which arises from primary functional ovarian hyperandrogenism (FOH) and primary functional adrenal hyperandrogenism (FAH) [15]. Extra glandular synthesis of androgens particularly in the adipose tissue was found to be involved in the pathophysiology of PCOS. Adipose tissue contains 17β-hydroxysteroid dehydrogenase type 5 (17HSD5), which can convert the weak androgen (androstenedione) to the more potent androgen (testosterone). The expression of 17HSD5 in subcutaneous adipose tissue is proportional to overall adiposity. Thus, excessive adiposity in PCOS women may contribute to increased peripheral production of testosterone [16]. Moreover, it is observed an increased 5α-reductase activity, which converts testosterone to the highly potent androgen dihydrotestosterone in the PCOS [17]. Its activity appears to correlate with both adiposity and insulin concentrations [18]. In women with PCOS, the liver production of sex hormone-binding globulin (SHBG) is deceased, thus increasing the free (biologically active) testosterone level is observed. These hormonal abnormalities might be related in part to obesity [19]. Other factors involved in the pathology of PCOS are abnormal pituitary function [20], oxidative stress [21] and sympathetic nerve activity [22].

Interleukin-18 (IL-18) is a proinflammatory cytokine that was first described in 1989 for its ability to induce interferon γ (IFN-γ) production, IL-18 functionally related to IL-12, but it is structurally similar to the IL-1 family of cytokines specifically IL-1β, So IL-18 is part of the IL-1 family [23]. The Pro IL-18 (an inactive precursor of IL-18) is stored in the intracellular space but after being cleaved and processed by caspase-1 into the biologically active cytokine IL-18, that is released into the extracellular [24]. IL-18 synthesis is elevated in hyperglycemic conditions [25]. The IL-18 expression is induced by nuclear transcription factor-κB (NFκB), interferon-gamma (IFN γ) [26],

catecholamines [27], angiotensin II and inflammation [28]. Interleukin-18 enhances the maturation of T-cells and natural killer cells and the production of cytokines, chemokines, cell-adhesion molecules, IFNγ and matrix metalloproteinases (MMPs) [26, 29]. IL-18 has been in focus amongst researchers in cardiovascular disease. On-the-other-hand, IL-18 is implicated in the pathogenesis of epithelial ovarian carcinoma (EOC) [30], endometriosis [31] and recurrent miscarriage [32].

Homocysteine is a non-protein forming sulfur-containing amino acid, formed as a primary intermediate during the metabolism of methionine, and Hcy is extremely important for optimal cellular function and survival [33]. The enzyme cystathionine-β-synthase and methylene tetrahydrofolate reductase (MTHFR) together with cofactor folic acid, vitamin B6 and vitamin B12, play a key role in the regulation of circulating homocysteine levels, hence a genetic defect in one of the enzymes or the cofactor of homocysteine metabolism can lead to metabolic disruption and potentially to Hyperhomocysteinemia [34]. Insulin resistance causes to increase the homocysteine levels in women with polycystic ovarian syndrome since insulin inhibit the hepatic cystathionine beta-synthase, which controls the breakdown of homocysteine to cystathionine in the transsulfuration pathway [35, 36].

In this study, we have focused on PCOS cases to measure IL-18 and homocysteine levels in serum. We aimed to find out the correlation of these two biomarkers together with the pathogeny parameters in PCOS and also for cardiovascular disease. Our study is the first attempt addressing the correlation between IL-18 and homocysteine, in our locality.

MATERIALS AND METHODS

This study is a case-control investigation, where a total of 300 females are recruited. One hundred fifty women participated in this study diagnosed positively as polycystic ovary syndrome by physician, based on the 2003 Rotterdam diagnosis consensus [11], while the other 150 participants are healthy control individuals, with respect to menstrual cycle regularity, absence of acne and hirsutism, and normal ranges for LH, FSH, TSH, Prolactin and Testosterone. All of the recruited females gave their informed consent for the study. To conduct this study, ethical permission was approved by the Ethics Committee at the College of Pharmacy, University of Sulaimani, Iraq.

