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Molecular Basis of Programmed Cell Death-1 and Their Association with Encode Protein and Interleukin-10 in Patients with Polycystic Ovary Syndrome in Kerbala Governorate

A Thesis

Submitted to the Council of the College of Medicine, University of Kerbala, in Partial Fulfillment of the Requirements for the Master Degree in Clinical Chemistry

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

فَتَعَالَى ٱللَّهُ ٱلْمَلِكُ ٱلْحَقُّ ۗ وَلَا تَعْجَلْ بِٱلْقُرْءَانِ مِن قَبْلِ أَن يُقْضَىٰٓ إِلَيْكَ وَحْيُهُۥ ۖ وَقُل رَّبِّ زِدْنِى عِلْمًا

صَدَقَ اللهُ العَلِيُّ العَظيم

(سورة طه / الاية 114)

Dedication

I dedicate My humble effort to my sweet and loving (AUNTS)

Whose affection, love, encouragement and prayers of day and nights make me able to get such success and honor along with

my life companion (Ashraf)

who patiently stood by me inspiring in me strength and confidence.

To my kids (Fatima, Dima, Mohammad).

To the pure hearts and loyal hands who assisted me in life

To my cousin (Yusur).

Hiba

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Finally, thank my colleagues for their stimulating discussions, for working together before deadlines, and for all the fun we have had for the last two years.

Hiba

Supervisor's Certification

We certify that this M.Sc. thesis entitled:

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was prepared under our supervision at the College of Medicine / University of Kerbala, as a partial requirement for the degree of Master in Clinical Chemistry (M.Sc.).

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We have examined the postgraduate student **(Hiba Aqeel Muslem Al-Quraishy)** in its M.Sc. thesis content and, in our opinion; it meets the standard for the degree of master in **(Clinical Chemistry)** and it is adequate with **(Excellent).**

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Summary

Polycystic ovarian syndrome (PCOS) is one of the most common endocrine and metabolic disorder. The etiopathogenesis of PCOS is complex genetic, environmental and lifestyle interaction.

Low-grade inflammation and inflammatory indicators have been linked to PCOS. Because ovulation is a semi-inflammatory process, visceral adipose tissue can induce an inflammatory response and sustain the inflammation in adipocytes by influencing the release of inflammatory cytokines. Out of this regulated inflammation, PCOS can arise.

Apoptosis in granulosa cell is the main cause of follicular atrophy. PCOS women display more maturing and following atrophic follicles that not pass into dominant follicles that lead to anovulation and infertility. Programmed cell death-1cytokin (PD-1) play very important inhibitory functions in inflammation and autoimmune diseases as well as tumors. Although, if the PD-1 gene polymorphisms (rs2229782) are associated with the incidence of PCOS has not been recorded yet.

This study was aimed to explore whether women with PCOS have any difference in PD-1 and IL-10 levels compared to control group. Also, to determine any association between PD-1 and IL-10 levels and hormonal parameters in PCOS women, and, to study the genetic polymorphism of PD-1 gene in PCOS and to determine the correlation between inflammatory markers and the resultant of genotypes.

A case-control study design of 160 subject with age ranged between (18 – 40) years involving 80 PCOS patients and 80 apparently healthy women as a control in childbearing age at the Reproductive Fertility consultant of gynecological and obstetric teaching hospital, Kerbala health directorate Iraq and College of Medicine, University of Kerbala Iraq during the duration from Dec., 2021 to Aug., 2022. The Rotterdam criteria-2003 was presumed to PCOS females. The hormonal levels of

each of Luteinizing hormone (LH), Follicular Stimulating Hormone (FSH) and prolactin were measured by the chemiluminescent automated immunoassay system. Free testosterone (FT) level was measured by competitive enzyme immunoassay using ELISA kit and the sandwich-ELISA principle was used to determined PD-1 level and IL-10 level. Body mass index (BMI) and Waist Hip Ratio (WHR) were measured in this study. Each blood sample had its genomic DNA extracted using the genomic DNA extraction kit. The genomic DNA was stored at -20 c until use. The SNP of PD-1 (rs2227982) was genotyped using Taqman real-time PCR. The graph pad Prism 9.0.0 and SPSS 25 were used for all statistical calculations.

The result of this study demonstrated significant elevations in LH concentrations ($p<0.001$), LH/FSH ratio ($p<0.001$) and free testosterone level ($p<0.001$), prolactin level ($p<0.0001$) and PD-1 level ($p=0.01$) were prevailed in the PCOS patients group when contrasted with the control group. while significantly decrease in FSH level ($P=0.01$) and IL-10 level ($P<0.0001$) during a comparable evaluation between PCOS patients and control group. PD-1 (rs2227982) GA genotype was related significantly with a higher frequency of PCOS p-value (0.0151), OR (2.728), CI 95% 1.230 to 5.977 and PD-1gene allele frequencies (rs2227982) A allele had significance p-value (0.0237), OR (2.393), CI 95% 1.197 to 4.815 with PCOS patient.

According to the present study concludes that PD-1 level increased in PCOS patients while IL-10 level decreased as compared with control. Also, the GA genotype of PD-1 gene (rs2227982) is significantly associated with a susceptibility of PCOS in Iraqi women and there is significant association between PD-1 level and PD-1 gene (rs2227982) GA genotype in the registered PCOS and control group.

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List of Abbreviations

Abbreviations	Full Nomenclature
AE	Androgen excess
AIH	Autoimmune hepatitis
AMH	Anti – Mullerian Hormone
APCs	Antigen presenting cells
AUC	Area under curve
AURC	Area under roc curve
B7	Biotin
BMI	Body mass index
c DNA	Complement strand DNA
CD	Cluster differentiation
CRP	C –reactive protein
CTLA-4	Cytotoxic T lymphocyte associated protein 4
DCs	Dendritic cells
ddH2O	DE nuclease distilled water
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DNA	Deoxyribonucleic acid
ds DNA	Double strand DNA
ECL	Electro chemiluminescence
EtBr	Ethidium bromide
Ex	Exon
FAI	Free androgen index
FSH	Follicular stimulating hormone
FT	Free testosterone

GnRH	Gonadotrophin releasing hormone
GWAS	Genome wide association studies
H2O2	Hydrogen peroxide
HA	Hyperandrogenemia
Hh signaling	Hedgehog signaling
HPO	Hypothalamic pituitary ovarian axis
HRP	Horseradish peroxidase enzyme
IFN	Interferon
IgC	Immunoglobulin C
IgV	Immunoglobulin V
IL	Interleukin
IL-10	Interleukin -10
IL-10 R1	Interleukin -10 receptor 1
IL-10 R2	Interleukin -10 receptor 2
IR	Insulin resistance
ITIM	Immune- receptor tyrosine- based inhibitory motif
ITSM	Immune- receptor tyrosine- based switch motif
JAK signaling	C –Jun amino terminal kinase
LH	Luteinizing hormone
MAPK	Mitogen –activated protein kinase
MES	(N –morpholino) ethane sulfonic acid
Mfg	Modified Ferriman –Gallwey scoring system
NF- κB	Nuclear factor κb
NIH	National institutes health
NK	Natural killer cells
NTC	No template control
OCP	Oral contraception pills

OD	Ovarian dysfunction
P	Proximal short arm of chromosome
PCOM	Polycystic ovary morphology
PCOS	Polycystic ovary syndrome
PD-1	Programmed cell death -1
PD-L1	Programmed cell death ligand 1
PD-L2	Programmed cell death ligand 2
PI3K/AKT	Phosphoinositide 3-kinase/ protein kinase B(AKT)
PRL	Prolactin
PTP	Protein tyrosine phosphatase
Py-ITSM	Python- immunoreceptor tyrosine based switch motif
Q	Quencher fluorophore
Q	Distal long arm of chromosome
qRT-PCR	Quantitative real time polymerase chain reaction
R	Reporter fluorescence
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
Ru(bpy)	Ruthenium –tris (2,2-bipyridyl) dichloride
SH2	Src homology 2
SHBG	Sex hormone binding globulin
SHP-2	Src homology phosphatase- 2
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
T1D	Type 1 diabetes
T2DM	Type 2 diabetes

Taq	Thermos aquaticus
TH1	T helper 1 cells
TH2	T helper 2 cells
TLR	Toll –like receptor
TMB	Tetra methyl benzidine
TNF	Tumor necrosis factor
TRIS	Tris amino methane
WHR	Waist hip ratio
Zap 70	Zeta chain associated protein kinase 70

1. General Introduction

Polycystic ovary syndrome (PCOS) is characterized by the unregulated menstrual cycle (oligomenorrhea or amenorrhea), elevated androgenic hormones levels (hyperandrogenism) and many cysts of the ovaries (polycystic ovaries). Other features include male pattern hirsutism, acne, increased skin pigmentation with tags sometimes, and obesity **(Zhang *et al.*, 2020)**. PCOS comorbid with insulin resistance, dyslipidemia, and obesity, it also carries significant risk for the development of cardiovascular and metabolic sequelae, including diabetes and metabolic syndrome **(Cincione *et al.*, 2021) .**

The existence of a fully developed hypothalamic – pituitary - ovarian axis and highly harmonized hormonal feedback circles are essential for the normal ovulatory menstrual cycle. Which consisting of three phases (follicular phase, ovulatory phase, and luteal phase), the normal menstrual cycle leads to the format of a mature follicle and liberate of an oocyte during each cycle, without fertilization menses would occur **(Itriyeva, 2022).** Every woman in reproductive age exhibit an FSH level rise at the luteal – follicular transition, stimulating a group of follicular growth in the initial follicular phase. The dominant follicle is specified in the mid - follicular phase, and as this dominant follicle grows it progressively secretes inhibin A and oestradiol for a week prior to ovulation and a later LH surge and oestradiol rise. The progesterone, oestradiol and inhibin A secreted by corpus luteum in response to LH pulses, and arrive at its peak in term of size, vascularization, and secretions **(Mihm *et al.*, 2011).**

1.1. Polycystic Ovary Syndrome

One of the most prevalent endocrine and metabolic illnesses, polycystic ovarian syndrome (PCOS), affects 5–15 percent of women of reproductive age

(Aslam *et al.,* 2022). It is a heterogeneous disorder characterized by chronic ovulatory dysfunction and hyperandrogenism. PCOS patients are also characterized by increase serum levels of luteinizing hormone (LH), which lead to hyperandrogenism and resulting in altered ratio between LH and the follicle stimulating hormone (FSH) **(Iervolino *et al.,* 2021)**. The definition of this disorder as a syndrome not a disease due to the presence of a cluster of symptoms which cannot be identified by a certain etiological factor or as a certificated pathophysiological axis, in addition, it is believed that hormones act on most of body tissues but in a variant rate which results in variation of clinical features due to hormonal disturbances and metabolic consequences **(Krug *et al.,* 2019).**

1.1.1. History and Etiopathogenesis of Polycystic Ovary Syndrome

Stein and Leventhal initially described polycystic ovary syndrome history in 1935 as a mix of amenorrhea, infertility, ovaries that were enlarged, hirsutism, obesity, and persistent anovulation **(Stein, 1935)**. Before the 10th edition of the International Classification of Diseases was published in 1990, the World Health Organization (WHO) previously combined PCOS with sclerotic ovary- Stein-Leventhal syndromes **(Organization, 1992).** The etiopathogenesis of PCOS is complex genetic, environmental and lifestyle interaction. Hyper-androgenism and insulin resistance (IR) are the major characteristics of PCOS **(Moghetti and Tosi, 2021).** PCOS manifests as a complex set of traits that are the consequence of the interaction of numerous hereditary and environmental variables. The shape of polycystic ovaries, elevated androgen levels, and insulin resistance with insulin secretion abnormalities are all heritable conditions as shown in **Figure (1.1).** Prenatal androgen exposure and the fetus's poor growth are environmental causes, while acquired obesity is a key postnatal issue. The diversity of pathways included and absence of a common course lead to the multifactorial

characteristics and heterogeneity of the syndrome **(Rosenfield and Ehrmann, 2016).**

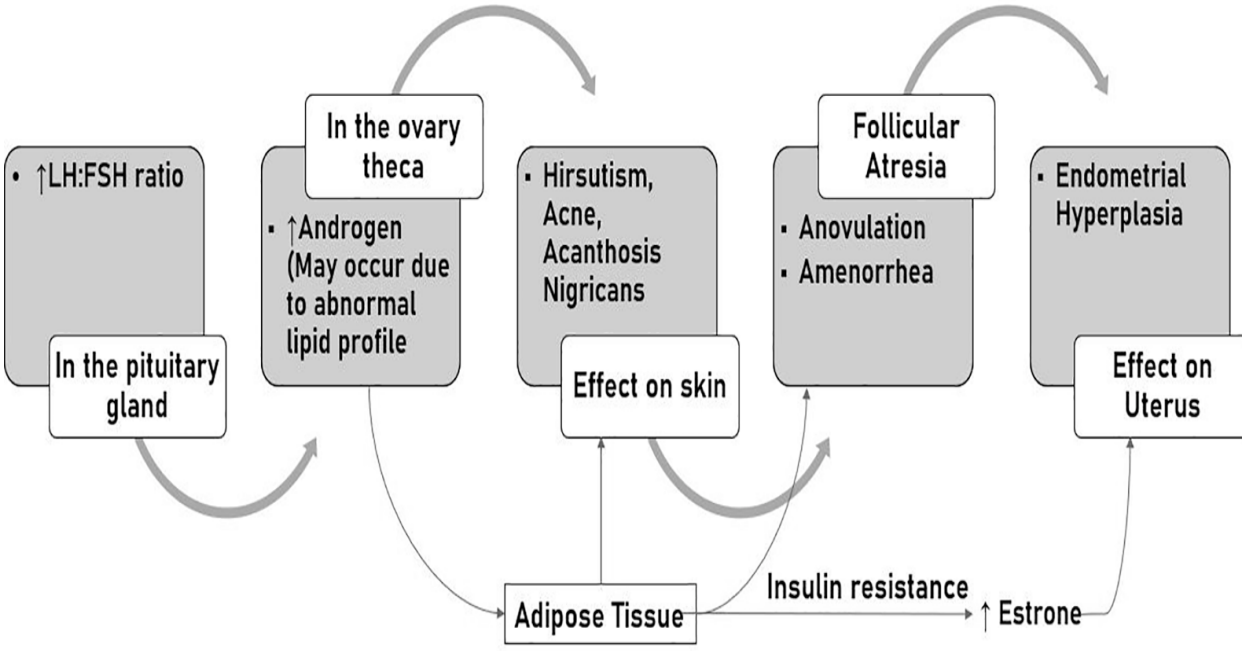


Fig. (1.1): Etiopathogenesis of PCOS (Corrie *et al.*, 2021).

1.1.2. Features of Polycystic Ovary Syndrome

Hyperandrogenism is an important feature for diagnosis to PCOS. In PCOS women, incidence rate of hyperandrogenism is as high as 60%-80%. Increase androgen level leads to (ovulatory disorder, menstrual disturbance, hirsutism, alopecia and acne vulgaris). hyperandrogenism is not only a clinical feature of PCOS, but also consider an important risk factor **(Ye *et al.*, 2021)**. Women with PCOS are most likely to progress metabolic syndrome, such as diabetes mellitus type II and cardiovascular disease in the long-term. uncontrolled ovarian steroidogenesis develops because of theca cell hyperplasia, which is lead to the fact that the ovary is a primary source of androgen excess between PCOS women. Anovulation is progress as a result of androgen excess that developed because of the follicular arrest. There is a significant association of decrease maturation rates

and developmental competency of oocyte with androgen excess **(Dadachanji *et al.*, 2018)**. Insulin resistance and hyperinsulinemia have the ability to develop endocrine and reproductive features of PCOS. Nevertheless, hyperandrogenemia may affect insulin action directly and / or through many changes take place in different body sites specially muscle and adipose tissue. Furthermore, excess body fat and obesity itself can aggravate the whole condition as shown in **Figure (1.2) (Abasian *et al.*, 2018)**. Anyhow, there are a lot of heartless circles in this processes, with bidirectional relations between hyperandrogenemia, adipose dysfunction, insulin resistance and different other factors such as inflammation and oxidative stress which crew the picture and make it extremely difficult to understand where the guiltiness lies **(Moghetti and Tosi, 2021)**.

