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# Study Some Immunological and Physiological Aspects in Iraqi Patients with Polycystic Ovary Syndrome

*A Thesis*

Submitted to the Council of the College of Education for Pure Science (Ibn Al-Haitham), University of Baghdad in Partial Fulfillment of the Requirements for the Degree of Master in Science of Biology /Immunology

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بِسْمِ اللهِ الرَّحْمَنِ الرَّحِيمِ

قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ

سورة البقرة (آية ٣٢ )

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I declare that this thesis was prepared under my supervision at the Department of Biology/ College of Education for Pure Science-Ibn Al-Haitham / University of Baghdad, in partial fulfillment of the requirements for the degree of Master in Biology/ Immunology.

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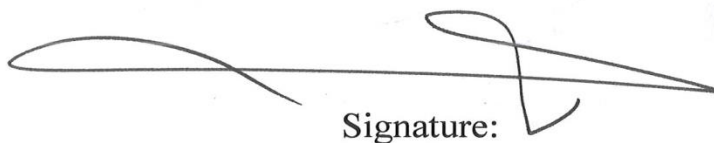**Assistant professor**

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# Certificate

We, the examining committee certify that we have read this thesis entitled **(Study Some Immunological and Physiological Aspects in Iraqi patients with polycystic Ovary Syndrome)** and examined the MSc. student **Mais Isam Mohammed** in its contents on / /2019, and that in our opinion is accepted as a thesis for the degree of Master of Science in Biology/ immunology with a grade (                ).

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# *Dedication*

*To…*

*My mother and father with deepest love…*

*Who provide me, the support, Encouragement and the power to continue my postgraduate study with my warmest love & appreciation….*

*To the bright side of my life, my dear husband yassir for his patience and endless support ….*

*To my lovely Sister & Brother….*

*To my Daughter Danya who provides me with limitless pleasure and give me my purpose in life.*

*Mais*

# Acknowledgement

## Summary

Polycystic ovary syndrome (PCOS) is one of the commonest endocrine disorders affecting many females in reproductive period which start from menarche till the menopause and commonly caused infertility around the world, and is characterized by irregular menstrual cycle, Hyperandrogensim and polycystic ovary; it can be considered a conditions involving reproductive, metabolic and cardiovascular components leading to lifelong health implication. Its prevalence among infertile women is between (15% to 20%). There is evidence that PCOS is a pro inflammatory disorder, characterized by the presence of low grade chronic inflammation that correlated with obesity or insulin resistance (IR). The present study aims to determine the role of immunological and physiological response in the pathogenesis of PCOS.

The current study included 66 females diagnosed with PCOS, there were recruited from Kamal Al-Samarrai hospital in Baghdad during August 2018 to March 2019; the diagnosis of polycystic ovary syndrome was based on the Rotterdam 2003 criteria. The control group consists of 22 fertile women who have regular menstrual cycle with no sign of hyperandrogenism and subjected to ultrasound examination and have normal hormonal levels. The age was identical in both groups and it was between (20-40) years. Body mass index (BMI) for both patients and control group was calculated. The questionnaire form has been filled for each patient and control. Hormones levels luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH) were estimated. The present study was carried out in two parts: immunological and physiological study. ELISA technique has been used to determine the serum level of Interlukine-18 (IL-18), Interferon gamma (IFN-γ), triiodothyronine (T3), thyroxine (T4) and insulin. Single radial immunodiffusion assay was performed to evaluate C3 complement component.

The results of immunological study demonstrated that IL-18 serum level was significantly increased (p<0.001) in PCOS patients in comparison to control group. The mean level of this interleukin in patients and control group were (609.04±34.94) pg/mL and 306.55±44.16) pg/mL respectively. IL-18 serum level recorded a highly significant difference (p<0.001) in obese patients with PCOS (281.30±14.13) pg/mL as compared to lean patients with PCOS (215.97± 9.33) pg/mL. Moreover, IL-18 level significantly elevated in patients with PCOS having hyperandrogenic state, with a mean of (276.56±13.04) pg/mL , and this elevation was statistically significant (p<0.05) in comparison to the level observed in patients with PCOS don't having hyperandrogenic states; the mean was (232.82±12.53) pg/mL. Furthermore, IL-18 serum level recorded a significant difference (p<0.05) in PCOS patients with irregular menstrual cycle (290.65±12.05) pg/mL compared to PCOS patients with regular menstrual cycle (200.09±11.35) pg/mL.

IFN-γ serum level was significantly increased (p<0.05) in PCOS patients (266.02±36.04) pg/mL as compared to control group (168.36±17.33) pg/mL. Furthermore, C3 serum level has a significant elevation (p<0.001) in PCOS patients (89.71±3.16) mg/dL as compared to control group (65.51±4.90) mg/dL.

The results for physiological study demonstrated that insulin serum level recorded a highly significant difference (p<0.001) in PCOS patients compared to control group, the mean level of this hormone for PCOS patients and control were (23.01±1.38) μIU/mL, (12.20±0.64) μIU/mL respectively. The present study was showed a significant increasing (p<0.001) in serum level of insulin in obese patients with PCOS (24.97±1.77) μIU/mL as compared to lean patients with PCOS (15.46± 1.63) μIU/mL. Also, there was a significant difference (p<0.05) in serum level of insulin in patients with PCOS having hyperandrogenic

state (23.44±1.83) µIU/mL compared to patients with PCOS don't having hyperandrogenic states (17.27±1.74) µIU/mL. The present study showed that there was a positive correlation (p<0.001) between BMI and IL-18, C3 and insulin. Also, a significant positive correlation (p<0.05) was found between IL-18 and C3. Moreover, a significant positive correlation was revealed between insulin and IL-18, IFN-γ and C3 with (p<0.001) except in IFN-γ with (p<0.05). While this study showed there were no significant differences in serum levels of T3, T4 and TSH between PCOS patients (1.01± 0.03. ng/mL, 7.81±0.11µg/dL, 2.94±0.15 µIU/mL) and control group (0.98±0.07 ng/mL, 7.37±0.25 µg/dL, 2.89±0.22 µIU/mL).

This study illustrate that the serum levels of IL-18, IFN-γ, C3 and insulin were elevated in PCOS comparing to healthy women and this high levels related to PCOS independent on the presence of obesity or IR.  There is a positive correlation between IL-18 and C3 and between these  parameters with obesity and hyperandroginsim, and there was a positive correlation between the insulin hormone with the three parameters IL-18, IFN-γ and C3 this may be due to the background of PCOS which considered as inflammatory disease.

According to the above; IL-18, IFN-γ and C3 may be a good tools to prognostic the cardiovascular disease in PCOS women, Furthermore, it may consider as a biomarkers to evaluate the progression of metabolic disturbance that may cause  prevention of ovulation which may lead to the infertility.

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# List of Abbreviations

| Abbreviation | Definition |
|---|---|
| AE-PCOS | Society of androgen Excess and PCOS Society |
| BMI | Body Mass Index |
| C3 | Complement 3 fraction |
| CLS | Crown-like structures |
| CRP | C-reactive protein |
| ELISA | Enzyme-linked Immunosorbent Assay |
| Ff | Follicle fluid |
| FSH | Follicle stimulating hormone |
| GnRH | Gonadotropin-releasing hormone |
| IFN-γ | Interferon gamma |
| IFN-γ R | IFN-γ receptor |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IL-1 | Interleukin-1 |
| IL-18 | Interleukin-18 |
| IL-4 | Interleukin-4 |
| IL-6 | Interleukin-6 |
| IR | Insulin resistance |
| IVF | In vitro fertilization |
| kDa | Kilo Dalton |
| Kg/m2 | Kilogram per square meter |
| LH | Luteinizing hormone |
| MBL | Mannose-binding lectin |
| NIH | National Institute of Health |
| NK cells | Natural killer cells |
| OCP | Oral contraceptive pill |
| PCOS | Polycystic ovary syndrome |
| RIDA | Redial immunodiffusion assay |
| S.E | Standard Error |
| SHBG | Sex hormone–binding globulin |
| T2DM | Type 2 diabetes mellitus |
| T3 | Triiodothyronine |
| T4 | Thyroxine |
| Th1 | T-helper1 |
| Th2 | T-helper2 |
| TNF-α | Tumor necrosis factor-Alpha |
| TSH | Thyroid stimulating hormone |

| WHO | World Health Organization |
|-----|---------------------------|

# Chapter One

# Introduction

## 1.1 Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous endocrine disorder that affects body systems and leads to reproductive and metabolic complications among women of reproductive age (Fauser *et al.*, 2012; Kollmann *et al.*, 2015). The pathophysiology of PCOS is largely unknown; evidence suggests that several genes are involved, as well as environmental and nutritional factors (Sedighi *et al.*, 2015). The characteristic features include menstrual abnormalities and clinical or biochemical features of hyperandrogenism, reduced fertility, polycystic ovaries and abnormalities of biochemical profile including raised LH, testosterone, and insulin levels while reduced in FSH levels (Dumesic *et al.*, 2015).

High level of androgen and the criterias of hyperandrogenism are main features of PCOS. About 80% of polycystic ovarian women who diagnosed by National Institute of Health (NIH) consensus criteria have elevated androgen levels (Roe and Dokras, 2011). Hyperandrogenemia is directly responsible for the signs and symptoms of PCOS which include hirsutism, acne, male pattern baldness and oligo ovulation or lack of ovulation. The main sources of excessive amount of androgen in PCOS are the ovaries, mainly theca cells, and the zona reticularis of the adrenal cortex (Lizneva *et al.*, 2016).

Despite PCOS being considered the most common endocrine disorder, the estimation of its prevalence is highly variable due to differences in the presentation of PCOS phenotypes (Wendy *al.*, 2010), but the prevalence in general population is about 20-33% (Sirmans and Susan, 2014).

Obesity is present in varying degrees in women with PCOS and is associated with hyperandrogenaemia and IR (Barber *et al.*, 2006). Although the mechanisms that linked obesity with PCOS are not fully understood. High body mass index is present in approximately 35% of PCOS patients in some studies and as high as 80% in other studies (Azziz

*et al.*,2004). It is strongly believed that increasing in the worldwide obesity may be responsible for apparent increasing in the prevalence of polycystic ovarian syndrome (Hanif *et al.*, 2015).

Inflammatory reactions which influenced by the high prevalence of obesity and overweight in PCOS females had been played an essential role in the progression of metabolic disturbance and IR and responsible for the high risk of coronary heart disease (Luque *et al.*, 2010).

IL-18 and IFN-γ are inflammatory cytokines; both have pleiotropic effects, and participate in the innate as well as in the adaptive immune response. Several studies reported that elevated level of both (IL-18 and IFN-γ ) were observed in PCOS patients, and both as inflammatory cytokine might participate in the etiology of PCOS (Qin *et al.*,2016 ; Al‑Musawy *et al.*,2018) .

The complement system is a crucial part of the immune system and consists of multiple categories of components. Complement component 3 (C3) is an acute-phase protein produced by the liver and also secreted by activated macrophages at inflammation sites and by adipocytes, it is highly associated with obesity and IR .It has been recently identified as predicting coronary or cardiovascular events but not independently of the established risk factors (Hernández *et al.*, 2007; Muscari *et al.*, 2007; Onat *et al.*, 2010).

Thyroid hormones are two hormones produced and released by the thyroid gland, namely T3 and T4. They are tyrosine-based hormones that are primarily responsible for regulation of metabolism. The production of T3 and T4 is regulated by TSH that release from thyrotrophic cell in anterior pituitary gland (O'Reilly, 2013).

It is evident that both PCOS and thyroid dysfunction conditions have multiple common presentations and both have profound effect on fertility and reproductive biology; more interestingly hypothyroidism can initiate, maintain or worsen PCOS (Ganvir *et al.*, 2017).

Insulin is a peptide hormone made by special cell, called beta cells in the pancreas; it is considered to be the main anabolic hormone of the body. It acts to regulate glucose homeostasis by stimulating glucose uptake by insulin responsive target tissue, adipocytes, skeletal and cardiac muscle. PCOS women have high insulin levels which play an important role (direct and indirect) in reproductive function; it can maintain and worsen PCOS (Pauli *et al.*, 2011; Bargiota and Diamanti-Kandarakis, 2012).

## 1.2 Aim of the study

PCOS has a great impact on the lives of women affected and associated with other problem such as androgen excess, infertility, insulin resistance/hyperinsulinemia and obesity , therefore these women are at risk of developing type two diabetes mellitus which in turn puts them at increased risk of developing cardiovascular disease, since the pathophysiology of PCOS is largely unknown and have several hypothesis , therefore this study aim to investigate the role of immune response in pathogenesis of PCOS and the effect of demographic factor on the PCOS, according to what is shown in the following :

1. Estimate the serum level of IL-18, IFN-γ, and C3 in PCOS women.
2. Evaluate the serum level of T3, T4, TSH and insulin in PCOS women.
3. Study the effect of demographic factors on PCOS.
4. Investigate the correlation between all the above parameters with the pathogenesis of PCOS.

# Chapter Two
# Literature Review

## 2. Literature Review

## 2.1 Ovarian function

The primary role of the ovary is the production of functional oocytes for reproduction through the growth and development of ovarian follicles. An ovary contains many tiny fluid-filled sacs called follicles, and each one contains an immature egg called an oocytes. Born girl already carries in her body about (400000) follicles. Only a small number of follicles (about 400) ever mature because a female usually produces only one egg per month during her reproductive years. The mature ovarian follicle is organized around the oocyte, which is surrounded by follicular fluid, granulosa cells, and an outer most layer of thecal cells (Mader, 2008) Figure (2-1). The ovary also serves as one of the major sites of steroid synthesis, producing progestins, androgens, and estrogens that are necessary for sexual differentiation, maturation, and maintenance of pregnancy. Follicular development and steroid Synthesis within the ovary is primarily controlled through the actions of pituitary gonadotrophins, LH and FSH, which are themselves regulated by ovarian hormones via pituitary feedback loops. The expression of LH receptors by theca cells, and FSH as well as by granulosa cells, allows the cells of the follicle to respond to hormonal stimulation by the pituitary (Knobil ,1980).
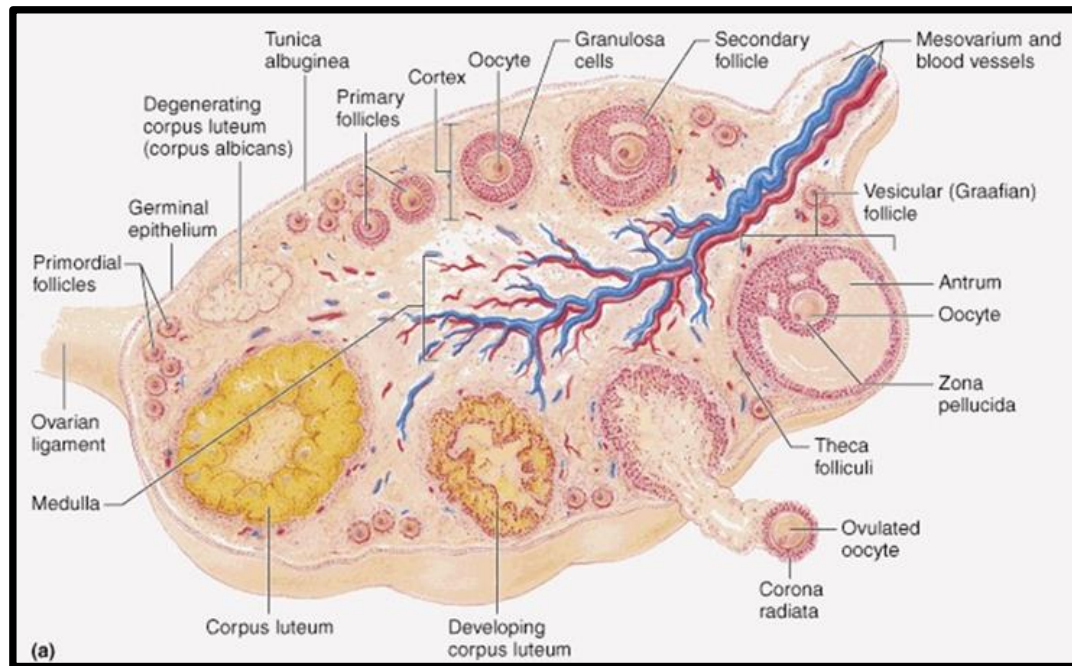
**Figure (2-1): The anatomy of the ovary. (Mescher,2016)**

## 2.2 Polycystic ovary syndrome (PCOS)

**Background**: PCOS was first described in 1935 by American gynecologists Irving F. Stein, and Michael L. Leventhal, from whom its original name of Stein-Leventhal syndrome is taken (Stein and Leventhal, 1935). In 1958 Mc-Arthur and Coworkers observed elevated LH levels in women with polycystic ovaries (Mc-Arthur *et al*., 1958). In the 1960, "Stein-Leventhal syndrome" represented a variety of clinical features. In the early 1970, the scientific community focused on increased serum levels of LH, elevated LH/FSH ratio, and the changed function in the hypothalamic – pituitary – ovarian axis (Rebar *et al*., 1976). The next milestone was the discovery of the association of PCOS and IR by (Burghen *et al*., 1980).

Then, ultrasound became central in visualizing polycystic ovaries (PCO) and diagnosing PCOS. The ultrasonographic finding of polycystic ovaries was described for the first time in 1981 (Swanson *et al*., 1981). In 1985, Adams insert a definition for the appearance of PCO in ultrasonographic as one diagnostic criterion of PCOS. This has been widely

used thereafter especially in Europe. The syndrome acquired its most widely used name due to multiple (poly) ovarian cysts which are diagnosed using ultrasound by the presence of eight or more follicular cysts, usually of equal size, which average 2-9 mm in diameter arranged around a dense stroma within PCOS ovary. These follicles may be oriented in the periphery, giving the appearance of a 'string of pearls'. The combination of multiple follicles and an increased amount of stroma contribute to the overall increase in the ovarian size (ovarian volume >10cm), that is 1.5 to 3 times larger than normal (Balen *et al*., 2003). These changes may be present in women without endocrine abnormality (Dunaif *et al*., 1997). (Figure 2-2)
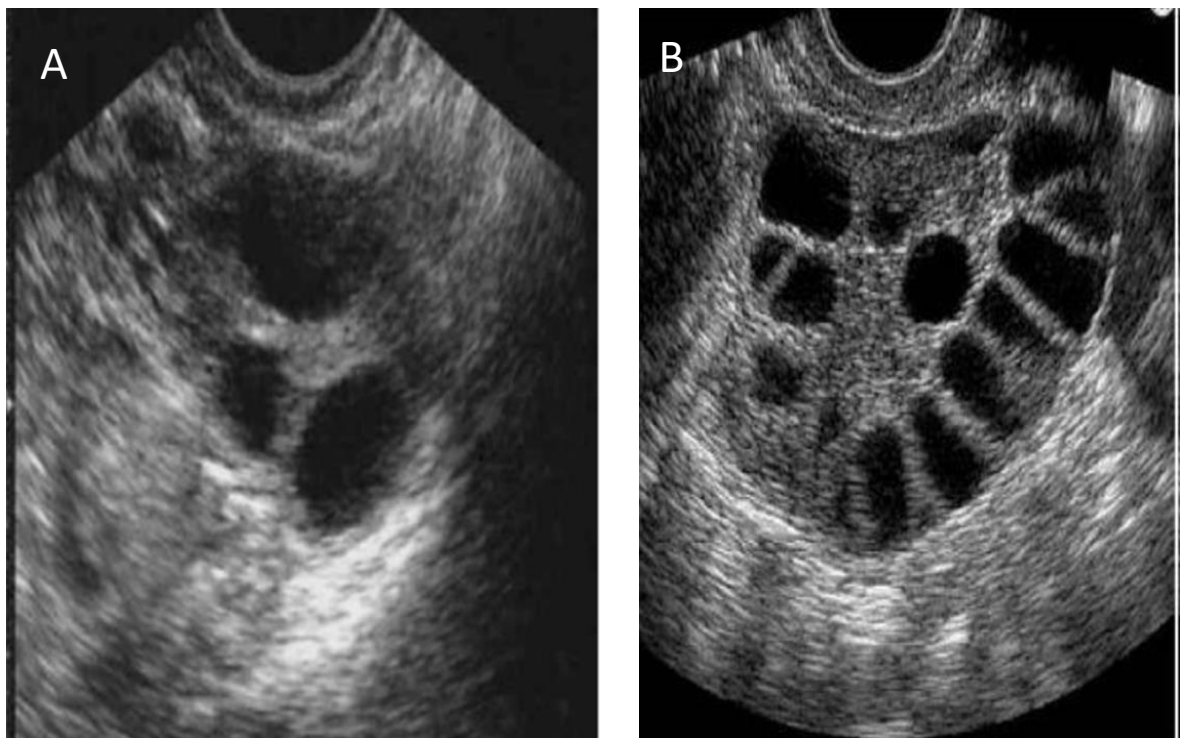


**A. Normal ovary**                    **B. Polycystic ovary**

**Figure (2-2): Comparison of normal ovary and polycystic ovary (Hiremath *and* Tegnoor, 2013). A. normal ovary  B. polycystic ovary.**

### 2.2.1 Clinical features of PCOS

It is important to appreciate that PCOS is a syndrome, not a disease, reflecting multiple potential etiologies and variable clinical symptoms (Christodoulopoulou *et al*., 2016).

Menstrual abnormality is most important feature seen in polycystic ovarian syndrome include oligomenorrhea (infrequent menses with less than 9 menstrual periods per year), amenorrhea (absence of menstruation for > 3 months without being pregnant), or erratic bleeding (loss of the cyclic menstrual pattern). 30% of females with PCOS will have typical menses and roughly 85–90 % of females with oligomenorrhea have PCOS, also 30%–40% of females with complete amenorrhea will have PCOS (Balen *et al*., 1995).

Hyperandrogenism, perhaps the most consistent and obvious diagnostic feature of PCOS is assessed clinically by hirsutism, acne and alopecia ( Farrell and Antoni, 2010).Hirsutism is the presence of excess body hair of male type on face and body in women (Mofid *et al*., 2008). The prevalence of hirsutism in PCOS varies with age, body weight and ethnic origin (Azziz *et al*., 2009). Huang *et al*., (2010) reported that hyperandrogenisim to be present in approximately 75% of females with PCOS. Moreover, It was estimated that between 50 and 70% of women with PCOS manifest IR (Legro *et al*., 2004), which supposed to be a vital part in both the pathogenesis and long term sequelae of the condition. There is strong epidemiological proof that insulin insensitivity presence would increase the danger of cardiovascular disease in PCOS ladies independence of other cardiovascular aggravating factors (Ford *et al*., 2002).

Another common feature of PCOS is the higher obesity prevalence up to 61%, including a higher waist-hip ratio (visceral obesity), which exacerbates the magnitude of irregular menstrual cycles and other metabolic alterations, including a high frequency of T2DM (Lim *et al*.,

2012). Stress and depression are the high-risk factors among the patients with PCOS along with the impaired metabolic and reproductive features. This high level of stress and anxiety in the patients with PCOS may be due to various reasons such as obesity, hirsutism, alopecia and infertility (Sadeeqa *et al.,* 2018). (Figure 2-3)
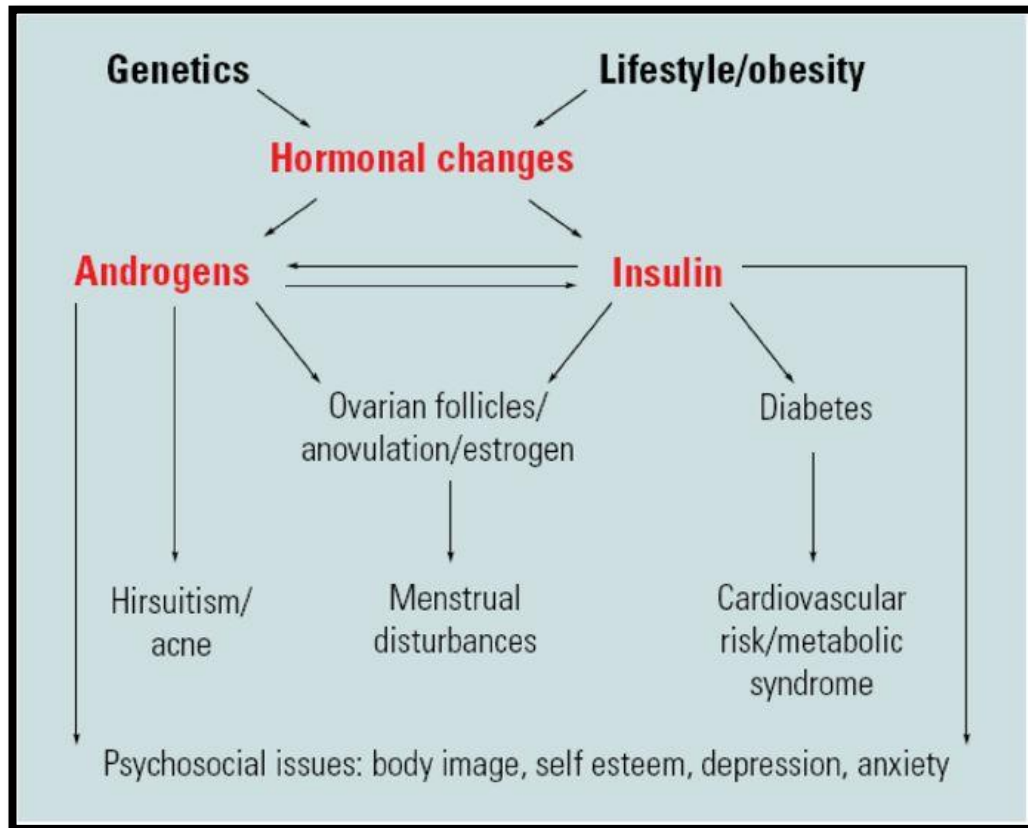


**Figure (2-3): Diagram of etiology and clinical features including menstrual, cardiovascular/metabolic and psychosocial issues of polycystic ovarian syndrome. (Teede *et al*., 2010).**

### 2.2.2 Diagnostic criteria of PCOS

Since first being described in the 1930s by Stein and Leventhal for PCOS, they first noted a combination of symptoms including obesity, hirsutism, and chronic anovulation, (Stein and 1935), later on both biochemical and radiographic imaging had been adopted to assist in diagnosis (Bachanek *et al*., 2015).

There have been three primary recommended criteria for PCOS. In 1990, a conference sponsored by the U.S. National Institute of Health

(NIH) put forward recommendations concerning diagnostic criteria for PCOS, which included clinical or biochemical hyperandrogenism together with chronic anovulation, and exclusion of other known disorders (Lucidi *et al.,* 2017).

The second, and most commonly used diagnostic criteria were established in 2003 entitled the Rotterdam criteria recognized PCOS as a syndrome that envelops a wide spectrum of signs and symptoms, including at least two of the following: ovulatory dysfunction, hyperandrogenism and polycystic ovarian morphology (PCOM) (Azziz *et al*., 2004). In 2006, androgen  Excess and PCOS  Society (AE-PCOS) published a  report emphasizing PCOS to be regarded primarily as a condition of androgen excess and defines the syndrome as follows; hyperandrogenism together with oligo/anovulation and/or polycystic ovaries which is the  third  criteria (Teede *et al*., 2010) (Table 2-1).

**Table (2-1): Diagnostic criteria for PCOS and accompanying component phenotypes.**

| Definition | Diagnostic criteria | Phenotypes |
|---|---|---|
| **NIH 1990** | presence of<br>1) Hyperandrogenism (HA)<br> 2) Chronic anovulation | HA+ anovulation |
| **Rotterdam 2003** | presence of at least two of<br>1) Hyperandrogenism<br>2) Oligo- and/or anovulation (OA)<br>3) PCO morphology (PCO) | HA + OA<br>HA + OA + PCO<br>HA + PCO<br>PCO + OA |
| **AE-PCOS 2006** | presence of<br>1) Hyperandrogensim<br>2) Ovarian dysfunction (OA or PCO) | HA + OA<br>HA + OA + PCO<br>HA + PCO |

*NIH,* **National Institute of Health;** *AE-PCOS,* **Society of Androgen Excess and Polycystic Ovary Syndrome (Azziz *et al*., 2004).**

## 2.2.3 Prevalence of PCOS globally and locally

In most studies, the prevalence of PCOS in fertile women is estimated to be between 5-10% (Goldenberg, N. and Glueck, 2008), but the prevalence rates reported are naturally dependent on the criteria used for its definition, and on the ethnicity of the studied population (Azziz *et al*., 2004; March *et al*., 2010). However, a recent study found that the prevalence of PCOS under NIH , Rotterdam and Androgen Excess Society (AE-PCOS) criteria were 6.1%, 19.9 % and 15.3%, respectively (Yildiz *et al*., 2012).

In Iraq , there are no proper studies on the prevalence of PCOS, only one local study that carried out in  the north of Iraq (Erbil), found that the prevalence of PCOS was 33%, observed among infertile women attending In vitro fertilization (IVF) center using the Rotterdam 2003 criteria for diagnosis (Hussein  and  Alalaf , 2013).