Inclusion and exclusion criteria

The inclusion criteria for 150 females diagnosed as PCOS, were combined menstrual dysfunction with either hyperandrogenemia or polycystic ovary morphology. The other 150 healthy control females, were clear from any clinical and/or biochemical symptoms for PCOS. The exclusion criteria for this study are postmenopausal women, pregnant women, thyroid disorder and any single inclusion criteria PCOS symptoms.

Sample collection

Using disposable syringes, five milliliters blood through cubital vein was obtained from all participants (is it no fasting), one milliliter collected in EDTA tubes for immediate HbA1c analysis. The other four milliliters transferred into serum gel separator tubes and leave at room temperature for 5 minutes to clot. The serum was separated after centrifugation for 20 minutes at 4000 rpm. The serum aliquoted into two parts, one part kept frozen at -70 ^{o}C for later IL-18 and homocysteine ELISA analysis. The other part utilized immediately for clinical laboratory determinations of LH, FSH, TSH, prolactin, testosterone, blood sugar and uric acid.

Serum IL-18 and homocysteine levels were measured using the ELISA technique (Human IL-18 ELISA Kit, eBioscience, USA, and Homocysteine ELISA kit, YH Bio search, China).

Statistical Analysis

Data entry was generated using an excel spreadsheet then the statistical analysis was performed by SPSS program, version 21 (IBM SPSS Statistical Package for the Social Sciences). The data presented in tabular forms showing the frequency and relative frequency distribution of different variables among both groups (polycystic ovarian syndrome cases and healthy controls). Chi-square tests were used to compare the categorical data between these two groups concerning different variables as indicated in the tables. For comparing the quantitative variables, the mean of cases and controls were compared using independent t-test and analysis of variance (ANOVA). The level of homocysteine and interleukin-18 within PCOS cases compared with the level of FSH, prolactin and other quantitative variables to determine the linear coefficient relationship (r) with (r2). P-values of ≤ 0.05 were used

as a cut-off point for significance and high-significance ≤0.001 of the statistical tests.

RESULTS

The level of serum IL-18 in PCOS cases

The double-sandwich ELISA technique was used for IL-18 determination with the dynamic range 0.1 ng/ml to 1 µg/ml. In all 150 PCOS samples, the mean and standard deviation of serum IL-18 was (378.3 ±181.2 pg/ml), while the corresponding value was (225.0± 131.9 pg/ml) in 150 control samples (Figure 1). According to these data, there was a highly significant difference (p-value ≤0.001) and high serum concentration of IL-18 in PCOS as compared to the control group.

The level of serum homocysteine (Hcy) in PCOS cases

In the next phase of the project, we have started to measure free serum Hcy in PCOS. The results of mean and standard deviation for serum concentration of Hcy was (10.4 ± 6.0 nmol/ml) and (5.2± 5.2 nmol/ml) for PCOS and the control group, respectively (Figure 2). These results indicated highly significant differences (P-value < 0.001) of the homocysteine levels in both PCOS and control groups.

Correlation between serum IL-18 with anthropological parameters and hormonal diagnosis in PCOS

We wanted to correlate anthropological and laboratory parameters with IL-18 levels in PCOS patients (Table 1). There was a significant positive correlation between serum IL-18 concentration with BMI (r=0.156, p-value=0.03) and FSH (r=0.18, p-value 0.026), while no significant positive correlation between serum IL-18 with age (r=0.08, p-value=0.17), and prolactin level (r=0.12, p-value 0.14) in PCOS.

Statistical analysis ANOVA were done as described in materials and methods and the values are given as R (Correlation coefficient), R2 (Coefficient of determination) and P-value, P-value significance ≤0.05, highly significance ≤0.001. There was highly significant positive correlation between serum IL-18 and LH level in PCOS patients (r=0.452, p-value=0.001), also there was a significant positive correlation of serum IL-18 concentration with BMI (r=0.156, p-value=0.03) and FSH (r=0.18, p-value 0.026), while no significant positive correlation between serum IL-18 with age (r=0.08, p-value=0.17), testosterone (r=0.124, p-value=0.07) and prolactin level (r=0.12, p-value 0.14) in PCOS.