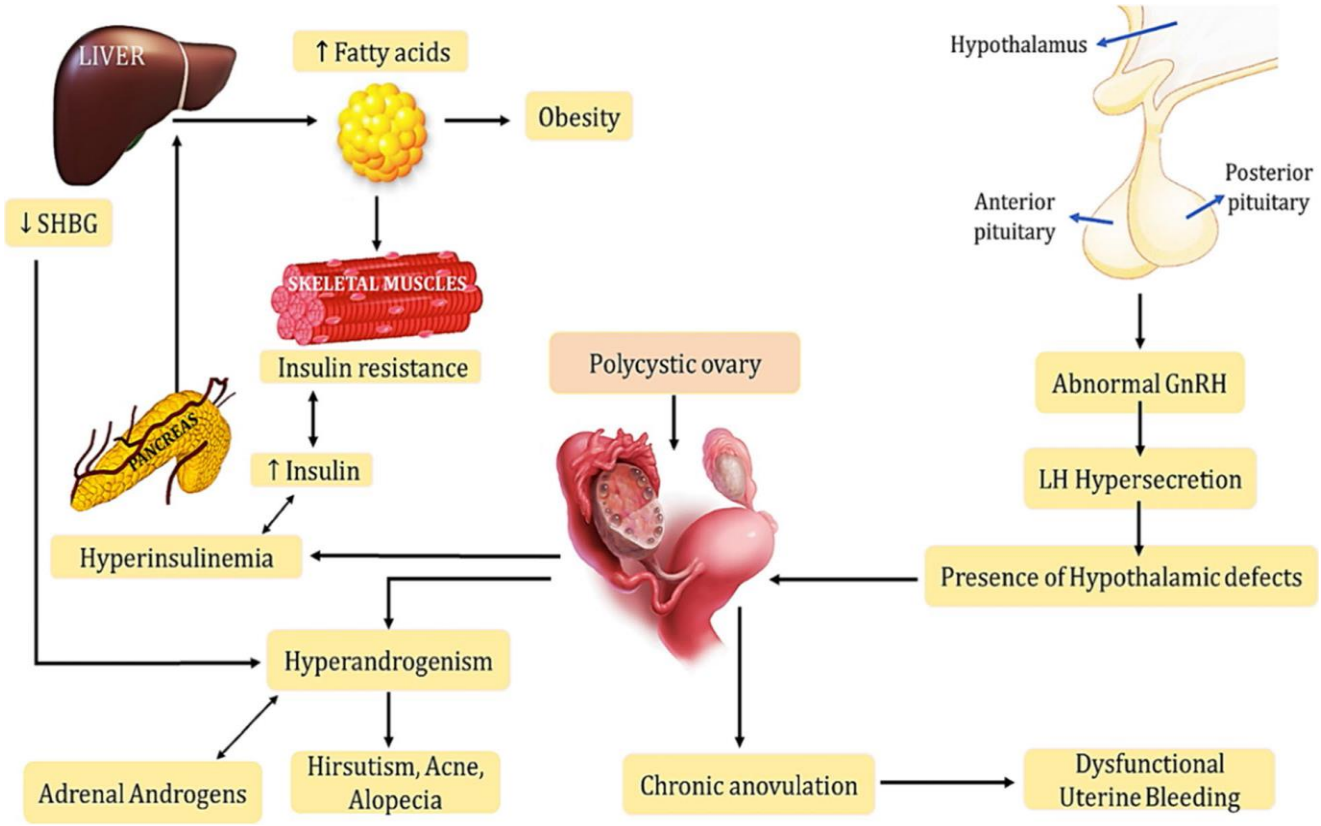


Fig. (1.2): The main pathogenic factors in PCOS (Chaudhary *et al.*, 2021).

1.1.3. Prevalence of Polycystic Ovary Syndrome

The main cause of infertility in Iraqi Arab female is PCOS which represent about 46% of all infertility cases **(Saeed *et al.*, 2021)**. The PCOS prevalence seems to be increasing due to changes in lifestyle, nutrition and obesity **(Fattah *et al.*, 2021)**. Polycystic ovary is a syndrome without much popular acknowledgment and PCOS women frequently do not request care. It is also must been mentioned that even in the same ethnic group (i.e. Middle East Women), there is huge difference in the prevalence of PCOS depend on different clinical symptom **Figure (1.3) (Abasian *et al.*, 2018)**.

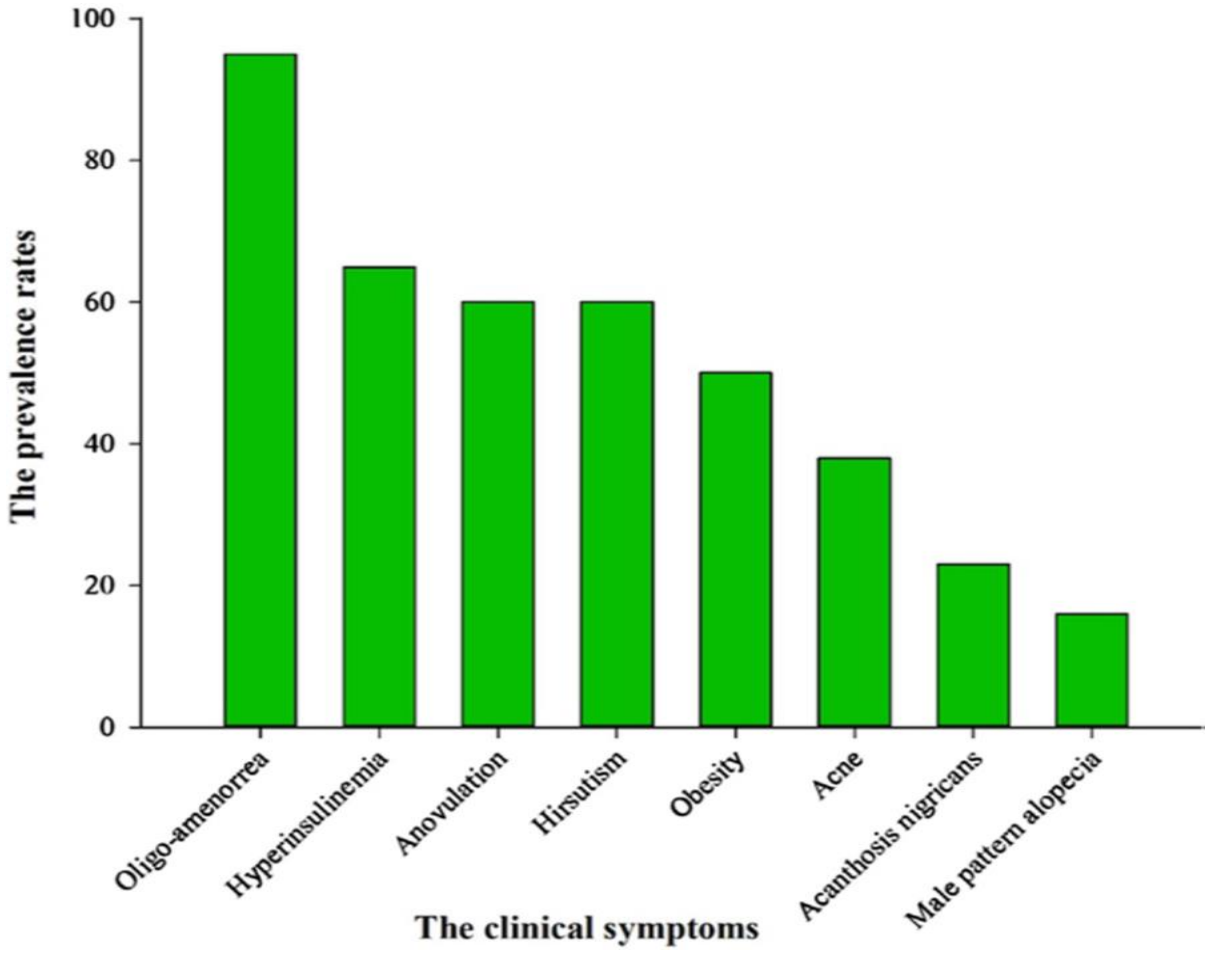


Fig. (1.3): The prevalence rates of clinical symptoms in PCOS patients.

1.1.4. Hypothalamic Pituitary Ovarian Axis

Gonadotropin-releasing hormone (GnRH), a neuropeptide that is released into the circulation and travels to the pituitary gland, initiates by the signaling process in the hypothalamus. In response to this signal, the pituitary releases the gonadotropin hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH). The anterior pituitary gland normally releases LH and FSH in a pulsatile pattern, in response to GnRH which release in a pulsatile pattern also from the hypothalamus **(Speroff and Fritz, 2005)**.

FSH and LH bind to the ovarian receptors, which lead to secretion of estrogen and progesterone. In the ovary, LH is a glycoprotein hormone fundamental for sexual development and reproduction binds to LH receptor on the theca-cells. Which stimulate theca cells to convert available cholesterol into androgen. This androgen then carried to the granulosa-cells where FSH The glycoprotein hormone binds to a receptor of FSH that stimulate the transformation of androgen into estradiol-β-17 using aromatase enzyme.

In order to assist oocyte development, the steroid hormone estrogen steadily rises during the follicular phase of the menstrual cycle. Once the oocyte is ruptured from the ovary, the level of estrogen slowly decreases at the luteal phase of the menstrual cycle and progesterone level start to increase **(McNATTY *et al.*, 1979)**.

Normally folliculogenesis depends on transformation of intra-ovarian androgens for estradiol synthesis, excess androgen production as a consequence of folliculogenesis disordered results in poor maturation of follicles and increased follicular atresia **(Balen *et al.*, 2005)**. Increase LH level enhance the production of androgen. Increased LH causes negative feedback at FSH levels, which may limit the transfer of androgen to estrogen and aggravate the excess androgen in the

ovaries **(Nardo *et al.*, 2008)**. This pattern of secretion gives rise to an abnormal LH/FSH ratio in many patients, making it likely a valuable diagnosis marker of PCOS **(Le *et al.*, 2019)**.

1.1.5. Polycystic Ovary Syndrome Diagnostic Criteria

For the diagnosis of PCOS in premenopausal women, there are three distinct sets of diagnostic criteria **Table (1.1) and (1.2) (Mumusoglu and Yildiz, 2020).**

Table (1.1): Polycystic ovary syndrome diagnostic criteria sets

PCOS features	(NIH) 1990/ National Institutes of Health	Rotterdam 2003	Androgen Excess 2006
Hyperandrogenism	✓	±	✓
Ovarian dysfunction	✓	±	±
Polycystic ovarian morphology	–	±	±

✓Essential diagnostic criteria; ± (optional) It may present or not; - Not identified

Table (1.2): Diagnosis statement of PCOS

National Institutes of Health (NIH) 1990	Rotterdam 2003	Androgen Excess and PCOS Society 2006
Hyperandrogenism and Ovarian Dysfunction are Essential clinical features	Any 2 of 3 features for diagnosis (Hyperandrogenism, Ovarian Dysfunction, Polycystic Ovarian Morphology)	Hyperandrogenism is the essential clinical feature and Ovarian Dysfunction and/or Polycystic Ovarian Morphology should be accompanied.

1.1.5.1. Ovulatory Dysfunction

Menstrual cycles that are irregular defined as having cycle durations of <21 or >35 days or having 8 menstrual cycles annually which are a clinical manifestation of ovulatory dysfunction. While amenorrhea mean absence of menstruation, more than 3 years post menarche **(Mumusoglu and Yildiz, 2020).**

1.1.5.2. Hyperandrogenism

Clinical and/or biochemical hyperandrogenism characterizes the majority of PCOS patients. Hirsutism is the main symptom of clinical hyperandrogenism. The modified Ferriman-Gallwey scoring system, which involves the observer rating nine different body regions (the upper lip, chin, chest, upper and lower abdomen, thighs, upper and lower back, and upper arms) on a scale of 0 to 4, should be used to assess hirsutism. This system takes into account the degree of excessive terminal hair growth. The cutoff for hirsutism is a total mFG score of 4-6, depending on ethnicity. A less precise indication of hyperandrogenism is acne and alopecia. Biochemical hyperandrogenism is the term used to describe elevated serum androgen levels **(Mumusoglu and Yildiz, 2020).**

1.1.5.3. Polycystic Ovarian Morphology

To diagnose polycystic ovary changes using ultrasound there were criteria like the presence of 12 follicles or more in ovaries with diameter measuring between 2 and 9 mm or increase in ovarian volume more than 10 cm^3 in one ovary at least. A classical features of PCOS ultrasound are the arrangement of follicles in periphery of ovaries giving the image of "string of pearls"**(Bozdag *et al.*, 2016)**.

1.1.6. Phenotypes of Polycystic Ovary Syndrome

Four phenotypes of PCOS from A to D were investigated according to Rotterdam criteria 2003 and 2006 AE-PCOS Society criteria **(Lizneva *et al.*, 2016) Table (1.3).** Type A is the most sever phenotype, and D is the least sever one. Types A and C are the most prevalent types **(Cree-Green, 2017)**.

1.1.6.1. Classic Polycystic Ovary Syndrome (phenotype A and B)

Phenotype A is frequently referred to as (Complete) form of PCOS, while phenotypes A and B are collectively referred to as (Classic) PCOS. When compared to ovulatory or non-hyperandrogenic phenotypes C and D, patients with classic PCOS appear to be more obese and hirsute, have more irregular menstrual cycles, and are more likely to have insulin resistance and dyslipidemia **(Azziz, 2018, Lim *et al.*, 2019).**

1.1.6.2. Ovulatory Polycystic Ovary Syndrome (Phenotype-C)

It is known as "ovulatory" PCOS since the 2003 Rotterdam criteria and the 2006 AE-PCOS Society standards both include an additional phenotypic, phenotype -C (HA and PCOM, but without OD). Patients with phenotype C have hirsutism, testosterone, and lipid levels that fall between those of phenotypes A-B and D **(Lizneva *et al.*, 2016)**.

1.1.6.3. Non-hyperandrogenic Polycystic Ovary Syndrome (Phenotype D)

The 2003 Rotterdam criteria added the Phenotype -D (OD and PCOM, without HA), known as "non hyperandrogenic PCOS." These individuals have normal levels of testosterone and the slightest form of endocrine malfunction, such as insulin resistance **(Lizneva *et al.*, 2016).**

Table (1.3): Possible phenotypes of PCOS (Lizneva *et al.*, 2016).

Phenotype	Hyperandrogenism	Ovarian Dysfunction	Polycystic Ovarian Morphology
Type A	+	+	+
Type B	+	+	
Type C	+		+
Type D		+	+

1.1.7. Biochemical Changes and Polycystic Ovarian Syndrome

Clinical and biochemical androgen excesses are major characteristics of women with PCOS. Studies have reported an association of PCOS with multiple endocrinal, reproductive and even metabolic risks that reduces quality of life through the lifelong of the affected female **(Ajmal *et al.*, 2019)**. For the diagnosis of PCOS, the basic parameters that must be considered are FSH, LH and androgen level. Raised LH level leads to an increase in androgen level that gives rise to the progression of PCOS. PCOS is being strongly associated with future development of type-2 Diabetes mellitus and hyperinsulinemia. The strongest association between abnormalities of ovulation associated with PCOS and elevated levels of the anti-Mullerian hormone (AMH) in the syndrome has been reported to the elevated LH concentration. It was proposed that a GnRH stimulation test could lead to LH excess. Additionally, increased LH: FSH ratio with GnRH stimulation may be a very helpful supplementary diagnostic technique for identifying PCOS **(Akram and Roohi, 2015).**

1.1.8. Complications of Polycystic Ovarian Syndrome

PCOS is understood to be a chronic illness. Menstrual abnormalities, hyperandrogenism, and/or infertility are the predominant symptoms throughout the first years of reproduction life. The lifelong implications of this disease, however, must be understood by both women and doctors. Indeed, in roughly 50% of individuals, metabolic abnormalities will eventually replace reproductive failure **(Peigné and Dewailly, 2014)**. Obesity, glucose intolerance, type 2 diabetes, cardiovascular disease, and endometrial cancer can all develop as people become older in age. Pregnancy problems like gestational diabetes and gestational hypertension are more common in women with PCOS, and there is also a higher risk of preterm birth and perinatal mortality **Figure (1.4) (Wang *et al.*, 2013)**.

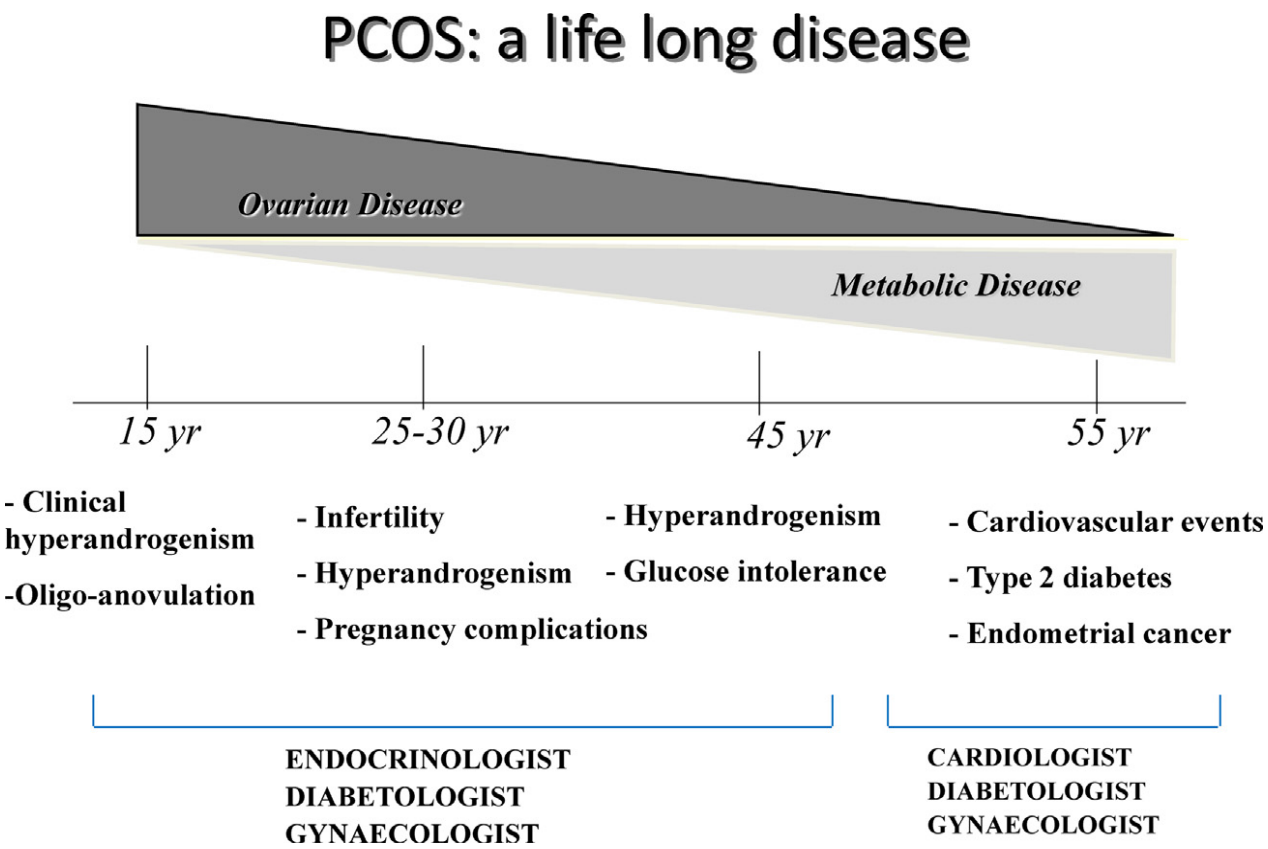


Fig. (1.4): PCOS symptoms and complication for life long (Peigné and Dewailly, 2014).