## 2.2.4 Pathophysiology of PCOS

Despite the diagnostic criteria, understanding of PCOS pathogenesis is still unclear, both genetic predisposition and environmental factors such as lifestyle, types of food, may contribute to the onset of PCOS features (Sedighi *et al.,* 2015). However, there are many different mechanisms cause PCOS first of all; there is a defect in the hypothalamic–pituitary axis lead to increased Gonadotropin-releasing hormone (GnRH) pulse frequency resulting in LH hyper secretion (Laven *et al*., 2002). As stated, LH stimulates the ovarian theca cells to produce androgens, such as testosterone. Because of a relative Follicle-stimulating hormone (FSH) deficiency, testosterone is incompletely aromatized by the granulosa cells, resulting in hyperandrogenemia (Nisenblat and Norman, 2009).

In the ovarian theca cells, androgen biosynthesis is mediated by cytochrome P-450c17 enzymes to form androstenedione which converted to testosterone by 17b-hydroxysteroid enzyme or aromatized to form estrone. Tsilchorozidou *et al*., (2004) showed that PCOS ovaries have an

increased cytochrome P-450c17 enzymatic activity, leading to enhanced the synthesis of androgenic precursors, and thereby testosterone. Moreover, although the ovaries are the main source of androgen excess in PCOS, also the adrenal glands contribute to the existing hyperandrogenism ( Nisenblat and Norman, 2009), these elevated androgen levels in PCOS patient can lead to symptoms of androgen excess such as hirsutism, acne and alopecia.

Insulin insensitivity in PCOS can be caused by a post binding deformity in insulin receptors signaling pathways, and higher insulin levels might raise the ovarian capacity for gonadotropins. Hyperinsulinemia might suppress the production of sex hormone–binding globulin (SHBG) in the liver that can worse androgenicity (Barber *et al.,* 2006; Wallace *et al*., 2013).

Briefly, in PCOS Hyperinsulinemia, hyperandrogenemia and altered intraovarian paracrine signaling can disrupt ovarian follicle growth, (Goodarzi *et al*., 2011). The consequent follicular arrest in PCOS is accompanied by menstrual irregularity, anovulatory, subfertility and the accumulation of small antral follicles within the periphery of the ovary, giving it a polycystic morphology (pcom) (Goodarzi *et al*., 2011) (Figure 2-4).
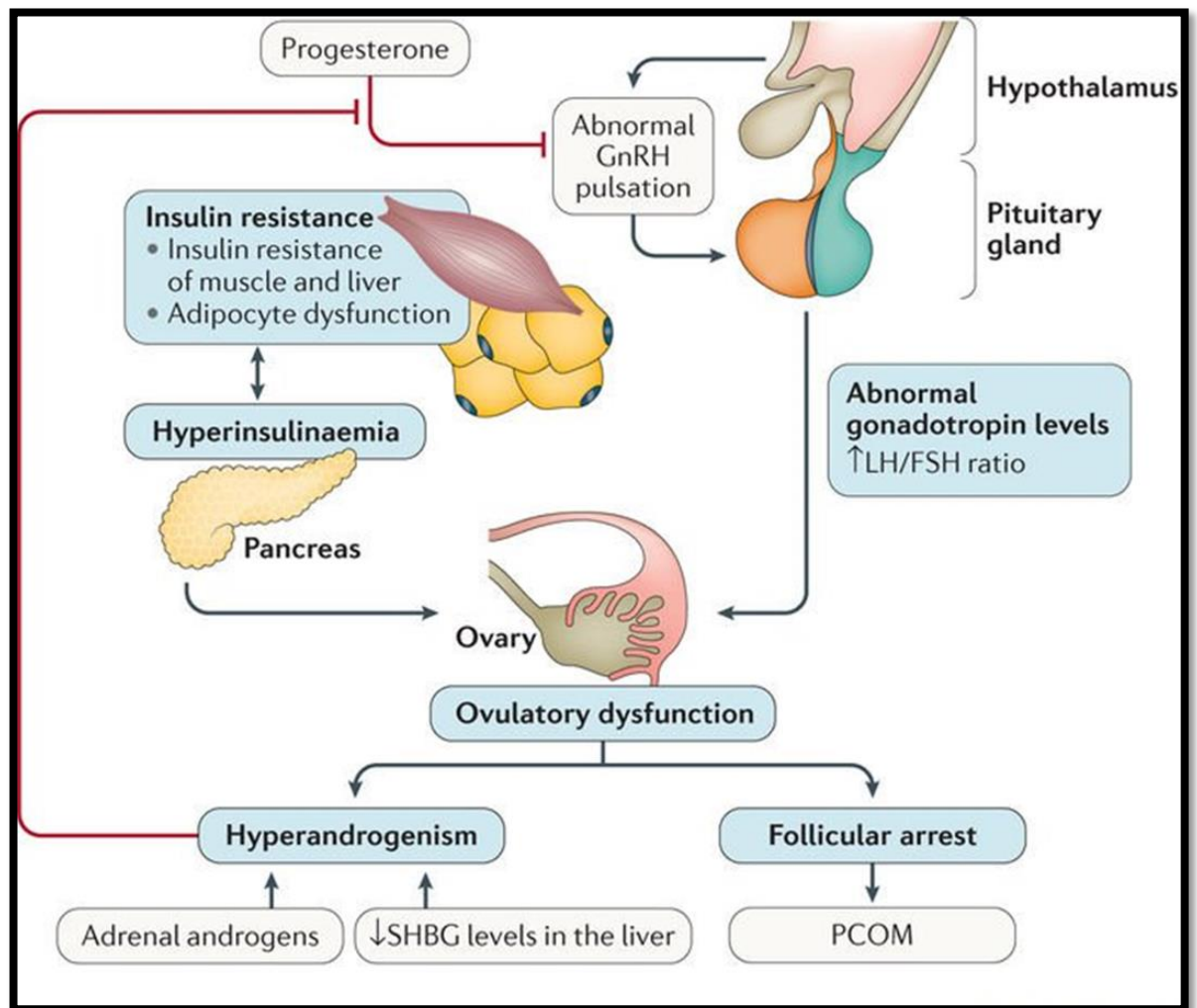
**Figure (2-4): The pathophysiology of polycystic ovary syndrome (Aziz *et al*., 2016).**

## 2.2.5 Treatment of PCOS

First line in the treatment of PCOS is the non-medication strategy, which emphasizes on the lifestyle modification, including diet and exercise; there is strong correlation between hyperinsulinemia and obesity with this disorder, which is mostly associated with decrease in ovarian function, weight loss may help in such cases; improves IR, correcting its deleterious consequences on hormonal milieu, ovulation and menstrual regularity (Domecq *et al*., 2013). Pharmacotherapy is also used in PCOS, including the oral contraceptive pill (OCP), insulin-sensitizing agents including metformin, cyclic progestin, anti-androgens and fertility treatment such as clomiphene citrate. Metformin have a positive effect in

PCOS; induces ovulation, regular menstrual cycle and increases the pregnancy rate (Naderpoor *et al*., 2015).

### 2.2.5.1 Metformin

Metformin or glucophage (commercial name) has a chemical structure ($C_4H_{11}N_5$) as shown in figure (2-5). It is the first line drug of choice for the treatment of T2DM, particularly in overweight and obese people and those with normal kidney function (Palomba *et al*., 2008).
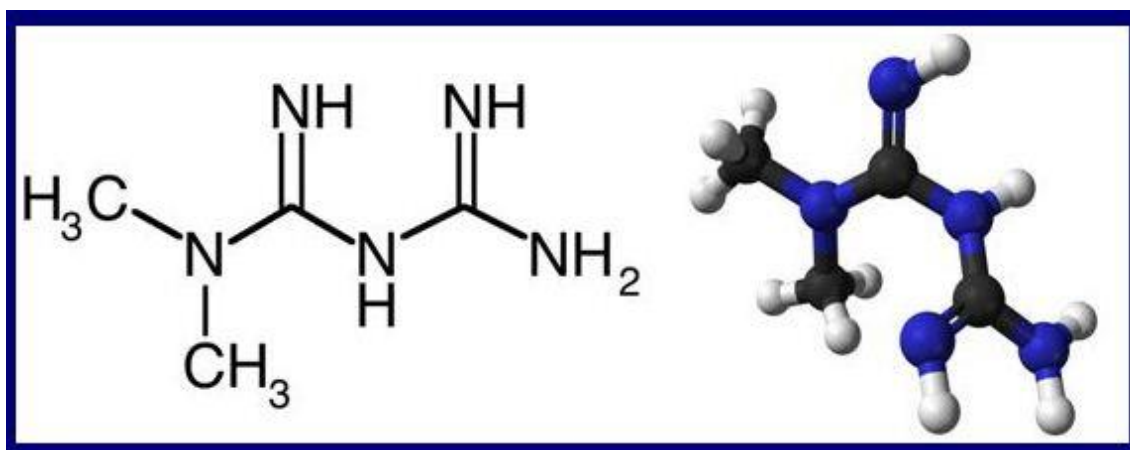


**Figure (2-5): Metformin structure (American Diabetes Association, 2007).**

Metformin is highly effective in PCOS management. Metformin therapy does not only reduce hyperinsulinemia and improves steroidogenic dysfunction, but also regulates menstrual cycles because it restores ovulatory menses in approximately 30%-50% of women with PCOS (Teede *et al*., 2010), it has also been shown decrease androgen levels, and reduce progression to overt T2DM in patients with PCOS. The main effect of metformin is to reduce the production of hepatic glucose, therefore reducing insulin secretion; it also reduces intestinal glucose absorption. Metformin has been shown to suppress lipolysis in adipose tissue leading to decreased circulating free fatty acids concentrations, thus lowering gluconeogenesis (Ferrannini, 2014). Furthermore, Metformin treatment had useful effects on lipid profile and blood pressure, and thus it could be helpful in the banning of cardiovascular complications in these females. So,

a six months course of Metformin treatment may promote menstrual cycle, ovulation rate, decreasing LH levels, therefor improving fertility in these females (Aruna *et al*., 2004).

## 2.3 Insulin Resistance

Insulin resistance is a condition in which the cells of the body become less sensitive to the insulin. Insulin is responsible for controlling the level of glucose. It was found that insulin insensitivity (insulin resistance) is a common criterion of the PCOS and both obese and lean women with this syndrome are more IR than normal women matched for age and weight; however, obese women with PCOS have significantly decreased insulin sensitivity compared with non-obese women who have PCOS. Beside the low insulin sensitivity, there is secretory dysfunction of β islets of pancreas had been detected in PCOS (Apridonidze *et al*., 2005). IR is known to precede the development of T2DM. Studies have been shown that 30%-40% of women with PCOS have impaired glucose tolerance, and as many as 10% develop T2DM by the age of 40 (Tsilchorozidou *et al*., 2004).

## 2.4 Obesity in PCOS

Obesity is a medical condition in which excess body fat has accumulated to an extent that it may have a negative effect on health. PCOS commonly associated with overweight or obesity, mainly abdominal adiposity; about 20-85% of women with PCOS are overweight (BMI 25 to30 kg/m2) or obese (BMI >30 kg/m2) (ESHRE/ASRM, 2012). Obese females with PCOS are characterized by severe hyperandrogenism, metabolic state disturbance, more menstrual disturbance, and lower pregnancy rates than lean females with PCOS, IR that present in most patients with PCOS and increased in its severity in the presence of obesity, was considered as a risk factor to develop T2DM  (Moran *et al*.,2012). Females with PCOS must be assessed for the presence of the risk factors

for both cardiovascular disease and metabolic syndrome by measuring their fasting lipid profile and plasma glucose concentration, fasting insulin and calculating their BMI (Carmina *et al*., 2003)

Chronic low-grade inflammation was played a significant role in the pathogenesis of obesity related diabetic syndromes. Leukocytes present in the circulation and adipose-tissue are capable of inducing insulin insensitivity in obesity and type II diabetes (Fernandez *et al.,* 2003).

## 2.5 Immunity in PCOS:

There was some evidence that PCOS is may be a pro inflammatory disorder characterized by presence of chronic low grade inflammation that correlated with obesity, IR or diabetes mellitus that found to be associated in PCOS (Yan Yang *et al*., 2011; Al-Musawy *et al*., 2018). The question whether chronic inflammation is an independent factor of PCOS development or rather secondary to obesity or IR that commonly present in women with this syndrome, is still open.

During normal ovulatory menstrual cycle in young healthy female, Estrogen promote the production of Interleukin4 (IL-4) from T-helper2 (Th2) lymphocytes, Interleukin1 (IL-1) from monocytes, Interleukin6 (IL-6) from T lymphocytes and IFN-$\gamma$ from T-helper1(Th1) cells, so follicular phase of menstrual cycle is characterized by increasing levels of these interleukins, while the level of IL-6 is decreased during luteal phase and show a negative correlation with the progesterone hormone (Angstwurm *et al.,* 1997). The stimulation of the immune response by estrogens hormone may be inhibited by progesterone hormone which is increased after ovulation. PCOS patients is presented with low level of progesterone due to oligo ovulation or un ovulation and high estrogen levels (especially estrone) due to extra glandular conversion from androgens; it is also has been reported that there is an increase in serum level of C-reactive protein (CRP) in patients with PCOS (Kelly *et al*., 2001). Gregor *et al*., (2011) reported that the obesity considered as a pro inflammatory state in which

both hypertrophied adipocytes and native immune cells (mainly macrophages and lymphocyte) were responsible for elevated levels of pro-inflammatory cytokines. The obesity-associated condition of chronic low grade inflammation, termed "metabolic inflammation," is thought to have a vital role in the physiology of both IR and T2DM in humans. Both liver and muscles show mild inflammatory responses induced by obesity, while the adipose tissue is the main site that mediate systemic inflammatory response (Odegaard *et al*., 2013) Figure (2-6). While Xiong *et al*., (2011) showed that PCOS ovarian tissue has more macrophages and lymphocytes when compared to healthy women; lymphocytes and macrophages secrete inflammatory cytokines like TNF-α and IL-6 which in turn, activate more lymphocytes and macrophages to enhance further cytokines secretion. It is postulated that this peripheral and ovarian inflammation might be the harbinger for IR, hypothalamic-pituitary ovarian dysfunction and anovulation (Xiong *et al*., 2011).
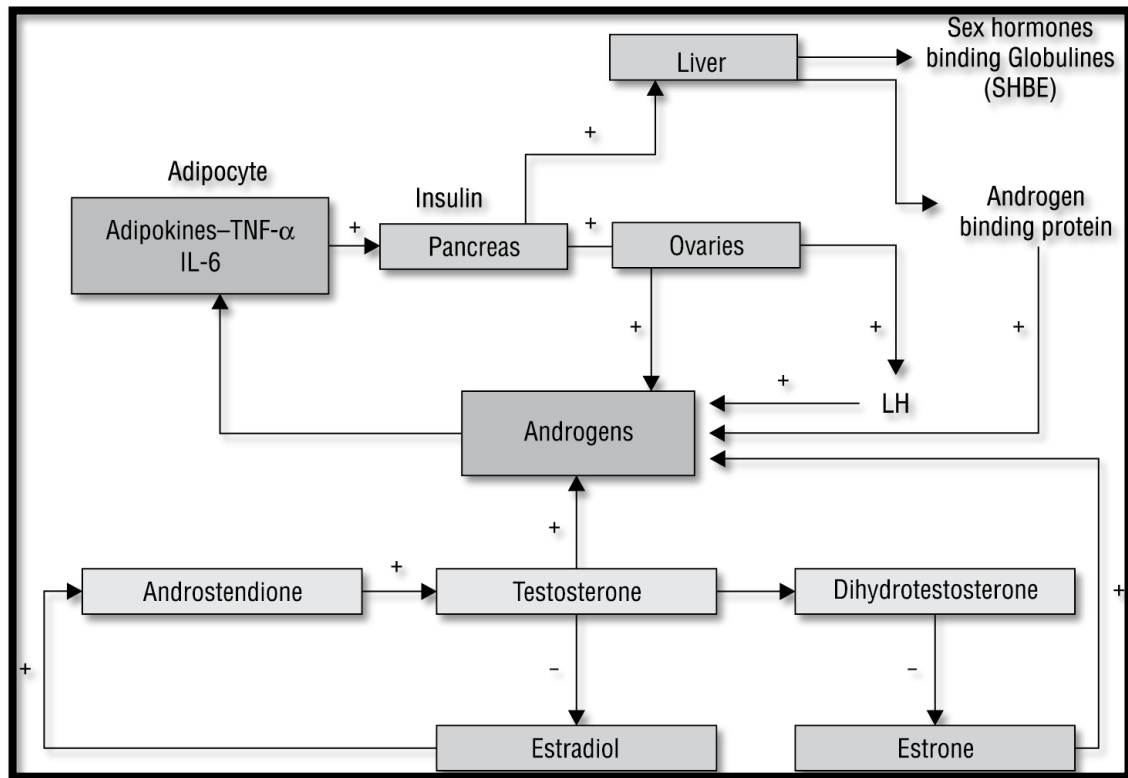
**Figure (2-6): Role of adipose cells in the polycystic ovary syndrome. TNF-α- tumor necrosis factor; IL-6- interleukin 6; LH -luteinising hormone (Ehrmann *et al*., 1999).**

## 2.6 Cytokines

Cytokines are large and heterogeneous group of secreted proteins produced by many different cell types, mediate and regulate all aspects of innate and adaptive immunity. It is very important to realize that cells in the body are never exposed to only single cytokine but they will be exposed to several cytokines, may be produced by a number of several cell types (Abbas and Lichtman, 2012).

Cytokine is a popular term; other terms include: lymphokine (which is cytokine produced by the lymphocytes), Monokine (which is the cytokine produced by monocytes), Chemokine is the cytokine that have chemotactic activities, and the interleukin is cytokine that produced by one leukocyte and acting on other leukocytes. Cytokines share many other general properties. One cytokine can act on diverse cell types rather than a single cell type, and have multiple biologic effects, a property that is

referred to as pleiotropism. Conversely, multiple cytokines may have the same action, and are said to be redundant. Each cytokine is produced by a group of cells in response to different types of stimuli; they stimulate a set of synergist, or antagonist actions that change target cell functions. Most cytokines act close to where they are produced, either on the same cell that secretes the cytokine (autocrine action) or on a nearby cell (paracrine action). Cytokines regulate the intensity and duration of the immune response by stimulating or inhibiting the activation, proliferation, and/ or differentiation of various cells and by regulating the secretion of antibodies or other cytokines (Abbas and Lichtman, 2012; Duque and Descoteaux, 2014).

There was an increasing evidence suggested that the defect in the regulation of effector cytokines activity which result in the maintenance of immune response and inflammatory activation is the baseline of autoimmune reactions. Normally, the immune response to infection or pathogens stimulates cytokine secretion which facilitates mechanisms for eliminating the invading organism. Once danger was eliminated, cytokine secretion was stopped and tissue damage resolved. In contrast, persistent, cytokines secretion resulted in increased tissue damage (Dinarello *et al*., 2004).

## 2.7 Interleukin-18

IL-18 is a pro-inflammatory cytokine which belongs to the (IL-1 superfamily), it is also known as IFN-γ Inducing Factor, is a cytokine with 17 kDa (157 amino acid). The major sources for IL-18 are macrophages and dendritic cells, but the precursor of IL-18 is constitutively expressed in epithelial cells throughout the whole body (Abbas and Lichtman, 2012). IL-18 is an important regulator of both innate and adaptive immune responses that is expressed at sites of chronic inflammation, in autoimmune diseases, a variety of cancers, and in the numerous infectious diseases. Receptors complex for IL-18 does not well identified. Novel

documentation assumed that the functional IL-18 binding component of this complex is IL-1 receptor-related protein, IL-18 was not a stimulant for production of IFN production only it also stimulates the production of TNF- α, which in turn promotes the synthesis of IL-6, and IL-6 adjust the synthesis of CRP within the liver. Like IL-6 and CRP, IL-18 is considered a strong risk marker for cardiovascular death (Blankenberg *et al*., 2002; Escobar *et al*., 2003; Tanaka *et al*., 2014).

IL-18 production is the primary response of the defensive innate immune reaction, and it was very important factor in initiation of both the T-helper 1 and the T-helper 17 dependent inflammatory immune response that is indispensable for eliminating the infectious or the stressful event and to re-establish homeostasis and there is an evidence suggested that IL-18 is involved in autoimmune pathogenesis, and it is essential for the first steps of autoimmune hyper activation (Dinarello *et al*., 2016).

Plasma IL-18 is found to be raised in case of obesity and also in females with polycystic ovarian syndrome and reduce after weight loss (Esposito *et al*., 2002). Also, serum IL-18 concentrations correlated with surrogate indexes of IR, such as the waist-to-hip ratio and fasting insulin levels (Esposito *et al*., 2003), suggested that the excess in serum IL-18 levels is related not exclusively to obesity but also to IR. Therefore, IL-18 might be a useful serum marker of the inflammatory process linked with obesity and IR (Esposito *et al*., 2003). Furthermore, IL-18 can be utilized as a marker of adipocyte production in PCOS and representing a reliable sign of cardiovascular risk in this syndrome (Dawood *et al*., 2018).

## 2.8 Interferon gamma

IFN-γ is a cytokine that plays an important role in inducing and modulating an array of immune responses, characterized as a homo-dimeric glycoprotein with pleiotropic immunologic function, described as a modulator of inflammation. It also called immune or type II, IFN (Tau and Rothman, 1999). IFN-γ is primarily produced by NK cells, CD4+ Th1

cells, and CD8+ T cells. IFN-γ receptor (IFN-γ R) consists of a complex of two receptor chains, designated IFN-γ R1 and IFN-γ R2. IFN-γ R1 binds IFN-γ with high affinity, allowing further stabilization of binding and productive signaling events through IFN-γ interactions with both chains (Marsters *et al*., 1995). The biological action of IFN-γ is macrophage-activating cytokine that provides the means by which T lymphocyte and NK cells activate macrophage to kill phagocytized microbes, promote the differentiations of naive CD4 T cells to Th1 subset and inhibit proliferation of Th2 cells, and acts on B cells to promote switching to certain IgG subclasses.

Accumulating evidences showed that the inflammation and immune regulation may be involved in the etiology of PCOS (Duleba and Dokras, 2012; Al Musawy *et al*., 2018) .Qin *et al*., (2016) was showed that the immune dominance of Th1 may be the immunological feature of the ovary in PCOS patients which confirmed that by enrich the lymphocytes from follicle fluid (ff) of ovary and detect Th1 /Th2-associated cytokine profile of lymphocytes subset in PCOS patients , and also found that the production of Th1 (IFN- γ, IL-2) cytokines in (ff) lymphocytes in PCOS patients were significantly higher than healthy women, while the production of Th2 (IL-4, IL-10) cytokines did not show statistic a differences between the two groups. Change level in inflammatory cytokine such as Th1 and Th2 cytokines contributes to ovarian follicle maturation; it may cause the poor quality of ovum and dysfunction of ovulation if imbalance occurs (Wegmann *et al*., 1993; Qin *et al*., 2016).

## 2.9 Complement System

The complement system is one of the major effector mechanisms of the innate immune system, composed up to more than 40 soluble factors, cellular receptors, and regulatory molecules present in blood plasma and on cell surfaces (Walport, 2001). It has many functions such as recognize and eliminate the 'foreign' microorganisms. Complement proteins act as a

cascade to opsonize pathogens and trigger a series of inflammatory responses, removing dead or dying cells, responses to viruses and maintain homeostasis (Merle *et al.*, 2015).There are three pathways: the classical, alternative and lectin pathway. The three routes meet at stage of the split the third component C3, that followed by the common terminal pathway. The antibody dependent pathway (classical pathway), was the first complement pathway discovered. It is initiated by binding of the C1 complex (consist of C1q, C1r and C1s) to the complement-fixing antibodies (IgM and IgG) bind with the antigen on the target surface (Wallis *et al.*, 2010), while the triggering alternative pathway can be initiated by any foreign molecules, including yeast, virus, and necrotic cells, and the final pathway (lectin pathway) is started by the binding of ficolins or mannose-binding lectin (MBL) to carbohydrate groups on bacterial cells surfaces (Holmskov *et al.*, 2003).

Complement C3 is an acute-phase protein synthesized predominantly in the liver, and raised levels may be a reflection of the metabolic disturbance associated with IR (Muscari *et al.*, 2007), it is also synthesize by adipocytes and predicts weight gain in humans, with levels diminishing after weight reduction, proposing that plasma C3 partly reflects fat mass (Scantlebury *et al.*, 2001; Muscari, 2007). Although complement C3 is associated with the main endogenous cardiovascular risk factors, it has been recently identified as predicting coronary or cardiovascular events but not independently of the established risk factors (Onat *et al.*, 2010).

Sadhu *et al.*, (2007) reported that iC3b is a cleavage fragment of complement activation product C3b bind to one receptor called CD11c, CD11c is a type I transmembrane protein and a member of the leuko integrin family, found at high level on most human dendritic cells, also on monocytes, macrophages, neutrophils, and some B cells that induce cellular activation, and have function in cell migration and cytokine production by monocytes and macrophages (Osugi *et al.*, 2002). On the other hand

Olefsky and Glass, (2010) reported that the adipose tissue in obese human is infiltrated by macrophages; these macrophages may be a major source of cytokines that further promote a local inflammatory response, resulting in systemic IR. The inflammatory macrophages in adipose tissue cluster in a class around dead adipocyte forming so-called crown-like structures (CLS) (Mannerås, 2010 ),  and CLS express the CD11c marker (Figure 2-7).

Later, Tao *et al.*, (2012) and Huang *et al.*, (2013) illustrate that the patients with PCOS associated with increase in CD11c expression and CLS density in adipose tissue, by increased levels of inflammatory cytokine and CD11c which bind to iC3b lead to activation of the complement cascade and initiate innate immunity in PCOS patients.
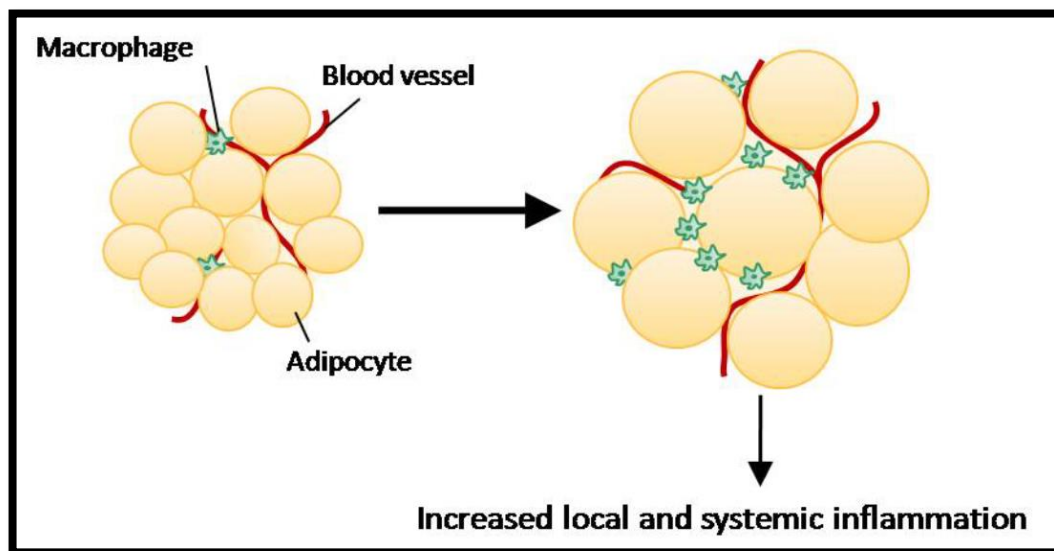


**Figure (2-7): Crown like structures in adipose tissue. Adipose tissue expansion, especially hypertrophic growth, is associated with an increased infiltration of macrophages. Many of the macrophages are aggregated around adipocytes, forming what are known as crown-like structures (CLS) (Mannerås, 2010).**

## 2.10 Thyroid hormone in PCOS (Thyroxine, Triiodothyronine and thyroid-stimulating hormone):

Thyroid gland is one of the largest endocrine glands in the body. It consists of two lobes lying on either side of the ventral aspect of the trachea

(Agur and Dalley, 2009). The thyroid gland controls how quickly the body burns energy, makes proteins, and how sensitive the body should be to other hormones. The thyroid gland participates in these processes by producing two principle iodinated hormones, thyroxine (T4) and triiodothyronine (T3) (Yen, 2001). T4 is the major secretory product of the thyroid gland, with a daily production rate of 80-100 µg. The ratio of T4 to T3 released in the blood is roughly 20 to 1. T4 is produced only by the thyroid gland. T3 is produced 80% by deiodination mechanism which converts T4 to T3 and 20% of T3 secreted by thyroid gland. The everyday production rate of T3 is 30-40µg . Most of the thyroid hormone circulating in the blood is bound to transport proteins (Santoro *et al*., 2002). Only a very small fraction of the circulating hormone is free (unbound) T4 0.03% and T3 0.3%, and biologically active. The production of T3 and T4 is regulated by TSH that release from thyrotrophic cell in anterior pituitary gland (O'Reilly, 2013).

Diseases of thyroid gland are manifested by alteration in the thyroid hormones secretion. Hypothyroidism results from suboptimal circulating level of one or both of thyroid hormones level, while hyperthyroidism is due to overproduction of thyroid hormones. Subclinical hypothyroidism, defined by elevated serum levels of TSH with normal levels of free thyroid hormones (Mohamed, 2016).Thyroid disorders and PCOS are two of the most common endocrine disorders in the general population, both pose a diagnostic dilemma as both share a common array of symptoms such as obesity, menstrual irregularities due to anovulation, acne, hirsuitism, infertility, carbohydrate intolerance in the form of IR; Therefore, thyroid profile should be analyzed along with the reproductive hormonal profile which may help in better understanding of the etiology and management of PCOS ( Nanda *et al*., 2014).