Correlation between serum Hcy with anthropological parameters and hormonal diagnosis in PCOS

Here, we wanted to identify any possible relationship between serum Hcy with anthropological parameters and also hormonal diagnosis in PCOS. The data shows a significant positive correlation between serum level of homocysteine with age (r=0.177, p-value=0.015), while no significant positive correlation of homocysteine level with BMI (r=0.093, p-value=0.13), FSH (r=0.006, p-value 0.94) and Prolactin level (r=0.038, p-value 0.94) in PCOS cases as shown in (Table 2).

Statistical analysis ANOVA were done as described in materials and methods and the values are given as R (Correlation coefficient), R2 (Coefficient of determination) and P-value, P-value significance ≤0.05, highly significance ≤0.001.The data shows a significant positive correlation between serum level of homocysteine with age (r=0.177, p-value=0.015), LH level (r=0.202, p-value=0.01) and testosterone level (r=0.176, p-value=0.016) in PCOS. However, we could not observe any significant correlation of homocysteine level with BMI (r=0.093, p-value=0.13), FSH (r=0.006, p-value 0.94) and Prolactin level (r=0.038, p-value 0.94).

The serum IL-18 and serum Hcy concerning LH and Testosterone in PCOS

There was a highly significant positive correlation between serum IL-18 and LH level in PCOS patients (r=0.452, p-value=0.001) (Figure 3). However, no significant positive correlation between serum IL-18 with testosterone level (r=0.124, p-value=0.07) in PCOS (Table 1).

On the other hand, there were a significant positive correlation between serum level of homocysteine with LH (r=0.202, p-value=0.01) (Figure 4) and testosterone level (r=0.176, p-value=0.016) (Figure 5).

Linear regression analysis, including luteinizing hormone as independent variable and serum IL-18 concentration as the dependent variable, and the data show a highly significant positive correlation of serum IL-18 with LH level in PCOS patients (r=0.452, p-value=0.001).

Linear regression analysis, including luteinizing hormone as the independent variable and serum homocysteine concentration as the dependent variable,

and the data show a significant positive correlation between serum level of homocysteine with LH (r=0.202, p-value=0.01).

Linear regression analysis, including serum homocysteine concentration as independent variable and testosterone hormone as an dependent variable, there was a significant positive correlation between serum level of homocysteine and testosterone level (r=0.176, p-value=0.016)

Correlation of testosterone with luteinizing hormone in polycystic ovarian syndrome

There was a significant correlation of testosterone with luteinizing hormone in polycystic ovarian syndrome (R2 =0.003).

Linear regression analysis, including serum luteinizing hormone concentration as independent variable and testosterone hormone as the dependent variable, There was a significant correlation of testosterone with luteinizing hormone in polycystic ovarian syndrome (R2 =0.003).

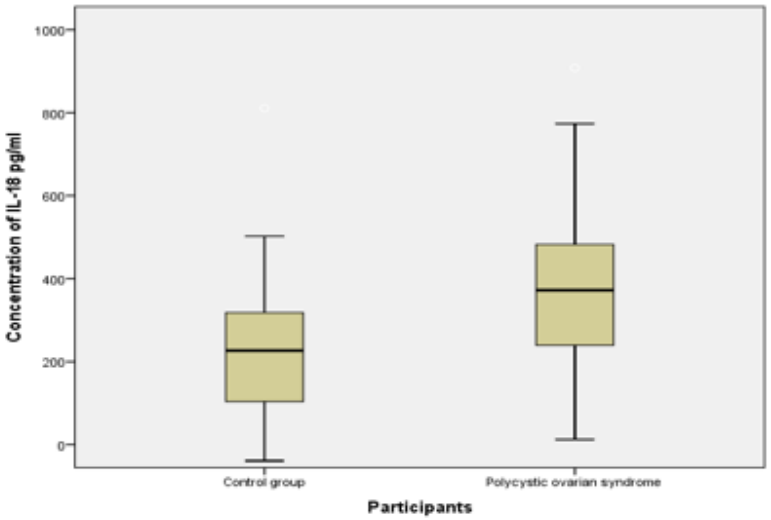

Figure 1. Concentration of serum IL-18 in PCOS patients and control group.