1.2. Interleukins

One of a group of related proteins made by leukocytes (white blood cells) and other cells in the body. Interleukins regulate immune responses **(Khosravi, Katuli *et al.*, 2021).** Interleukins play a critical role in the control of the innate and adaptive immune responses **(Chen *et al.*, 2006).**

1.2.1. Interleukin-10

Interleukin-10 (IL-10), which was first discovered in 1991, is an immunosuppressant and an anti-inflammatory cytokine that is essential for the body's defense processes **(Moore *et al.*, 2001, Gnanadass *et al.*, 2021).** IL-10 belongs to the class-2 group of cytokines, which also includes IL-19, IL-20, IL-22, and IL-24 **(Wei *et al.*, 2019)** and interferons type-I (IFN-alpha, -beta, -epsilon, -kappa, -omega), type-II (IFN-gamma) and type-III (IFN-lambda) **(Liu *et al.*, 2021)** . Reduce IL-10 concentration is lead to obesity and metabolic syndrome **(Scarpelli *et al.*, 2006).** Decrease in IL-10 level was present in PCOS patients **(Talaat *et al.*, 2016)**. IL-10 is a critical biomarker for poor disease outcome **(Huang *et al.*, 2020).**

1.2.2. Structure of Interleukin-10

The IL-10 cytokine is a homodimer; with 178 amino acid in each of its subunits **(Nissar *et al.*, 2021).** IL- 10 is a member of T helper cells (TH2) and inhibit the activity of (TH1)cells members **(Rasquinha *et al.*, 2021).** Monocytes and lymphocytes, specifically type-II T helper cells (TH2), mast cells, regulatory T cells, and a specific proportion of activated T cells and B cells, produce the majority of the human IL-10 protein, which is encoded by the IL-10 gene on chromosome (1) **(Eskdale *et al.*, 1997)**.

1.2.3. Production and Function of Interleukin - 10

Interleukin-10 is an inflammatory suppression cytokine which excreted by activated immune cells. IL-10 can be produced by monocytes upon triggering in these cells **(Said *et al.*, 2010).** as well as non-immune cells such as epithelial or neuronal cells **(Sözen *et al.*, 2017).**

IL-10 take action by way of a trans-membrane receptor complex, which is composed of IL-10R1 and IL-10R2, and control the functions of lymphocytes, macrophages, and many other cells **(Ralston and De Crombrugghe, 2006).**

It limits the secretion of proinflammatory cytokines, such as TNF, IL-1, IL-6, IL-12 and IL-2 , it regulate the differentiation and proliferation of immune cells, such us macrophages, T cells and B cells; and decreased monocyte activation **(Bakiri and Mingomataj, 2019).**

1.2.4. Interleukin-10 and Polycystic Ovary Syndrome

Pro-inflammatory cytokine from TH1 cells and macrophage expression is down-regulated by the anti-inflammatory cytokine interleukin-10. To keep the ovary functioning properly, it's crucial to maintain a balance in the levels of inflammatory markers.

Enhanced production of IL-6 and TNF-α and decreased production of IL-10 may produce imbalance between pro-inflammatory and anti-inflammatory cytokines which lead to altered steroidogenesis, delayed follicular maturation and ovarian dysfunction as shown in **Figure (1.5) (Vural *et al.*, 2010).**

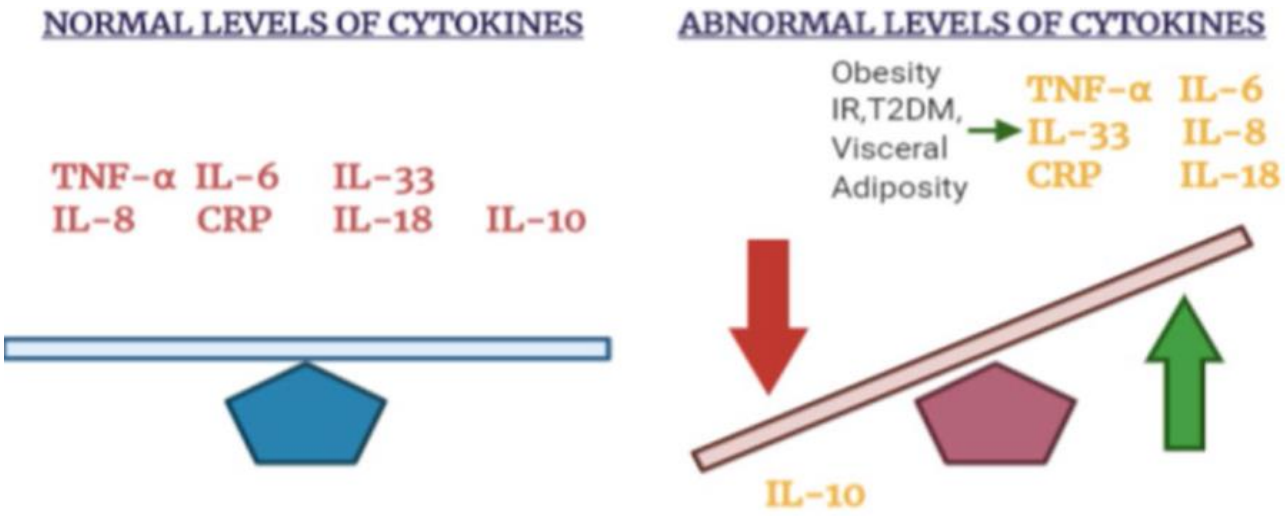


Figure (1.5): Inflammatory marker concentrations in PCOS and normal ovaries. Inflammation-promoting cytokines are expressed more, while anti-inflammatory cytokines are expressed less (Abraham Gnanadass *et al.*, 2021).

Obesity and the metabolic syndrome are linked to decreased IL-10 levels. The fact that PCOS patients had lower plasma levels of IL-10 suggests that clomiphene citrate increases IL-10 and helps PCOS women become pregnant and ovulate more frequently **(Abraham Gnanadass *et al.*, 2021).**

1.2.5. Chronic Inflammation in Polycystic Ovary Syndrome

Low-grade inflammation and inflammatory indicators have been linked to PCOS. Understanding the pathophysiology of PCOS and its therapy by inhibition or regulation of associated pathways can be improved by looking into the inflammatory mediators involved for its development. Because ovulation is a semi-inflammatory process, visceral adipose tissue can induce an inflammatory response and sustain the inflammation in adipocytes by influencing the release of inflammatory cytokines. Out of this regulated inflammation, PCOS can arise **(Rostamtabar *et al.*, 2021)**.

Chronic low grade inflammation may be relate not only to adiposity, but also to androgens concentration **(Rudnicka *et al.*, 2021)**. Obesity, especially the visceral type, is seriously common in PCOS patients **(Milanović *et al.*, 2020)**. Androgens trigger the differentiation of pre-adipocytes to adipocytes, mainly in the abdomen area and cause visceral-type obesity **(de Medeiros *et al.*, 2021)**.

Obesity is a metabolic condition with chronic low- grade inflammation and higher pro-inflammatory cytokines, chemokines levels. According to that , inflammatory markers are higher in PCOS patients **(Rudnicka *et al.*, 2021)**.

Immune cells of a particular type produce communication proteins called cytokines that have a big impact on other cells. It is excreted from the ovary by leukocytes, oocytes, and follicular cells **(Abraham Gnanadass *et al.*, 2021)**. These signaling molecules were produced in the ovary, which suggests that they can monitor ovarian function in a paracrine and autocrine manner **(Qiao and Feng, 2011, Abraham Gnanadass *et al.*, 2021)**. They regulate the synthesis of gonadal hormones, maturation of the ovarian follicle, steroidogenesis, maturation of corpus luteum and differentiation of the ovum **(Field *et al.*, 2014)**.

The T- cells can be classified into three classes: T- helper cells (CD3, CD4 T- helper) and T- cytotoxic cells (CD3, CD8 T- cytotoxic) and Regulatory T-cells, which produce IL-10 also **(Gagliani and Huber, 2017)**. However, it has been discovered that human pre-ovulatory follicles include T-cells and there is very active interaction between subtypes **(Gallinelli *et al.*, 2003)**.

A careful balance between pro- and anti-inflammatory responses is necessary to generate an efficient immune response to an infection while also limiting tissue damage **(Cicchese *et al.*, 2018)**. Initial research showed that IL-10 deficiency, caused by disruption of the IL-10 gene, or IL-10 signaling, via antibody blockage of the IL-10 receptor, caused the majority of intracellular

infections to be better controlled or removed more quickly. However, pathogens can also take advantage of the immunosuppressive abilities of IL-10 to aid in their own survival **(Ouyang and O'Garra, 2019).**

1.3. Programed Cell Death Protein-1

Programed Cell Death Protein-1 (PD-1) is present on the surface of T- cells and B- cells. It is responsible for organizing how the immune system reacts to human self-cells by down-regulating the immune system and encouraging self-tolerance by suppressing inflammatory activity of T-cells **(Annibali *et al.*, 2018).** Immune checkpoint PD-1 exhibit defense against autoimmunity. It causes more antigen-specific T lymphocytes to undergo apoptosis (programmed cell death) **(Janssens and Cools, 2020).** PD-1 is an inhibitor of both adaptive and innate immune responses, and is expressed on activated T-cells, natural killer (NK) and B lymphocytes, macrophages, dendritic cells (DCs) and monocytes **(Ahmadzadeh *et al.*, 2009).**

1.3.1. Programmed Cell Death-1 Structure

Programmed cell death–1 is a transmembrane protein containing 288 amino acids with an extracellular N-terminal IgV like domain a membrane-permeating domain and a cytoplasmic tail located at the N and C ends, respectively, with two tyrosine base **(Neel *et al.*, 2003).** Honjo investigate it as an apoptosis-associated gene in 1992 at Kyoto University **(Ishida *et al.*, 1992, Patsoukis *et al.*, 2020b).** Later research from the same team made it clear that T and B cell antigen receptors signaled to produce PD-1 production **(Patsoukis *et al.*, 2020b).** This was a part of the suppression of immunological responses **(Patsoukis *et al.*, 2020b).** The ligands of PD-1 The type I transmembrane glycoproteins PD-L1 and PD-L2, which have an extracellular domain of an immunoglobulin as their

primary structural pattern, a transmembrane region, and a cytoplasmic tail that works as an anchor site for signaling or scaffolding proteins **(Zhang *et al.*, 2004).** Programmed death–1 contains a front β sheet face and a back β sheet face. It is necessary for the front "sheets" of the interacting molecules to be present for PD-1 to bind to its ligands. PD-L2 has a higher affinity for PD-1 than any of its other ligands, which is attributable to its particular sequence properties. This is supported by variations in the interactions between PD-1 and each of its ligands **(Lázár-Molnár *et al.*, 2008).**

1.3.2. Mechanisms and Targets of Programed Cell Death Protein- 1 Signaling

The cytoplasmic tail of PD-1 have two tyrosine-based structural motifs, an immune-receptor tyrosine-based inhibitory motif (ITIM), and an immunoreceptor tyrosine-based switch motif (ITSM) **(Shinohara *et al.*, 1994).** Mutational studies have shown that PD-1 inhibitory function is dependent on the ITSM phosphotyrosine, which acquire (Src homology 2) domain containing phosphatase-2 (SHP-2), resulting in dephosphorylation and reduce regulation of downstream signaling pathways **(Yokosuka *et al.*, 2012).** SHP-2 has two SH2 domains, N-terminal (N-SH2) and C-terminal (C-SH2), followed by a phosphatase [protein tyrosine phosphatase (PTP)] domain, and a C-terminal hydrophobic tail with two tyrosine phosphorylation sites. At the major state, the N-SH2 domain of SHP-2 folds into an auto inhibitory blocked modification to directly shut off the active PTP site. Interaction of the N-SH2 domain with phosphotyrosine peptide breaks this interaction and activates the enzyme. Binding of both SH2 domains is in demand for full SHP-2 enzymatic activation, with the C-SH2 domain play a part in binding energy **(Neel *et al.*, 2003).** Alternative mode of PD-1: SHP-2 interaction preferentially occurred in live cells, whereas, after PD-1 phosphorylation, SHP-2, through its N-SH2 and C-SH2 domains, could link

two phosphorylated pY-ITSM residues on two PD-1 molecules centering at the plasma membrane, forming a PD-1:PD-1 dimer as shown in **Figure (1.6) (Patsoukis *et al.*, 2020a).** Programmed death–1 signaling can be overcome by interleukin-2 and that only cytokine that activate signal transducer and activator of transcription 5 (STAT5) can assist PD-1 inhibition **(Carter *et al.*, 2002).**

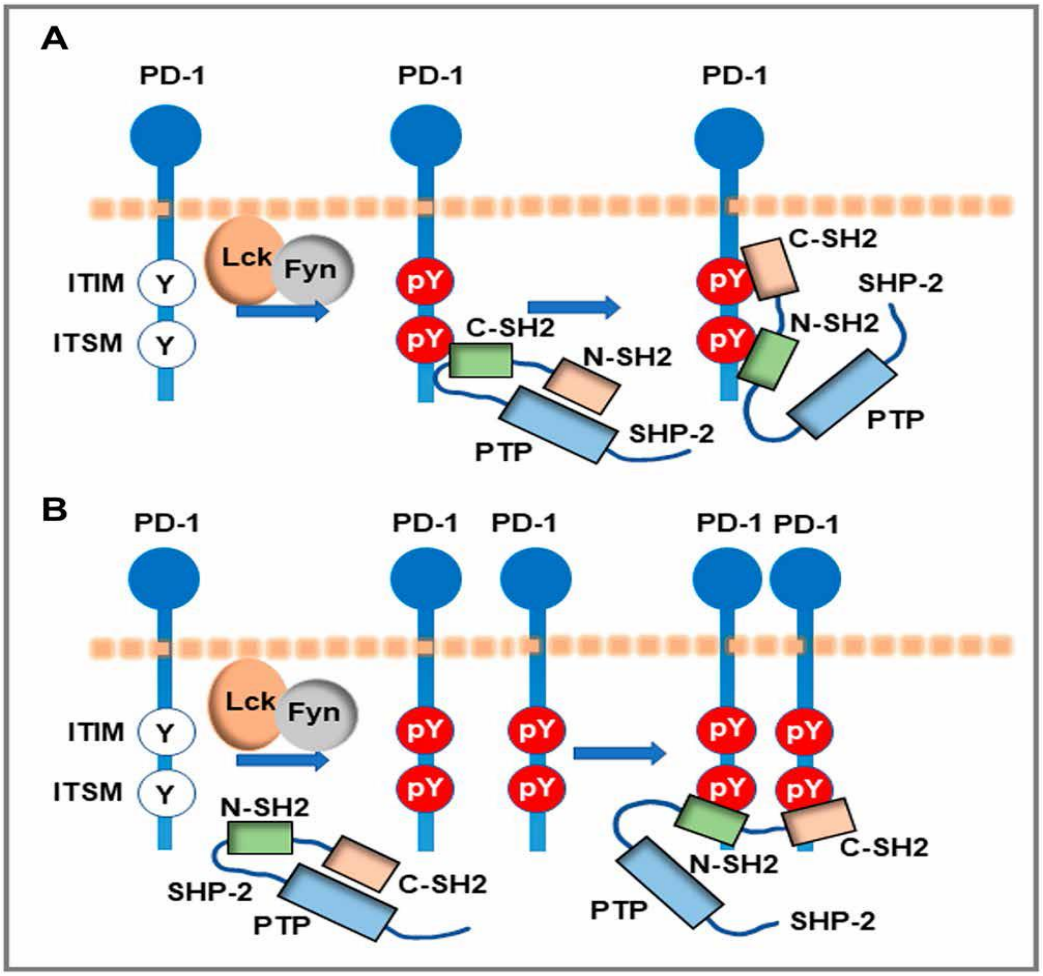


Fig. (1.6): PD-1/SHP-2 interaction modes. (A) Two-step binding model, according to which SHP-2 C-SH2 binds to PD-1 pY-ITSM with strong affinity, recruiting PD-1 to SHP-2, while PD-1 pY-ITIM binds to N-SH2, displacing it from the PTP site to activate the phosphatase. (B) Dimerization model, according to which SHP-2 bridges two pY-ITSM residues on two PD-1 molecules via its N-SH2 and C-SH2 domains forming a PD-1: PD-1 dimer and inducing SHP-2 activation. (Patsoukis *et al.*, 2020b).