## 2.11 Insulin

Insulin is a pancreatic peptide hormone produced by β-cells of islets of Langerhans (Charles, 2001). It acts to regulate glucose homeostasis by stimulating glucose uptake by insulin responsive target tissues, adipocytes, skeletal and cardiac muscle, as well as by suppressing hepatic glucose production .Insulin is composed of two amino acid chains (A chain: 21 amino acids; B chain 30 amino acids) in human which are linked together by disulfide bond (Thomas,1999). Insulin receptor is a transmembrane glycoprotein containing two α β-dimers associated by disulfide bonds. The extra cellular α-subunits contain the insulin binding sites and the intracellular components of the membrane-spanning β-subunits contain intrinsic protein tyrosine kinase activity (Thomas, 1999). The initial phase in glucose take-up by the muscle cell is started by the binding of insulin to the α-subunit of the insulin receptor that leads to stimulation of the tyrosine kinase action in the β-subunit starting a cascade of intracellular protein phosphorylation (Thomas, 1999).

Insulin levels are increased in PCOS women, they mostly have IR that lead to hyperinsulinemia, so these women are under the risk of T2DM (Bargiota and Diamanti-Kandarakis, 2012), the elevated level of insulin in PCOS patients may be related to ethnic background and different life styles (Wijeyaratne *et al.*, 2002) or may be due to the defect of post binding in insulin signaling, especially in the major insulin target tissues like adipocytes and skeletal muscles (Corbould *et al.*, 2005).

Insulin effect was mediated through receptor of protein known as tyrosine kinase as mention above (Thomas, 1999), tyrosine autophosphorylation increased the activity of tyrosine kinase, while serine phosphorylation decrease its activity. An important mechanism of insulin insensitivity in PCOS females is related to high serine phosphorylation for insulin receptors (Dunaif *et al.,* 1997). It has been found that phosphorylation of insulin receptors substrate by serine was the same

mechanism of the TNF-α mediated IR in obesity (Rosen and Spiegelman, 1999).

It was found that insulin play a role in the regulation of ovary, beside the pituitary and adrenal gland; insulin appear to increase the sensitivity of pituitary gonadotropes to GnRH action and to potentiate the ovarian steroidogenic response to gonadotropins, by a mechanism probably related to an increase of LH receptor number (Poretsky,1999). Insulin acts synergistically with LH to enhance androgen production in the ovarian theca cells via cytochrome P450C 17 alpha. Also, insulin promotes ovarian growth and cyst formation. Moreover, Insulin decreases hepatic synthesis and secretion of sex hormone-binding globulin, the hormone that binds with testosterone in the circulation, thus increasing the amount of free testosterone that is biologically available (Tsilchorozidou *et al*., 2004).

These findings that described above, together with clinical evidence that a huge number of PCOS women show a condition of IR and hyperinsulinemia, suggested that insulin play a vital role in promotion or maintence of PCOS (Poretsky, 1999).

## 2.12 Researches on immunological markers in PCOS in Iraq

• Alteia *et al*.,(2013). Estimated serum level of some pro inflammatory cytokines in obese women with PCOS after metformin therapy and they found, there was an increasing levels of the IL-6 and TNF-α and metformin improve these cytokines levels.

• AL-Hadithi, (2013). Studied The proinflammatory IL-6 and serum glucose in PCOS and she found that Serum IL-6 levels was significantly higher among PCOS women than in healthy group, while PCOS patients had high blood sugar when compared to control group, A positive association were seen between serum IL-6 and Fasting blood sugar.

• Waheed et al., (2013). Evaluated of some cytokines and hormones in a sample of Iraqi women with PCOS and their relation to obesity and they concluded that serum vaspin and apelin levels increased in PCOS women in the same manner particularly the obese. These data suggest their involvement in the pathogenesis of PCOS.

• Wadood *et al*., (2015). Estimated serum level of Immunoglobulin IgG, IgA, IgM, complement C3 and C4 in sera of patients with PCOS and the risk of cardiovascular diseases and they found that the increasing in the circulating immunoglobulin and complement component might serve as a signal for the presence of an immune response that may increase cardiovascular risk in PCOS patients

• Al-Assadi *et al.,* (2017). Evaluated Serum level of TNF-α synergetic with IR potentially contributes to the development of PCOS and they conducted that TNF-α in path mechanism of PCOS being the basis of increase body weight which lead to development of IR.

• Fathi, (2018). Evaluated  Serum level of CRP and adiposity in women with PCOS and he found that CRP was one of biomarkers in obese women with PCOS and its high level associated with adiposity rather than PCOS itself.

• Ibrahim and AL-saffar, (2018). Estimated serum level of IL-18 in obese women with PCOS and they found  there was an increasing in the level of the inflammatory cytokine IL-18 in all the PCOS patient subgroups (normal weight, overweight and obesity).

• Al-Musawy *et al*.,(2018) evaluated Levels of cytokines profile in PCOS and they found IL-6, IL-18, and TNF-α were highly statistically significant in PCOS comparing to normal women, and these high levels were related to PCOS independent on the presence of obesity or hyperandrogenism.

# Chapter Three

# Materials and Methods

# 3. Materials and Methods

## 3.1 Subjects

A total number of 66 females with PCOS were involved in this case control study (Figure 3-1). Patients were collected from Kamal Al-Samarrai infertility treatment and in vitro fertilization hospital, whom age range from (20-40) years, and 22 match ages of the apparently healthy women. The clinical assessment of patients with PCOS was evaluated by physician according to sonograph and laboratory assessment; hormones levels FSH, LH and TSH were estimated (taken from the hospital).The questionnaire form has been filled for each patient and control as shown in appendix (A).

## **The inclusion criteria used for the recruitment of PCOS subjects**

PCOS was diagnosed according to the 2003 Rotterdam criteria (ESHRE/ASRM., 2004b). Two out of three of the following criteria were met for the diagnosis:

**(1)** Clinical and/or biochemical signs of hyperandrogenism.

**Clinical:** Hirsutism was considered as the indicator for hyperandrogenism. Modified Ferriman-Gallwey (MFG) score test was taken as significant in locations (upper lip, face, jaw and neck, upper back, lower back, upper arm, thigh, chest, upper abdomen, lower abdomen and perineum) when the hair distribution score ≥8 of 36 (Tehrani *et al*., 2013). (Figure 3-2), **Biochemical:** increased testosterone >0.8ng/mL.

**(2)** Oligo ovulation and /or non-ovulation; Cycle ranged from 35-45 days (oligomenorrhoea) or absence of menstruation for more than 3 months (amenorrhoea).

**(3)** Appearance of polycystic ovaries on ultrasound. In 3$^{rd}$ or 4$^{th}$ day of the menstrual cycle, ultrasonic evaluation was performed with transvaginal ultrasound to check the morphological appearance of ovaries. Test was

used by ultrasound specialist physician.
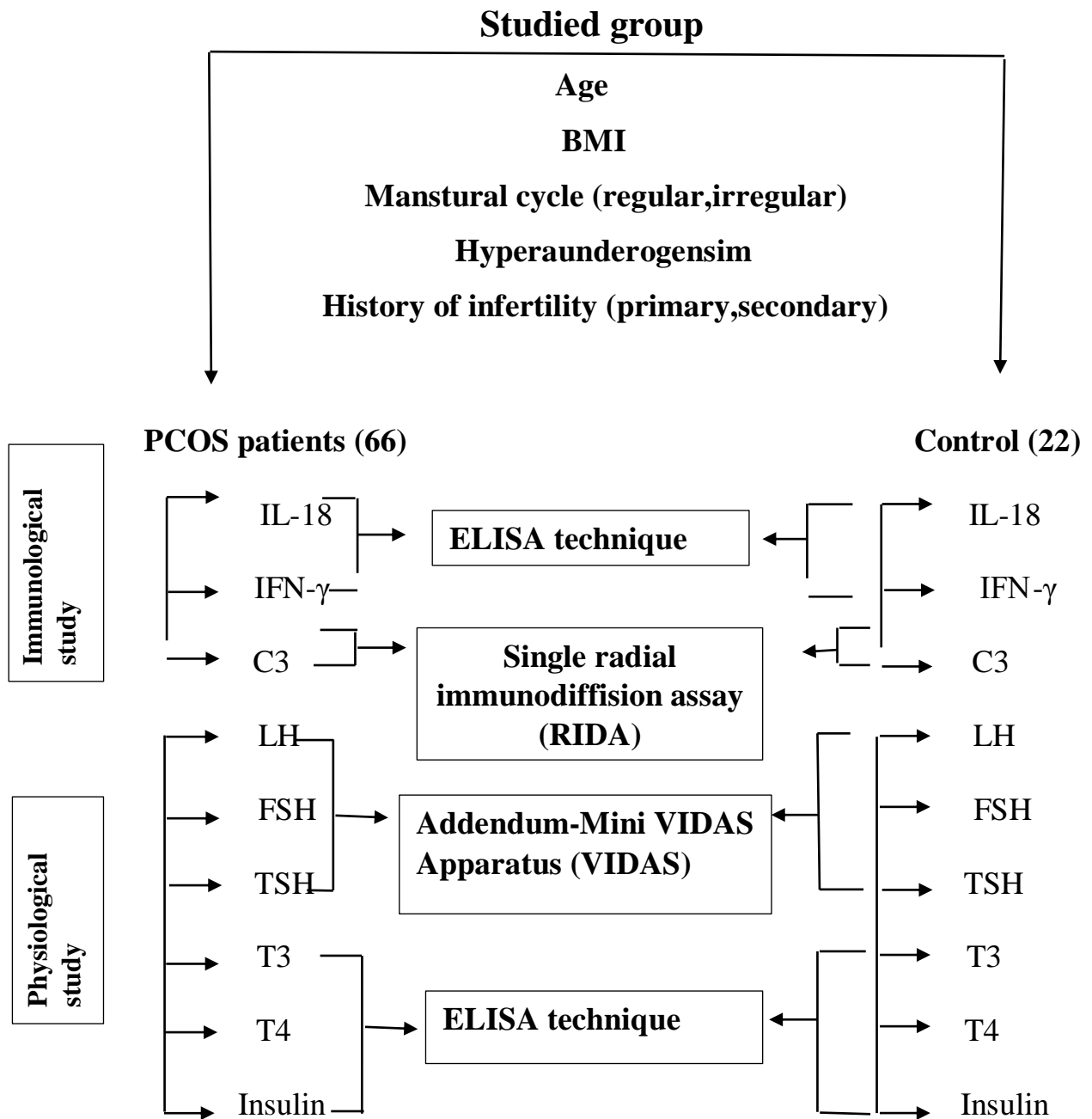


**Figure (3-1): Diagram of the study and the groups used in it.**
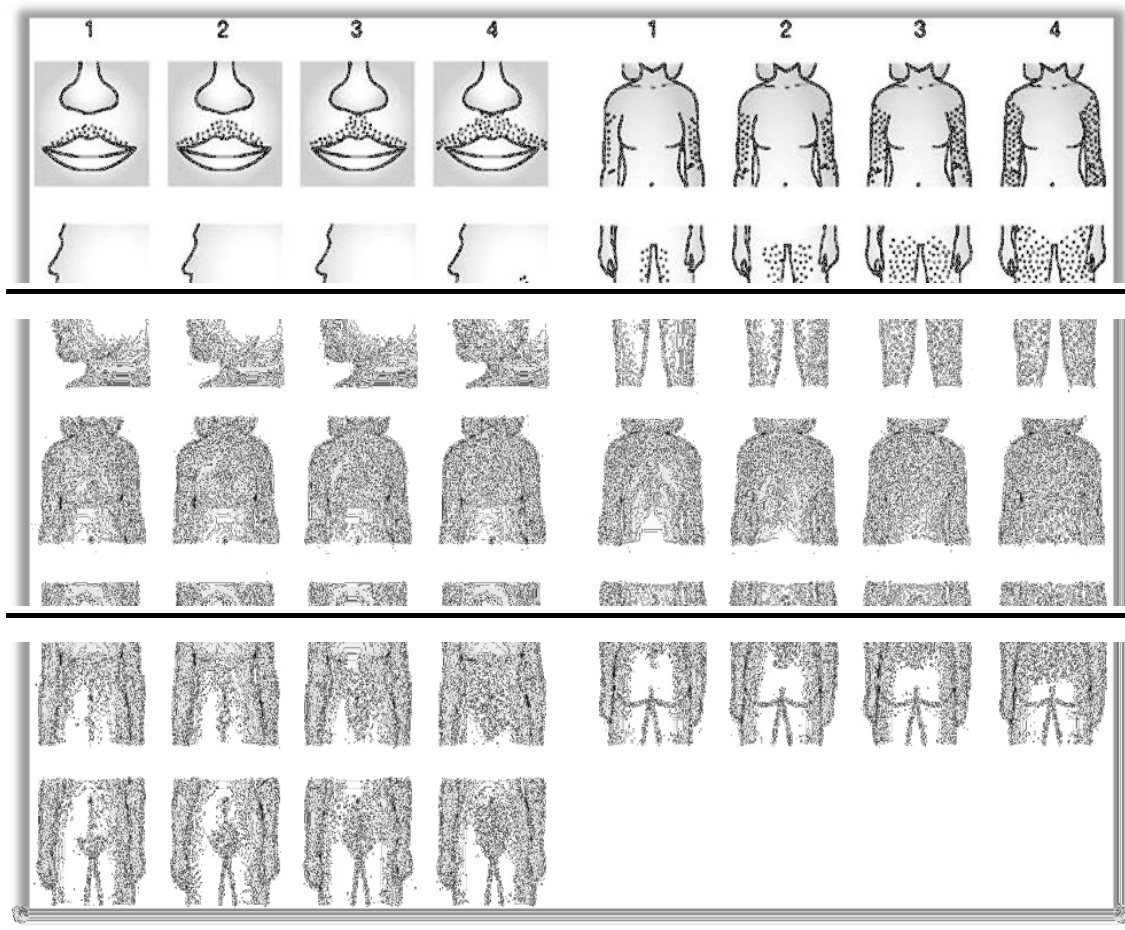
**Figure (3-2): Ferrimane Gallwey hirsutism scoring system (Hatch *et al*., 1981).**

## The inclusion criteria used for the recruitment of control group

1. The length of their menstrual cycles ranged between 22-35days.

2. History of spontaneous conception and should be fertile with at least two children.

3. They had no clinical or biochemical signs of hyperandrogenism and no polycystic ovaries at any stage of life.

Body mass index (BMI) for both patients and healthy control group was measured as following:

$$\frac{\text{weight (kilogram)}}{\text{Height (m}^2)}$$

The measurement was easy and simple so there is widespread use of the BMI as a marker of adiposity not for epidemiological study only but in clinical procedure also (Pasco et al., 2014). The most commonly used definitions, established by the World Health Organization (WHO) in 1995

and published in 2000, with some modification done in 2004. Provide the values listed in the table (3-1).

While the infertility for patients divided in two group primary and secondary infertility:

primary infertility refers to couples who have not become pregnant after at least 1 year having sex without using birth control methods.

secondary infertility refers to couples who have been able to get pregnant at least once, but now are unable.

**Table (3-1): The International Classification of adults of normal weights overweight and obesity according to BMI.**

| Classification | BMI(kg/m$^2$) |
|---|---|
| Normal range | 18.50 - 24.99 |
| Overweight | ≥25.00 |
| Pre-obese | 25.00 - 29.99 |
| Obese | ≥30.00 |
| Obese class I | 30.00 - 34.99 |
| Obese class II | 35.00 - 39.99 |
| Obese class III | ≥40.00 |

(WHO, 1995; WHO, 2000 and WHO, 2004)

## 3.2 Equipments and Materials

### 3.2.1 Equipments

Equipment used in this study shown in table (3-2).

**Table (3-2):  The general Equipments and tools utilized in this study.**

| Equipment | Company | Country |
|---|---|---|
| Cold box | Cosmoplast | UAE |
| Eppendorf tubes 1.5mL | Ataco | China |
| Gel tube (6mL) | BIO ZEK medical | Netherlands |
| Length measurement meter | _____ | China |
| Micropipette | Eppendorf | Germany |
| Multichannel pipette | Genomx | USA |
| Syringes (5mL) | Jiangyin Changqiang | China |
| Sensitive electronic balance | Beurer | Germany |

### 3.2.2 Devices

The devices used in this study shown in table (3-3).

**Table (3-3): General devices used in this study.**

| Devises | Company | Country |
|---|---|---|
| Centrifuge | Fisher Scientific | USA |
| ELISA printer | HP | China |
| ELISA Reader | Human | Germany |
| Lens | LTA | Italy |
| Oven | Funi | China |
| Refrigerator | Hitachi | Japan |

### 3.2.3 General kits used in this study

General kits used in this study shown in table (3-4).

**Table (3-4): Total kits used in immunological and physiological study.**

| Kits | Company | Country |
|---|---|---|
| Human IL-18 ELISA Kit | mybiosource | USA |
| Human IFN-gamma ELISA Kit | Komabiotech | Korea |
| C3 Complement component single radial immunodiffusion plate | LTA | Italy |
| Triiodothyronine (T3) ELISA Kit | Monobind Inc. | USA |
| Thyroxin( T4) ELISA Kit | Monobind Inc. | USA |
| Thyroid stimulating hormone (TSH) | Biomerieux | France |
| Insulin ELISA KIT | LDN | Germany |

## 3.3 Methods

The blood sample was obtained in the morning subsequent to an overnight fasting and during the early follicular phase of a menstrual cycle (days 3–5). Blood samples were collected in clot activator tubes, and the serum was separated after centerfugation and divided in to five Eppindroff tubes to avoid multiple freezing and thawing and kept frozen until time of analysis.

## 3.4 Immunological study

### 3.4.1 Human Interleukin- 18 (IL-18) assay.

### ● Principle Assay of Human IL-18 ELISA Kit.

This measurement employs the quantitative sandwich enzyme immunoassay technique.

**Table (3-5): Contents of Human IL-18 ELISA Kit.**

| Items | Specifications(48T/96T) |
|---|---|
| ELISA Microplate(Dismountable) | 8×6 /8×12 |
| Lyophilized Standard | 1 vial/2 vial |
| Sample / Standard Dilution Buffer | 0mL/20mL |
| Biotin-labeled Antibody (Concentrated) | 60ul/120ul |
| HRP-Streptavidin Conjugate(SABC) | 60ul/120u |
| SABC Dilution Buffer | 5mL/10mL |
| TMB Substrate | 5mL/10mL |
| Stop Solution | 5mL/10mL |
| Wash Buffer (25X) | 15mL/30mL |

### ● Assay procedure

Before starting the procedure, human IL-18 ELISA kit components were left at a room temperature. All standard, samples and reagents were prepared according to test preparation in kit leaflet, and the procedure illustrated in the table (3-6). The assay Range:15.625 -1000 pg/mL.

**Table (3-6): Procedure of IL-18 ELISA kit.**

| Number | Steps of test | Procedure |
|--------|---------------|-----------|
| 1 | Washing | The plate washed 2 times before added standard, sample and control. |
| 2 | Adding | 100 µL of standard and sample were added to each well and incubated for 90 min at 37. |
| 3 | Washing | The plate was aspirated and washed 2 times. |
| 4 | Add | 100 µL of Biotin-labeled antibody working solution were added to each well and incubate for 60 minutes at 37°C. |
| 5 | Washing | The plate was washed and aspirated 3 times. |
| 6 | Add | 100µL of SABC working solution were added to each well incubated for 30 minutes at 37°C. |
| 7 | Washing | The plate was aspirated and washed 5 times. |
| 8 | Add | 90µL of TMB Substrate were added to each well then incubated15 -30 minutes at 37°C. |
| 9 | Add | 50µL of stop solution were added to each well. |
| 10 | Reading | The plate was read at (450nm) wave length. |

## 3.4.2 Human IFN-γ Assay

### ● Principle Assay of Human IFN-γ ELISA kit

This measurement employs the quantitative sandwich enzyme immunoassay technique.

**Table (3-7): Contents of Human IFN-γ ELISA Kit.**

| Reagents | Quantity |
|----------|----------|
| Pre-Coated 96 well ELISA microplate | 1 Plate |
| Biotinylated Affinity Purified Detection Antibody (Lyophilized ) | 2 EA |
| Streptavidin-HRP Conjugate (0.6 mL) | 1 EA |
| Assay Diluent (50 mL) : 1% BSA in PBS | 1 EA |
| Assay Dileunt G (10 mL) : N/A | N/A |
| TMB or pink-ONE Solution (10 mL) | 1 EA |
| Stop Solution (10 mL) | 1 EA |
| Wash Buffer Concentrate (20X, 50 mL) to make 1 liter | 1 EA |
| Wash Buffer Concentrate (20X, 50 | 3 EA |

### ● Assay procedure

Before starting the procedure, human IFN-γ ELISA kit components were lifted at a room temperature, all standard, samples and reagents were

prepared according to test preparation in kit leaflet, and the procedure illustrated in the table (3-8). The assay Range for IFN-γ: 23.4–1500 pg/mL.

**Table (3-8): Procedure of IFN-γ ELISA kit.**

| Number | Steps of test | Procedure |
|--------|---------------|-----------|
| 1 | Washing | 200 μL of washing solution was added to each well. Then the wells were aspirated to remove liquid and wash the plate three times using 300 ul of washing solution. |
| 2 | Reaction | 100 μL of standard and sample were added to each well .then Covered the plate with the Plate Sealer. Incubated at room temperature for at least two hours. |
| 3 | Washing | Aspirated the wells to remove liquid and washed the plate four times. |
| 4 | Detection | 100 μL of the diluted detection antibody was added to per well. Then covered the plate and incubate at room temperature for 2 hours. |
| 5 | Washing | The plate was aspirated and washed four times |
| 6 | Conjugates | 100 μL of the diluted streptavidin-HRP was added to per well. Then Covered the plate and incubated 30 minutes at room temperature (or at 37°C for 30 min). |
| 7 | Washing | Each well were aspirated and washed 4 times. |
| 8 | Color Development | 100 μL of TMB or pink-ONE TMB solution was added to each well then incubated at room temperature for a proper color development and added 100 ul of the stop solution to each well. |
| 9 | Reading | Micro plate reader was used and measured observance at 450 nm. |

### 3.4.3 Estimation of C3 Complement Component

### ● C3 serum level was estimated by using single redial immunodiffusion assay (SRIDA)

The examined protein, diffusing in agarose gel containing a specific antibody will form an immune- complex, visible as a ring around the well. The ring diameter is direct proportional to the concentration of the analyzed protein. The proportion corresponds to the diffusion time. In fact, at the end (72h), the square of diameter will be in linear proportion to the concentration of the sample. With the plate is supplied a reference table in which each diameter of the halo is associated a concentration.

● **Procedure of C3 complement component:**

The plates were removed from its envelope and leave to stand at room temperature for few minutes so that any condensed water in the wells can evaporate. The wells were filled with 5 µL of sample and/or controls and waited, it has been completely adsorbing before handling the plate. The plate was placed in a moist chamber for 72 hours. The assay range for C3: 26 -300 mg/dL.

● **Reading:**

The precipitating ring were measured with an appropriate ruler or measuring lens however a system which provides a maximum error of 0.'1mm. the reading were on enclosed reference table the concentration value corresponding to the precipitating ring diameter.
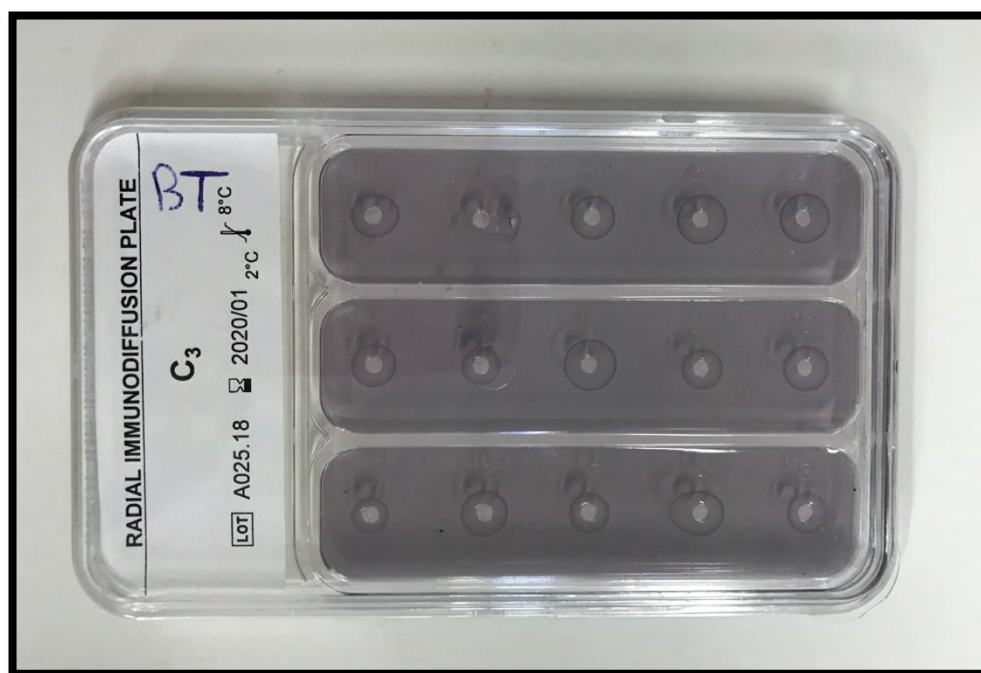


**Figure (3-3): Show the precipitating rings of C3 complement component**

## 3.5 Physiological study

### 3.5.1 Thyroxin T4 and Triidothyroxin T3 Assay

● **Principle Assay of T3 and T4 ELISA kit**

This measurement employs the competitive enzyme immunoassay technique.

**Table (3-9): Contents of T3 and T4 ELISA kit**

| Reagents | Quantity |
|---|---|
| Pre-coated 96 well ELISA microplate | 8×6 /8×12 |
| Human serum references | 1mL/6 vial |
| T3/T4 conjugate buffer | 13mL/10mL |
| HRP-Conjugate | 1.5mL/1 vial |
| Substrate A TMB | 7mL/1vial |
| Substrate B (hydrogen peroxide) in buffer | 7mL/1vial |
| Stop solution | 8mL/1vial |
| Wash buffer | 20mL/1 vial |

● **Assay procedure**

Before starting the procedure, T3 and T4 ELISA kit components were left at a room temperature, all standard, samples and reagents were prepared according to test preparation in kit leaflet, and the procedure illustrated in the table (3-10). The assay range for T3: 0.52 -1.85 ng/mL, the assay range for T4: 4.4 -11.6 µg/dL.

**Table (3-10): Procedure of T3 and T4 ELISA kit.**

| Number | Steps of test | Procedure |
|---|---|---|
| 1 | Format | The microplates' wells for each serum reference were formatted for patients and controls |
| 2 | Pipette | For T3 kit: pipette 50 µL of the appropriate serum reference, control were pipetted into the assigned well. For T4 kit: pipette 25 µL of the appropriate serum reference, control were pipetted into the assigned well. |
| 3 | Adding | 100 µL of working reagent A, hormone enzyme reagent were added to all wells. |
| 4 | Incubate | Incubation has been done 60 min at room temperature. |
| 5 | Discard | The contents of the microplates discarded by aspiration. |
| 6 | Adding | 350 µL of wash buffer was added to wells, repeated 2 additional times for total of three washes. |
| 7 | Adding | 100 µL of working substrate solution was added to all wells. then Incubated for 15 min. |
| 8 | Adding | 50 µL of stop solution was added to each well. |
| 9 | Reading | The absorbance was read in each well at 450nm in micro plate reader. |

## 3.5.2 Thyroid Stimulating Hormone (TSH), Luteinizing hormone (LH) and Follicle Stimulating Hormone (FSH) Assay

### ● Principle Assay of the VIDAS ®TSH, LH and FSH

Hormones analysis was performed by using Addendum-Mini VIDAS apparatus (VIDAS), through an enzyme linked fluorescent assay (ELFA) technique. The normal range for the TSH, LH and FSH illustrated in the table (3-11).

**Table (3-11): Normal range of TSH, LH and FSH in female**

| Parameter | Normal value |
|-----------|--------------|
| Serum TSH | Euthyroid :0.25-5 µIU/mL<br>Hyperthyroid:<0.15µIU/mL<br>Hypothyroid:>7µIU/Ml |
| LH | 1.5-8 mIU/ml |
| FSH | 2.9-9 mIU/mL |

## 3.5.3 Insulin Assay

### ● Principle Assay of Insulin ELISA kit

This measurement employs the quantitative sandwich enzyme immunoassay technique.

**Table (3-12): Contents of Insulin ELISA kit.**

| Reagents | Quantity |
|----------|----------|
| Pre-coated 96 well ELISA microplate | 8×6 /8×12 |
| Insulin standard | 1mL/6 vial |
| Enzyme conjugate | 5mL/10mL |
| Streptavidin-HRP complex | 7mL/1 vial |
| TMB substrate | 14mL/1vial |
| Stop solution | 14 mL/1vial |
| Wash buffer | 30 mL/1 vial |

### ● Assay procedure

Before starting the procedure, insulin ELISA kit components were left at a room temperature, all standard, samples and reagents were prepared according to test preparation in kit leaflet, and the procedure illustrated in the table (3-13). The assay range for insulin: 1.76-100 µIU/mL. The cut-off value of insulin ≥25 µIU/Ml.