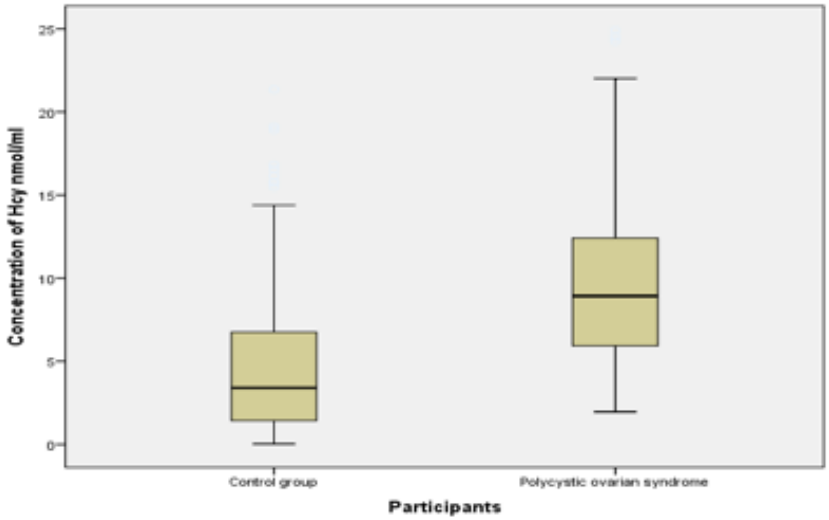

Figure 2: Concentration of serum homocysteine in PCOS patients and control group.

Table 1. Correlation of IL-18 with Age, BMI, LH, FSH, prolactin, and testosterone level in PCOS.

Variable	R	R^2	P-Value
Age	0.08	0.006	0.17
BMI	0.156	0.024	0.03
LH	0.452	0.205	< 0.001
FSH	0.18	0.033	0.026
Testosterone	0.124	0.015	0.07
Prolactin	0.12	0.008	0.14

Table 2. Correlation of homocysteine with Age, BMI, LH, FSH, prolactin, and testosterone level in PCOS.

Variable	R	R^2	P-Value
Age	0.177	0.031	0.015
BMI	0.093	0.009	0.13
LH	0.202	0.041	0.01
FSH	0.006	0.00004	0.94
Testosterone	0.176	0.031	0.016
Prolactin	0.038	0.001	0.94

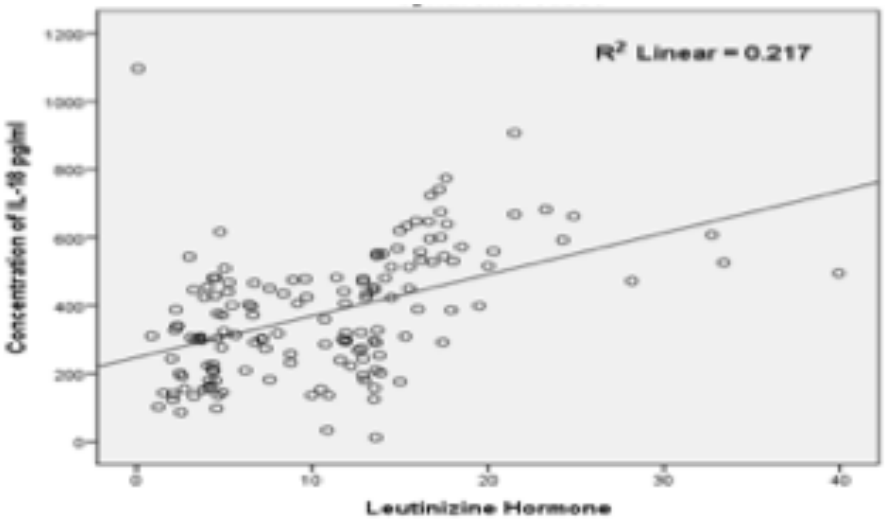

Figure 3. Correlation of interleukin-18 with luteinizing hormone in polycystic ovarian syndrome.