1.3.3. Programed Cell Death -1 Signaling Pathways

Programed Cell Death -1 performed various pathways as shown in Figure 1-5 and listed below:

A. PI3K / AKT signaling pathway

Cell proliferation and activities related to apoptosis are regulated by the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway **(Han *et al.*, 2020, Ruchi Sharma *et al.*, 2017).**

B. MAPK signaling pathway

The mitogen-activated protein kinase (MAPK) signaling It controls cell proliferation, differentiation, invasion, metastasis, and death by phosphorylation activation and is linked to the conversion of extracellular signals to intracellular responses **(Han *et al.*, 2020, Xu *et al.*, 2018).**

C. JAK - STAT signaling pathway

A number of cytokines, IFNs, growth factors, and related substances use JAK signaling to activate the STAT pathway **(Han *et al.*, 2020, Banerjee *et al.*, 2017).**

D. WNT signaling pathway

WNT signaling promotes tumor growth, malignant transformation, and resistance to traditional cancer therapies **(Han *et al.*, 2020, Harb *et al.*, 2019).**

E. NF-κB signaling pathway

The expression of the PD-L1 gene can be induced by Toll-like receptor (TLR) or IFN-γ-driven nuclear factor that may play a dual role: targeting tumor cell proliferation and survival, as well as tumor immune checkpoints **(Han *et al.*, 2020).**

F. Hedgehog signaling pathway

For substrate cells to proliferate, the Hedgehog (Hh) signaling system is crucial, and abnormalities in this route may result in the development of tumors **(Han *et al.*, 2020).**

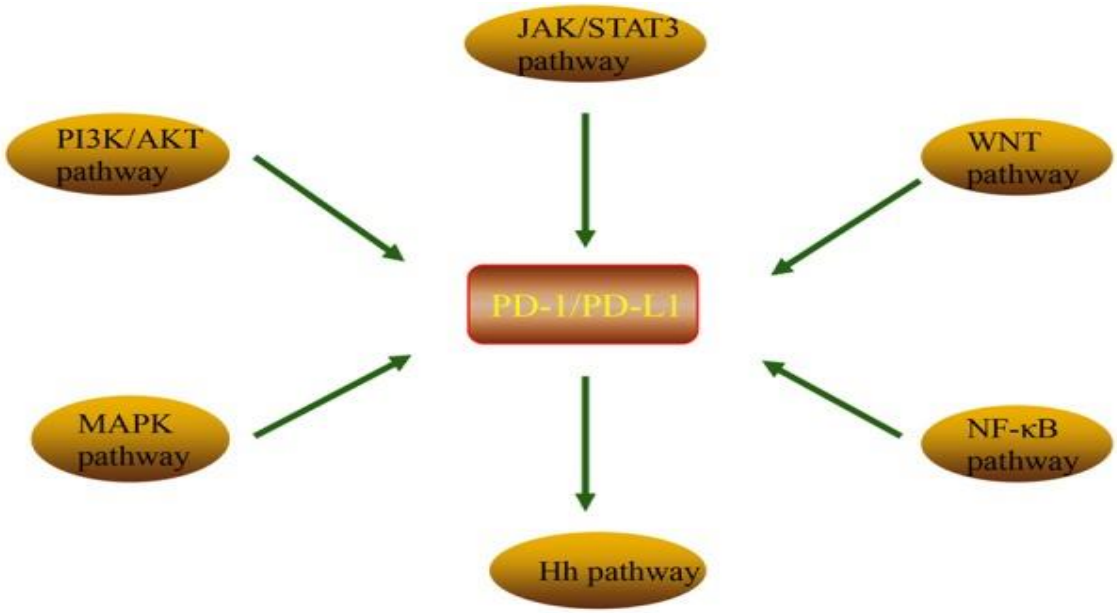


Fig. (1.7): Various pathways regulation of PD-1/PD-L1 expression (Han *et al.*, 2020).

1.3.4. Programmed Cell Death-1 Protein Ligands

PD-L1 and PD-L2 are the two ligands for PD-1. They are transmembrane protein receptors. Lieping Chen and colleagues identified and cloned human B7-H1 gene in 1999 and recognized the molecule of having inhibitory effects on T cells **(Qin *et al.*, 2019).** With the discovery of interaction of PD-1 and B7-H1 molecule, it was renamed as PD-L1. Structurally, PD-L1 is a 290-amino acid protein receptor encoded by Cd274 gene, comprising of 7 exons, and located on chromosome 9 in humans. It is composed of 2 extracellular domains, IgV- and IgC-like domains; a transmembrane domain; and a cytoplasmic (intracellular) domain **(Ibañez-Vega *et al.*, 2021).** The intracellular domain of PD-L1 is short

comprising of 30 amino acids, and there is no known function for this domain. The protein is constitutively expressed on many cell types, including antigen-presenting cells (APCs), T cells, B cells, monocytes, and epithelial cells, and is upregulated in a number of cell types after the activation in response to proinflammatory cytokines such as IFNγ and IL4 through signal transducer and activator of transcription-1 (STAT1) and IFN regulatory factor-1 **(Zhao *et al.*, 2013).**

PD-L2 is encoded by Pdcd1lg2 gene adjacent to Cd274 gene separated by 42 kb of intervening genomic DNA in human. It is composed of 273 amino acid residues and comprised of 7 exons which consist of IgV-like domain, IgC-like domain, transmembrane domain, and cytoplasmic (intracellular) domain. In contrast to PD-L1 expression, PD-L2 is restricted largely to antigen-presenting cells APCs and it is inducible expressed on dendritic cells DCs, macrophages, and bone marrow-derived mast cells **(Akinleye and Rasool, 2019).**

The upregulation of PD-Ls is associated with non-small cell lung cancer, melanoma, and renal cell carcinoma whereas downregulation is associated with inflammatory and autoimmune diseases such as type 1 diabetes (T1D), encephalomyelitis, inflammatory bowel disease (IBD), rheumatoid arthritis (RA), autoimmune hepatitis (AIH), systemic lupus erythematosus (SLE), systemic sclerosis, myocarditis, and multiple sclerosis (MS) **(Ibañez-Vega *et al.*, 2021).**

PD-1 delivers a negative signal when bound to its ligands, PD-L1 and PD-L2. PD-1 suppresses T cell activation through the recruitment of phosphatase SHP-2 and the subsequent inactivation of Zap 70, which plays a critical role in T-cell receptor signaling **(Hui *et al.*, 2017).** Though PD-L1 is expressed infrequently in normal human tissues its expression is upregulated by IFN-γ and other

cytokines that are released by activated T cells **(Berger and Pu, 2018).** The expression of PD-L1 in peripheral tissues is crucial to prevent immune-mediated damage at the time of an inflammatory response, as the activation of PD-1 inhibits T-cell effector functions that would otherwise be carried out against the target cell **(Francisco *et al.*, 2010)**

1.3.5. Apoptosis in Polycystic Ovary Syndrome

Apoptosis is a type of programmed cell death that take place in multicellular organisms **(Nakao *et al.*, 2022).** Biochemical phenomenon result in characteristic morphological cell changes and death. These changes include cell shrinkage and nuclear fragmentation **(Vicar *et al.*, 2020).** Granulosa cells supply the nutrients for oocyte and regulate its growth. on the other hand, the central oocyte stimulates growth and differentiation of granulosa cells **(Alam and Miyano, 2020).** These bidirectional mutual actions between the oocyte and granulosa cells have an effect on follicle maturation. According to this, apoptosis within the granulosa cells is an essential part of folliculogenesis. So there is greater incidence of apoptosis in between PCOS women **(Strączyńska *et al.*, 2022).** Granulosa cell apoptosis is a complex process which can be triggered by lots of factors which includes inflammatory cytokines **(Wang *et al.*, 2020).**

1.3.6. Polycystic Ovary Syndrome and Programed Cell Death Protein - 1

Programmed cell death 1 (PD-1) also known as (PD CD-1) play important roles in inhibition of inflammation, autoimmune diseases, and tumors **(Ponce de León *et al.*, 2019)**. PCOS patients have a uniform clinical feature of chronic inflammation. T cell activation is prevented by the suppression signal molecule PD-1 **(Han *et al.*, 2021).** PD-1 ligand, can induce T-cells apoptosis and trigger cells to secrete IL-10, that induce immunosuppressive effects **(Yan *et al.*, 2014)**.

Both CD4+ and CD8+ T lymphocytes from infertile PCOS patients had higher levels of PD-1 **(Li *et al.*, 2019b).**

The host is protected from disease by the immune system, which is a defense mechanism made up of numerous biological components. When the immune system of the body is compromised, a number of diseases can result. Immune systems play a role in controlling polycystic ovary syndrome. According to studies, patients with PCOS have excessive numbers of leukocytes, endothelial dysfunction, and a disturbance of the proinflammatory cytokines. They also have a chronic low-grade inflammation condition. Human preovulatory follicles contain significant numbers of immunocompetent cells, such as T-cells, B-cells, macrophages, and dendritic cells. T-cells, which make up the majority of lymphocytes, perform a number of biological tasks, the majority of which are connected to the body's cellular immune response. They have the ability to directly kill target cells or to increase and broaden the immunological effect by releasing lymphatic factor **(Li *et al.*, 2019b).**

1.4. Genetic Basis of Polycystic Ovary Syndrome

The PCOS is heterogeneous condition with uncertain cause. Genetic studies have shown that PCOS is inherited in an autosomal dominant way, with a 50% likelihood of inheritance from mother to daughter. Cooper and colleagues published the first study on the genetic basis of PCOS in 1968. Multiple siblings and relatives have been documented in PCOS studies in families with an autosomal dominant inheritance.

The prevalence of PCOS in the first-degree relative of the individual, which was reported to be between 55 and 60 percent in a number of small families, reinforced the idea that the condition being inherited autosomal dominantly. Later, single-gene causes of male pattern baldness, oligomenorrhea in PCOS

women, and hirsutism were discovered **(Khan *et al.*, 2019).** Obesity and IR are linked to PCOS, which ultimately causes T2D **(Pelanis *et al.*, 2017).** A single gene defect may be associated with autosomal dominant transmission, although PCOS is acknowledged as a polygenic disorder. One of the possible causes, where a certain gene in any family may have a predominating influence and trigger the phenotypic manifestation, may be the reason why it happens **(De Leo *et al.*, 2016).**

Women with PCOS will have higher amounts of testosterone and other androgen hormones, which also causes irregular menstruation, acne, and weight gain, which can result in obesity and infertility **(Ollila *et al.*, 2017).** The etiology of PCOS has been identified to have a genetic basis **(Diamanti-Kandarakis and Piperi, 2005).** Familial and twin studies have identified that PCOS is an inherited trait **(Vink *et al.*, 2006).**

A recent large genome meta-analysis identified 14 gene loci related to the remarked metabolic, reproductive and pathophysiologic findings in PCOS women **(Day *et al.*, 2018).**

PCOS is a complex illness that has several anomalies as causes. All genes and mutations that directly or indirectly impact the ovaries are linked to PCOS. In patients from the same family, various genes have distinct genetic susceptibilities. Case-control studies with a larger population size and genome-wide association studies (GWAS) can be useful in identifying potential associations in such a disease. Parental analysis in these disorders is frequently impractical, although the disease's recognized risk can be approximated **Figure 1-8** shows a summary of the genetic profile of PCOS **(Khan *et al.*, 2019).**

It has not been revealed, nevertheless, if the PD-1 gene polymorphisms are connected to the prevalence and progression of PCOS **(Han *et al.*, 2021).**

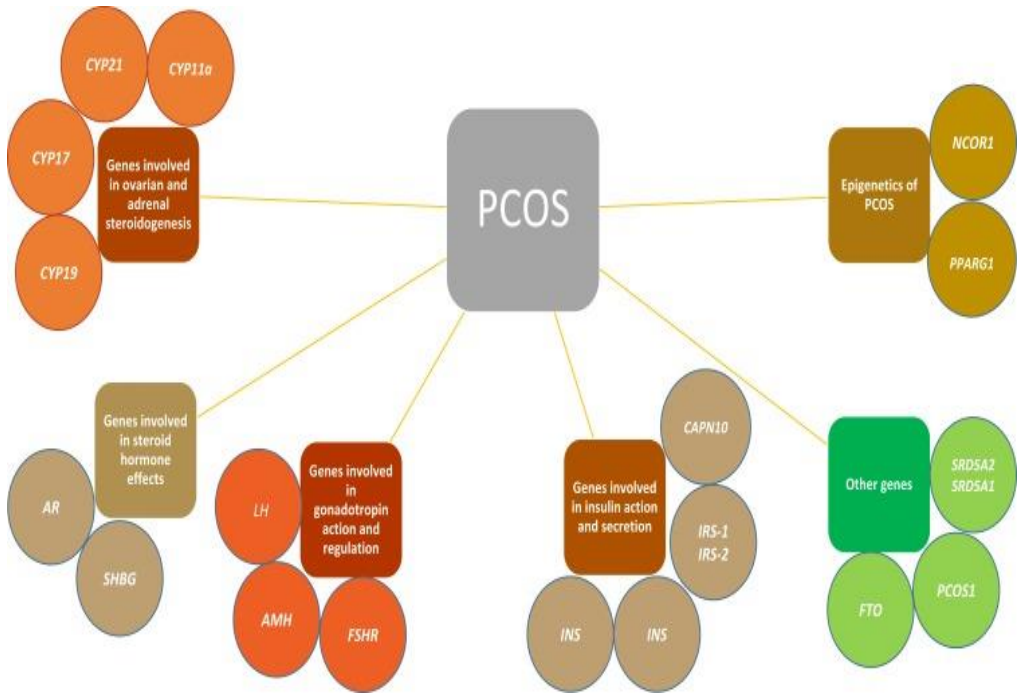


Fig. (1.8): A list of the PCOS genes demonstrates how complex the condition is (Khan *et al.*, 2019).

1.4.1. Gene Polymorphism

The genomics rise and its effect on human health established in 1990, the Human Genome Project was one of the most expensive and cooperative project ever undertaken in science. It complete since Ten years but still provide a lot of novel information **(Karki *et al.*, 2015).**

If more than one allele appears at the gene's locus throughout a population, the gene is considered to be polymorphic. In addition to having several alleles at a single location, polymorphism requires that each allele exist in the population at a rate of 1% **(Annear *et al.*, 2021).** The protein coding genes occupy only 1-2% of the human genome. Because of that, a large number of polymorphic variations will not have direct effect on gene activity **(Tung *et al.*, 2020).** but, if a polymorphic variation settles in the gene regulatory region, it can cause

significant functional involvements **(Gong *et al.*, 2021).** Any area of the genome can have gene polymorphisms. Most polymorphisms are silent and have no impact on a gene's function or expression **(Sameer *et al.*, 2021).** A polymorphic forms of a gene can result in abnormal expression or to an abnormal protein production; this abnormality may be associated with disease **(Jaunmuktane and Brandner, 2020).**

1.4.2. Single Nucleotide Gene Polymorphism

Double-stranded DNA (dsDNA), consist of two paired DNA strands that are complementary in their base sequence, DNA carries all of the genetic information for life**.** In recent decades, a lot of researches has been done for DNA analysis because of its importance in clinical diagnosis of hereditary diseases**.** Single nucleotide polymorphisms (SNPs), caused by mutation in a single base-pairing in the dsDNA genome **(Gao *et al.*, 2019).**

The accurate detection of SNPs can give clinical diagnosis of disease genetic predispositions and help distinguish the genome of each individual **(Gao *et al.*, 2019).** SNPs exist through the entire genome, within coding and noncoding regions sequences. SNPs outside coding regions can occur in untranslated regions (intronic regions) and associated noncoding regions such as promoters and transcription factor binding sites **(Hunt *et al.*, 2009).**

1.4.3. Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (RT-PCR) has emerged as a dependable and well-liked tool for biological investigation due to its capacity to identify and quantify extremely minute amounts of certain nucleic acid sequences. This technology has many uses as a research tool, including the quick and precise evaluation of gene expression variations brought on by physiology, pathology, or

development **(Valasek and Repa, 2005).** Real-time PCR can be used to assess the presence of cancer and monitor viral or bacterial loads. The groundbreaking PCR technique, invented by Kary Mullis in the 1980s, is the foundation of real-time polymerase chain reaction (PCR), which enables scientists to amplify particular DNA fragments more than a billion times.

The first real-time PCR demonstration was completed by Higuchi and colleagues from Roche Molecular Systems and Chiron. By including the widely used fluorescent dye ethidium bromide (EtBr) in the PCR and conducting the reaction under UV light, which causes EtBr to glow, they were able to observe and record the accumulation of DNA using a video camera. Since 1966, it has been understood that the binding of nucleic acids to EtBr increases its fluorescence; nonetheless, real-time PCR, as it was initially known, could only be developed by combining this fluorescent chemistry with PCR and real-time videography. Several instruments initially made real-time PCR equipment commercially available, and it was used for clinical diagnostics **(Valasek and Repa, 2005).**

Real-time PCR technology is fundamentally based on traditional PCR. By using the PCR method, DNA can be replicated and amplified. Utilizing short, sequence-specific oligonucleotides as primers, PCR uses DNA polymerases to amplify particular DNA fragments. Taq DNA polymerase is the first and most widely utilized of these enzymes (from Thermus aquaticus). This enzyme is helpful for PCR because it has two basic properties: (1) it can create new DNA strands using a DNA template and primers, and (2) it is heat resistant The latter quality is crucial because, following each round of DNA copying, the produced double-stranded DNA (dsDNA) must be "melted" into single strands at high temperatures inside the reaction tube (95 °C). In order for the oligonucleotide

primers to attach to the now single-stranded template DNA and direct the DNA polymerase enzyme to begin elongation by adding a single complementary nucleotide to create a new complete strand of DNA, the reaction must first cool. Thus, dsDNA is created. The melting away of this new dsDNA is required for the next round of copying **(Valasek and Repa, 2005).**

Real-time PCR offers a number of advantages. The capacity to quantify nucleic acids over an extraordinary wide dynamic range is likely its most significant feature. All real-time platforms are also generally rapid, and some of them allow for high-throughput automation. Last but not least, real-time PCR is carried out in a closed reaction vessel with no post-PCR adjustments, decreasing the possibility of cross contamination in the lab.