**Table (3-13): Procedure of Insulin ELISA Kit.**

| Number | Steps of test | Procedure |
|--------|---------------|-----------|
| 1 | Add | 25 µL of each standard, control and samples were added into appropriate wells. |
| 2 | Distribution | 25 µL of enzyme conjugate was distributed into each well, then incubated for 30 minutes at room temperature. |
| 3 | Washing | The wells were rinsed 3 times with diluted wash solution (400 µL per well) |
| 4 | Distribution | 50 µL of enzyme complex was distributed to each well .Then incubated for 30 minutes at room temperature. |
| 5 | Washing | The wells were rinsed 3 times with diluted wash solution (400 µL per well). |
| 6 | Distribution | 50 µL of substrate solution was distributed to each well. Then incubate for 15 minutes at room temperature. |
| 7 | Adding | 50 µL of stop solution was added to each well.to Stop the enzymatic reaction. |
| 8 | Determination | The absorbance of wells were determined at 450±10 nm with a microtiter plate reader. |

## 3.6 Statistical Analysis

The statistical analysis of the results was performed using SPSS program 18 (IBM SPSS Statistics software). ONE way ANOVA test was used to find P value by using Least significant differences of mean (LSD) for all results. Pearson correlation coefficient was used to investigate the correlation between studied parameters. A difference of $P<0.05$ was considered statistically significant. All data were presented as Mean±S.E.

# Chapter four

# Results

# 4. Results

## 4.1 Basic Characteristics of Study Group:

This study included 66 patients with PCOS obtained from Kamal Al-Samarai hospital in Baghdad at the period between August 2018 to March 2019. These were compared with age matched 22 apparently healthy controlss.

Table (4-1) illustrates the basic characteristics of PCOS patients and controls groups. There were no statistically significant differences found in the mean age between patients (28.09±0.59) and controls (29.13±0.71), and there was a significant difference in the mean of BMI (P<0.001) between patients (26.99±0.38) and controls (24.84±0.34), while the mean of Luteinizing hormone (7.49±0.44 mIU/mL) was higher than Follicular Stimulating hormone (5.20±0.2 mIU/mL) in patients group. (60 %) of the patients have an evidence of hyperandrogenism and (30.3%) have regular menstrual cycle and (69.7%) have irregular (oligomenorrhea and amenorrhea), while the history of infertility in patients was (71.2%).

**Table (4-1): Basic Subjects Characteristic**

| Parameters | Patients (N=66) | Controls (N=22) | P- value |
|---|---|---|---|
| Age ( years) Mean ± S.E. | 28.09±0.59 | 29.13±0.71 | 0.267 |
| BMI(Kg/m2) Mean ± S.E. | 26.99±0.38[**] | 24.84±0.34 | 0.001 |
| FSH  Mean ± S.E. | 7.49±0.44[**] | 2.83±0.12 | 0.001 |
| LH  Mean ± S.E. | 5.20±0.2 | 5.75±0.176 | 0.643 |
| History of infertility: No (%) | 47 (71.2%) | 0(0.0%) | _____ |
| Hyperandrogenism: No (%) | 39 (60 % ) | 0(0.0%) | _____ |
| Menstrual cycle: Irregular Regular | 46 (69.7%) 20 (30.3 %) | 0 (0.0%) 22 (100%) | _____ |

**Table (4-2): The education level, feeding, geographic distribution and stress for patients and controls.**

| Parameters | Patients (N=66) | Controls (N=22) |
|---|---|---|
| **Education level** | | |
| **Primary school** | 40 (60%) | 0 (0%) |
| **Secondary school** | 20 (31%) | 0 (0%) |
| **Graduated** | 6 (9%) | 22 (100%) |
| **Feeding** | | |
| **Fresh chicken** | 46 (69 %) | 16 (73%) |
| **Freezing chicken** | 20 (31%) | 6 (25%) |
| **Geographic distribution** | | |
| **Urban** | 48 (73%) | 22 (100%) |
| **Rural** | 18 (27%) | 0 (0%) |
| **Stress** | 56 (84%) | 0 (0%) |

Table (4-2) illustrates the education level of PCOS patients which were (60%, 31%, 9%) for primary school, secondary school and graduated respectively. While the feeding of PCOS patients was fresh chicken in (69%) and freezing chicken in (31%). The geographic distribution of PCOS patients were (73%, 27%) for urban and rural respectively, while the state of stress for PCOS patients was (84%) based on questionnaire form.

## 4.2 The immunological and physiological study

The concentration levels of IL-18, IFN-γ, C3, T3, T4, TSH and insulin were estimated in the serum of the studied samples using ELISA technique except C3 was estimated using single radial immunodiffusion assay (RIDA) and TSH by Addendum-Mini VIDAS Apparatus (VIDAS).

### 4.2.1 Serum levels of IL-18, IFN-γ and C3 in patients and controls.

Table (4-3) illustrates the serum levels of IL-18, IFN-γ and C3 in PCOS patients and controls group. The results for the three parameters IL-18 , IFN-γ and C3 for PCOS patients were (609.04±34.94 pg/mL, 266.02±36.04 pg/mL, 89.71±3.16 mg/dL) respectively; while the result for the three parameters IL-18 , IFN-γ and C3 for controls group were (306.55± 44.16 pg/mL; 168.36±17.33 pg/mL; 65.51± 4.90 mg/dL) respectively, all

results were with high significant differences (P<0.001) as compared to controls group , except in IFN-γ has a significant difference (P<0.05) in patients as compared to controls group .

**Table (4-3): Serum levels of IL-18, IFN-γ and C3   in patients and controls.**

| Parameters | PCOS patients N=66 | Controls N=22 | P-value |
|---|---|---|---|
| **IL-18 (pg/mL)** | 609.04± 34.94[**] | 306.55± 44.16 | 0.001 |
| **IFN-γ (pg/mL)** | 266.02± 36.04[*] | 168.36±17.33 | 0.017 |
| **C3 (mg/dL)** | 89.71± 3.16[**] | 65.51± 4.90 | 0.001 |

* (P≤0.05) ,** (P≤0.001)

## 4.2.2 Serum levels of hormones (T3, T4, TSH and insulin) in patients and controls.

Table (4-4) revealed the serum levels of T3, T4, TSH and insulin in PCOS patients and controls. The result for the four parameters T3, T4, TSH and insulin  for  PCOS patients were (1.01± 0.03. ng/mL, 7.81±0.11μg/dL, 2.94±0.15 μIU/mL, 23.01±1.38 μIU/mL)  respectively, while the result in the controls group for T3, T4, TSH and insulin were (0.98±0.07 ng/mL; 7.37±0.25 μg/dL; 2.89±0.22 μIU/mL ;12.20±0.64 μIU/mL) respectively, there was a highly significant difference (P<0.001) in insulin level in PCOS patients compared to controls group, while there was no significant difference in T3 , T4, TSH levels between the two group .

**Table (4-4):  Hormones levels (T3, T4, TSH and insulin) in patients and controls.**

| Parameters | PCOS patients N=66 | Controls N=22 | P-value |
|---|---|---|---|
| **T3 (ng/mL)** | 1.01± 0.03 | 0.98± 0.07 | 0.703 |
| **T4 (µg/dL)** | 7.81± 0.11 | 7.37± 0.25 | 0.121 |
| **TSH (µIU/mL)** | 2.94±0.156 | 2.89±0.22 | 0.861 |
| **Insulin (µIU/mL)** | 23.01± 1.38[**] | 12.20± 0.64 | 0.001 |

** (P≤0.001)

### 4.2.3 Relationship of obesity with studied parameters (IL-18, IFN-γ and C3) in PCOS patients.

Table (4-5) demonstrates the effect of obesity on the serum levels of IL-18, IFN-γ and C3 in PCOS patients; there was a highly significant difference (P<0.001) in the serum levels of IL-18 between obese and non-obese women with PCOS, while there was no significant difference in the serum levels of IFN-γ and C3 between the two groups. The result for the three parameters IL-18, IFN-γ and C3 for (BMI $\leq$ 25 Kg/m$^2$) were (215.97±9.33 pg/mL, 237.86±14.27 pg/mL, 87.88±4.68 mg/dL) respectively, while the results for three parameters for (BMI > 25 Kg/m$^2$) were (281.30±14.13 pg/mL, 243.79±14.35 pg/mL, 91.13±4.32 mg/dL) respectively.

**Table (4-5): Effect of obesity on IL-18, IFN-γ and C3 in PCOS patients.**

| Parameters | Normal (BMI $\leq$ 25 Kg/m$^2$) N=29 | Overweight and obese (BMI > 25 Kg/m$^2$) N=37 | P-value |
|---|---|---|---|
| IL-18 (pg/mL) | 215.97± 9.33 | 281.30± 14.13[**] | 0.001 |
| IFN-γ (pg/mL) | 237.86±14.27 | 243.79±14.35 | 0.929 |
| C3 (mg/dL) | 87.88± 4.68 | 91.13± 4.32 | 0.612 |

** (P$\leq$0.001)

### 4.2.4 Relationship of obesity with hormone levels (T3, T4, TSH and insulin) in PCOS patients

Table (4-6) demonstrates the relation of obesity with levels of hormones in PCOS patients; there was no significant differences in serum levels of T3 and T4, TSH between obese females with PCOS and non-obese females with PCOS, while there was a significant difference (p<0.001) in the level of insulin between obese and non-obese females with PCOS. The results for the four parameters T3, T4, TSH and insulin for (BMI $\leq$ 25 Kg/m$^2$) were (0.98±0.05 ng/mL, 7.86±0.18 μg/dL, 2.87±0.17 μIU/mL, 15.46±1.63 μIU/mL) respectively, while the result for the four

parameters for (BMI > 25 Kg/m$^2$) were (1.05±0.04 ng/mL, 7.84± 0.15 µg/dL, 2.93±0.23 µIU/mL, 24.97±1.77 µIU/mL) respectively.

**Table (4-6) Effect of obesity on hormone levels in PCOS patients.**

| Parameters | Normal (BMI ≤ 25 Kg/m$^2$) N=29 | Overweight and obese (BMI > 25 Kg/m$^2$) N=37 | P-value |
|---|---|---|---|
| **T3 (ng/mL)** | 0.98± 0.05 | 1.05± 0.04 | 0.397 |
| **T4 (µg/dL)** | 7.86± 0.18 | 7.84± 0.15 | 0.959 |
| **TSH (µIU/mL)** | 2.87±0.17 | 2.93±0.23 | 0.829 |
| **Insulin (µIU/mL)** | 15.46± 1.63 | 24.97± 1.77[**] | 0.001 |

** (P≤0.001)

## 4.2.5 Relationship of infertility with studied parameters (IL-18, IFN-γ and C3) in PCOS patients

Table (4-7) reveals the effect of infertility on serum levels of IL-18, IFN-γ and C3 in PCOS patients , there was no significant differences in serum levels of IL-18, IFN-γ and C3 in PCOS women with infertility or in PCOS women without infertility .The results for the three parameters  IL-18 , IFN-γ and C3 for infertility group  were (300.16± 10.52 pg/mL, 253.74±14.01  pg/mL, 84.98± 3.88 mg/dL) respectively, while the result for the three parameters in without infertility group were (291.05±18.47 pg/mL, 267.65±16.24 pg/mL, 85.08±5.93 mg/dL) respectively.

**Table (4-7): Effect of infertility on IL-18, IFN-γ and C3 in PCOS patients.**

| Parameters | With Infertility N=47 | Without infertility N=19 | P-value |
|---|---|---|---|
| **IL-18 (pg/mL)** | 300.16±10.52 | 291.05±18.47 | 0.671 |
| **IFN-γ (pg/mL)** | 253.74±14.01 | 267.65±16.24 | 0.857 |
| **C3 (mg/dL)** | 84.98±3.88 | 85.08± 5.93 | 0.898 |

## 4.2.6 Relationship of infertility with hormone levels (T3, T4, TSH and insulin)

Table (4-8) illustrates the effect of infertility on hormone levels in PCOS women, there was no significant differences in the serum levels of T3, T4, TSH and insulin in PCOS women with infertility or in PCOS

women without infertility. The result for the four parameters T3, T4, TSH and insulin for with infertility group were (0.98±0.04 ng/mL, 7.77± 0.15 µg/dL, 2.92±0.167 µIU/mL, 20.49±1.50 µIU/mL) respectively, while the result for the four parameters in without infertility group were (1.06±0.06 ng/mL, 7.61±0.15 µg/dL, 2.98±0.33 µIU/mL, 20.83±2.56 µIU/mL) respectively.

**Table (4-8): Effect of infertility on hormone levels in PCOS patients.**

| Parameters | With Infertility N=47 | Without infertility N=19 | P-value |
|---|---|---|---|
| T3 (ng/mL) | 0.98± 0.04 | 1.06± 0.06 | 0.355 |
| T4 (µg/dL) | 7.77± 0.15 | 7.61± 0.15 | 0.47 |
| TSH (µIU/mL) | 2.92±0.167 | 2.98±0.33 | 0.864 |
| Insulin(µIU/mL) | 20.49± 1.50 | 20.83± 2.56 | 0.909 |

## 4.2.7 Relationship of infertility sub group (primary and secondary) with studied parameters (IL-18, IFN-γ and C3) in PCOS patients

Table (4-9) reveals the comparison between serum levels of IL-18, IFN-γ and C3 between PCOS patients with primary and secondary infertility. The results showed that the difference in the mean levels of IL-18, IFN-γ and C3 between polycystic women with primary infertility and polycystic women with secondary infertility are not significant. The mean for the three parameters IL-18 , IFN-γ and C3 for primary infertility group were (299.95±13.36 pg/mL, 243.08±15.01 pg/mL, 82.97±5.59 mg/dL) respectively, while the mean of the three parameters for secondary infertility group were (315.82±15.77 pg/mL, 279.96±16.32 pg/mL, 83.06±5.03 mg/dL) respectively.

**Table (4-9): levels of IL-18, IFN-γ and C3 in PCOS patients with primary and secondary infertility.**

| Parameters | Primary infertility N=26 | Secondary infertility N=21 | P-value |
|---|---|---|---|
| IL-18 (pg/mL) | 299.95±13.36 | 315.82±15.77 | 0.447 |
| IFN-γ (pg/mL) | 243.08± 15.01 | 279.96± 16.32 | 0.65 |
| C3 (mg/dL) | 82.97± 5.59 | 83.06± 5.03 | 0.991 |

## 4.2.8 Relationship of infertility sub group (primary and secondary) with hormone levels (T3, T4, TSH and insulin) in PCOS patients

Table (4-10) illustrates the comparison between serum level of hormones between PCOS women with primary and secondary infertility. The results shows that the difference in the mean levels of T3 , T4, TSH and  insulin between polycystic women with primary infertility and polycystic women with secondary infertility are not significant. The mean for the four parameters T3, T4, TSH and insulin for primary infertility group were (0.99±0.07 ng/mL, 7.79±0.23 µg/dL, 2.85±0.22 µIU/mL, 22.94± 2.48 µIU/mL) respectively, and the mean for the four parameters in secondary infertility group were (0.97±0.05 ng/mL, 7.76±0.22 µg/dL, 2.9±0.22 µIU/mL, 23.01±2.18 µIU/mL) respectively.

**Table (4-10): Level of hormones in PCOS women with primary and secondary infertility**

| Parameters | Primary infertility N=26 | Secondary infertility N=21 | P-value |
|---|---|---|---|
| T3 (ng/mL) | 0.99± 0.07 | 0.97± 0.05 | 0.824 |
| T4 (µg/dL) | 7.79± 0.23 | 7.76± 0.22 | 0.929 |
| TSH (µIU/mL) | 2.85±0.22 | 2.9±0.22 | 0.867 |
| Insulin (µIU/mL) | 22.94± 2.48 | 23.01± 2.18 | 0.984 |

## 4.2.9 Relationship of hyperandrogenism with studied parameters (IL-18, IFN-γ and C3) in PCOS patients

Table (4-11) demonstrates the effect of hyperandrogenism on serum levels of IL-18, IFN-γ and C3 in PCOS women. There was a significant difference ($P<0.05$) in the serum level of IL-18 in women with PCOS having hyperandrogenic state compared to women with PCOS without hyperandrogenic states, while there was no significant differences in serum levels of IFN-γ and C3 between the two groups. The mean for the three parameter IL-18, IFN-γ and C3 for with hyperandrogenism group were (276.56±13.04 pg/mL, 309.85±15.21 pg/mL, 88.72± 4.60 mg/dL)

respectively, while the mean for the three parameters for without hyperandrogenism group were (232.82±12.53 pg/mL, 202.72±14.06 pg/mL, 91.12±4.01 mg/dL) respectively.

**Table (4-11): Relationship of hyperandrogenism with IL-18, IFN-γ, and C3 levels in PCOS patients.**

| Parameters | With Hyperandrogenism N=39 | Without Hyperandrogenism N=27 | P-value |
|---|---|---|---|
| IL-18 (pg/mL) | 276.56± 13.04 | 232.82± 12.53[*] | 0.016 |
| IFN-γ (pg/mL) | 309.85±15.21 | 202.72±14.06 | 0.126 |
| C3 (mg/dL) | 88.72± 4.60 | 91.12± 4.01 | 0.696 |

* ($P \leq 0.05$)

## 4.2.10 Relationship of hyperandrogenism with hormone levels (T3, T4, TSH and insulin) in PCOS patients

Table (4-12) illustrates the effect of hyperandrogenism on serum levels of T3, T4, TSH and insulin in PCOS patients. There was no significant differences in the levels of T3, T4 and TSH in women with PCOS in the presence or absence of hyperandroginc status, except in insulin level there was a significant differences ($P<0.05$) between these two state in patients group. The mean for the four parameters T3, T4, TSH and insulin in hyperandrogenism group were (0.95± 0.03 ng/dL, 7.64±0.15 μg/dL, 2.89±0.21 μIU/mL, 23.44±1.83 μIU/mL) respectively, while the mean for the four parameters in the group without hyperandrogenism were (1.09±0.06 ng/dL, 8.05±0.18 μg/dL, 2.9±0.23 μIU/mL, 17.27±1.74 μIU/mL) respectively.

**Table (4-12) Relationship of hyperandrogenism with hormone levels in PCOS patients.**

| Parameters | With Hyperandrogenism N=39 | Without Hyperandrogenism N=27 | P-value |
|---|---|---|---|
| T3 (ng/mL) | 0.95± 0.03 | 1.09± 0.06 | 0.079 |
| T4 (μg/dL) | 7.64± 0.15 | 8.05± 0.18 | 0.098 |
| TSH (μIU/mL) | 2.89±0.211 | 2.9±0.23 | 0.998 |
| Insulin (μIU/mL) | 23.44± 1.83 | 17.27± 1.74[*] | 0.018 |

* ($P \leq 0.05$)

## 4.2.11 Relationship of menstrual cycle (regular and irregular) with studied parameters (IL-18, IFN-γ and C3) in PCOS patients

Table (4-13) illustrates the comparison between serum levels of IL-18, IFN-γ and C3 in PCOS women with regular and irregular menstrual cycle. There was a significant increasing ($p<0.001$) in the serum levels of IL-18 in women with PCOS who have irregular menstrual cycle compared to women with PCOS who have regular menstrual cycle , while there were no significant differences in serum levels of IFN-γ and C3 between these two groups .The mean for the three parameters IL-18 , IFN-γ and C3 for regular menstrual cycle group were ($200.09\pm11.35$ pg/mL, $280.43\pm15.09$ pg/mL, $90.35\pm6.28$ mg/dL) respectively, and the mean for the three parameters for irregular menstrual cycle group were ($290.65\pm12.05$ pg/mL, $271.60\pm14.38$ pg/mL, $91.07\pm4.46$ mg/dL) respectively .

**Table (4-13): levels of IL-18, IFN-γ and C3 in PCOS patients with regular and irregular menstrual cycle.**

| Parameters | Regular menstrual cycle N=21 | Irregular menstrual cycle N=45 | P-value |
|---|---|---|---|
| **IL-18 (pg/mL)** | $200.09\pm11.35$ | $290.65\pm12.05$ [**] | 0.001 |
| **IFN-γ (pg/mL)** | $280.43\pm15.09$ | $271.60\pm14.38$ | 0.909 |
| **C3 (mg/dL)** | $90.35\pm6.28$ | $91.07\pm4.46$ | 0.926 |

** ($P\leq0.001$)

## 4.2.12 Relationship of menstrual cycle (regular and irregular) with hormones levels (T3, T4, TSH and insulin) in PCOS patients

Table (4-14) illustrates the comparison between level of hormones T3, T4, TSH and insulin in PCOS women with regular and irregular menstrual cycle; there was no significant differences in serum levels of T3, T4, TSH and insulin in PCOS women who have regular or irregular menstrual cycle. the mean for the four parameters T3, T4, TSH and insulin for regular menstrual cycle group were ($1.02\pm0.06$ ng/mL, $7.87\pm0.23$

µg/dL, 2.96±0.33 µIU/mL, 17.74±1.86 µIU/mL) respectively, and the mean for the four parameters for irregular menstrual cycle group were (0.99±0.045 ng/mL, 7.78±0.14 µg/dL, 3.01±0.21 µIU/mL, 22.82± 1.83 µIU/mL) respectively.

**Table (4-14): Levels of hormone in PCOS patients with regular and irregular menstrual cycle.**

| Parameters | Regular menstrual cycle N=21 | Irregular menstrual cycle N=45 | P-value |
|---|---|---|---|
| T3 (ng/mL) | 1.02±0.06 | 0.99±0.045 | 0.754 |
| T4 (µg/dL) | 7.87±0.23 | 7.78±0.14 | 0.759 |
| TSH (µIU/mL) | 2.96±0.33 | 3.01±0.21 | 0.91 |
| Insulin (µIU/mL) | 17.74±1.86 | 22.82±1.83 | 0057 |

## 4.3 Correlation

Correlation of study parameters with BMI and age is shown in (Table 4-15) and (Table 4-16) respectively. There was a significant correlation (p<0.001) between BMI and IL-18, C3 and insulin, while there was no significant correlation of IFN-γ, T3, T4 and TSH to BMI. On the other hand, no correlation was observed between age and other parameters except with T4 levels which have a negative correlation with age.

Furthermore, a significant positive correlation was found between IL-18 and C3 but not with IFN- γ; also a significant correlation was revealed between IL-18 and insulin while no correlation was detected with T3, T4 and TSH (Table 4-17). Moreover, no significant correlation was observed between IFN- γ and other studied parameters except with insulin hormone (Table 4-18). C3 recorded a significant correlation with insulin only but not with other studied parameters (Table 4-19). However, no correlation was found between insulin and the three hormones (T3, T4 and TSH) (Table 4-20). Furthermore, no correlation was found between the

three hormones (T3, T4 and TSH) (Table 4-21).All figures of result and correlation in Appendix A

**Table (4-15): Correlation of BMI and other studied parameters.**

| Parameters | Pearson Correlation coefficient | P value |
|---|---|---|
| **BMI and IL-18** | 0.383** | 0.001 |
| **BMI and IFN-γ** | 0.196 | 0.067 |
| **BMI and C3** | 0.311** | 0.003 |
| **BMI and T3** | -0.027 | 0.806 |
| **BMI and T4** | 0.068 | 0.53 |
| **BMI and TSH** | 0.019 | 0.861 |
| **BMI and Insulin** | 0.531** | 0.001 |

** (P≤0.001)

**Table (4-16): Correlation of age and other studied parameters.**

| Parameters | Pearson Correlation coefficient | P value |
|---|---|---|
| **Age and IL-18** | 0.051 | 0.635 |
| **Age and IFN-γ** | -0.02 | 0.855 |
| **Age and C3** | 0.092 | 0.393 |
| **Age and T3** | 0.061 | 0.57 |
| **Age and T4** | -0.307* | 0.004 |
| **Age and TSH** | 0.084 | 0.435 |
| **Age and Insulin** | 0.062 | 0.567 |

* (P≤0.05)

**Table (4-17): Correlation of IL-18 and other studied parameters.**

| Parameters | Pearson Correlation coefficient | P value |
|---|---|---|
| **IL-18 and IFN-γ** | 0.163 | 0.128 |
| **IL-18 and C3** | 0.242* | 0.023 |
| **IL-18 and T3** | -0.09 | 0.405 |
| **IL-18 and T4** | 0.096 | 0.405 |
| **IL-18 and TSH** | 0.065 | 0.549 |
| **IL-18 and Insulin** | 0.305** | 0.004 |

* (P≤0.05) ,** (P≤0.001)

**Table 4-18: Correlation of IFN-γ and other studied parameters.**

| Parameters | Pearson Correlation coefficient | P-value |
|---|---|---|
| IFN-γ and C3 | 0.188 | 0.079 |
| IFN-γ and T3 | 0.006 | 0.957 |
| IFN-γ and T4 | -0.08 | 0.458 |
| IFN-γ and TSH | -0.005 | 0.96 |
| IFN-γ and Insulin | 0.263[*] | 0.013 |

* (P≤0.05)

**Table (4-19): Correlation of C3 and other studied hormones.**

| Parameters | Pearson Correlation coefficient | P-value |
|---|---|---|
| C3and T3 | 0.015 | 0.829 |
| C3 and T4 | -0.011 | 0.92 |
| C3 and TSH | 0.102 | 0.345 |
| C3 and Insulin | 0.483[**] | 0.001 |

** (P≤0.001)

**Table (4-20): Correlation of insulin and other studied hormones.**

| Parameters | Pearson Correlation coefficient | P value |
|---|---|---|
| Insulin and T3 | 0.019 | 0.858 |
| Insulin and T4 | 0.131 | 0.224 |
| Insulin and TSH | 0.041 | 0.707 |

**Table (4-21): Correlation between studied parameters (T3, T4, and TSH)**

| Parameters | Pearson Correlation coefficient | P-value |
|---|---|---|
| T3 and T4 | 0.007 | 0.945 |
| T3 and TSH | 0.01 | 0.923 |
| T4 and TSH | 0.017 | 0.875 |

# Chapter five
# Discussion

# 5. Discussion

Polycystic ovary syndrome is one of the most popular endocrine disorders among females. In fact, Emphasis on its importance rise from increased prevalence of cardiovascular diseases and higher cardiovascular morbidity even in young and thin women with PCOS this may be partially due to low grade inflammation. Excessive amount of visceral or ectopic fat, a feature of most women with PCOS played an essential role in cardiovascular disorders and atherosclerotic changes (Mathieu *et al.*, 2008).

Evidence of low-grade chronic inflammation in PCOS is indicated by the presence of several elevated markers such as inflammatory cytokines (i.e. IL-6, IL-18 and IFN-γ, in addition to C3) (Thyagaraju, 2014; Qin *et al.*, 2016; Ibrahim and Al-Saffar, 2018).

Immune system is influenced by estrogen /progesterone ratio (Khan and Ansar, 2016). Patients with PCOS are presented with low progesterone level as a result of oligo ovulation or anovulation, so the immune system could be overstimulated by excess estrogen leading to promote production of several cytokines in these patients (Angstwurm *et al*., 1997).

## 5.1 Basic Characteristics of Study Groups:

In this study which involved 66 women with PCOS , the average age of patients was higher than controls (Table 4-1). This result is in agreement with (Yan Yang *et al*., 2011) and slightly lower than (Alfatlawi, 2017) and lower than (ELMekkawi *et al*., 2010 ) and higher than (Ateia *et al.*, 2013).The similarities between these studies regarding the same age group because PCOS appeared at menarche and the women became symptomatic later but most women with PCOS are diagnosed when their age between 20-30 yrs (Bronstein *et al*., 2011).

BMI in PCOS females was higher than controls (Table 4-1).This result is in agreement (Ateia *et al*., 2013) who showed that there is high prevalence of obesity in PCOS women. Obesity is a common feature in PCOS women, the relation between adiposity with menstrual disturbance and hyperandrogenic status in PCOS is confirmed by data that detect an improvement in these parameters with weight loss (Caren *et al*., 1999). Obesity may play a pathogenetic role in the development of the syndrome in susceptible individuals, this due to endocrine function of adipose tissue. Adipose tissue is an endocrine organ populated by different cell types, such as pre adipocytes, mature adipocytes and macrophages (Romacho *et al*., 2014). Adipose tissue involved in innate and adaptive immune response by producing cytokines such as TNF-α, IL-6 and IL-18 which contribute to the establishment of IR of systemic inflammatory status and of other cardio metabolic risk factors in the long term. The present study showed that women with PCOS in our country have a higher body weight than other counterparts (Azziz *et al*., 2001; Carmina *et al*., 2003), which may be due to the different nutritional habits in Iraq than other countries and the lack of exercise among Iraqi women.

In the present study, it was found a highly significant difference (P<0.001) in serum level of LH between PCOS patients and control group , while there was no significant difference in serum level of FSH between PCOS patients and control group (Table 4-1), this result is in agreement with studies done by AL-Razzaq, (2014) and Al-Musawy *et al*., (2018). Study by Taylor *et al*., (1997) reported that 75% of women with clinical evidence of PCOS have an elevated LH level. LH and FSH synthesis and secretion are highly dependent on the pattern of the GnRH stimulus, with rapid frequencies favoring LH and slower pulses FSH synthesis and secretion. The underlying cause of this pattern of gonadotropin secretion is linked to an accelerated GnRH pulse generator

activity and heightened pituitary response to GnRH, which in turn results in an increase in the LH/FSH ratio (Hayes *et al*., 1998).