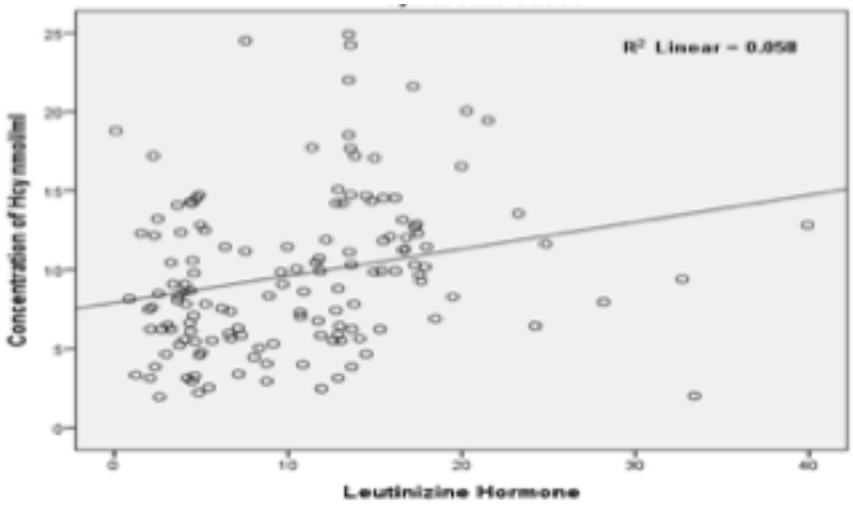

Figure 4. Correlation of homocysteine with luteinizing hormone in polycystic ovarian syndrome.

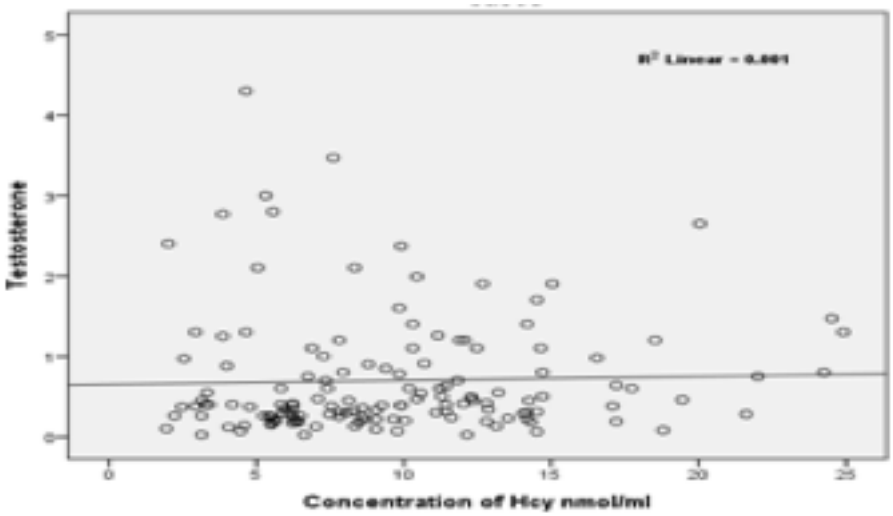

Figure 5. Correlation of homocysteine with testosterone in polycystic ovarian syndrome.

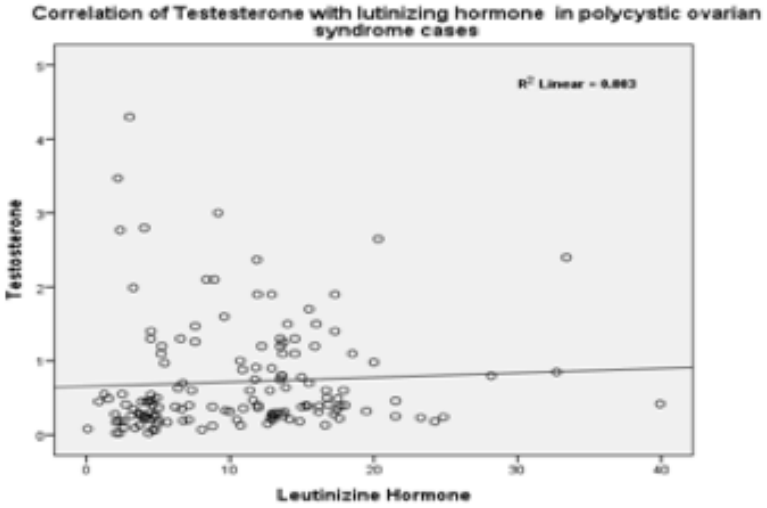

Figure 6. Correlation of testosterone with luteinizing hormone in polycystic ovarian syndrome.