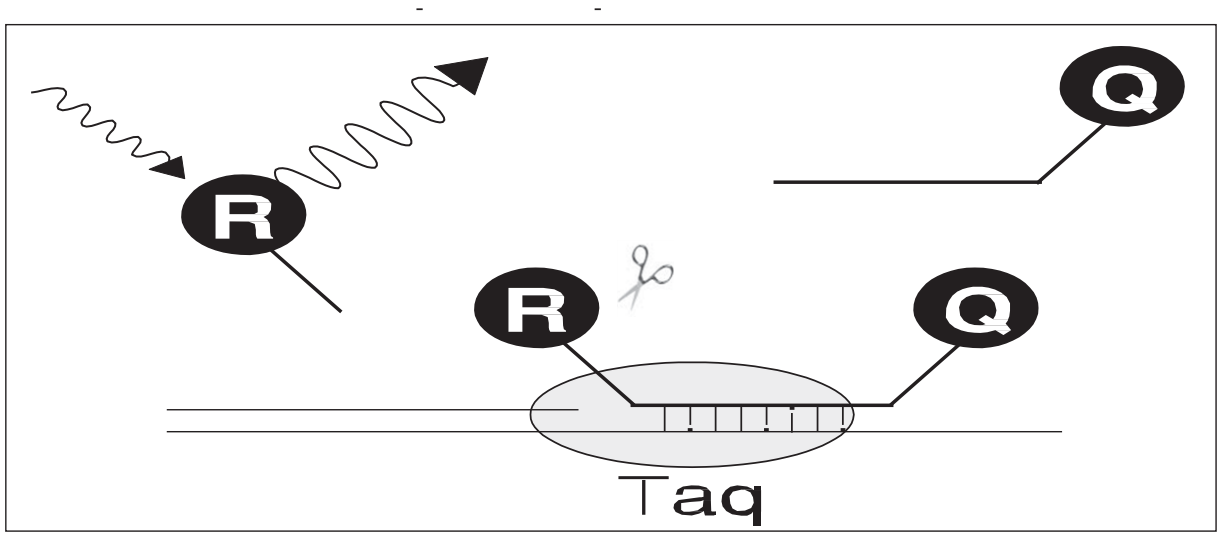


Fig. (1.9): The reporter fluorescence (R) in the Taqman probe produces light at a wavelength that is absorbed by the quencher fluorophore (Q). The DNA polymerase (Taq) cleaves the probe during PCR amplification, releasing the reporter from the quencher and enabling quantifiable fluorescence (Valasek and Repa, 2005).

However, real-time PCR techniques have a number of limitations. Most of these can be found using any PCR- or RT-PCR-based method. Real-time PCR can be inhibited by substances found in specific biological samples. For instance,

inhibitors present in some body fluids, including hemoglobin or urea, may have an impact on real-time PCR applications in the clinical and forensic fields. Other restrictions mostly relate to real-time PCR-based gene expression analysis. More problems could arise as a result of the additional enzymatic step that requires the usage of RNA. However, human error—inadequate assay development, wrong data analysis, or erroneous conclusions—resides as real-time PCR's most significant current constraint **(Valasek and Repa, 2005).**

1.4.4. Programed Cell Death -1 Gene

Human PD-1 homologue's complete cDNA sequence, gene structure, and genomic organization were discovered. Its distinct immune regulatory effects were observed, and they were reported in the journal Gene in November 1997 **(Berger and Pu, 2018, Finger *et al.*, 1997).**

More than 30 single nucleotide polymorphism sites (SNPs) are found in the promoter, intron, exon, and 3'UTR regions of the PD-1 gene, which is found on chromosome 2 in the q37.3 region **(Wang *et al.*, 2018).** Each human cell typically has 23 pairs of the 46 chromosomes that make up the human genome. One of the pairs contains two copies of chromosome 2, one from each parent **(Sameer *et al.*, 2021, Yahaya *et al.*, 2021)**. The second-largest human chromosome, Chromosome 2, covers over 243 million base pairs of DNA, accounting for close to 8% of all DNA found in cells **(Teama, 2018 and Logsdon *et al.*, 2020) Figure 1-10**.

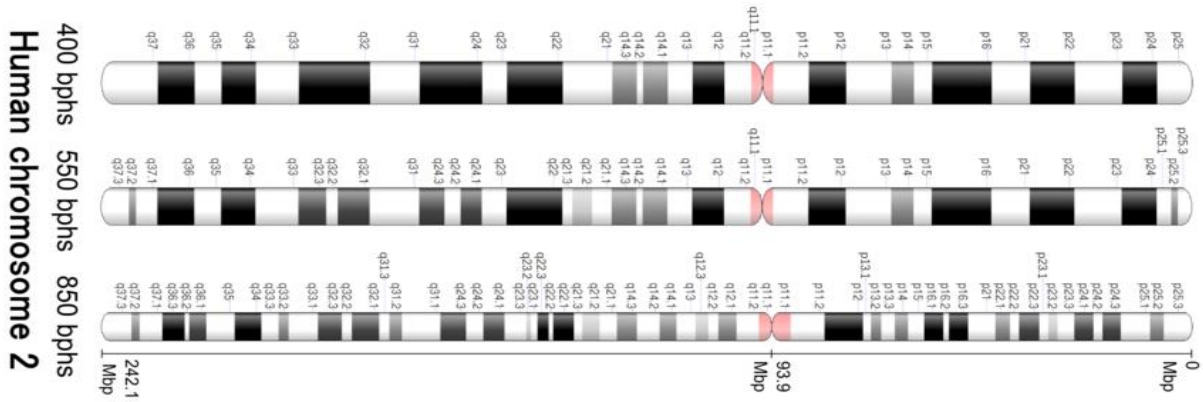


Fig. (1.10): Humane chromosome number 2 PDCD-1 gene in the q37.3 regions, (p) proximal short arm of chromosome, (q) distal long arm of chromosome by NCBI website.

The inhibitory signals provided by the PD-1 linkage and its ligands, PD-L1 and PD-L2 are provided to maintain balance for T-cell activation, Tolerance, and immune-mediated tissue damage, and inhibition of its function can reverse the function of T cells and their effective re activity **(Raziorrouh *et al.*, 2014).**

The reported association between single nucleotide polymorphisms (SNPs) in the human PD-1 gene and susceptibility to systemic lupus erythematosus (SLE), type 1 diabetes, rheumatoid arthritis, and the presence of nephropathy in SLE patients supports an important role of PD-1 in maintaining the peripheral self-tolerance and preventing autoimmunity **(Nielsen *et al.*, 2005)**.

In addition to raising the risk of kidney and thyroid cancer, genetic changes in PD-1 are also strongly linked to the development, clinical stage, and metastasis of the tumor and can even alter how kidney transplants work. Additionally, the PD-1 gene polymorphism was linked to the development and prognosis of all malignancies and can influence a person's vulnerability to ankylosing spondylitis.

As a result, the PD-1 gene polymorphism is intimately associated with the development of disorders like cancer. However, it is unknown if the PD-1 gene polymorphisms are connected to the formation and progression of PCOS **(Han *et al.*, 2021).**

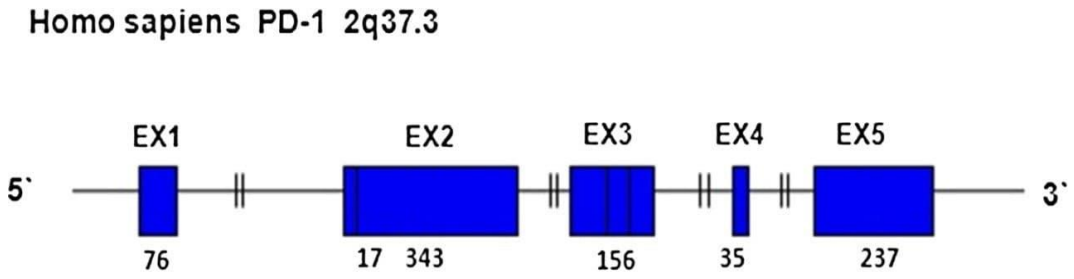


Fig. (1.11): Schematic structure genomic organization of PD-1 gene. The bars represent the exons (EX) and the lines represent introns (Shi *et al.,* 2013).

1.4.5. Programed Cell Death -1 Gene Polymorphism (rs2227982)

A/G single-nucleotide variation near gene PDCD1 (rs2227982) SNP (PD-1.9) in gene exon 5region. Exon 5 controls the immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-switch motif (ITSM) in the cytoplasmic domain of PD-1 **(Takahashi *et al.,* 2013)**.

PD-1.9 (rs2227982) polymorphism lead to a nonsynonymous mutation which produce an amino acid exchange (Alanine to Valine) through protein biosynthesis, which affect the PD-1 cytokine function **(Huang *et al.,* 2019)**. An example of a genetic variety is single nucleotide polymorphism (SNP), which is a mutation in a single base scattered throughout the genome. SNP influence the protein function that was encoded by the same gene **(Huang *et al.,* 2019)**.

Various studies detected that PD-1 gene (rs2227982) was act as a risk factor in various cancers for examples, leukemia**,** gastric adenocarcinoma **(Tang *et al.,* 2017),** esophageal squamous cell carcinoma **(Zhou *et al.,* 2016)** and ovarian cancer **(Tan *et al.,* 2018).** Immune tolerance, infection resistance, and cancer immunity are all mediated by PD-1. The immune system is a defense system since

it consists of several biological components that protect the body from disease. When the immune system is compromised, a number of diseases can result **(Li *et al.*, 2019b)**. New studies have revealed that immune system mechanisms are included in polycystic ovary syndrome **(He and Li, 2020)**.

PCOS patients were established to be immersed in a chronic low-grade inflammation condition, which include increase leukocytes, dysfunction of endothelium, and the proinflammatory cytokines disturbance. Human preovulatory follicles contain significant numbers of immunocompetent cells, such as T- and B-cells, dendritic cells, and macrophages **(Li *et al.*, 2019b).** There are several single nucleotide polymorphisms (SNPs) in PD-1; these SNPs can alter how the PD-1 gene is expressed, which can result in disease **(Li *et al.*, 2019a).**

The association of rs2227982 with human diseases has been reported in Asian populations: ankylosing spondylitis in Korean and Chinese, and type 1 diabetes mellitus in Japanese. These findings imply that in Asian people, rs2227982 is one of the risk factors for autoimmune illnesses. The exclusively Asian cultures whose rs2227982 is linked to autoimmune illnesses raises the possibility that the genetic background may affect how an SNP in PDCD1 affects pathogenesis **(Takahashi *et al.*, 2013).**

Aim of the study:

The presented work aims included the following:

1. To explore whether women with PCOS have any difference in PD-1 level compared to women without PCOS.
2. To determine the level of interleukin-10 (IL-10) as inflammatory marker in PCOS patients and compared with apparently healthy control.
3. To focus on determination of any association between PD-1 levels and IL-10 levels with hormonal-metabolic parameters in women with PCOS.
4. To study the genetic polymorphisms for PD-1 gene in women with PCOS as early prognosis for complication and to see its role in pathogenesis of PCOS.
5. To determine the correlation between inflammatory markers IL-10 levels and PD-1 levels with the resultant data of genotypes.

2. Materials and Methods

2.1. Subjects

2.1.1. Study Design and Ethical approval

A case-control study design, for a total 160 subjects were collected throughout the period from Dec., 2021 to Aug., 2022. The College of Medicine, University of Kerbala, and Kerbala Health Directorate validated the study's ethical approval. Approval also taken from administration of gynecological and obstetric teaching hospital and from each patient after explaining the nature and purpose of study as shown in **Figure (2.1).**

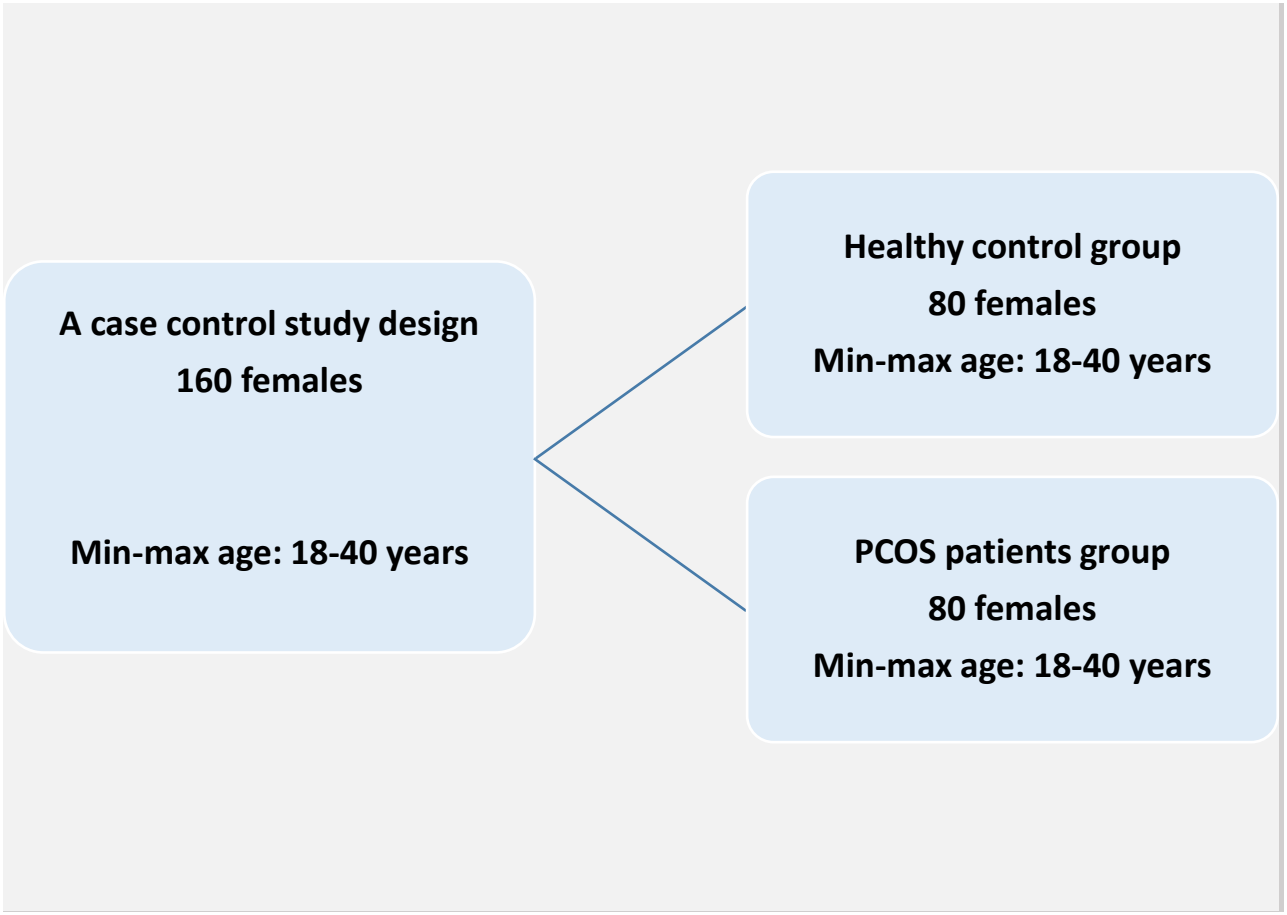


Fig. (2.1): Scheme of the study.

2.1.2. Patients

Whole blood samples of eighty PCOS patients of childbearing age women were collected at the reproductive fertility consultant of teaching hospital for obstetrics and gynecology, Kerbala health directorate, Iraq with age ranged between 18 – 40 years. An exhaustive interview addressing personal history, family history, demographic information and laboratory examination was performed. A questionnaire was formed to get the data of the patient set which contain the age, weightiness, height, hip circumference, waist circumference, menstrual regularity, fertility and hirsutism. The measurements in these studies included anthropometry: waist to hip ratio (WHR) and body mass index (BMI).

- **Inclusion criteria**

The Rotterdam criteria-2003 was assumed to eighty PCOS females (N=80) with ages reached between (18 - 40 years). It is possible to diagnose patients with any two of the subsequent three conditions: Hyperandrogenemia, Ovarian Dysfunction, ovarian volume greater than 10 ml on ultrasonography, and fewer than 12 follicles measuring 2 to 9 mm in diameter **(Geisthövel, 2003 and Freeman et al., 2022)**.

- **Exclusion criteria**

Women suffered from diseases (autoimmune disease, diabetes mellitus, thyroid disease, cardiovascular disease, hypertension, chronic renal failure and malignant diseases) were excluded and history of receiving any other medication (lipid reduction, ovulation stimulation, corticosteroids, antidiabetic and antihypertensive medications) within 6 months were excluded also.