The GnRH pulse frequency designates the preferential production of LH via high frequency pulses versus FSH via low frequency pulses in normal adult women. The pulse frequency is regulated by progesterone in presence of estradiol, such that increased progesterone production by corpus luteum slows LH pulse frequency to favor FSH production, which aids in follicular development for the next menstrual cycle (Hayes et al., 1998). The cause of LH hypersecretion in PCOS is probably due to enhanced pituitary sensitivity to GnRH or to changes in GnRH secretion patterns rather than increased GnRH secretion. It appears to be a result of an acquired impaired sensitivity of the hypothalamic pulse generator to the negative feedback of estrogen and progesterone in PCOS, possibly by chronic estrogen exposure. An abnormal feedback mechanism by ovarian estrogen is blamed to play role in this discriminated increase in LH release, and there is no cyclic production of progesterone by a corpus luteum in PCOS, in addition the pituitary and hypothalamus are less sensitive to the inhibitory effect of exogenous progesterone on LH secretion in PCOS (McKenna, 1988).

Altered sex steroid production, metabolic dysfunction, and obesity may all contribute to the changes in LH secretion pattern (Patel *et al*., 2004), Insulin resistance/hyperinsulinemia may directly or indirectly contribute to the abnormal gonadotropin secretion (Allahbadia *et al*., 2011).

It was found that PCOS women had infertility rate 66% in the study of  Joham *et al*., (2015) and 68%  in Al-Musawy *et al*., (2018) study and in the present study it was 71.2%, but these studies are based on data collected from fertility clinics and hospitals and there's no study determined the natural history of the prevalence of infertility in PCOS, so further study is needed.

High percentage of hirsutism in PCOS women which was recorded in this study (table 4-1). This result is in agreement with Barberri *et al.*, (1986) who explained that all patients with PCOS have an increased sensitivity to androgens, up to 70% have elevated levels, and the other 30% are in the normal range. Main sources of excessive amount of androgen in PCOS are the ovaries, mainly theca cells and the zona reticularis of the adrenal cortex (Lizneva *et al* .,2016), In the ovarian theca cells, androgen biosynthesis are mediated by cytochrome P-450c17 enzymes to form androstenedione which converted to testosterone by 17b-hydroxysteroid enzyme or aromatized to form estrone. Tsilchorozidou *et al.*, (2004) showed that PCOS ovaries have an increased cytochrome P-450c17 enzymatic activity, leading to enhanced the synthesis of androgenic precursors and thereby testosterone. Many researchers found an association between elevated testosterone, blood pressure, abnormal lipids metabolism with cardiovascular diseases in PCOS, suggesting that hyperandrogenism increases the cardiovascular risk in PCOS (Phillips *et al.*, 1997). However, the association between hyperandrogenism and cardiovascular risk is not universally accepted (Verthelyi, 2001). Therefore many studies may be useful to find out the interaction between immune signals and androgens that may provide biomarkers for a pre-disease existence in women with PCOS at risk to develop cardiovascular diseases.

Menstrual irregularity that present in our study might be considered as a marker for IR in PCOS. Irregular menstrual cycle (Oligomenorrhea and amenorrhea) has been associated with hyperinsulinemia and with increased prevalence and future risk of type 2 diabetes mellitus (Solomon *et al.*, 2002). The irregularity of the menstrual cycle in PCOS subject is due to anovulatory cycle. The state of anovulation will cause no formation of corpus luteum, consequently no progesterone will be secreted so the endometrium continues its

proliferative phase excessively (Ehrmann, 2005). As a result; menstrual disturbance, amenorrhea, oligomenorrhea, or dysfunctional uterine bleeding and infertility will occur, for the same reason dysmenorrhea occurs, since cystic ovaries have also been described in women with venous congestion resulting in pelvic pain. This condition is thought to arise from abnormal relaxation of the pelvic veins and may respond to progesterone therapy (Beard et al., 1988).

In this study, 73% of PCOS patients were live in urban with low level of education, in addition to the feeding habit ; 69% of PCOS patients depended on farm fresh chicken, and according to the questionnaire form most patients with PCOS 84% suffer from stress (Table 4-2).

Stress and anxiety are common in women with PCOS but are often overlooked and therefore left untreated. In the present study, 84% of PCOS women were suffered from stress (Table 4-2), this high level of stress and anxiety may be due to various reasons such as hirsutism, alopecia , infertility and obesity which is the most prominent feature causing an elevation in emotional stress level, and this state in long term lead to depression among these women. Factors such elevated cortisol levels, and low level of serotonin are associated with both insulin and depression. As a result of the PCOS consequence, secretion of cortisol will be thwart the effect of insulin and increased blood sugar levels which lead to IR in PCOS women (Rasgon et *al*., 2003; Sadeeqa *et al.,* 2018).

## 5.2 Immunological study

## 5.2.1 Serum levels of IL-18, IFN-γ and C3 in patients and controls

### 5.2.1.1 Serum level of IL-18 in patients and controls

It was found that the average level of IL-18 significantly higher (p<0.001) in PCOS women than in control group (Table 4-3), it is the strongest predictor of PCOS among the studied parameters by using stepwise multi pearson correlation coefficient, the increase in IL-18 level found in PCOS patients is probably related to the visceral adiposity, and IR, frequently found in those patients (Escobar-Morreale *et al*., 2004).

Moreover, in this study it was found a highly significant difference (p<0.001) in the serum level of IL-18 between obese females with PCOS and non-obese females with PCOS (Table 4-5), this result is in agreement with Esposito *et al.,* (2003) who reported that serum IL-18 concentrations are increased in obese women and decrease after weight loss. But this result is disagree with other studies performed by Yan Yang *et al*., (2011) and Al-Musawy *et al*., (2018 ) which reported that serum levels of IL-18 was elevated in both lean and obese women with PCOS. This disagreement may be due to the effect of different demographical factors and inclusion criterias. Also, it was found that the average serum level of IL-18 was significantly increased (P<0.05) in PCOS women with hyperandrogenism than in PCOS women without hyperandrogenism (Table 4-11), this result is in agreement with study performed by Escobar-Morreale *et al*., (2007) and disagree with another study performed by Al-Musawy *et al*., (2018) which reported that there was no significant difference in serum level of IL-18 in PCOS women in the presence or absence of hyperandroginc status. To explain the high level of IL-18 in the presence of hyperandroginc status; in this study, it was recorded 59% of PCOS women have evidence of

hyperandrogenism, it refers to increased blood levels of androgens. Escobar-Morreale et al., (2007) reported that androgen excess, as a feature of PCOS women induce adipocytes to be prone to hypertrophy. This hypertrophic adipose tissue release several adipokines and inflammatory mediators such as TNF-α, IL-6 and IL-18, and these cytokines that associate with insulin resistance/ hyperinsulinemia, which promotes additional increase in androgen secretion by the ovary.

In the present study, it was found a significant difference ($P<0.001$) in serum levels of IL-18 between regular menstrual cycle and irregular menstrual cycle (Table 4-13) in PCOS females and this result is in agreement with Iraqi study performed by Mehde, (2009) and disagree with another Iraqi study performed by Al-Musawy *et al*., (2018) who found that there was no significant difference in serum level of 1L-18 between regular and irregular menstrual cycle in PCOS patients. These differences may be due to the effect of different demographical factors and inclusion criteria. moreover, IL-18 has positive correlation with BMI ($P<0.001$) in obese and non-obese PCOS females (Table 4-15), this result is in agreement with Mehde, (2009) and Yan Yang *et al.*, (2011), while disagree with Al-Musawy *et al*., (2018 ) who reported that IL-18 had positive correlation with BMI in non-obese PCOS females .This difference may be due to the difference in inclusion criteria and inability to exclude all factor that elevate serum IL-18 levels. Moreover, in this study it was found a positive correlation ($P<0.001$) between IL-18 and insulin in PCOS patients (Table 4-17), this result agree with Esposito *et al*., (2002) who found that serum IL-18 concentrations correlated with surrogate indexes of IR, such as the waist-to-hip ratio and fasting insulin levels, suggesting that the increase in serum IL-18 levels is related not only to obesity but also to IR. Therefore, IL-18 might be a useful serum marker of the inflammatory process associated with obesity and IR.

In present study, it was observed a positive correlation (P<0.001) between IL-18 and C3(Table 4-17). In fact IL-18 is a pro inflammatory cytokine that induce the production of TNF- α, which in turn promote the synthesis of IL-6, and as we known IL-6 is consider a hepatic cell inducer to produce acute phase protein like CRP and complement component C3 (Moshage *et al.*, 1988; Paszkiewicz, 1993)

Elevation of IL-18 has been postulated to have several deleterious effects. Higher serum IL-18 levels appear to be associated with atherosclerosis (Yamagami *et al.*, 2005). Serum IL-18 levels are associated with cardiovascular death in patients with ischemic heart disease and with coronary events (Mallat *et al.*, 2001).

Increased risk of ischemic heart disease has been reported in PCOS women in comparison with healthy control (Guzick *et al.*, 1996). PCOS is associated with endothelial dysfunction, decreased vascular compliance, and early carotid atherosclerotic changes. It is possible that these findings are due in part to increased IL-18 levels

### 5.2.1.2 Serum level of IFN-γ in patients and controls

In the present study it was found that the level of IFN-γ is significantly increased (p<0.05) in PCOS women as compared to control group (Table 4-3). There are no previous published data link between serum level of IFN-γ and demographic factor in PCOS patients. one study performed by Qin *et al.*, (2016 ) which reported that the immune dominance of Th1 may be the immunological feature of the ovary in PCOS patients , confirmed that by enrich the lymphocytes from follicle fluid (ff) of the ovary and detect the Th1 /Th2-associated cytokine profile in PCOS patients , and found that the production of Th1 (IFN-γ, IL-2) cytokines in (ff) lymphocytes in PCOS patients were significantly higher than healthy women, while the production of Th2 (IL-4, IL-10) cytokines did not show statistic differences between the two groups .Additionally, change level in inflammatory cytokines that produce by

Th1 and Th2 are contributes to ovarian follicle maturation; it may cause the poor quality of ovum and dysfunction of ovulation if imbalance occurs.

Similar to other pro-inflammatory cytokines, IFN-γ appears to be association with cardiovascular disease (Mattina et al., 2019), and as we know PCOS patients under the risk of cardiovascular disease even in young and thin women , so it is possible that this state  are due in part to increased IFN-γ levels.

Moreover, the present study showed that there was no significant difference in level of IFN-γ between obese and non-obese  women with PCOS (Table 4-5). This result shows the reason for elevation level of IFN- γ in PCOS women could be due to factors other than obesity. To clarify more, immune system is affected by estrogen/progesterone ratio (Khan and Ansar, 2016), and women with PCOS are presented with low progesterone level as a result of oligo ovulation or anovulation therefore the immune system could be overstimulated by excess estrogen leading to stimulate the production of IL-4 in Th2 lymphocyte, IL-1 in monocytes, IL-6 in T-lymphocytes and IFN-γ in Th1 cells (Angstwurm *et al*., 1997). on the other hand, this study revealed that IL-18 was elevated in PCOS women; IL-18 was first described as IFN-γ inducing factor, and has multiple functions which include of the synthesis of IFN-γ by T cells and NK cells, promotion of Th1-type immune response, augmentation of proliferative response and cytokines production of activated T Cells (Okamura *et al*., 1995; Yan Yang *et al.*, 2011).

### 5.2.1.3 Serum level of C3 in patients and control

In our study, serum level of C3 was estimated, as a marker of low grade chronic inflammation, as suggested in previous studies. It was found that the average level of C3 significantly higher ($P<0.001$) in PCOS women in comparison to control group (Table 4-3). This indicates an increase in the level of inflammation in PCOS women. These results

was in agreement with Iraqi study done by Wadood *et al.,* (2015), and disagree with Iranian study done by Dehdashtihaghighat *et al.*, (2013) who found that there was no significant difference in serum level of C3 between PCOS women and healthy women. This difference may be due to variation in BMI between our study and the Iranian study which done by Dehdashtihaghighat *et al.*, (2013). Moreover, this study revealed there was no significant difference in serum level of C3 between obese and non-obese PCOS women and this result agree with Thyagaraju, (2014) and disagree with Yang *et al.*, (2011) who reported that the level of C3 was more in obese PCOS women as compared to non-obese PCOS women. On the other hand, in the comparison of all PCOS women (obese and non-obese) group (Table 4-15), a positive correlation (P<0.001) of C3 to BMI was observed (the indicators of obesity). This result emphasizes that C3 is an acute-phase reactant produced not only by the liver but also by adipocytes and predicts weight gain in humans, suggesting that plasma C3 partly reflects fat mass (Scantlebury *et al.*, 2001; Muscari, 2007). It's a hepatic production, is induced by cytokines like IL-1 and TNF-α which already elevated in PCOS women as evident by previous studies, as considered PCOS as a low-grade chronic inflammation, and these cytokines may be interfere with insulin receptor functioning and cause IR (Marette, 2002; Dehdashtihaghighat *et al.*, 2013), and this may be illustrate the positive correlation (P<0.001) between complement C3 and insulin that observed in this study (Table 4-15). These results agree with (Yang *et al.*, 2011) who reported that C3 might be a stronger inflammatory marker of IR in women with PCOS.

Higher serum C3 levels appear to be associated with the main endogenous cardiovascular risk factors, and associated with tissue damage at the site of myocardial infarctions (Onat *et al.*, 2010). Also, increased risk of cardiovascular disease has been reported in PCOS women in comparison with healthy women (Talbott *et al.*, 1995), PCOS

was associated with endothelial dysfunction, decreased vascular elasticity, and early carotid atherosclerotic changes, as mentioned earlier. It is possible that these findings are due in part to increased C3 levels.

## 5.3 Physiological study

## 5.3.1 Serum levels of hormones (T3, T4, TSH and insulin) in patients and controls.

### 5.3.1.1 Serum levels of (T3, T4 and TSH) in patients and controls

In the present study, there is no statistical significant differences in the serum levels of T3, T4 and TSH between PCOS females and control (table 4-4), this result is consistent with Iraqi study performed by AlFaisal, and Al-Deresawi, (2013) and disagree with another Iraqi study performed by Zwain and Aziz, (2016) who found that thyroid disorders were detected in 16% of PCOS women. Moreover, this study revealed that there are no significant differences in serum levels of T3, T4 and TSH in all studied demographic factors. Both the thyroid and ovaries are part of the endocrine system and belong to a common hormonal axis consisting of hypothalamus-pituitary–thyroid–ovaries, and according to recent studies, many evidences showed that women who suffered from PCOS present in most cases, thyroid disorders which is often associated with hypothyroidism or at risk of future hypothyroidism (Allahbadia and Merchant, 2011). The hypothyroidism may lead to lower levels of sex hormone binding globulin (SHBG), which in turn leads to high concentrations of testosterone, one of the factors that contribute to the onset of some symptoms of PCOS such as infertility, polycystic ovaries, hirsutism and acne (Dittrich *et al*., 2009) . In the current study, no significant changes were found in the thyroid hormones among PCOS patients which may link the PCOS with another factor such as obesity and IR.

### 5.3.1.2 Serum level of insulin in patients and control.

In the present study, it was found that the serum level of insulin to be significantly higher (P<0.001) in PCOS females than in control group and this result is in agreement with almost studies that measure serum level of insulin in PCOS patients ( Wesen, 2008; Mehde, 2009 and YanYang *et al*., 2011). In our results, there are 53.3% of PCOS patients who had abnormal insulin value according to cut-off value ≥25 µIU/mL. Other studies showed that this percentage was 81.22% as in the study of Carmina and Lobo, (1999) , 93.75% in Wesen, (2008) study and 96.67% in Mehde, (2009) study of patients with abnormal insulin and PCOS. The different percentage of insulin in PCOS between studies might be due to selection of different clinical and endocrine features associate PCOS.

This study revealed that the serum level of insulin was increased significantly (P<0.001) in obese women with PCOS than lean women with PCOS (Table 4-6), this result agree with previous study performed by Dale *et al*., (1992) and disagree with other study performed by Gupta *et al*., (2015) who reported that the differences in levels of insulin between obese and lean women with PCOS is not significant, while Yildizhan *et al*., (2016) found higher insulin levels in lean women with PCOS as compared to normal women. The elevated level of insulin in PCOS patients may be related to ethnic background and different life styles (Wijeyaratne *et al*., 2002) or may be due to the defect of post binding in insulin signaling, especially in the major insulin target tissues like adipocytes and skeletal muscles (Corbould *et al*., 2005). Elevated fasting insulin level greatly than 20 µIU/mL may alone indicate of IR (Traub, 2011). However, in the past 20 years there has been growing evidence supporting that the defects in insulin actions or in the insulin signaling pathways are central in the pathogenesis of PCOS. In fact most of these females are metabolically IR, in part due to genetic

predisposition and in part secondary to obesity. But some women with typical PCOS do not display IR, which supports the hypothesis of a genetic predisposition specific to PCOS that would be revealed by the development of IR and compensatory hyperinsulinemia in most, but not all women with PCOS (Baptiste *et al.*, 2010). However, these hypotheses are not yet appropriately confirmed, and more research is still needed to unravel the true pathogenesis underlying this syndrome.

In present study, it was found that serum level of insulin significantly increased ($P<0.05$) in PCOS females with hyperandrogenism than in PCOS females without hyperandrogenism (Table 4-12). This result was in agreement with several studies that associate between insulin and hyperandrogensim in PCOS patients (Baptiste *et al.*, 2010; Allahbadia and Merchant, 2011; Al-Watify, 2014), and these studies reported that hyperinsulinemia which has been proposed as the primary event leading to hyperandrogenism .At the level of ovary, insulin acts synergistically with LH to enhance androgen production of the theca cells via cytochrome P450C 17 alpha. Furthermore, Insulin has been shown to decrease secretion of (SHBG) which in turn increase the amount of unbound (free) or bioactive testosterone. Moreover, free testosterone stimulates androgen receptors of the pilosebaceous unit which can lead to the clinical findings of hyperandrogenism such as hirsutism and acne (Tsilchorozidou *et al.*, 2004).

The elevation of these inflammatory markers (IL-18, IFN- γ and C3) in polycystic women are signals for alteration of immune function and this may help in identification subjects with increased risk of cardiovascular disease and metabolic syndrome and also help in prevention of this disease and may give a hint on the use of anti-inflammatory agents to reduce these marker and prevent complications (Al-Musawy *et al.*, 2018).

Finally, the present study and other previous studies that performed in Iraq and in another country showed that the disturbance of the immune system was a common feature for the PCOS and might led to production of antibodies and development of autoimmunity (Escobar-Morreale *et al*., 2004; Yan Yang *et al*., 2011; Ateia *et al*., 2013; Wadood *et al*., 2015; Al-Musawy *et al*., 2018).

# Conclusions
# and
# Recommendations

# Conclusions and Recommendations

## Conclusions

1. The levels of IL-18, IFN-γ, and C3 were highly statistically significant in PCOS comparing to normal women and these high levels were related to PCOS independent on the presence of obesity, or IR.

2. IL-18 may be a useful serum marker of the inflammatory process associated with obesity and IR.

3. No significant changes were found in the thyroid hormones among PCOS patients and control which may link the PCOS with another factor such as obesity and IR.

4. The level of insulin was highly significant in PCOS comparing to normal women, this high level may be related to inflammatory cytokines which elevated in PCOS women that interfere with insulin receptor functioning and cause IR.

5. There is a positive correlation between IL-18 and C3, and between these two parameters with obesity and hyperandroginsim, also there was a positive correlation between insulin and the three parameters (IL-18, IFN-γ and C3), this may be due to the back ground of PCOS which consider as inflammatory disease.

**Recommendation**

1. The three parameters (IL-18, IFN-γ and C3) is a good tool to prognostic the cardiovascular disease in PCOS women, also it may consider as a biomarker to evaluate the progression of metabolic disturbance that may cause anovulation which may lead to the infertility.

2. Study the effect of ovulation drugs on the level of IL-18, IFN-γ, and C3 in PCOS women.

3. Evaluate the levels of IL-18, IFN-γ, and C3 during different stage of menstrual cycle.

4. A molecular and genetic study for all pro inflammatory cytokines include the understudied cytokines are requires.

5. Increase the awareness among women with reproductive age by following a balanced diet and exercise to avoid obesity, which have a great impact on causing PCOS.

6. Study the cytokine profile in order to get a clear picture for the immune disturbance in PCOS patients.

7. Investigate the T cell count by using flow cytometer technique to follow the deviation of immune response.

8. Study the role of stress in immune response in PCOS patients.

9. Ministry of health classified PCOS as a non-inflammatory disease and the present study and other previous studies that performed in Iraq and in another country showed that the PCOS was inflammatory disease that lead to development of autoimmunity .

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# Appendices

# Appendices

## Questionnaire for female patients

File number..........
Religion ……….
Ethnicity……….
Name ……….  Age ………. years
Height ……….cm, weight……….kg
City origin ……….
Education level ……..
Married? …… years
Number of children ……..
History of infertility …….
Have you history of abortion?
Have you been diagnosed with other disease?
Have you taken any drug?
Smoking ……?
Are the family member have infertility problem?
Feeding? (Type of food)
Stress? ……
Menstrual cycle?  Regular ……  Or  irregular ……
PCOS diagnosed by:
1- Ultra sound
2- FSH……, LH……
3-TSH ……
4-Othe symptoms
Hirsutism?
When diagnosed ?
Husband's health ?

**Appendix (1): Questionnaire for female patients.**

# Figure of serological study
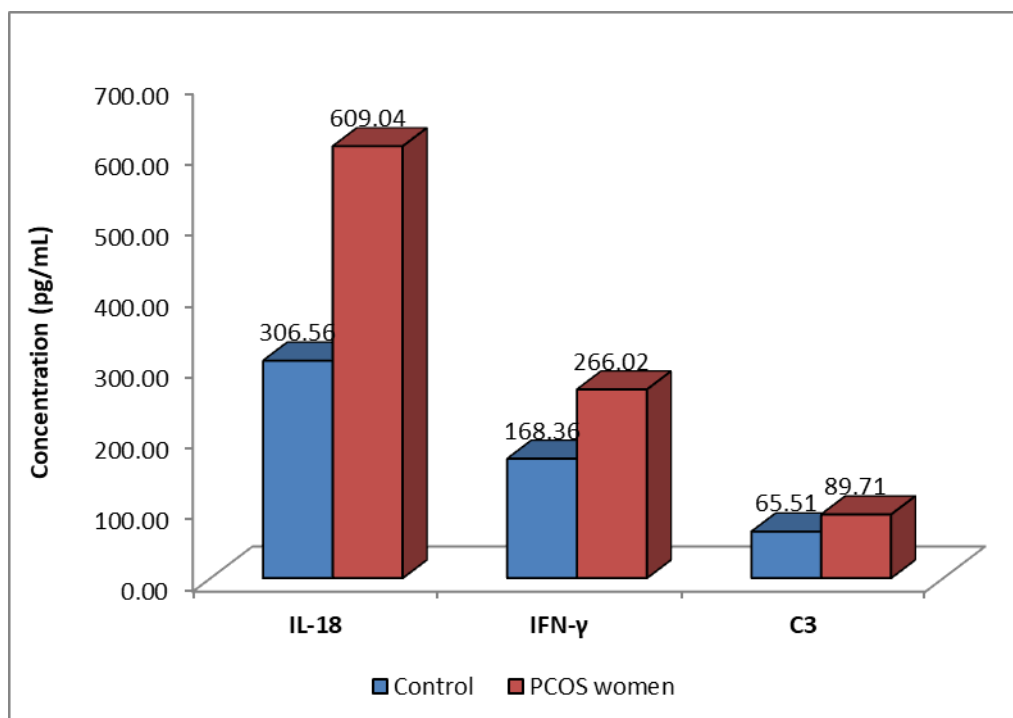


**Appendix (2) : Serum levels of IL-18, IFN-γ and C3 in patients and control.**
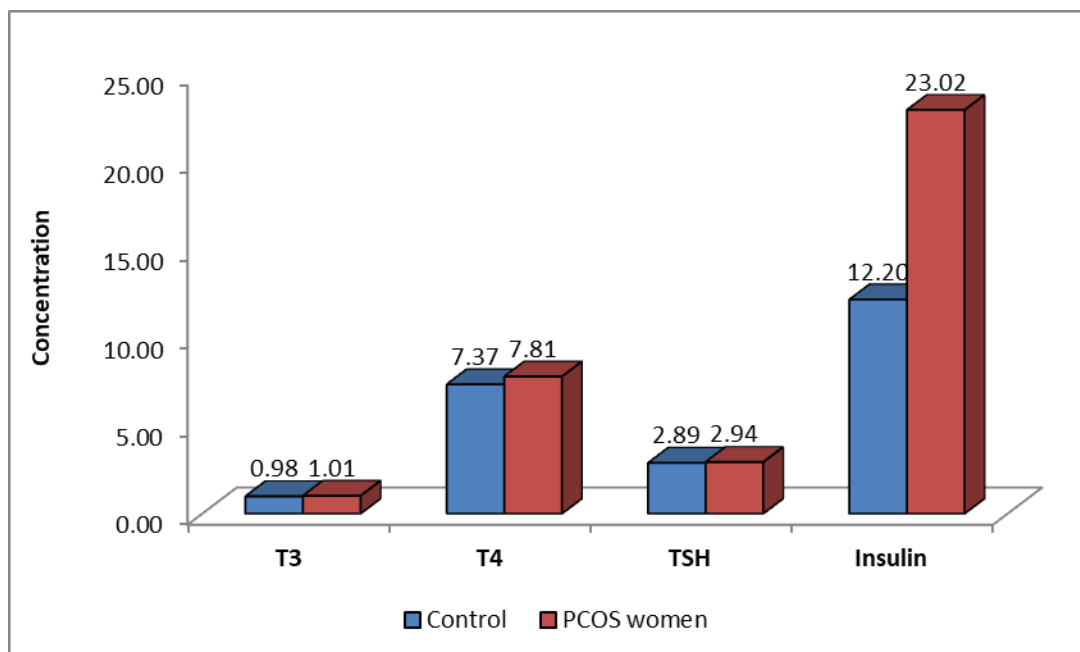


**Appendix (3): Hormones levels in patients and control.**
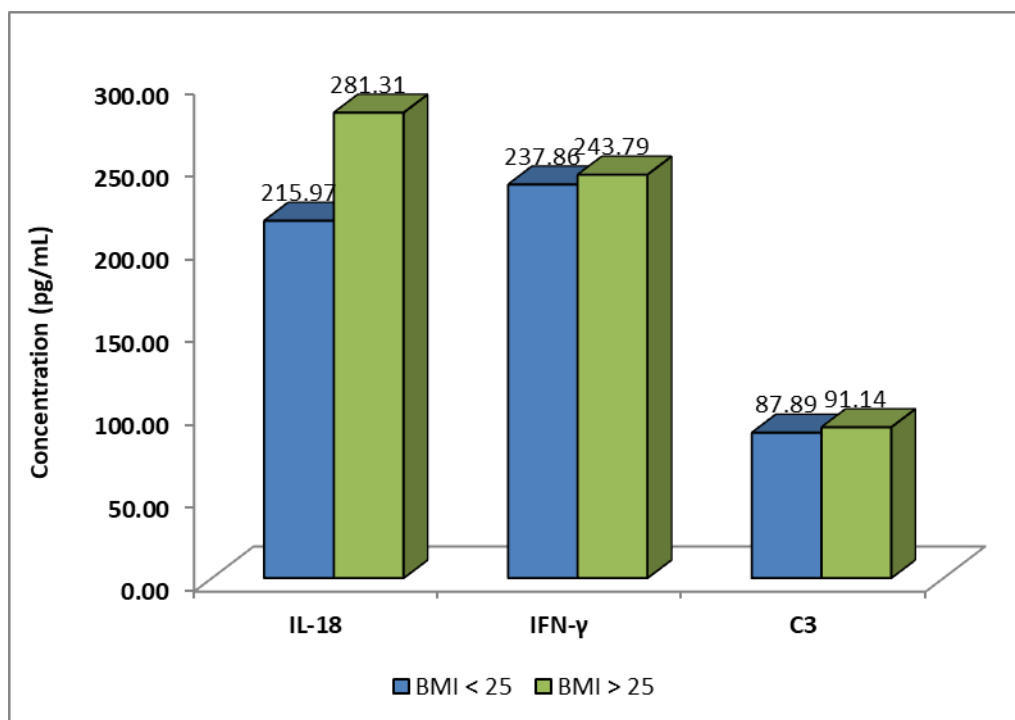
**Appendix (4): Effect of obesity on IL-18, IFN-γ and C3 in PCOS patients.**
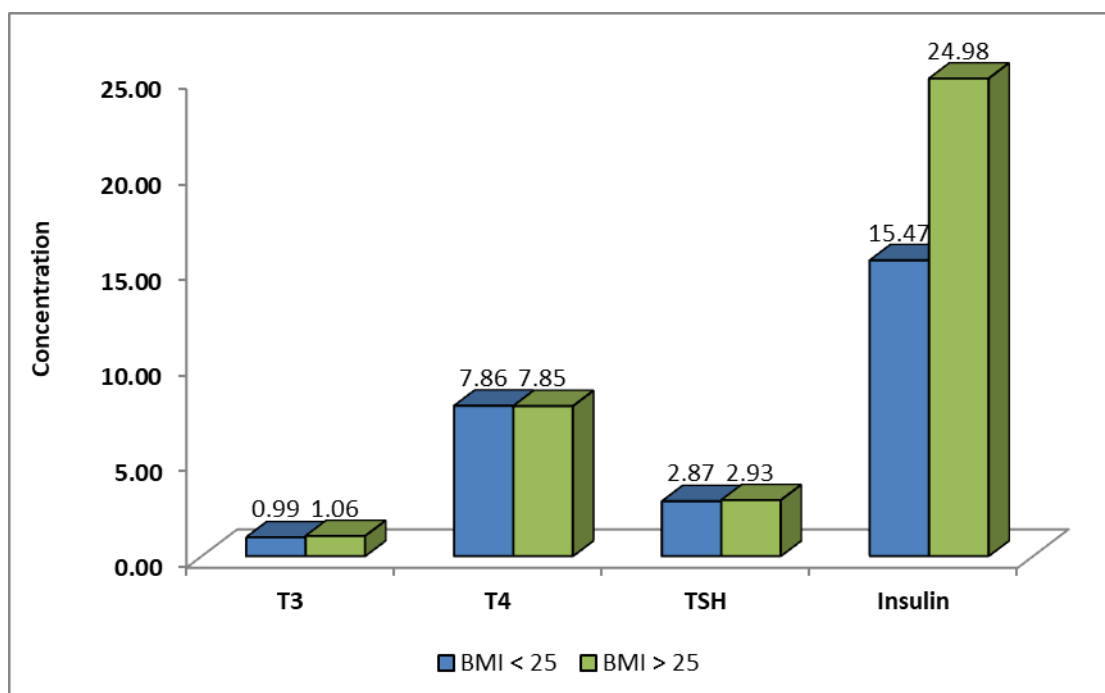