DISCUSSION

The level of Serum IL-18 in PCOS cases

As shown in (Figure 1), there was a highly significant difference (p-value ≤0.001) and high serum concentration of IL-18 in PCOS as compared to the control group. This result is an agreement with one previous finding [37]. In which IL-18 which is considered as a strong risk marker for cardiovascular disease [38], significantly increased in PCOS patient

The level of Serum Hcy in PCOS cases

according to our results data in (Figure 2) mean and standard deviation of Hcy in PCOS was a statistically significant difference (p-value < 0.001) and higher-level compared to control group. These results agree with other studies which also notice that serum Hcy concentrations were elevated and risk of CVD has been increased in patients with PCOS compared with healthy controls [39-43].

Correlation between serum IL-18 with anthropological parameters and hormonal diagnosis in PCOS

The present study (Table1) showed the correlation of serum IL-18 with age, BMI, LH, FSH, PRL, and Testosterone. There was a significant positive correlation between serum IL-18 concentration with BMI (r=0.156, p-value=0.03) and FSH (r=0.18, p-value 0.026), These results were in agreement with others previous studies, which found correlation of IL-18 with BMI and FSH level (Escobar-Morreale et al., 2004; Esposito et al., 2002), while no significant positive correlation between serum IL-18 with age (r=0.08, p-value=0.17) and prolactin (r=0.12, p-value 0.14) in PCOS this results consistent with other studies (Zhang et al., 2006, Kowalska et al., 2006), who's also couldn't found correlation between serum IL-18 with age and prolactin level.

Correlation between serum Hcy with anthropological parameters and hormonal diagnosis in PCOS

The data shows a significant positive correlation between serum level of homocysteine with age (r=0.177, p-value=0.015), This result is a disagreement with an earlier study in PCOS [42], but some of the other studies support our findings [44]. However, we could not observe any significant correlation of homocysteine level with BMI (r=0.093, p-value=0.13), FSH (r=0.006, p-value 0.94) and Prolactin level (r=0.038, p-value 0.94) (Table 2), This was an agreement with previous studies which reported serum Hcy is significantly elevated in both lean and obese PCOS patients which significantly related to insulin resistance and not correlated to body weight [45, 46].

The serum IL-18 and serum Hcy concerning LH and Testosterone in PCOS

There was a highly significant positive correlation between serum IL-18 and LH levels in PCOS patients (r=0.452, p-value=0.001) (Figure 3). These results were in agreement with other previous studies, which found a significant correlation of IL-18 with LH level (Yang et al., 2011, Kowalska et al., 2006). However, no significant positive correlation between serum IL-18 with testosterone level (r=0.124, p-value=0.07) in PCOS (Table 1). This result consistent with other studies (Zhang et al., 2006, Kowalska et al., 2006) which also couldn't found a correlation of serum IL-18 with testosterone level.

On the other hand, there was a significant positive correlation between serum level of homocysteine with LH (r=0.202, p-value=0.01) (Figure 4). This finding is an agreement with this study [47], Interestingly, there was a significant positive correlation between serum level of homocysteine and testosterone level (r=0.176, p-value=0.016) (Figure 5). This finding is in agreement with the results from the previous study, which also found a correlation between Hcy and testosterone levels [48].

Correlation of testosterone with luteinizing hormone in polycystic ovarian syndrome

There was a significant correlation of testosterone with luteinizing hormone in polycystic ovarian syndrome (R2 =0.003). This result in agreement with other studies [49, 50], which also found a correlation of testosterone with LH level in PCOS.

In conclusions, most common symptoms of PCOS in Sulaimani city were hirsutism, while the lest common symptoms were oligomenorrhea, acne, infertility, amenorrhea, and alopecia. PCOS was more common in age 20-29 years and a family history of PCOS increased the risk of the disease. Most PCOS enrolled in this study have a high concentration of LH and testosterone levels, which were critical for the diagnosis of PCOS.

In PCOS cases, the serum IL-18 elevated and had a positive correlation with LH, testosterone, FSH, Prolactin levels and BMI. Furthermore, the serum Hcy

was found increased in PCOS and the Hcy levels were correlated with age, LH and testosterone.

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