2.1.3. Control

Eighty apparently healthy women were selected as control group, which ages ranged between (18-40 years). They have regular menstruation period, with normal ovaries as they were observed by the gynecologists. The questionnaire for the control group was the age, weight and height, hip and waist circumferences and menstrual regularity with fertility and hirsutism. Not smokers with no kidney disease, liver disease, cancer, strokes, any acute or chronic inflammatory disease, cerebrovascular accidents, alcoholics, rheumatoid arthritis, autoimmune disease, patients with type 1 and 2 diabetes mellitus and no history of contraceptive drugs. All patients and healthy women included in this study were married.

2.1.4. Blood Samples Collection

Disposable syringes and needles were used for blood collection (5 mL). Blood samples were obtained from PCOS women and control groups by vein puncture in day two of the cycle. Each sample obtained will be divided into two parts:

A. The foremost tube contained 3 milliliters of blood, set in gel tubes. Next, it stayed for 10 to15 minutes at room temperature for clotting. Then centrifuged the blood for 10 to 15 minutes at 2000 x g. for separation of serum and divided into 4 parts and put in Eppendorf tubes then stored at -15 ˚C till examination of the biomolecules in this study.

B. Another portion was applied for the study of genetic analysis. It comprised 2 milliliters of blood specimen that have been placed in EDTA tube. Then this tub saved freezing at -30 ˚C till investigation of PD-1 gene polymorphisms.

2.2. Chemicals and Kits

The kits used in this study summarized in **Table (2.1).**

Table (2.1): Chemicals and kits are used in this study and their suppliers

No.	Chemicals and Kits	Company and Country
1	Absolute Ethanol	Fluka/ Germany
2	AddPrep Genomic DNA Extraction kit 10023	Addbio/Korea
3	Follicular Stimulating Hormone Elecsys reagent kit	Roche/Germany
4	Free Nuclease Distil Water	Bioneer/ Korea
5	Free Testosterone Hormone kit 5325-300	Monobind/USA
6	GoTaq Probe qPCR Master Mix A610A	Promega/USA
7	Human IL-10 (Interleukin 10) kit E-EL-H6154	Elabscience/USA
8	Human PD-1 (Programmed Cell Death Protein1) kit E-EL-H1534	Elabscience/USA
9	Luteinizing Hormone Elecsys reagent kit	Roche/Germany
10	Primers	Macrogen/Korea
11	Probes	Macrogen/Korea
12	Prolactin II Hormone Elecsys reagent kit	Roche/Germany

2.3. Instruments and Lab Equipment

The instruments and laboratory tools used in this study summarised in **Table (2.2).**

Table (2.2): Instruments and Lab Equipment that used in this study

No.	Instruments and Lab Equipment	Company and country
1	Centrifuge	Kokusan /Japan
2	Cobas, e 411 analyzer	Roche / Germany
3	Dry micro tubes incubator	Ae /UK
4	ELISA system, ELx800	Bio Tek /USA
5	Incubator, TPM-900	Siroca crossline/ Japan
6	Microfuge	Beckman Coulter/Germany
7	Pipettes, tubes	DARWELL / China
8	Stratagen Real Time Thermal Cycler, MX 3005 P	Stratagen /USA
9	Vortex Mixer	Clay Adams/ Germany
10	Water Distillator	Lab Tech/Korea

2.4. Methods

2.4.1. Body Mass Index measurement

Obesity was categorized using the body mass index (BMI) which was calculated from the following equation **(Keys *et al.*, 1972):**

$$\textbf{BMI (Kg/m}^2\textbf{) = Weight (kg) / Height (m}^2\textbf{).}$$

WHO classification was used for BMI evaluation**.** Normal BMI level was ranged between (20-24.9) kg/m^2 while for overweight was ranged between (25-29.9) kg/m^2 and when BMI $\geq$ 30 kg/m^2, the woman is considered as obese **(Namjou *et al.*, 2021).**

2.4.2. Waist Hip Ratio measurement.

The Waist Hip Ratio (WHR) ≥ 0.85 diagnostic standard for abdominal obesity in women **(Bassi *et al.*, 1970, Lee *et al.*, 2022).**

2.4.3. Hirsutism Assessment

The modified Ferriman-Gallwey scoring system is used to determine whether a person has hirsutism. Nine different body parts (the upper lip, chin, chest, upper and lower abdomen, thighs, upper and lower back, and upper arms) are scored from 0 to 4, a score 0 represent the absence of terminal hair growth, and a score 4 represent extensive growth. A total mFG score ≥ of 4-6, depending on the patient's race, is used to define hirsutism. **(Mumusoglu and Yildiz, 2020).**

2.4.4. Determination of Luteinizing, Follicle Stimulating, and Prolactin Hormones

Luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin hormones levels were measured by the chemiluminescent automates immunoassay system (ECL) **(Cobas e 411, Roche diagnostic, Germany).**

General Assay Principle:

A completely automated, random access, software-controlled system for immunoassay analysis is the Roche Diagnostic Cobas e411 Immunoassay System **(Black and da Silva Costa, 2018).** Three test principles are offered on the system: a bridging principle to identify antibodies in the sample, a sandwich principle (one or two steps) for larger analytes, and a competitive principle for extremely small analytes **(Nivedhita and Brundha, 2020).** Applying voltage to the immunological complexes connected to the streptavidin-coated microparticles causes the chemiluminescent reactions that result in the emission of light from the ruthenium complex to begin electrically **(Toboc *et al.*, 2018).**

Procedure:

1. Assay cups and assay tips were loaded in the instrument (cobas e411).
2. Regents of each parameter (LH, FSH, PRL), loading in reagent rack.
3. Calibration and control of each reagent were made.
4. The sample of serum was loaded in to sample rack then the instrument automatically began analysis.

2.4.4.1. Luteinizing Hormone Level Determination

A. Test Principle

Sandwich principle of the Elecsys LH level assay employs two monoclonal antibodies specifically directed against human LH.

B. working solutions and Reagents

The label on the reagent rack pack reads LH.

M Microparticles with 0.72 mg/mL streptavidin coating.

R1 Anti-LH-Ab~biotin: Mouse monoclonal anti-LH antibody that has been biotinylated 2.0 mg/L in TRIS (Trisaminomethane) buffer at pH8.0.

R2 Anti-LH-Ab~Ru(bpy): Mouse monoclonal anti-LH antibody labeled with ruthenium complex at a concentration of 0.3 mg/L in TRIS buffer at a pH of 8.0.

C. Procedure

The assay took 18 minutes to complete.

1. 1^{st} incubation: A sandwich complex was created from 20 µL of sample, a monoclonal LH-specific antibody that has been biotinylated, and a monoclonal LH-specific antibody that has been labeled with ruthenium complex.

2. 2nd incubation: Following the addition of microparticles coated in streptavidin, the complex was linked to the solid phase by an interaction between biotin and streptavidin.

3. The measurement cell receives the reaction mixture by aspiration, and the microparticles were magnetically attracted to the electrode's surface there. Pro Cell/Pro Cell M was then used to eliminate any remaining unbound materials. A photomultiplier measures the chemiluminescent emission that is caused when a voltage is applied to the electrode.

2.4.4.2. Follicular Stimulating Hormone Level Determination

A. Test principle

Sandwich principle of the Elecsys FSH level assay employs two monoclonal antibodies specifically directed against human FSH.

B. working solutions and Reagents

The label on the reagent rack pack reads FSH.

M Microparticles with 0.72 mg/mL streptavidin coating.

R1 Anti-FSH-Ab- biotin: Mouse monoclonal FSH antibody that has been biotinylated 0.5 mg/L in MES (morpholino)ethane sulfonic acid buffer at pH6.0.

R2 Anti-FSH-Ab- Ru(bpy): Mouse monoclonal anti-FSH antibody labeled with ruthenium complex at a concentration of 0.8 mg/L in MES buffer at a pH 6.0.

C. Procedure

Total duration of assay: 18 minutes.

1. 1st incubation: A sandwich complex was created from 40 µL of sample, a monoclonal FSH-specific antibody that has been biotinylated, and a monoclonal FSH-specific antibody that has been tagged with a ruthenium complex.
2. 2nd incubation: The complex was bonded to the solid phase after the addition of microparticles coated in streptavidin through the interaction of biotin and streptavidin.
3. The measurement cell receives the reaction mixture by aspiration, and the microparticles were magnetically attracted to the electrode's surface there. Pro Cell/Pro Cell M was then used to eliminate any remaining unbound materials. A photomultiplier measures the chemiluminescent emission that is caused when a voltage is applied to the electrode.

2.4.4.3. Prolactin Hormone Level Determination

A. Test principle

Two monoclonal antibodies designed specifically against human prolactin were used in the sandwich principle of the Elecsys Prolactin Level Assay.

B. Reagents - working solutions

The label on the reagent rack pack reads "PRL II."

M Microparticles with 0.72 mg/mL streptavidin coating.

R1 Anti-Prolactin-Ab- biotin: Mouse monoclonal anti-prolactin antibody that has been biotinylated, 0.7 mg/L in phosphate buffer at pH 7.0.

R2 Anti-Prolactin-Ab- Ru(bpy): Mouse monoclonal anti-prolactin antibody labeled with ruthenium complex at a concentration of 0.35 mg/L in phosphate buffer at a pH of 7.0.

C. Procedure

The assay took 18 minutes to complete.

1. 1st incubation: A initial complex was formed by 10 µL of material and a monoclonal prolactin-specific antibody that has been biotinylated.
2. 2nd incubation: An interaction between streptavidin and biotin causes a sandwich complex to develop with the addition of a monoclonal prolactin-specific antibody tagged with a ruthenium complex and microparticles coated in streptavidin.
3. The measurement cell receives the reaction mixture by aspiration, and the microparticles were magnetically attracted to the electrode's surface there. Pro Cell/Pro Cell M was then used to eliminate any remaining unbound materials. A photomultiplier measures the chemiluminescent emission that is caused when a voltage is applied to the electrode.

2.4.5. Free Testosterone Hormone Level Determination

A. principle of test

Enzyme-based Competitive Immunoassay (TYPE 5). Immobilized antibody, enzyme-antigen conjugate and natural antigen were the fundamental substances needed for a solid phase enzyme immunoassay. The natural antigen in serum and the enzyme-antigen conjugate compete for a limited number of insolubilized binding sites and combined to immobilized antibody.

B. Reagents – working solutions

1. Icons A-G on Free Testosterone Calibrators, 1 mL/vial Seven vials of free testosterone-containing serum referance with concentrations of 0 (A), 0.2 (B), 1.0 (C), 2.5 (D), 7.5 (E), 20 (F), and 60 (G) pg/mL.
2. Free Testosterone Controls – 1mL/vial – Icons L, M, N three vials of free testosterone serum reference at low, middle, and high established concentrations.
3. Free Testosterone Enzyme Reagent – 13 mL/vial – Icon One vial of Testosterone (Analog)-horseradish peroxides (HRP) conjugate in a protein stabilizing matrix with dye.
4. Testosterone antibody was coated on a 96-well microplate in a free testosterone coated plate that is wrapped in an aluminum bag with a drying agent.
5. One vial of a surfactant in buffered saline makes up the 20 mL-per-vial Wash Solution Concentrate.
6. Tetramethylbenzidine (TMB) in buffer was present in one vial of SubstrateA, which was 7 mL in volume.
7. Hydrogen peroxide ($H_2 O_2$) in buffer was contained in one vial of SubstrateB, which has a volume of 7 mL.
8. Strong acid was contained in one vial of the Stop Solution (8 mL) (1N HCl).

C. Reagent preparation

1. **Wash Buffer:** Using distilled water and a suitable storage container, dilute the wash solution's contents to 1000 mL.
2. **Working Substrate Solution:** The contents of the amber vial marked "Solution A" were transferred to the clear vial marked "Solution B."

D. Procedure

1. The microplates' wells were formated for each serum reference, control and patient specimen to be assayed.
2. In the designated well, 20 µL of the proper serum reference, control, or sample was pipetted.
3. Avolume of (100 µL) of Free Testosterone Enzyme Reagent was inserted into each well.
4. Gently swirled the microplate for 20–30 seconds to mix.
5. At room temperature, covered, and incubated for 60 minutes.
6. Decantation was used to discard the microplate's contents.
7. There was decanted and added 350 µL of wash buffer. For a total of three washes.
8. To each well, 100 µL of the working substrate solution were added.
9. Incubated for fifteen minutes at room temperature.
10. Each well received a volume of (50 µL) of stop solution, which was added and carefully mixed for 15-20 seconds.
11. A microplate reader was used to measure the absorbance in each well at 450nm.

E. Calculation

1. The absorbance obtained from the printout of the microplate reader was recorded.
2. On a sheet of linear graph paper, the absorbance for each serum reference was plotted against the appropriate Free Testosterone concentration in pg/mL.
3. A best-fit curve was used to connect the points.
4. The average absorbance of the samples for each unknown was placed on the graph's vertical axis, the point of intersection was determined on the curve,

and the concentration (in pg/mL) was read off the horizontal axis to determine the unknown's Free Testosterone concentration.

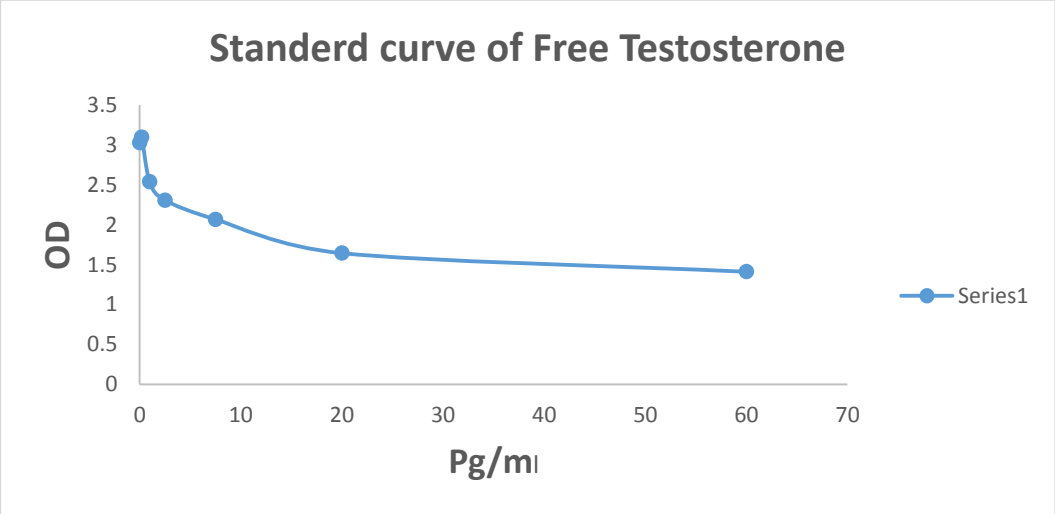


Fig. (2.2): The standard curve for free testosterone level determination.

2.4.6. Interlukin-10 Level Determination

A. principle of the test

The Sandwich-ELISA method was employed by this ELISA kit. This kit's micro ELISA plate has been pre-coated with an antibody that recognizes human IL-10. The micro ELISA plate wells were filled with samples (or standards) and the particular antibody. After that, each microplate well receives an addition of an Avidin-Horseradish Peroxidase (HRP) conjugate and a biotinylated detection antibody specific for human IL-10 and incubated. Free parts were removed during washing. To each well, the substrate solution was applied. There will only be blue coloration in the wells that have human IL-10, biotinylated detection antibody, and Avidin-HRP conjugate. The addition of stop solution stops the enzyme-substrate reaction, and the color changes to yellow. At a wavelength of 450 ±2 nm, the optical density (OD) is determined spectrophotometrically. The OD value varies in direct proportion to the level of human IL-10. By comparing the OD of

the samples to the standard curve, the human IL-10 concentration in the samples was obtained.

B. Reagents – working solutions

1. Microplate ELISA.
2. Reference standered.
3. Concentrated Biotinylated Detection Ab.
4. Concentrated HRP cojugate.
5. Sample Diluent and Referance standerd.
6. Diluent for biotinylated detection Ab.
7. Concentrated Wash Buffer.
8. Substrate Reagent.
9. Stop Solution.

C. preparation of reagents

1. Prior to use, all reagents were purged and brought to room temperature (18–25 °C).
2. Wash Buffer: 750 mL of wash buffer was created by diluting 30 mL of concentrated wash buffer with 720 mL of deionized or distilled water.
3. The standard was centrifuged at 10,000 Xg for one minute. To get the working solution after adding 1 mL of the reference standard and sample diluent, the mixture was let to stand for 10 minutes before being gently inverted several times. With a pipette, it was properly blended once it had completely dissolved. The workable solution after reconstitution is 100 pg/mL. In accordance with the suggested dilution

gradient, the following serial dilutions were made: 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.0 pg/mL.

Dilution method: Seven Eppendorf tubes were used, and each one received 500 µL of Reference Standard and Sample Diluent. 500 µL of the 100 pg/mL working solution were pipetted into the first tube, where they were combined to create the 50 pg/mL working solution. In accordance with this procedure, 500 µL of the solution were pipetted from the former tube into the later tube.

The final tube was considered a blank. There was no solution pipetted into it from the previous tube.