**Appendix (5): Effect of obesity on hormones levels in PCOS patients.**
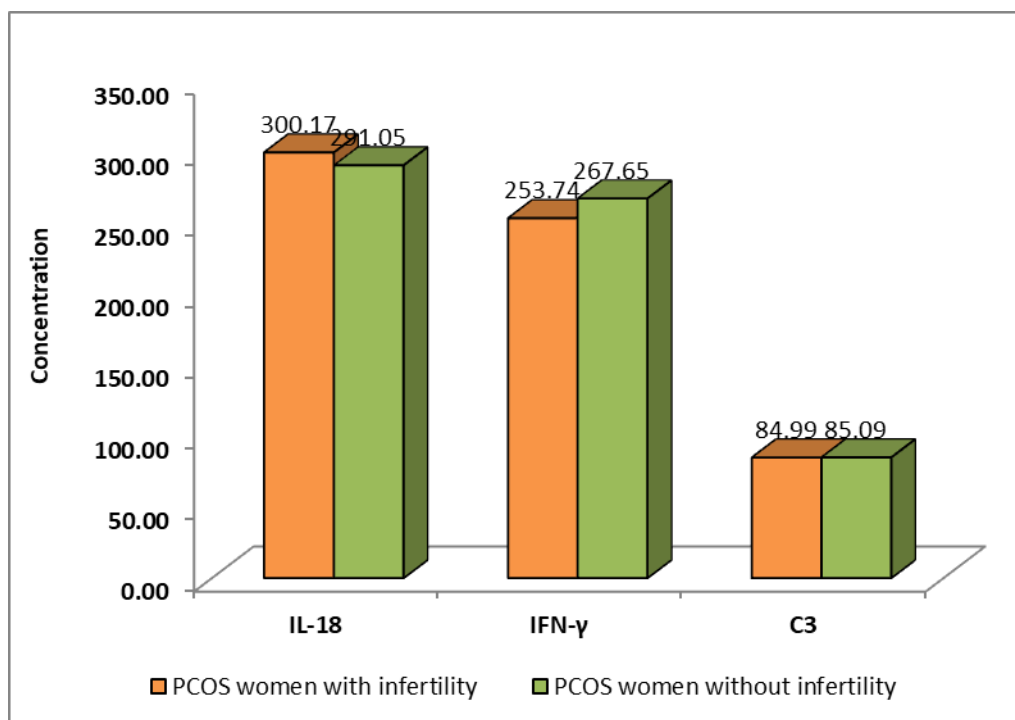
**Appendix (6): Effect of infertility on IL-18, IFN-γ and C3 in PCOS patients.**
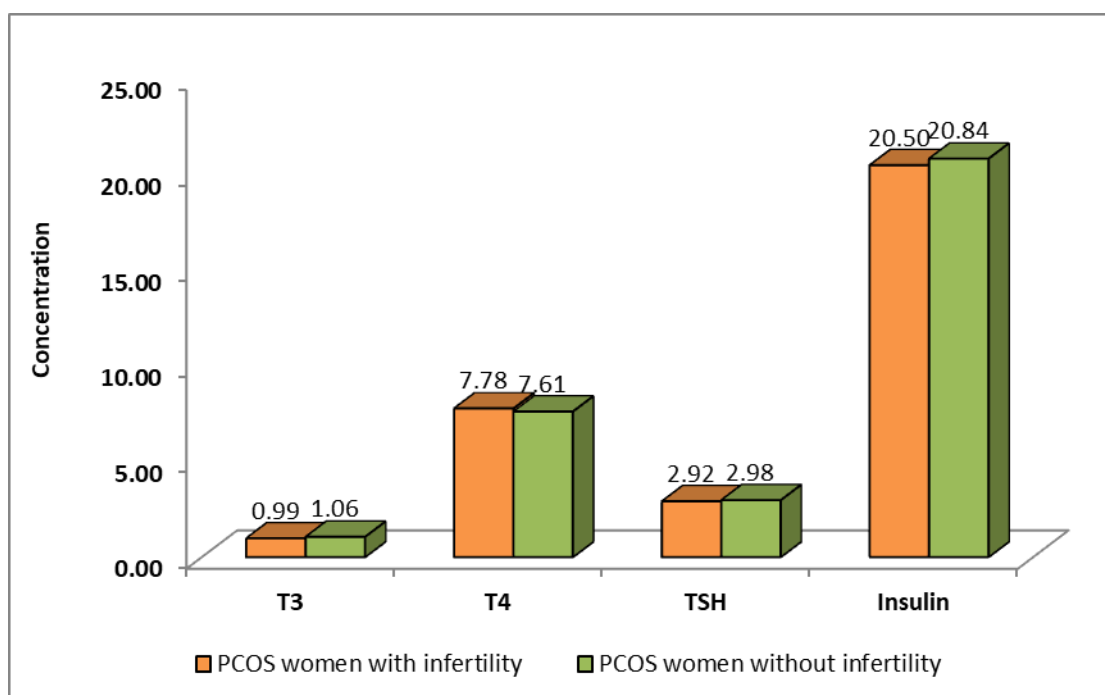


**Appendix (7): Effect of infertility on hormones levels in PCOS patients.**
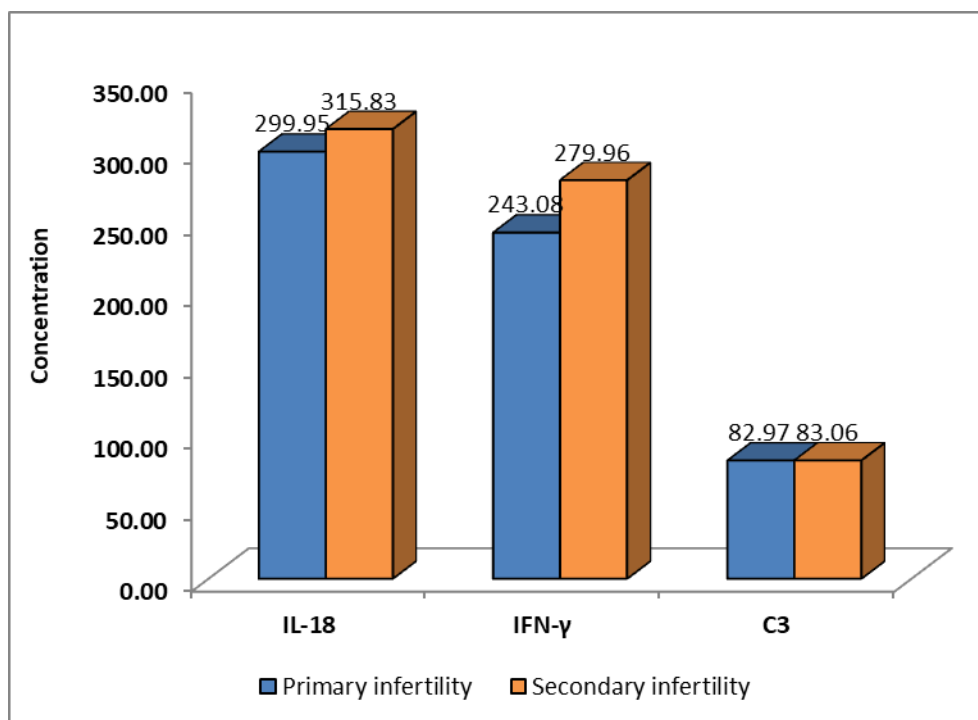
**Appendix (8): Levels of IL-18, IFN-γ and C3 in PCOS patients with primary and secondary infertility.**
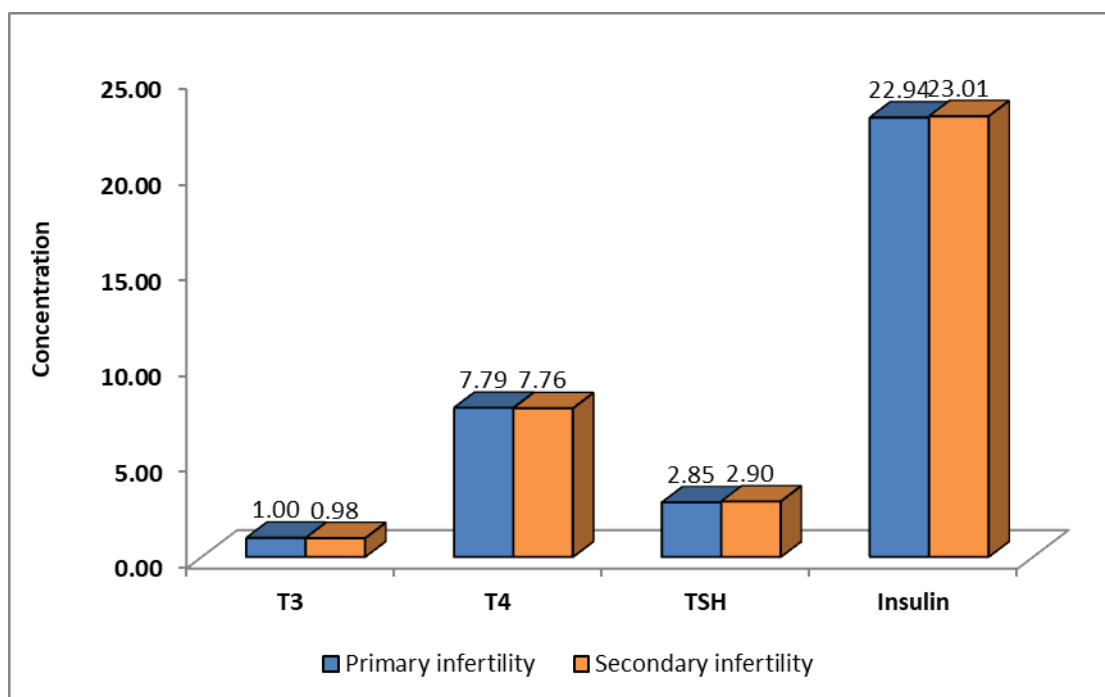


**Appendix (9): levels of hormones in PCOS women with primary and secondary infertility.**
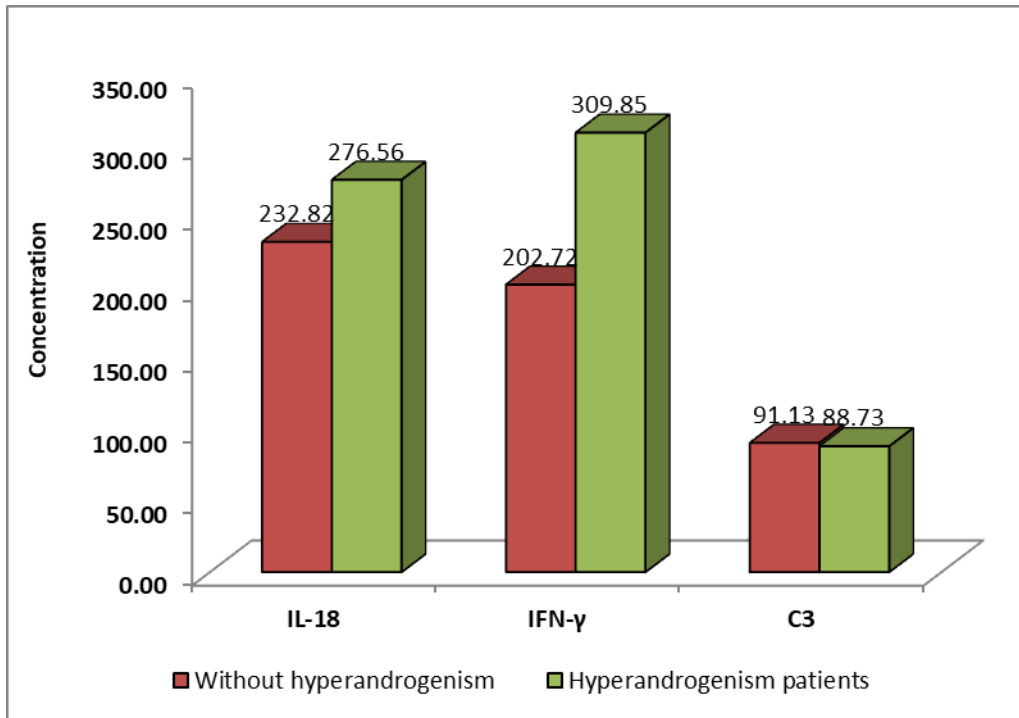
**Appendix (10): Relationship of hyperandrogenism with IL-18, IFN-γ and C3 levels in PCOS patients.**
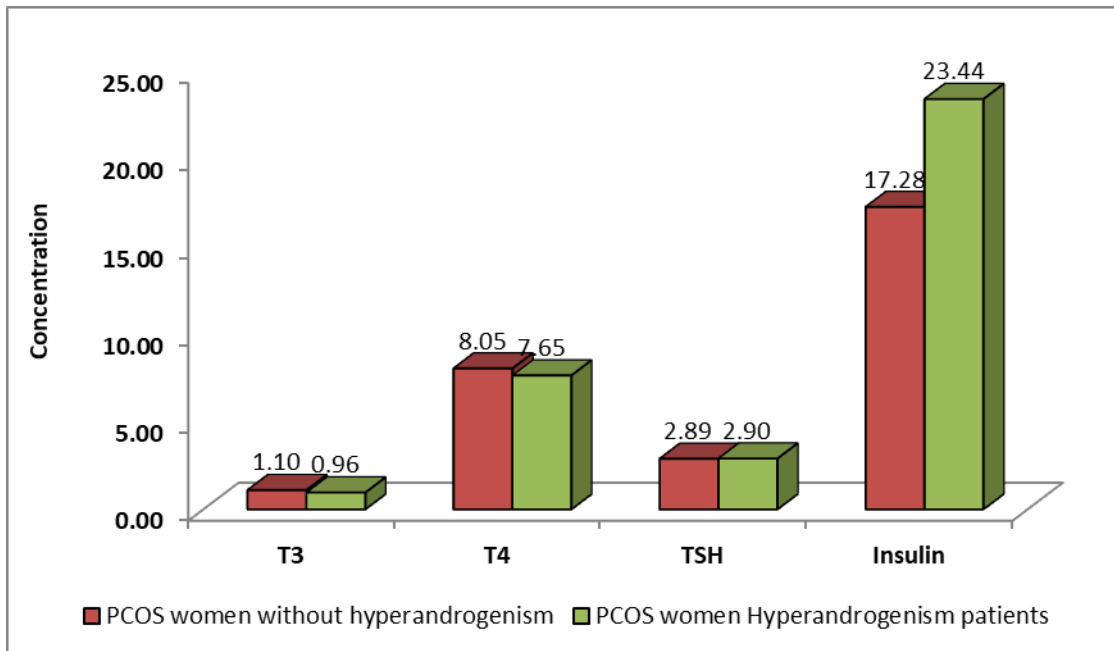


**Appendix (11): Relationship of hyperandrogenism with hormones levels in PCOS patients.**
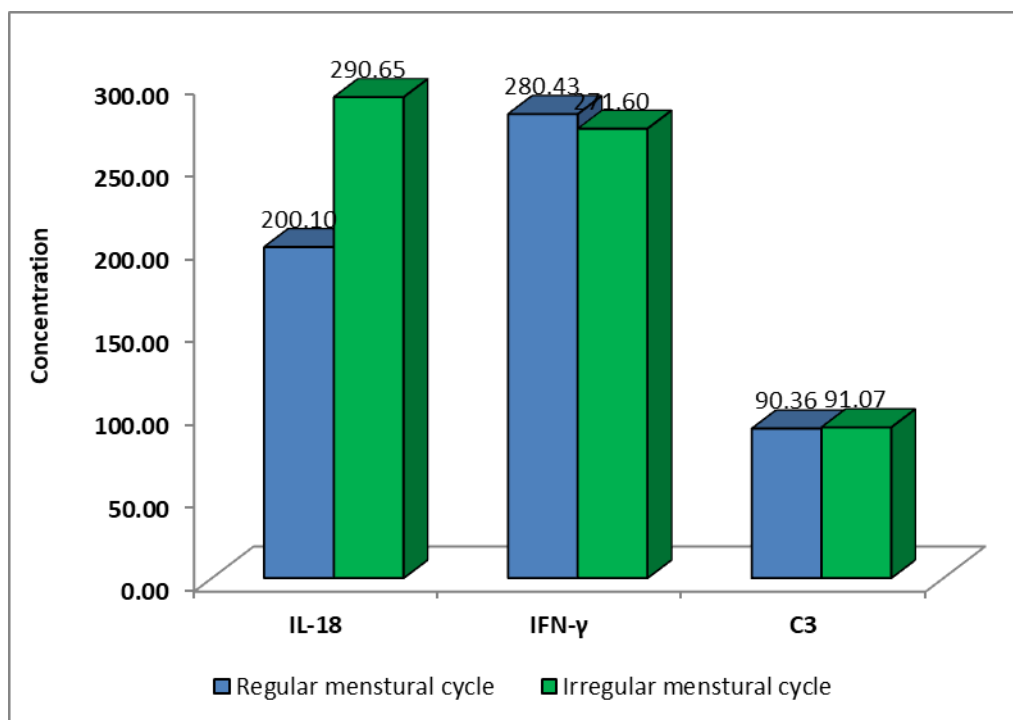
**Appendix (12): Levels of IL-18, IFN-γ and C3 in PCOS patients with regular and irregular menstrual cycle.**
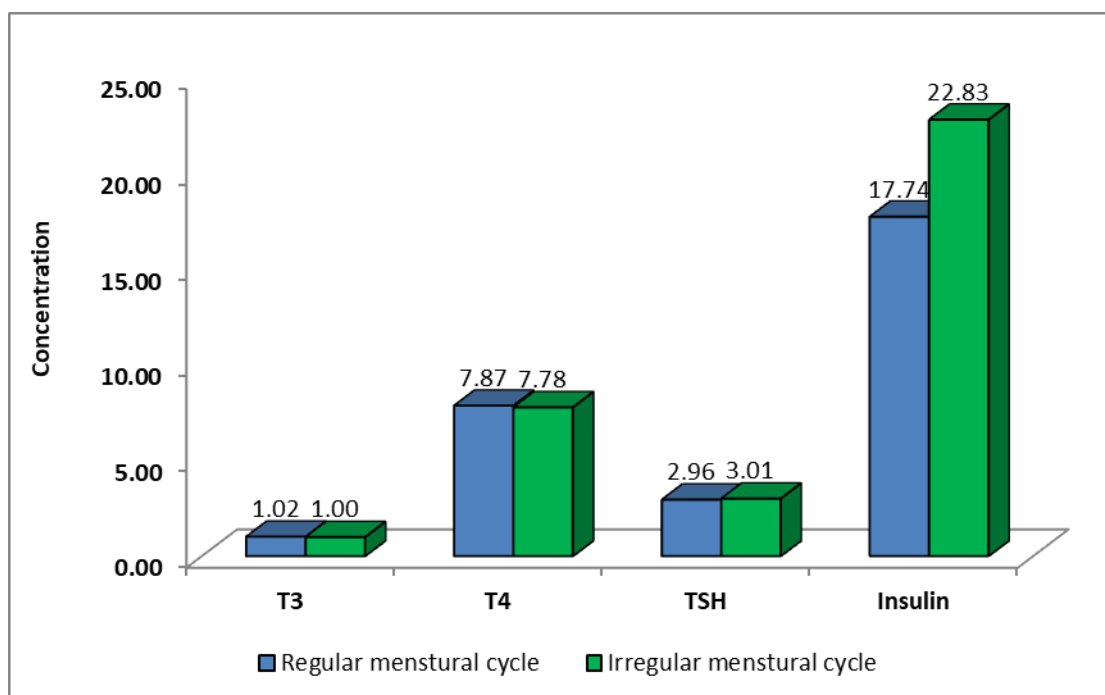