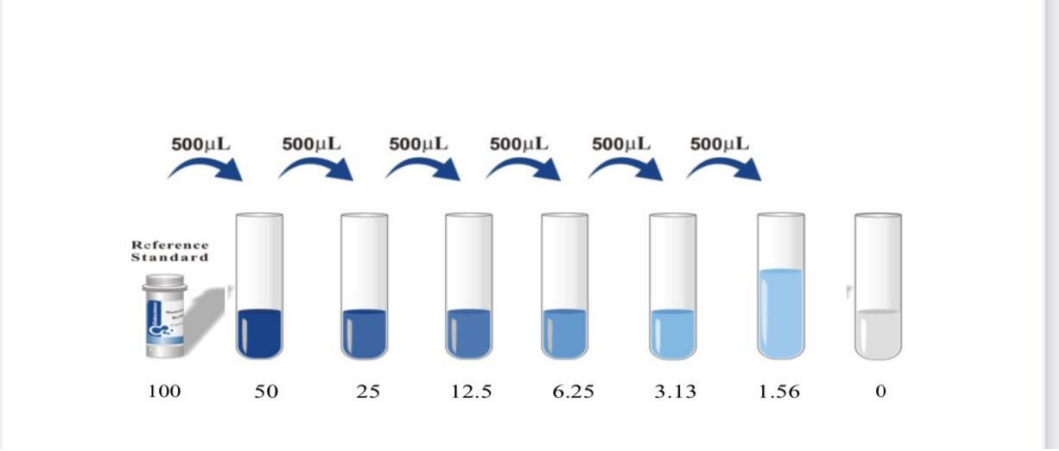


Fig. (2.3): Serial Dilution method for Interlukin-10 standard.

4. Biotinylated Detection Ab working solution: the required amount was calculated before the experiment (100 µL/well). The Concentrated Biotinylated Detection Ab was centrifuged at 800 X g for one minute, and then dillute the 100 X Concentrated Biotinylated Detection Ab to 1 X working solution with Biotinylated Detection Ab Diluent (Concentrated Biotinylated Detection Ab: Biotinylated Detection Ab Diluent= 1: 99).

5. The necessary volume (100 µL/well) for the HRP Conjugate working solution was estimated prior to the experiment. After centrifuging the Concentrated HRP Conjugate at 800 X g for 1 min, it was diluted the 100 X concentrated

HRP conjugate to 1 X working solution with HRP conjugate diluent (Concentrated HRP Conjugate: HRP Conjugate Diluent = 1: 99).

D. Procedure

1. The weels received a volume of 100 µL of standard or sample, which was then incubated for 90 minutes at 37 ^{o}C.
2. After discarding the liquid, each well received 100 µL of the Biotinylated Detection Ab working solution, which was then incubated for 60 minutes at 37 ^{o}C.
3. Three washes and aspirations were performed on the plate by added 350 µL of wash buffer and soak for 1 minutes.
4. After added 100 µL of HRP conjugate working solution and incubated at 37^{o}C for 30 minutes the plate was decanted and washed 5 times.
5. Substrate reagent in avolume of 90 µL was added and incubated at 37^{o}C for 15 minutes.
6. The stop solution was added in a volume of 50 µL.
7. The result was determined immediately after the plate was read at 450 nm.

E. Calculations

The readings for each standard and sample was calculated, and the average optical density of the zero standard was then subtracted. On log-log graph paper, a four parameter logistic curve was drawn, with standard concentration on the x-axis and OD values on the y-axis.

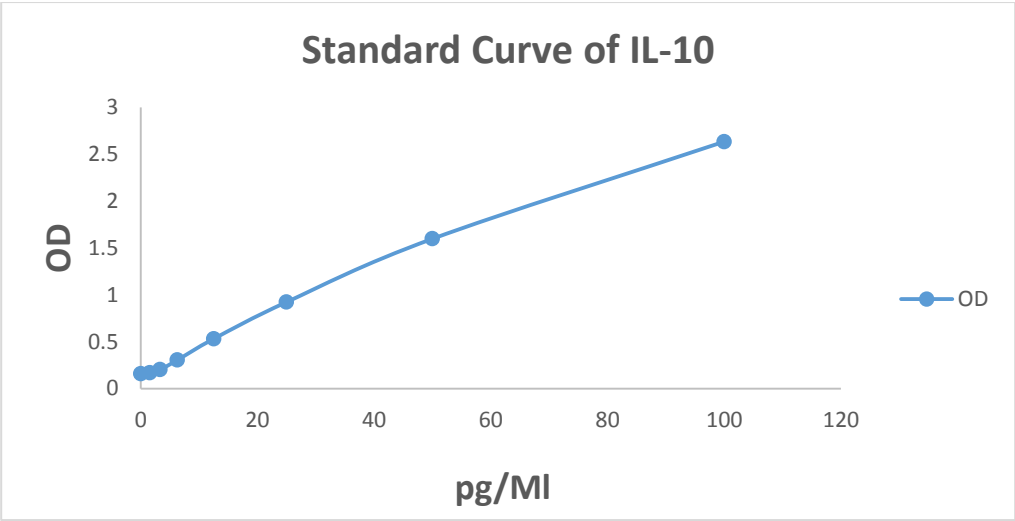


Fig. (2.4): The Standard curve for Interlukin-10 level measurement.

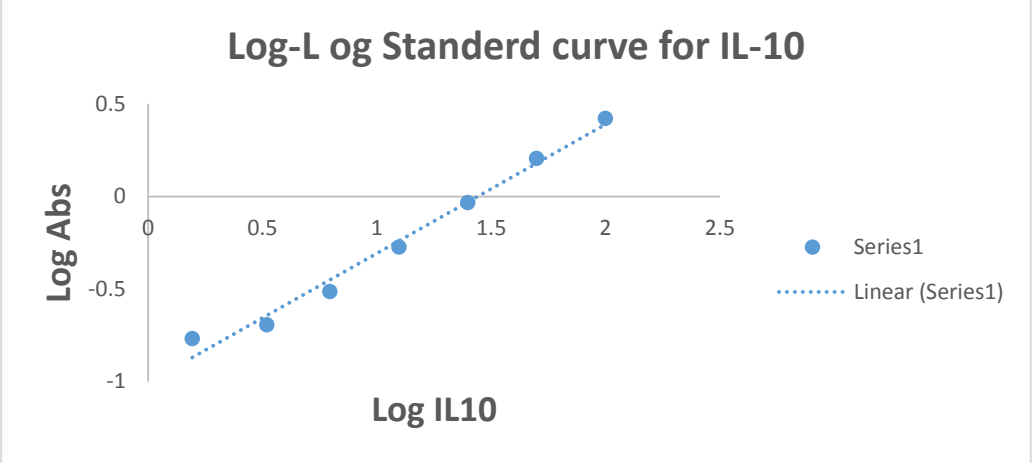


Fig. (2.5) : Log-Log Standard curve for Interleukin 10

2.4.7. Programmed Cell Death-1 Level Determination

A. Principle of the test

The Sandwich-ELISA method was employed by this ELISA kit. This kit's micro ELISA plate has been pre-coated with a human PD-1-specific antibody. The micro ELISA plate wells were filled with samples (or standards) and the particular antibody. After that, each microplate well receives an addition of an Avidin-Horseradish Peroxidase (HRP) combination and a biotinylated detection

antibody specific for Human PD-1. Free parts were removed during washing. To each well, the substrate solution was applied. There will only be blue coloration in the wells that have Human PD-1, biotinylated detection antibody and Avidin-HRP conjugate. The addition of stop solution stops the enzyme-substrate reaction, and the color changes to yellow. At a wavelength of 450 ±2 nm, the optical density (OD) is determined spectrophotometrically. The Human PD-1 concentration was correlated with the OD value. By contrasting the OD of the samples with the standard curve, you may determine the amount of human PD-1 present in the samples.

B. Reagents - working solutions

1. Microplate ELISA.

2. Reference standered.

3. Concentrated Biotinylated Detection Ab.

4. Conjugated concentrated HRP.

5. The sample diluent and reference standard.

6. Diluent for biotinylated detection ab.

7. Diluent for HRP conjugate.

8. concentrated wash buffer.

9. Substratum Reagent.

10. The Stop solution.

C. preparation of reagents

1. Prior to use, all reagents were purged and brought to room temperature (18-25°C).

2. Wash Buffer: 750 mL of wash buffer was created by diluting 30 mL of concentrated wash buffer with 720 mL of deionized or distilled water.

3. The standard was centrifuged at 10,000 X g for one minute. To get the working solution after adding 1 mL of the reference standard and sample diluent, the mixture was let to stand for 10 minutes before being gently inverted several times. With a pipette, it was properly blended once it had completely dissolved. The workable solution after reconstitution is 10 ng/mL. In accordance with the suggested dilution gradient, the following serial dilutions were made: 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16, and 0.0 ng/mL.

Dilution method: Seven eppendrofe tubes were used, and each one received 500 µL of Reference Standard and Sample Diluent. 500 µL of the 10 ng/mL working solution were pipetted into the first tube, where they were combined to create the 5 ng/mL working solution. In accordance with this procedure, 500 µL of the solution were pipetted from the first tube into the second.

The final tube was considered a blank. There was no solution pipetted into it from the previous tube.

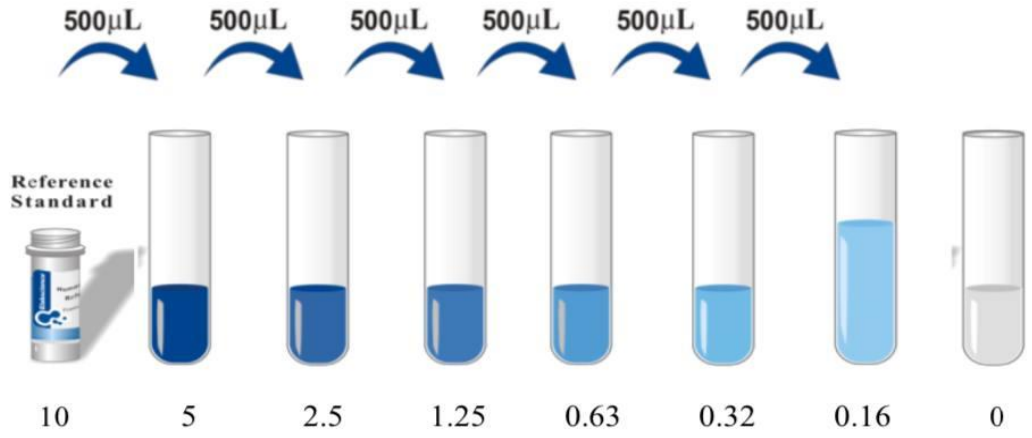


Fig. (2.6): Serial Dilution method for programmed cell death -1 standard.

4. Biotinylated Detection Ab working solution: the required amount was calculated before the experiment (100 µL/well). The Concentrated Biotinylated Detection Ab was centrifuged at 800 X g for one minute, and then dilute 100 X Concentrated Biotinylated Detection Ab to 1 X working solution with Biotinylated Detection Ab Diluent (Concentrated Biotinylated Detection Ab: Biotinylated Detection Ab Diluent= 1: 99).

5. The necessary volume (100 µL/well) for the HRP Conjugate working solution was estimated prior to the experiment. After centrifuging the Concentrated HRP Conjugate at 800 X g for 1 min, then diluted the 100 X concentrated HRP conjugate to 1 X a working solution with HRP conjugate diluent (Concentrated HRP Conjugate: HRP Conjugate Diluent = 1: 99).

D. Procedure

As mentioned in 2.4.6.D

E. Calculation

As mentioned in 2.4.6.E

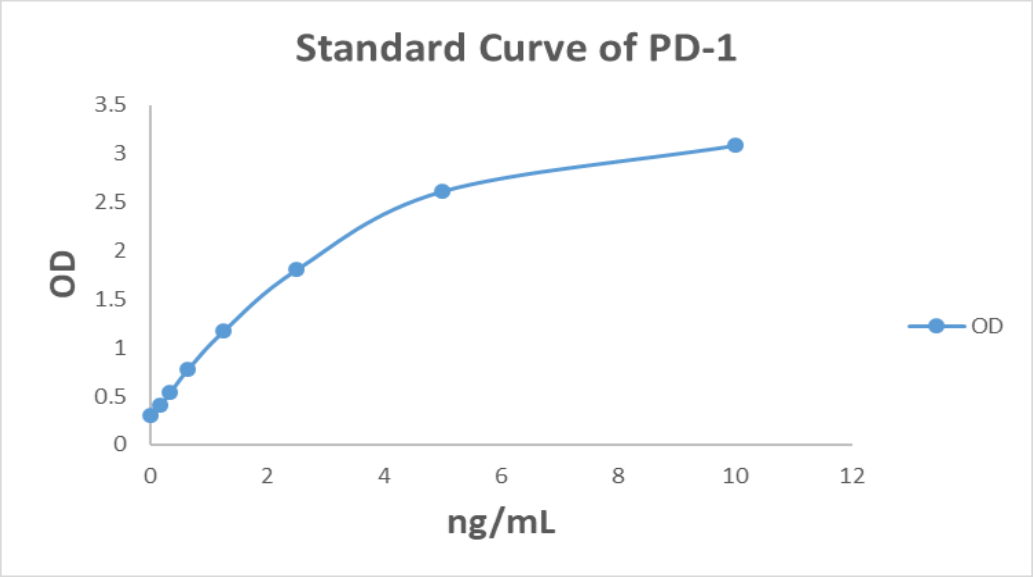


Fig. (2.7): The Standard curve for Programmed Cell Death -1 level measurement.

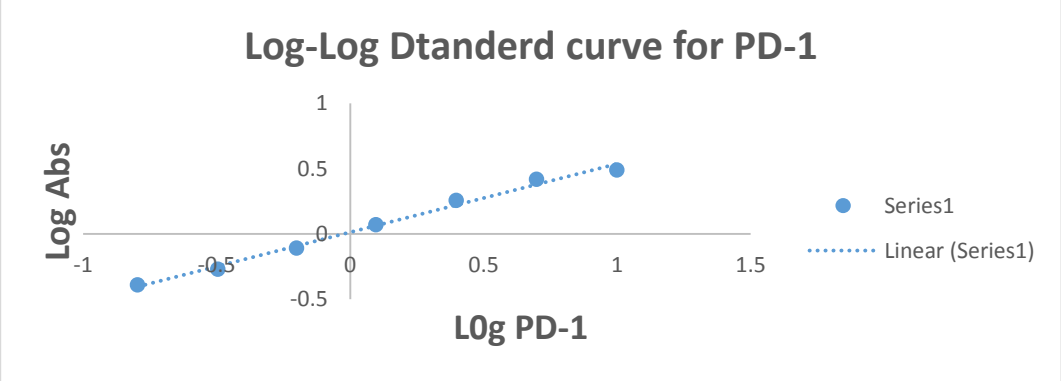


Fig. (2.8): Log-Log Standard curve for Programmed Cell Death Protein 1

2.5. Genotyping Studies

2.5.1. Genomic DNA Extraction

The samples of whole blood which were collected in EDTA-tubes, then by using the Extract System AddPrep Genomic DNA Extraction Kit was extracted from blood samples.

2.5.2. Extraction protocol for blood

1. A volume of 200 µL of blood were transferred into a 1.5 mL microcentrifuge tube, 200 µL of lysis solution were added, supernatant were discarded and

200 µL of lysis solution were added, and resuspended the cell pellet by pipetting.

2. A volume of 20 µL of Proteinase K solution (20 mg/mL) was added to sampel tube, mixed thoroughly using a vortex mixer, and then left to sit at 56°C until the tissue was completely lysed.
3. The tube was briefly spined down to eliminate any droplets that may have formed inside the sample tube cover.
4. The sample tube received 200 µL of the binding solution, which was added and well mixed by pulse-vortexing mixer for 15 seconds.
5. Ten minutes of incubation at 56 °C.
6. By pulse-vortexing mix for 15 seconds, 200 µL of 100% ethanol was introduced and thoroughly mixed. After completing this step, quickly spined down to collect the drips that clung to the lid.
7. With a 2.0 mL collecting tube and with care, the lysate was transferred into the upper reservoir of the spin column without wetting the rim.
8. Centrifuged for one minute at 13,000 rpm: poured off the flow-through and put the spin column and 2.0 mL collection tube together.
9. Avolume of 500 µL of washing One solution was put to the spin column with the collecting tube, and it was centrifuged at 13,000 rpm for one minute as follows: Poured off the flow through and put together the spin column with the 2.0 mL collection tube.
10. A volume of 500 µL of the washing 2 solution was introduced to the spin column with the collecting tube, and it was centrifuged for one minute at 13,000 rpm: Poured off the flow through and put the 2.0 mL collecting tube in the spin column.
11. To eliminate any remaining ethanol, another centrifugation at 13,000 rpm for 1 minute dried the spin column.

12. The new 1.5 mL micro-centrifuge tube received the spin column.

13. Elution solution in a volume of 100 µL was introduced to the spin column with the micro-centrifuge tube and allowed to stand for at least one minute.

14. Centrifugation was used to elute the genomic DNA for 1 minute at 13,000 rpm.

2.5.3. Primers Preparation

In accordance with the primer synthesiser company's instructions, the primers and Probes (which had been lyophilized initially) were dissolved in free denuclease-distilled water (ddH_2O) to obtain a final concentration of 100 µM/µL, which served as a stock solution that was kept at -20 ° C. From the stock primers and Probes, a work primers and probes with a concentration of 10 µM/µL were created.

Table (2.3): Primers and Probes used in this study SNP rs2227982 G/A (RT-PCR assay).