**Appendix (13): Levels of hormones in PCOS patients with regular and irregular menstrual cycle.**

## Correlation of serological study
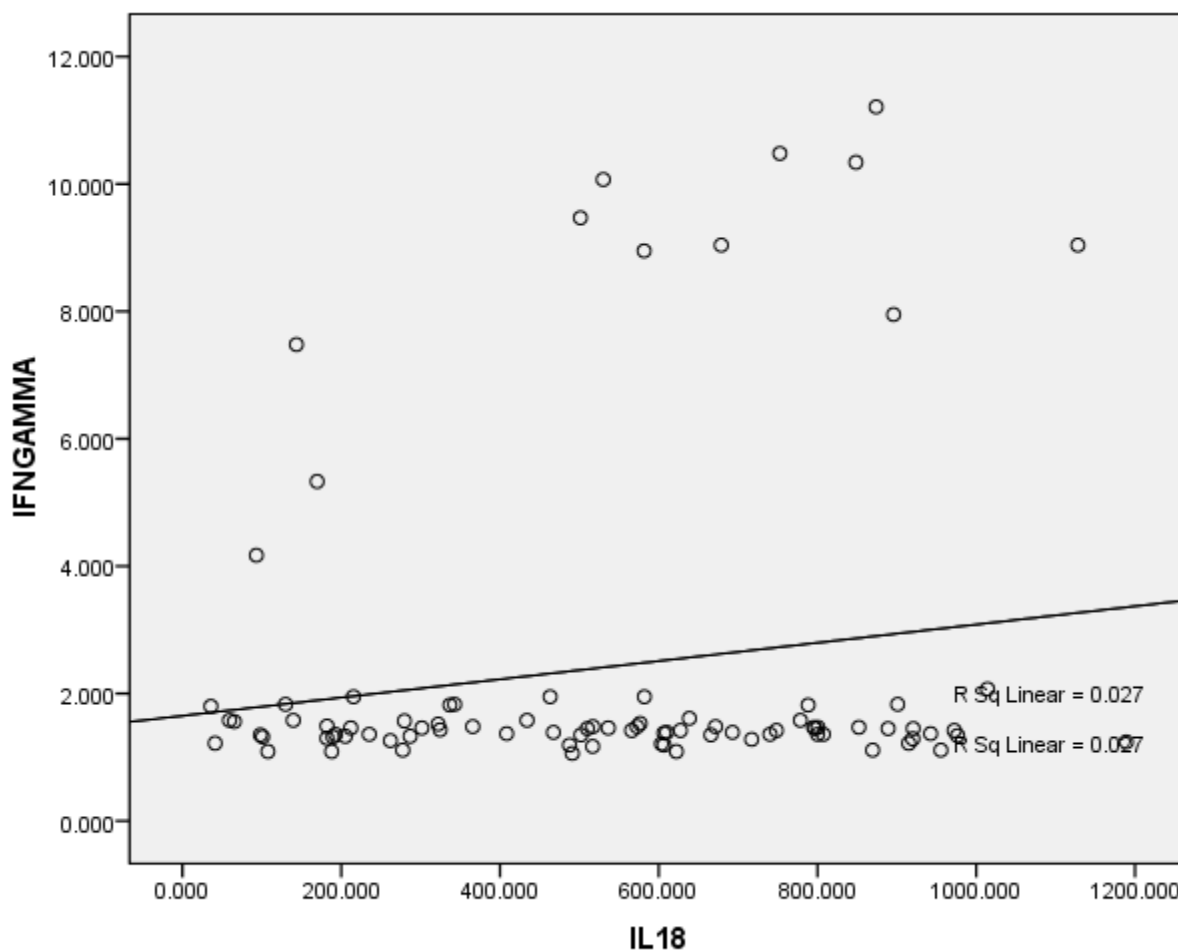


**Appendix (14): Correlation between IL-18 and IFN-γ**
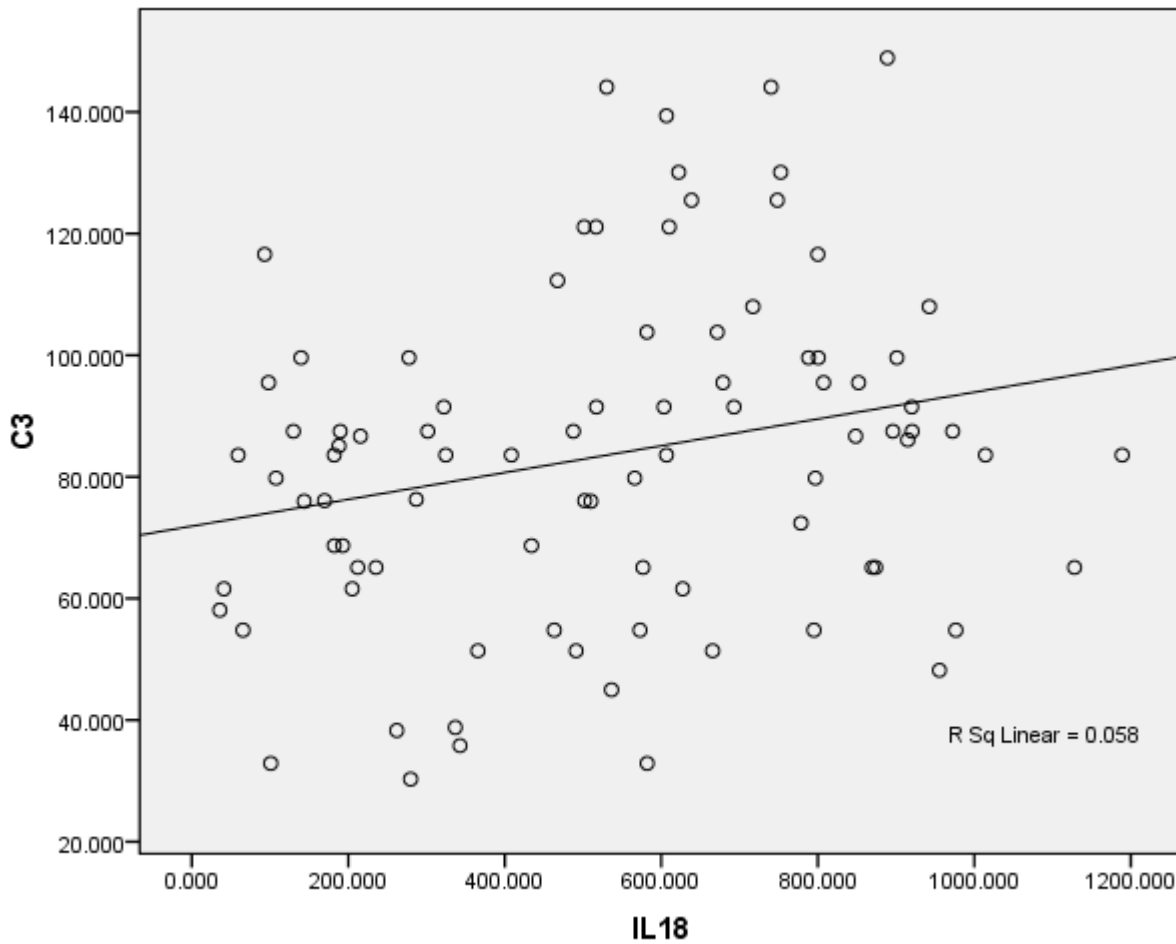
**Appendix (15): Correlation between IL-18 and C3.**
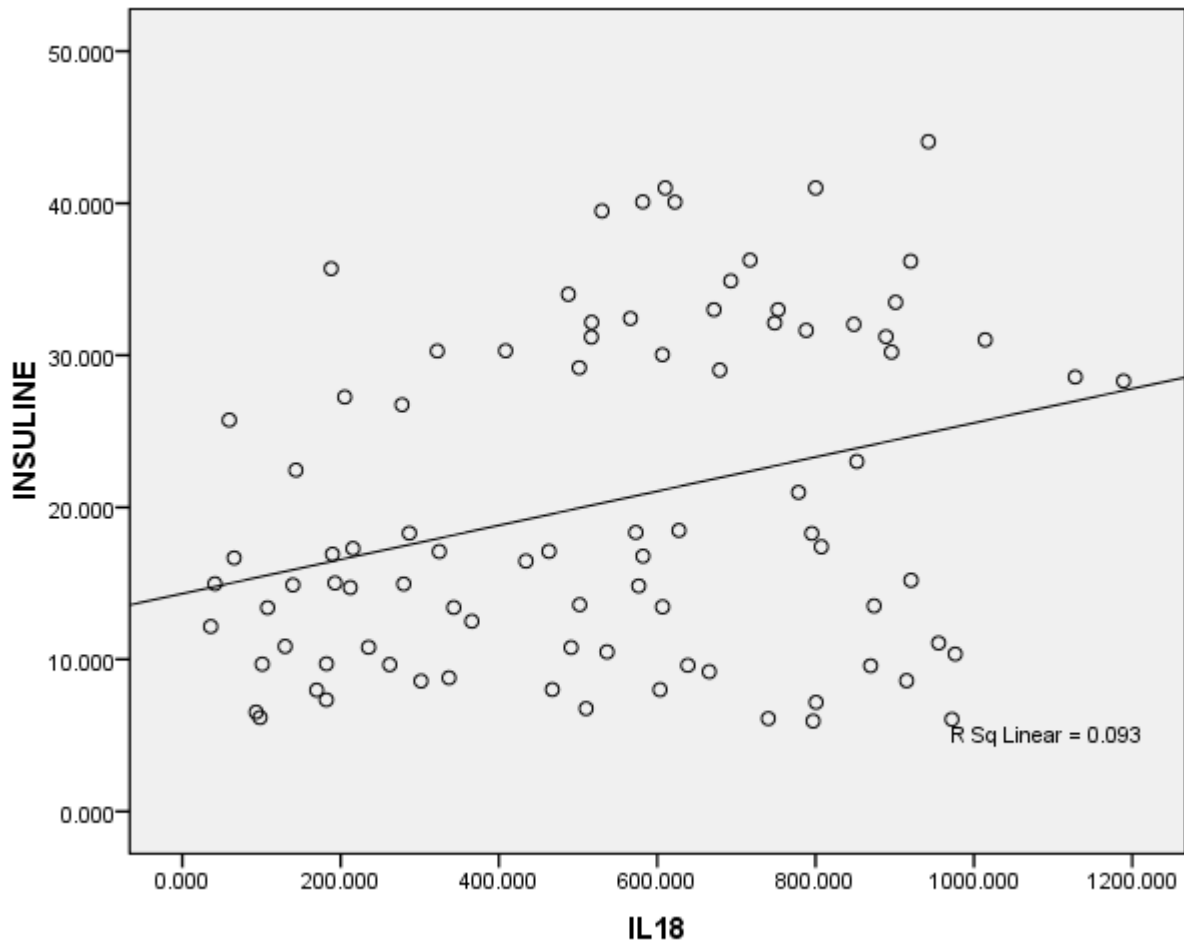
**Appendix (16): Correlation between IL-18 and insulin.**
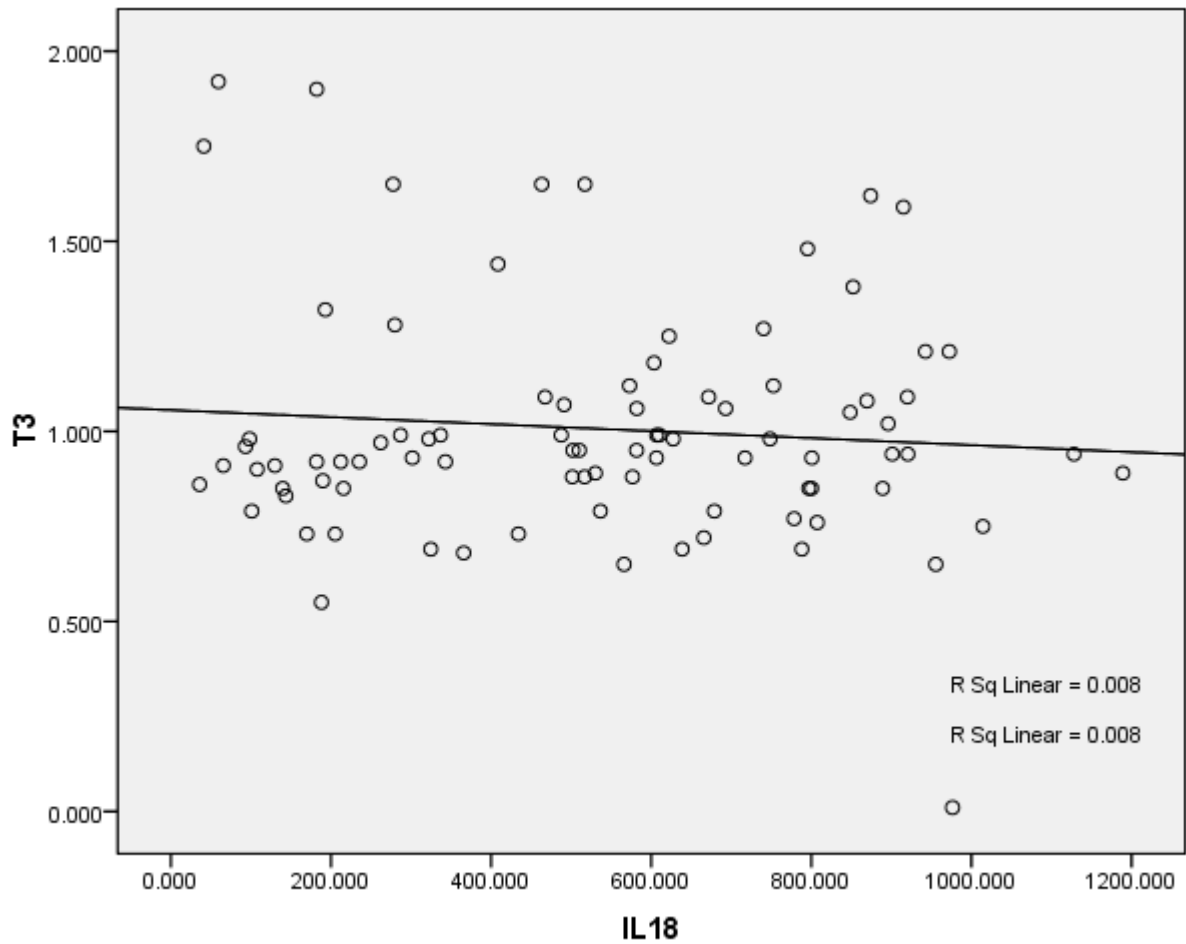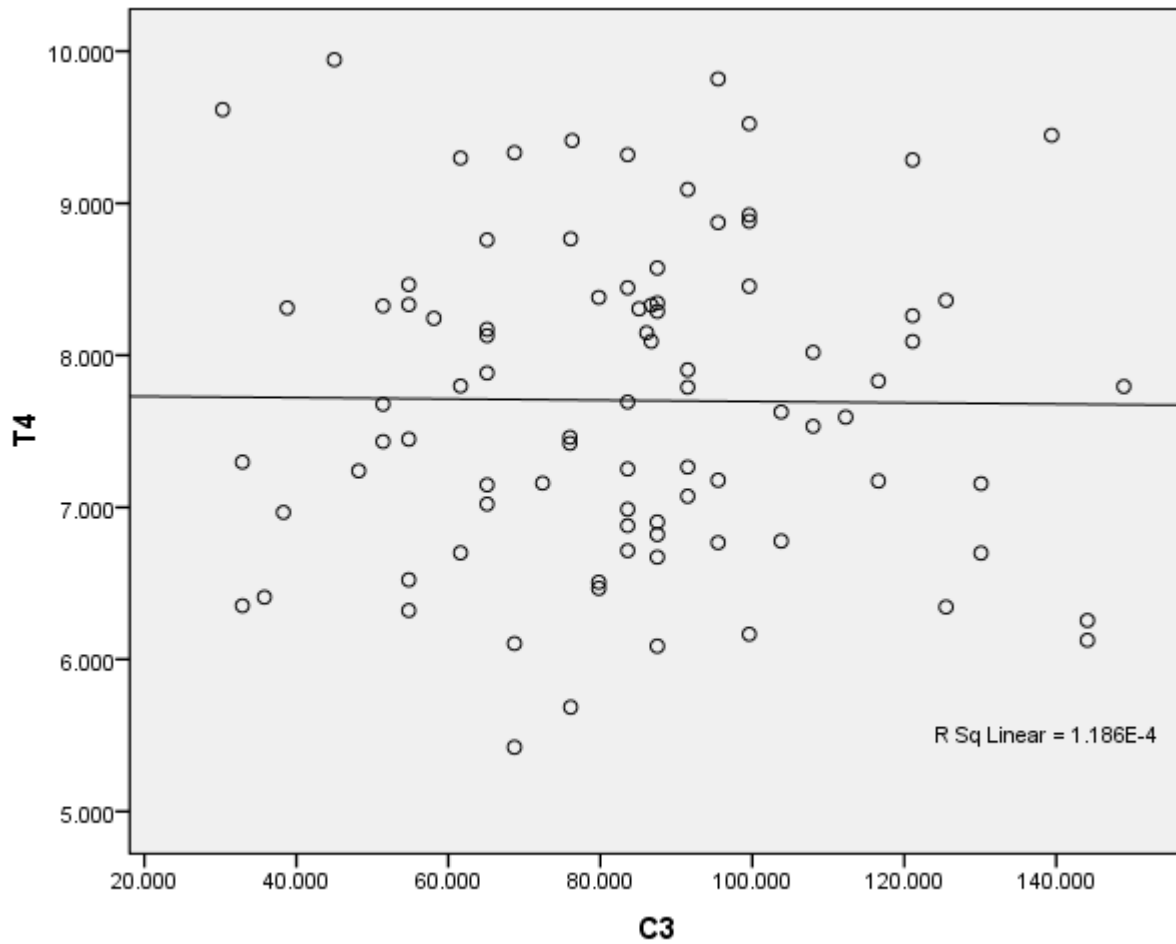
**Appendix (17): Correlation between IL-18 and T3.**
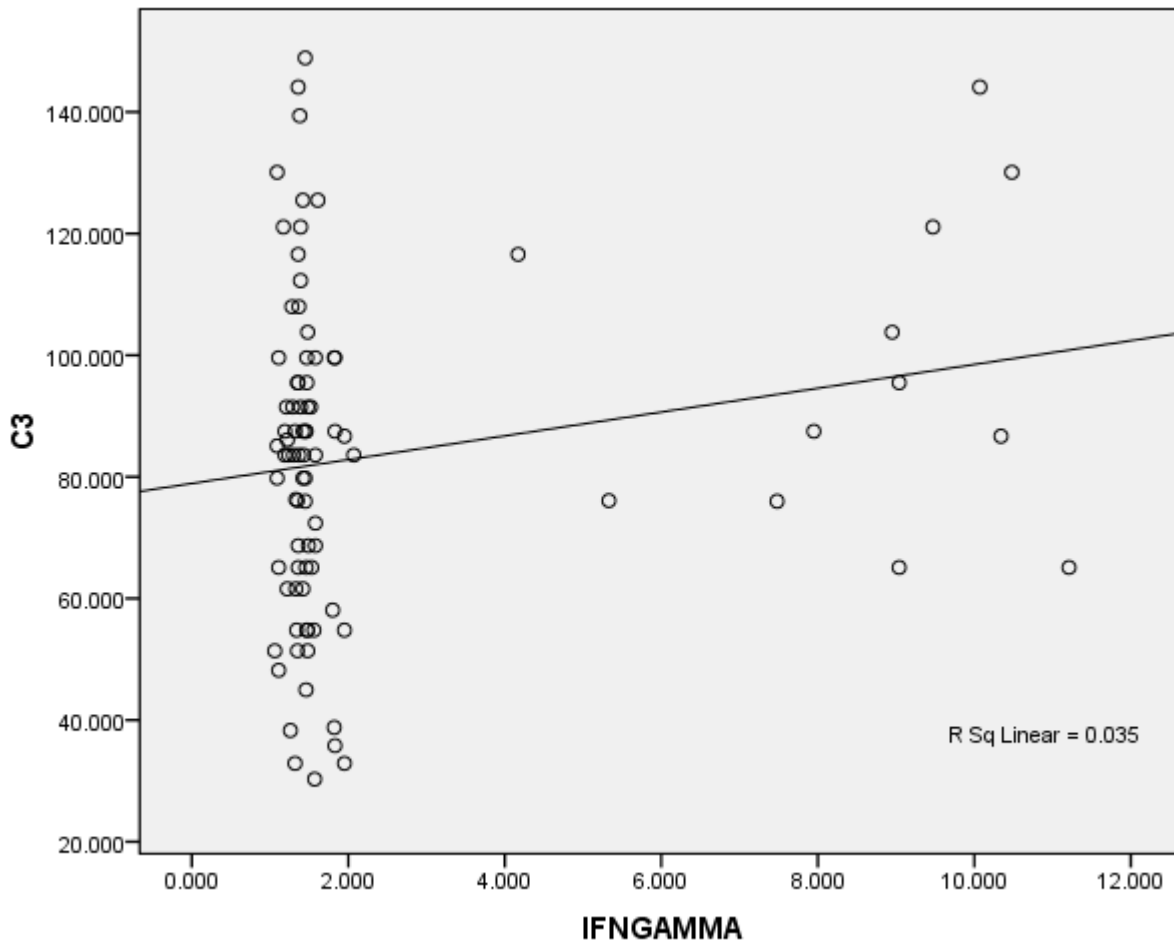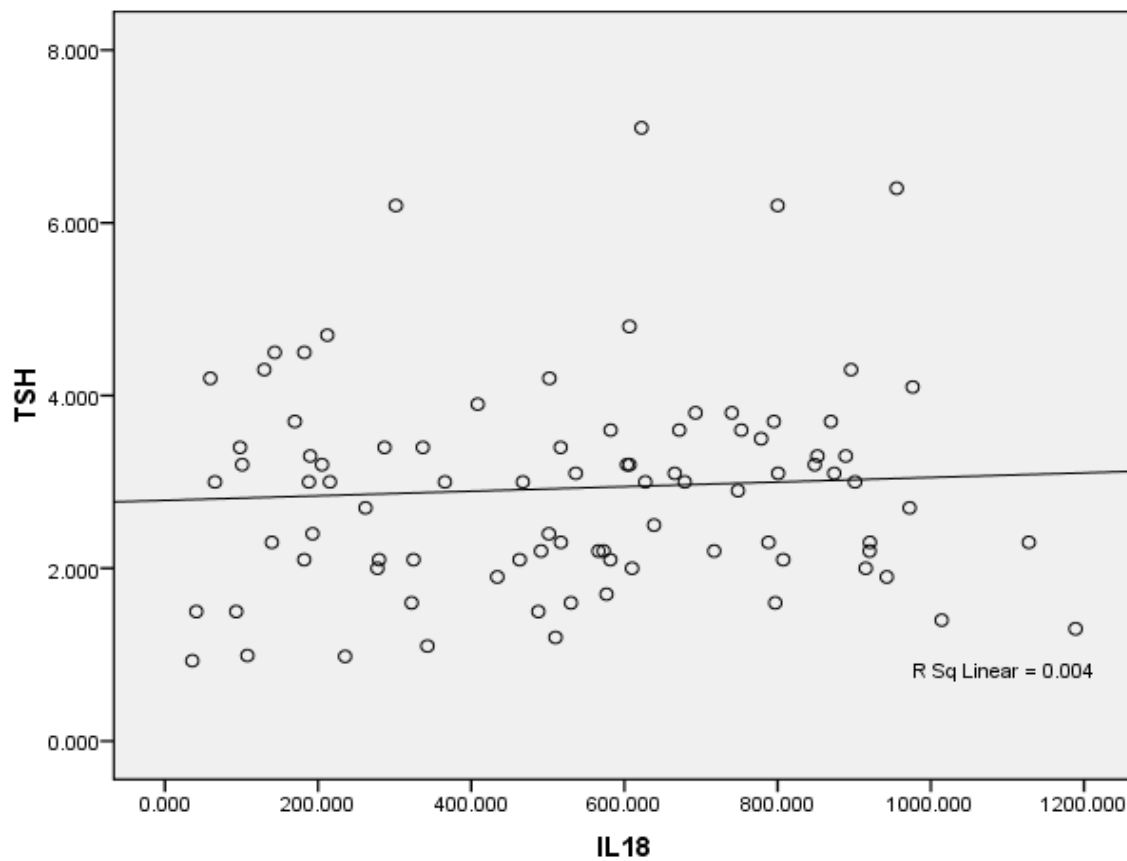
**Appendix (18): Correlation between IFN-γ and C3.**
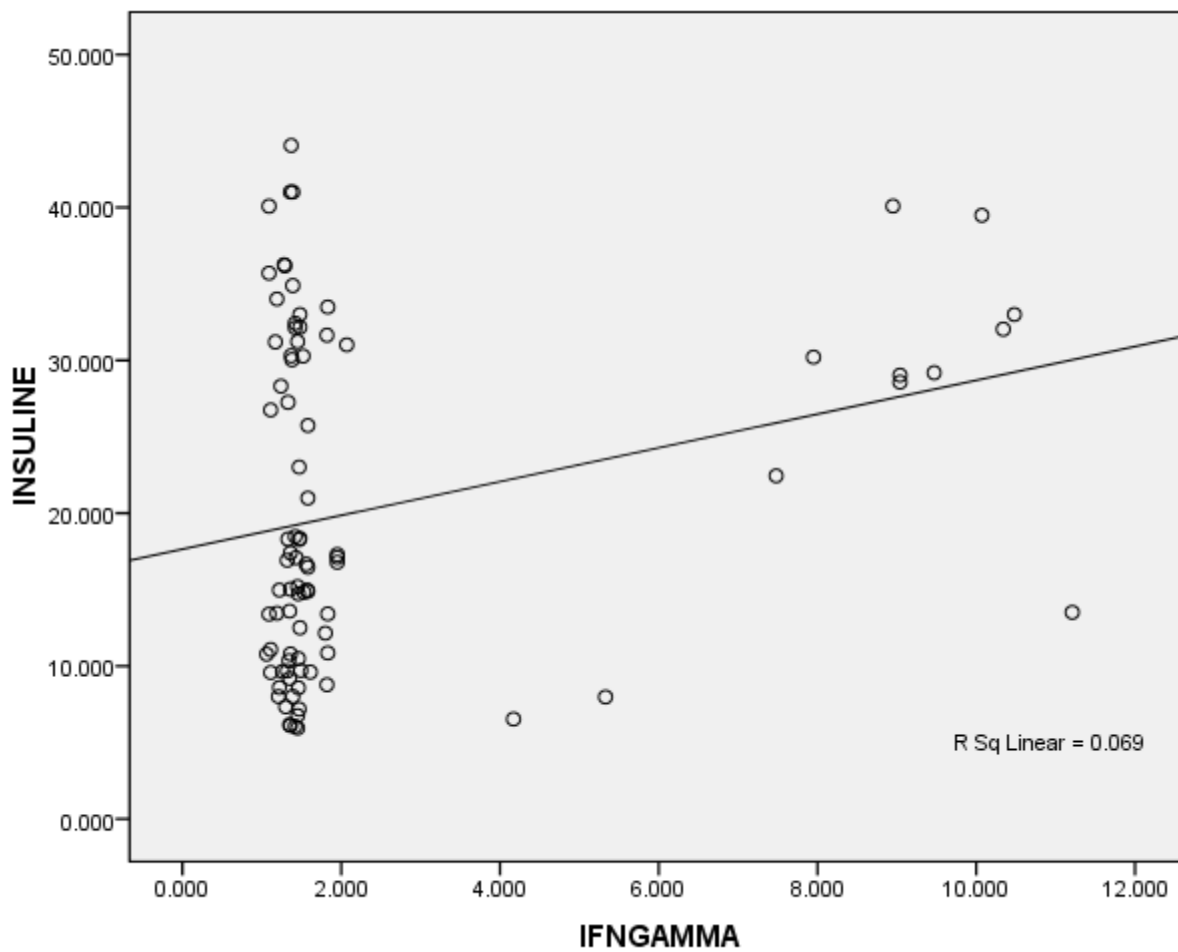
**Appendix (19): Correlation between IFN-γ and insulin.**
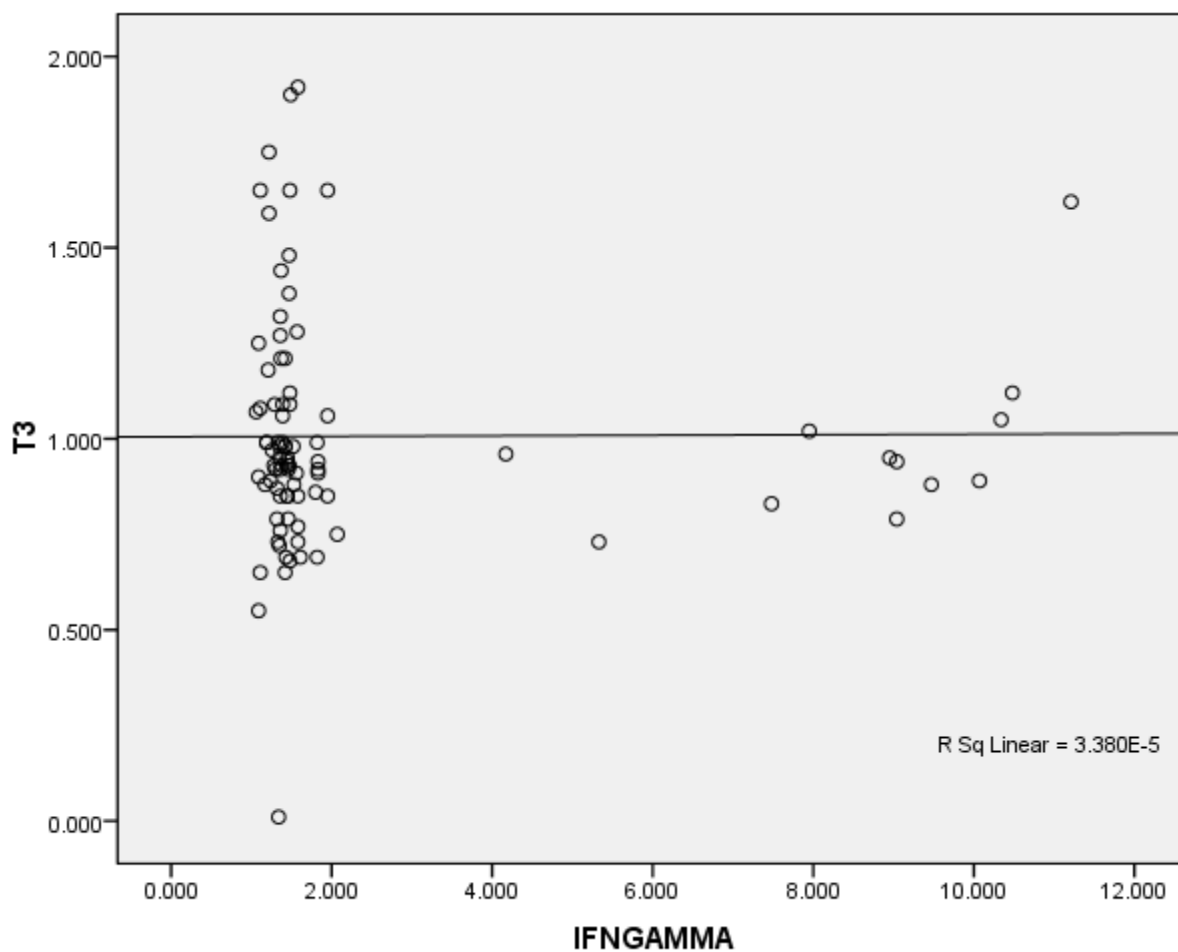
**Appendix (20): Correlation between IFN-γ and T3.**
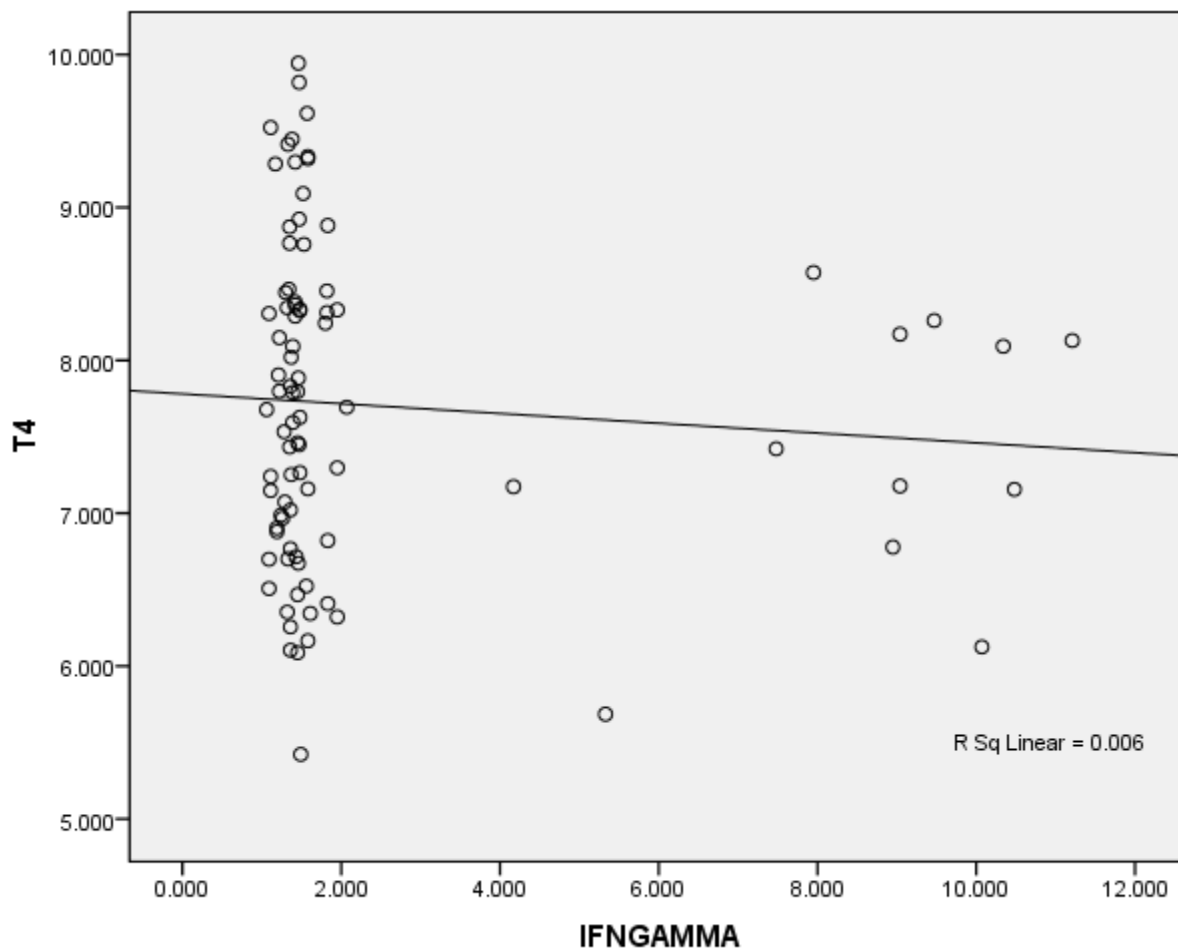
**Appendix (21): Correlation between IFN-γ and T4.**
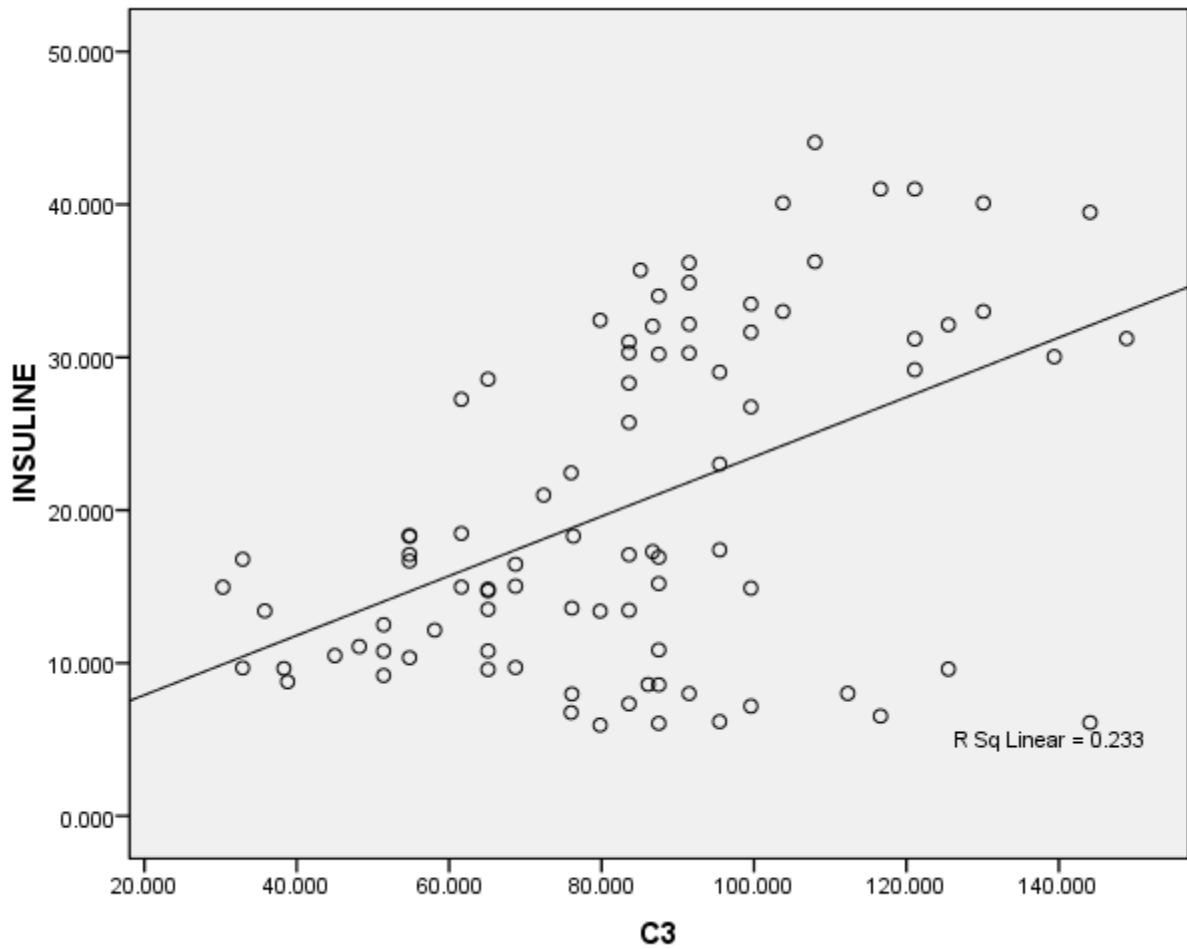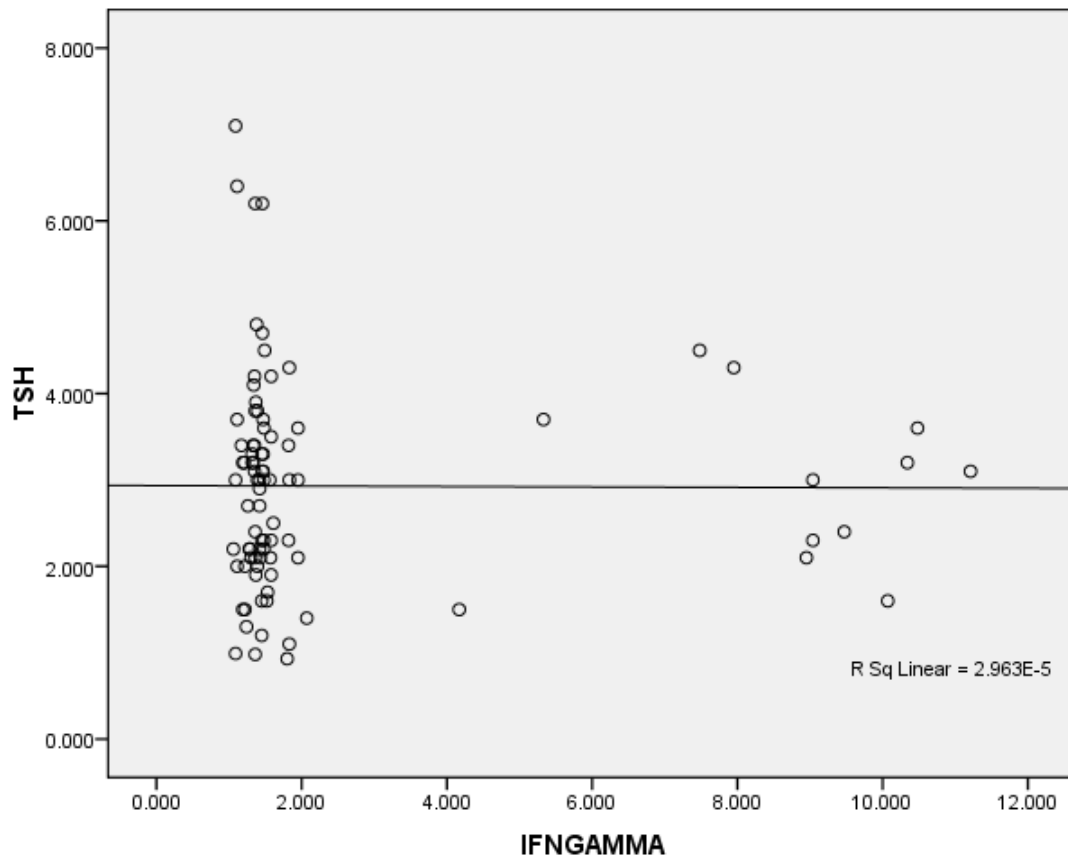
**Appendix (22): Correlation between C3 and insulin.**
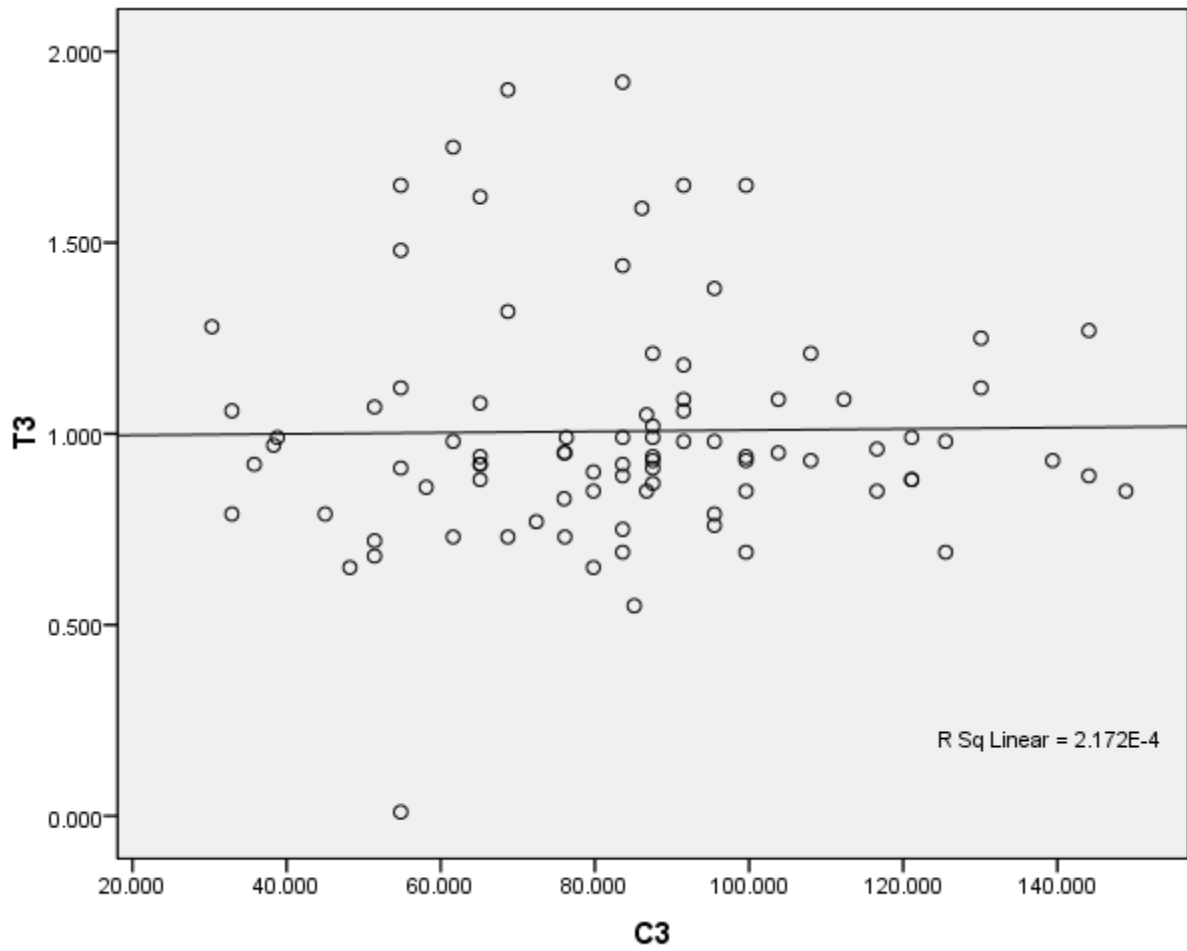
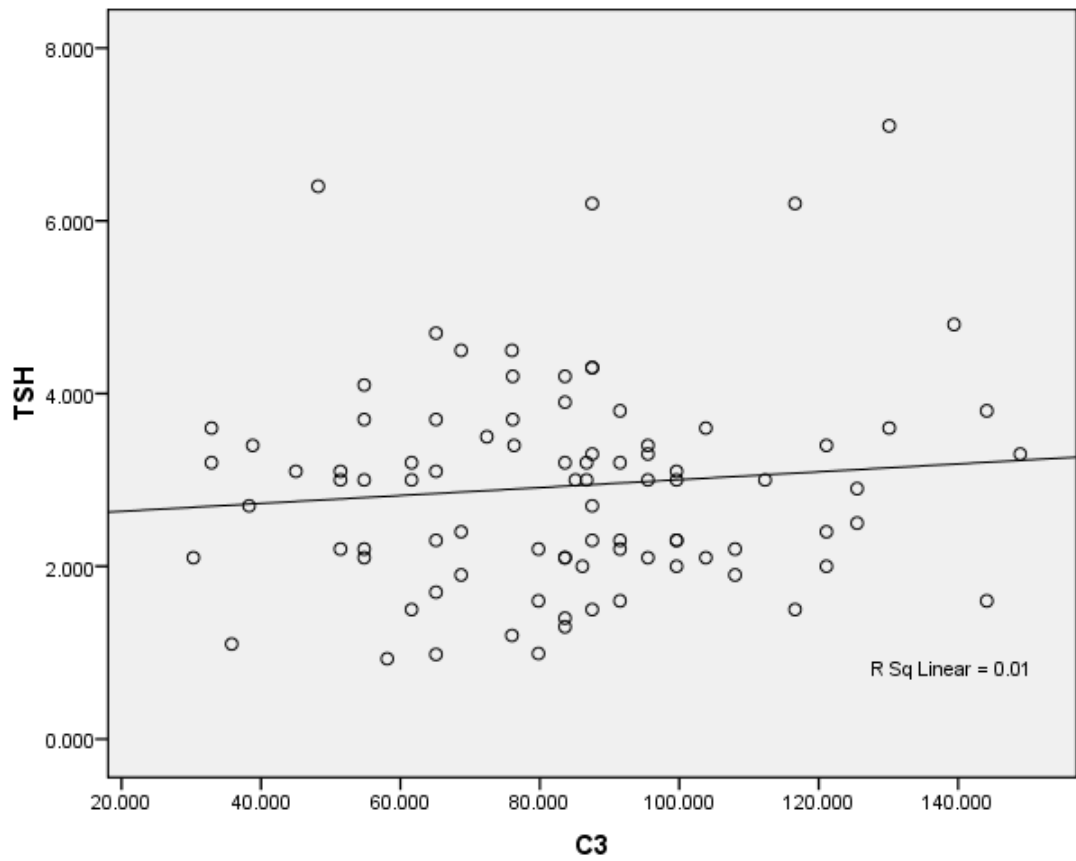**Appendix (23): Correlation between C3 and T3.**

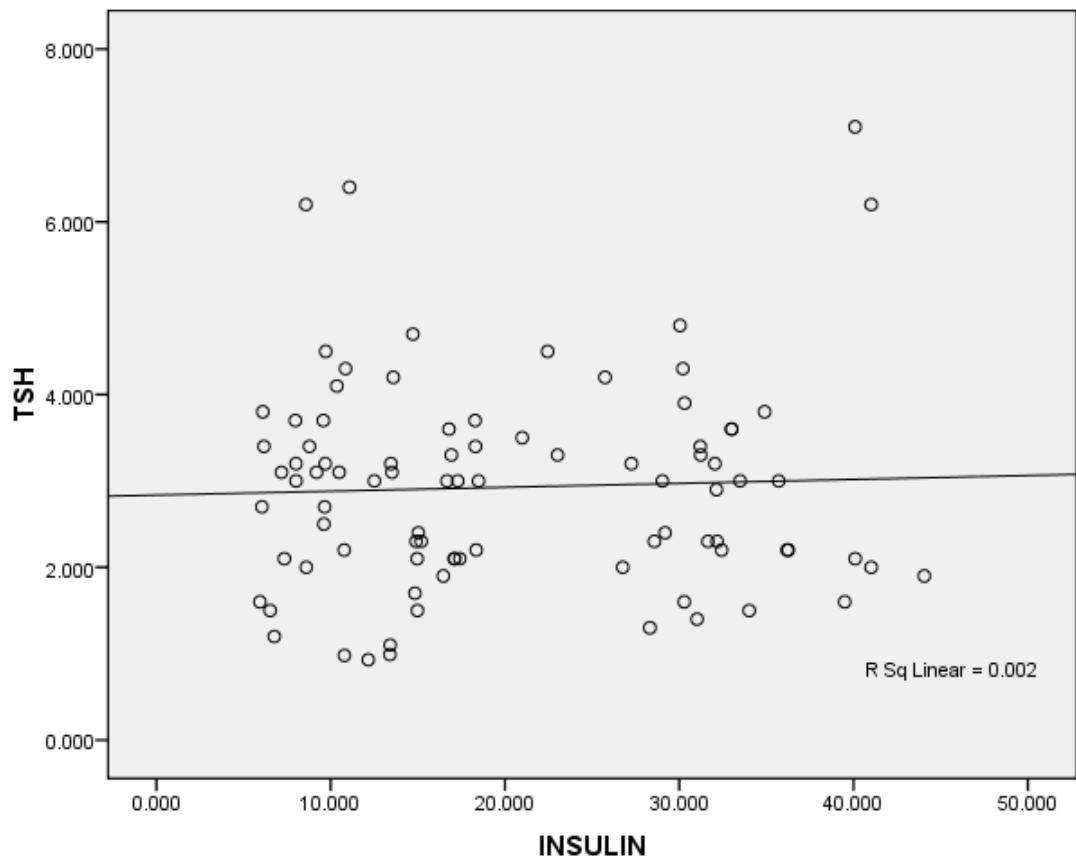**Appendix (24): Correlation between C3 and T4.**

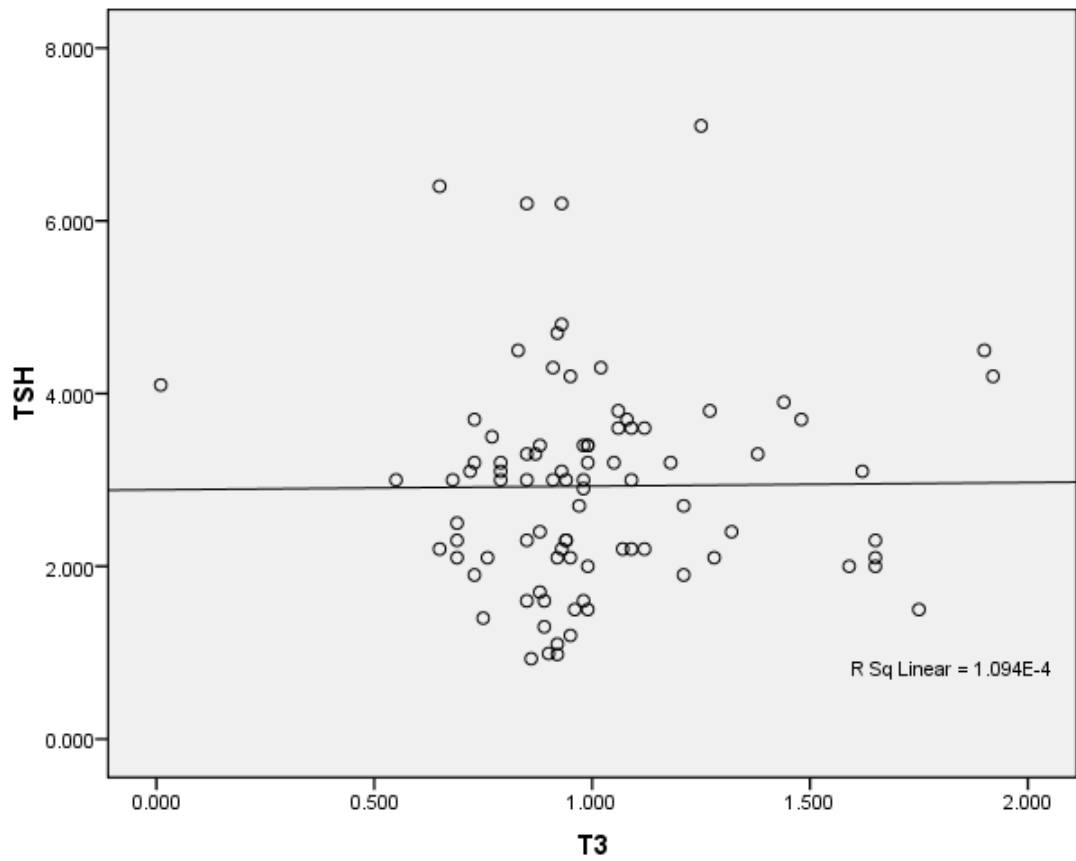**Appendix (25): Correlation between TSH and IL-18**

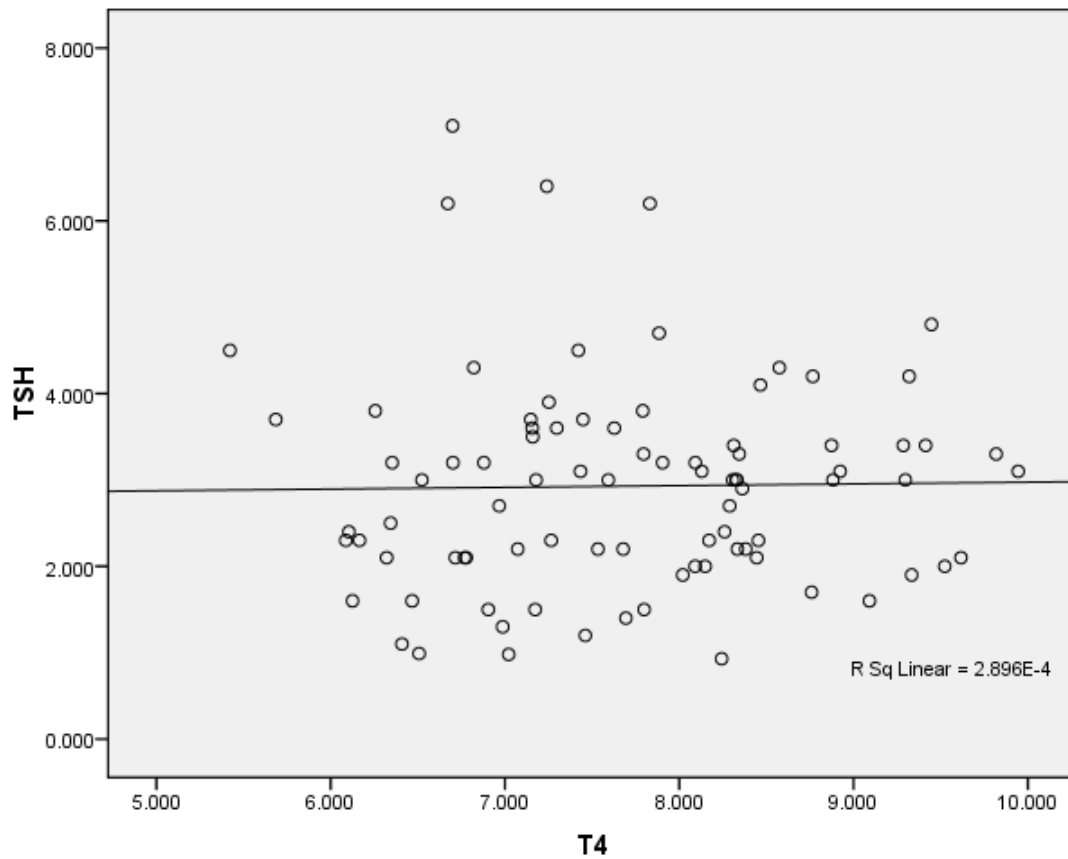**Appendix (26): Correlation between TSH and IFN-γ**

**Appendix (27): Correlation between TSH and C3**

**Appendix (28): Correlation between TSH and insulin**

**Appendix (29): Correlation between TSH and T3**

**Appendix (30): Correlation between TSH and T4**

## الخلاصة

تعد متلازمة تعدد الاكياس المبيضي احدى اعتلالات الغدد الصم الاكثر شيوعا التي تصيب العديد من الإناث في فترة التكاثر والتي تبدأ من اول حيض وحتى سن اليأس وتسبب العقم بشكل شائع في جميع أنحاء العالم ، وتتميز بدورة طمث غير منتظمة ، فرط الاندروجينية, وتعدد الاكياس المبيضي ويمكن اعتباره متلازمة تؤثر على الإنجاب، والتمثيل الغذائي، ومكونات القلب والأوعية الدموية ما يؤدي إلى اعتلالات صحية مدى الحياة. نسبة إنتشاره بين النساء المصابات بالعقم تتراوح بين (15-20%). هناك ادلة على ان متلازمة تعدد الاكياس المبيضي هو اضطراب التهابي، يتميز بوجود التهاب مزمن منخفض الدرجة يرتبط بالسمنة او مقاومة الانسولين. تهدف هذه الدراسة إلى تحديد دور الاستجابة المناعية والفسيولوجية في التسبب بمتلازمة تعدد الاكياس المبيضي.

تضمنت الدراسة الحالية 66 مريضة ( تم تشخيصهم بمرض متلازمة تعدد الاكياس المبيضي) من المراجعات لمستشفى كمال السامرائي في بغداد, خلال الفترة من أغسطس 2018 الى مارس 2019، بالإضافة إلى المجموعة الضابطة التي تضم 22 امرأة خصبة لديهن دورة طمث منتظمة مع عدم وجود علامة على فرط الأندروجينية واللاتي تعرضن للفحص بالموجات فوق الصوتية ولديهن مستوى هرموني طبيعي. كان العمر متطابقًا في كلا المجموعتين بين (20-40) سنة. تم حساب مؤشر كتلة الجسم (BMI) لكل من المرضى والمجموعة الضابطة. تم فحص مستوى الهرمونات LH, FSH, TSH وقد أجريت هذه الدراسة في جزئين: دراسة مناعية ودراسة فسيولوجية استخدمت تقنية الامتزاز المناعي المرتبط بالأنزيم ELISA لتحديد مستوى المصل من الوسائط IL-18 و IFN-γ و لهرمونات الغدة الدرقية T4, T3 و هرمون الانسولين وتم إجراء فحص الانتشار المناعي الشعاعي المفرد لتقدير تركيز مكون المتمم C3.

أظهرت نتائج الدراسة المناعية أن مستوى IL-18 زاد بشكل ملحوظ (P<0.001) في مصل مرضى متلازمة تعدد الاكياس المبيضي مقارنةً بالمجموعة الضابطة. وكان المستوى المتوسط لهذا البين ابيضاضي في المرضى والمجموعة الضابطة (34.94 ±609.04) بيكوغرام/مل مقابل 44.16 ±306.55) بيكو غرام/مل على التوالي. وسجلت نتائج IL-18 فرقاً معنويا كبيراً (P<0.001) في امصال مرضى متلازمة تعدد الاكياس المبيضي االلواتي يعانين من السمنة (14.13 ±281.30) بيكوغرام/ مل مقارنة بمرضى متلازمة تعدد الاكياس المبيضي الهزيلات (9.33 ±215.97) بيكوغرام/ مل. فضلاً عن ذلك، ارتفعت مستويات IL-18 في مرضى متلازمة تعدد الاكياس المبيضي اللواتي يعانين من حالة فرط الأندروجينية ، بمتوسط (13.04 ±276.56 ) بيكوغرام/ مل وكان هذا الارتفاع ذو دلالة إحصائية (P <0.05) بالمقارنة مع المستوى المسجل في مرضى متلازمة تعدد الاكياس المبيضي اللواتي لا يعانين من حالة فرط الأندروجينية بمتوسط (232.82±12.53) بيكو غرام/ مل.

علاوة على ذلك، ارتفع مستوى IL-18 بشكل ملحوظ ( P<0.05) في امصال مرضى متلازمة تعدد الاكياس المبيضي اللواتي يعانين من دورة طمث غير منتظمة (12.05 ±290.65) بيكوغرام/ مل بالمقارنة مع مرضى متلازمة تعدد الاكياس المبيضي اللواتي لديهم دورة طمث منتظمة (11.35 ± 200.09 ) بيكوغرام/مل.

وسجلت مستويات IFN-γ ارتفاعا معنويا (P<0.05) عند مجموعة مرضى متلازمة تعدد الاكياس المبيضي(36.04 ±266.02) بيكوغرام/مل بالمقارنة مع مجموعة الاصحاء ظاهريا البالغ متوسطها ( 17.33 ±186.36 بيكوغرام/مل)، فيما كانت مستويات مكون المتمم C3 مختلفة معنويا بين مجموعتي الدراسة ( 3.16 ±89.71) ملغ/ ديسيلتر ,(4.90 ± 65.51) ملغ/ ديسيلتر المرضى والمجموعة الضابطة على التوالي (P<0.001).

اظهرت نتائج الدراسة الفسيولوجية ان مستويات الأنسولين سجلت فرقاً معنويا كبيراً (P<0.001) في امصال مرضى متلازمة تعدد الاكياس المبيضي مقارنةً بالمجموعة الضابطة، وكان المتوسط لهذا الهرمون للمريضات والمجموعة الضابطة (1.38 ± 23.01) ميكرو وحدة دولية/مل, (0.64 ±12.20) ميكرو وحدة دولية/مل على التوالي، وأظهرت الدراسة الحالية زيادة معنوية كبيرة ( P>0.001) في مستوى الأنسولين في مصل مريضات متلازمة تعدد الاكياس المبيضي اللواتي يعانين من السمنة (1.77 ±24.97 ) ميكرو وحدة دولية/ مل بالمقارنة مع مريضات متلازمة تعدد الاكياس المبيضي الهزيلات (15.46±1.63) ميكرو وحدة دولية/مل.

أيضاً كان هناك اختلاف معنويا (P<0.05) في مستوى الأنسولين في مصل مريضات متلازمة تعدد الاكياس المبيضي الواتي يعانين من حالة فرط الأندروجينة (1.83 ±23.44) ميكرو وحدة دولية/ مل مقارنة بمريضات متلازمة تعدد الاكياس المبيضي اللواتي لا يعانين من حالة فرط الأندروجينة (17.27±1.74) ميكرو وحدة دولية/مل.

أظهرت الدراسة الحالية وجود علاقة إيجابية (P<0.001) بين مؤشر كتلة الجسم وIL-18 وC3 والأنسولين (P<0.001). وكذلك تم تسجيل علاقة إيجابية بين IL-18 و C3 (P<0.05). بلاضافة الى ذلك ، تم تسجيل علاقة إيجابية بين الأنسولين وIL-18 وIFN-γ وC3 مع (P<0.001) باستثناء IFN-γ مع (P<0.05). في حين أظهرت هذه الدراسة عدم وجود فروق ذات دلالة أحصائية في مستويات T3, T4, TSH في امصال مرضى متلازمة تعدد الاكياس المبيضي (2.94±0.15 ميكرو وحدة دولية/مل, 7.81±0.1 ميكروغرام/ديسيلتر, 1.01±0.03 نانوغرام/مل) بالمقارنة مع المجموعة الضابطة (2.89±0.22 ميكرو وحدة دولية/مل ، 7.37±0.25 ميكرو غرام/ديسيلتر ، 1.98±0.07 نانوغرام/مل) على التوالي.

نستنتج أن مستويات IL-18 ,C3, IFN-γ والأنسولين كانت مرتفعة في امصال مرضى متلازمة تعدد الاكياس المبيضي مقارنةً بالمجموعة الضابطة وهذه المستويات المرتفعة ترجع الى متلازمة تعدد الاكياس المبيضي بالاعتماد على وجود السمنة او مقاومة الانسولين. وكان هناك ارتباط إيجابي بين IL-18 و C3 وبين هذه المعلمات IL-18 وC3 مع السمنة وفرط الأندروجينة ، و بين هرمون الأنسولين و المعلمات الثلاث IFN-γ , IL-18 و C3 من جهة اخرى, و قد يكون ذلك بسبب اعتبار متلازمة تعدد الاكياس المبيضي مرض التهابي.

وفقا لما ورد اعلاه ; تعتبر هذه المعلمات كاداة جيدة للتنبؤ بحدوث امراض القلب والاوعية الدموية في متلازمة تعدد الاكياس المبيضي, كما انها قد تعد وسيلة كشف لتطور اضطراب التمثيل الغذائي الذي يسبب عدم الاباضة والتي قد تودي الى العقم.

# دراسة بعض الجوانب المناعية والفسلجية لمصابات عراقيات بمتلازمة تعدد الاكياس المبيضي

رسالة مقدمة

**الى مجلس كلية التربية للعلوم الصرفة (ابن الهيثم) – جامعة بغداد**

**وهي جزء من متطلبات نيل درجة ماجستير علوم في**

**علوم الحياة / علم المناعة**

من قِبَل

# ميس عصام محمد

بكالوريوس علوم الحياة/ كلية التربية للعلوم الصرفة (ابن الهيثم) / جامعة بغداد (2009)

**باشراف**

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ذو الحجة/1440هـ

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