Target gene	Primers and Probes name	Sequence (5'-3')	Ta (°C)	Product size	Reference	Accession number
PDCD1	F	GCTGACTCCCTCTCCCTTTCTC	56	103 bp	U-Gene lab	NG-012110.1
	R	GTCTTCTCTCGCCACTGGAA				
	Allele G	FAM-5'-CTCAGCCGTGCCTG-3'-BHQ1	60			
	Allele A	HEX-5'-CCTCAGTCGTGCCTG-3'-BHQ1	60			

2.5.4. Real-Time Polymerase Chain Reaction

The real-time polymerase chain reaction (RT-PCR) entails several steps, including DNA denaturation to separate double stranded DNA, primer association with the DNA target via hydrogen bonds in a single chain, and primer extension by synthesis of the complementary strand, which is catalyzed by DNA polymerase. A real-time quantitative PCR (qRT-PCR), a variation of standard PCR, has become crucial in molecular diagnostics over the past ten years due to its great sensitivity. Real-time PCR is becoming more and more useful in a variety of functional genomics applications, as well as in molecular medicine, virology, microbiology, and biotechnology. This method is used to simultaneously amplify and quantify a particular DNA molecule area **(Rebouças *et al.*, 2013)**. While in traditional PCR the amplification result is evaluated using electrophoresis, in (qRT-PCR) the chemical reaction is carried out using fluorescent molecules, allowing the amplicon to be quantified. Comparing the TaqMan method to the traditional quantitative PCR approach reveals various benefits. The technique becomes at least seven orders of magnitude more sensitive when fluorescent dye-labeled probes are used **(Nolan *et al.*, 2006)**. The system picks up the fluorescence that the sample produces. Each light stimulus produced by the apparatus now corresponds to the fluorophore's second light emission. As a result, the amount of product produced in the reaction tube correlates directly with the light emission. This in turn relates to how many initial targets there are in the amplification reaction **(Bustin *et al.*, 2005).**

2.5.5. Real-Time Polymerase Chain Reaction Protocol

All the suspected samples were used in the implementation of the Taqman RT-PCR. One pair of specific primers was used in the amplification process of the conserved region of gene PD1 of human. In order to genotype the SNP

rs2227982 (G/A), appropriate primers and probes were used. 2 ng of genomic DNA and a GoTaq Probe qPCR Master Mix were used in the reactions, which were carried out in 0.2 µL wells in a total volume of 20 mL (Promega, USA). The wells were then put in a thermal cycler (made by Stratagene, USA) and heated for 10 minutes at 95 degrees before 40 cycles of 95 degrees for 15 seconds and 60 degrees for one minute were performed. With a calculated error rate based on PCR duplicates of less than 1%, the genotyping success rate was greater than 95%.

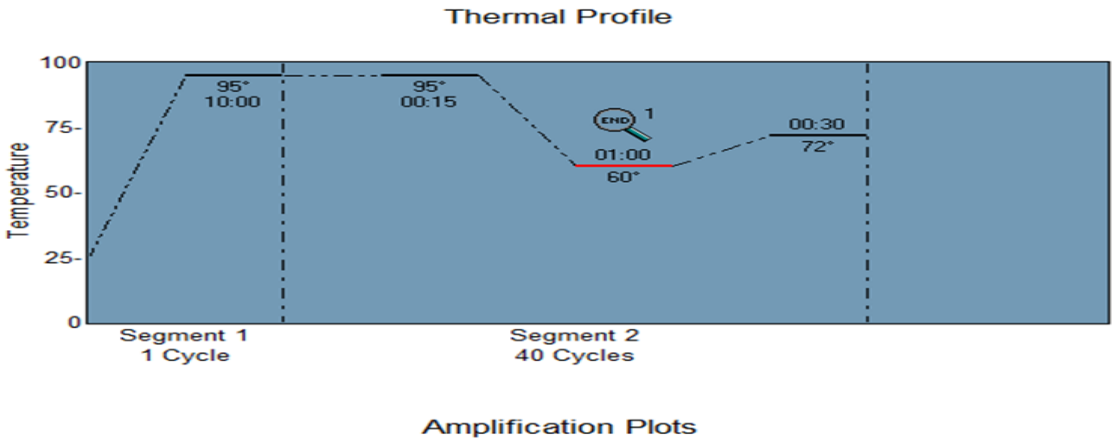


Fig. (2.9): The amplification plots for samples plus an NTC in the RT-PCR experiment.

Table (2.4): Preparation of PCR solutions GoTaq Probe qPCR

Components	Concentration	Volume (20 µl)
ddH_2O	-	4 µL
DNA	40 ng	2µL
Forward primer	10 µM/µL	1 µL
GoTaq Probe qPCR Master Mix	1X	10 µL
Probe 1	10 µM/µL	1 µL
Probe 2	10 µM/µL	1 µL
Reverse primer	10 µM/µL	1 µL

Table (2.5): The RT-PCR conditions.

Phase	Tm (°C)	Time	Cycles
Initial denaturation	95 °C	10 min	1 X
Denaturation	95 °C	15 sec.	40 X
Annealing	60 °C	1 min	
Final Extension	72 °C	30 sec	

2.6. Biostatistical Analysis

All statistical analyses were performed with the Graph Pad Prism 9.0.0 was released on October 28, 2020 and Spss version 25. The results were presented as Mean ± SD. Unpaired t-test was used to compare the data with normal distributions. ROC analysis was performed to determine the scpecificity and sensitivity of the data observed for patients and control groups. Skewed and abnormally distributed data the nonparametric Mann-Whitny U test was used to verify the association of biochemical parameters in patients relative to the control group.

The Spearman r test was applied for normally distributed data and Pearson r correlation was used to abnormally distributed data to evaluate the relationships of the changes of the biochemical parameters, in relevance to various factors related to the PCOS patients. Genotypic and allelic frequencies of PDCD1were calculated by direct gene counting method. The association between the healthy controls and patients with PCOS using Fisher's exact test and the probability value (P value), Odds Ratios (OR) and 95% Confidence Intervals (95% CI) were compute for each analysis to express the significance connecting the studied groups.

In statistical analysis the highly significant value is ($P < 0.01$) and the significant value is ($P < 0.05$).

3. Results and Discussion

3.1. Clinical study

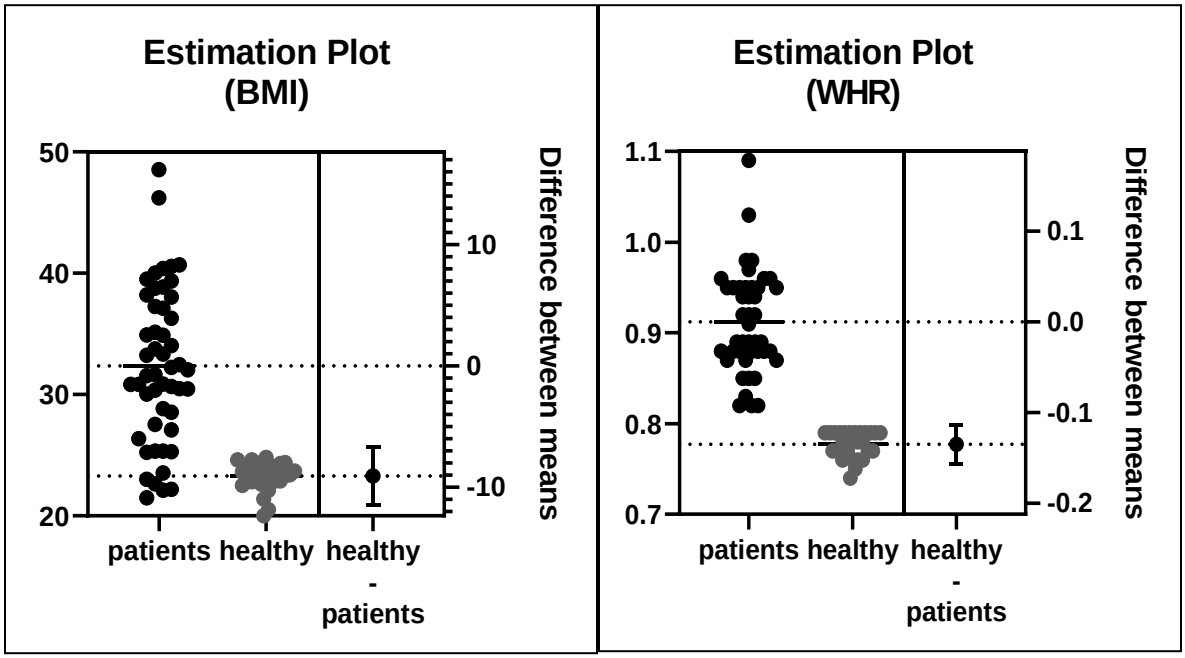
3.1.1. Demographics and Base Line Characteristics in PCOS Patients and Control groups

The characteristics of the study groups are presented in **Table (3.1)** and **Figure (3.1)**, which consists of the data of both patients with PCOS and the control group. They include the number of women, the mean ± SD of age, BMI and WHR as well as the number of patients with hirsutism and those without hirsutism, those with primary infertility and those with secondary infertility, menstruation pattern (regular and irregular). It is evident that the two groups are approximately well matched, thus results obtained could be considered creditable.

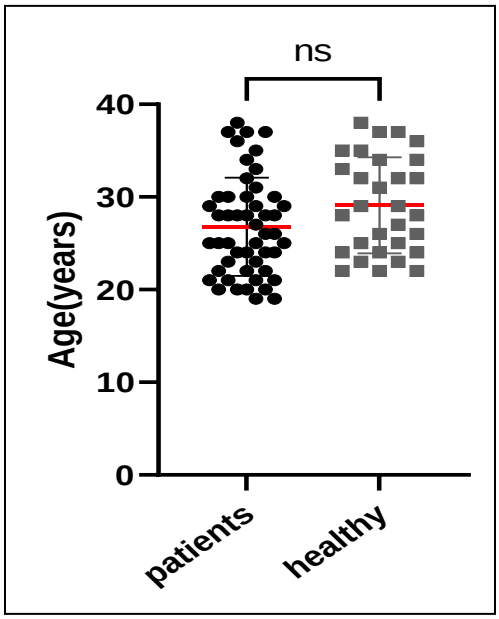
Table (3.1): Host information of the patients and the control groups

Parameters	Control Mean ± SD N = 80	Patients Mean ± SD N = 80
Age	**26 ± 5.175**	**26.1 ± 5.3**
BMI	**23.3 ± 1.156**	**32.36 ± 6.53**
WHR	**0.77 ± 0.78**	**0.89 ± 0.92**
With Hirsutism	**-----**	**67**
Without Hirsutism	**80**	**13**
Primary infertility	**-----**	**52**
Secondary infertility	**-----**	**28**
Menstruation Pattern (regular)	**80**	**17**
Menstruation Pattern (Irregular)	**------**	**63**

The data represented as mean ± SD, SD: standard deviation, No.: number, BMI: body mass index, and WHR: waist hip ratio



A. BMI **B. WHR**



C. age

Fig. (3.1): Estimation plot of: (A) BMI, (B) WHR and (C) age in women with PCOS and healthy control group

3.1.2. The Biochemical Parameters of Patients and Healthy Control Group

Comparisons between biochemical parameters of the study groups revealed in **Table (3.2) and Figure (3.2).** A significant elevation in levels of LH (P<0.0001), LH / FSH ratio (P<0.0001), Prolactin (P<0.0001) and Free Testosterone (P<0.0001) were obtained in PCOS patients group compared with healthy women. While, significantly lower level of FSH (p=0.01) was noticed during comparable evaluation in PCOS women and healthy women.

Table (3.2) Biochemical characteristics of the patients and control groups.

Parameters	Control group Mean ± SD	Patients group Mean ± SD	P- value
LH (m.IU/mL)	**5.16 ± 0.55**	**11.89 ± 3.18**	**<0.0001**
FSH (m.IU/mL)	**6.73 ± 0.65**	**5.36 ± 1.36**	**0.01**
LH / FSH	**0.98 ± 0.04**	**2.41 ± 0.37**	**<0.0001**
Prolactin (ng/mL)	**12.19 ± 2.92**	**16.14 ± 4.05**	**<0.0001**
FT (pg/mL)	**2.97 ± 1.81**	**18.7 ± 14.98**	**<0.0001**

The data represented as mean ± SD, SD: standard deviation, LH: luteinizing hormone, FSH: follicular stimulating hormone and FT: free testosterone.

Elevated LH is a frequent symptom of polycystic ovarian syndrome (PCOS), however it is not required for diagnosis. LH is known to enhance ovarian androgen production in addition to inducing ovulation and luteinization, and it is one of the primary factors contributing to hyperandrogenism in PCOS patients. Luteinizing hormone stimulates the production of androgens largely in ovarian theca cells that have LH receptors **(Ashraf *et al.*, 2019)**. Higher LH concentrations appear to be related to more severe types of PCOS. Previous research has demonstrated a positive

relationship between follicle number and ovarian volume, with higher LH concentrations in PCOS patients being associated with more severe cycle disruptions and a higher likelihood of infertility. LH hypersecretion in PCOS women also reflects how severe the condition is **(Hendriks *et al.*, 2008).**

Numerous follicles in the theca cells of the ovaries become mostly pre-antral and antral stages arrested due to increased LH stimulation, leading to hyperplasia of theca cells and accumulation of follicular fluid that forms cyst-like structures along the periphery of the ovary giving it a string of pearls appearance. In contrast, a relative deficit in FSH impairs follicular development **(Ashraf *et al.*, 2019).** Increased LH pulse frequency impairs estrogen and FSH synthesis, thus inhibiting follicle growth and ovulation and finally contributes to the formation of polycystic ovaries in PCOS patients **(Liao *et al.*, 2021).** Consistently rapid (GnRH) pulsatility, which favors pituitary LH over FSH synthesis and leads to the elevated LH concentrations and consequently changed LH / FSH ratios typical of PCOS, is thought to be a neuroendocrine feature of the condition. Follicular development is hampered by low FSH levels, while ovarian androgen production is enhanced by high LH levels **(Malini and George, 2018)**.

In PCOS, FSH levels may be increase, fall below their normal levels or remain constant **(Rebar *et al.*, 1976, Mohammed and Qasim, 2021)**. The occurrence of the disorder in the LH and FSH hormones is the result of hypothalamus pituitary gland, which causes the difference in the level of these hormones in the affected women **(Taylor *et al.*, 1997 and Mansour *et al.*, 2020)**. The pathogenesis of polycystic ovarian disease has been forced to include abnormality of the hypothalamic-pituitary-ovarian or adrenal axis. The relative increase in LH to FSH release is caused by a disruption in the gonadotrophin-releasing hormone's (GnRH) pattern of production.

The ovarian granulosa cells are affected by FSH, which transforms the androgens produced in the theca cells into estrogens, primarily estradiol, which is necessary for the formation of follicles **(Ashraf *et al.*, 2019).** The aberrant feedback mechanism that increased LH release was brought on by ovarian estrogen. LH to FSH ratios in healthy women typically range between 1 and 2. This ratio is inverted in women who have polycystic ovarian disease, and it may even increase to 2 or 3 **(Saadia, 2020).**

Either free or coupled to proteins like SHBG and albumin, testosterone is present. Normal testosterone levels are as follows: 80% of it is bound to sex hormone-binding globulin, 19% of it is tied to albumin, and only 1% is free to circulate. According to the Rotterdam agreement, circulating free testosterone or FAI measures should be used instead of serum total testosterone to detect hyperandrogenism in women with PCOS **(Ashraf *et al.*, 2019).** The hyperplasia of theca cells in PCOS women's ovaries and the deregulation of steroid production pathway enzymes are both factors in the rise in androgen. Increased androgens prevent the hypothalamic-pituitary axis from receiving any negative feedback, increasing the frequency of GnRH pulses. LH production is more favored by elevated hypothalamic GnRH than FSH production **(Ashraf *et al.*, 2019)**.

The first impact of androgen excess in PCOS is impaired folliculogenesis. Increased androgens in the early gonadotropin-independent stage stimulate the formation of primordial follicles and increase the number of small antral follicles **(Dewailly *et al.*, 2016)**. High LH encourages the ovary to secrete too many androgens in PCOS patients, while increased FSH may prompt the ovary's cells to convert too much androgens to estrogen **(Malini and George, 2018)**. The diagnosis of PCOS and/or phenotypic should be made when the presence of clinical indicators of hyperandrogenemia is uncertain or missing. This can be done by testing calculated

free testosterone, free androgen index, or calculated bioavailable testosterone in clinical practice. Despite the fact that 78 percent of PCOS patients visiting clinics exhibit biochemical hyperandrogenemia **(Mumusoglu and Yildiz, 2020)** Therefore, The main factor believed to be responsible for this disorder's symptoms and indications developing is an excess of androgen **(Özen and Cander, 2022).**

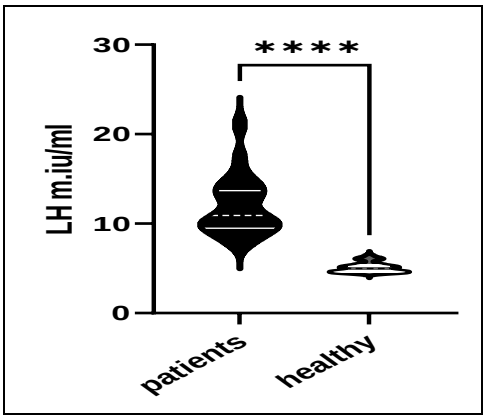
Clinical manifestations of hyperandrogenism in women with PCOS include hirsutism, acne, and androgenic alopecia. Increased androgen excess can also cause additional symptoms like weight gain, irregular menstruation, acanthosis nigricans, and insulin resistance **(Mahajan *et al.*, 2021)**. Hyperandrogenism is the most consistent and prominent diagnostic component of PCOS. The Androgen Excess Society, currently known as the Androgen Excess and PCOS Society, put out the most recent addition to the PCOS diagnostic criteria in 2006. Given how subjective clinical assessments of hyperandrogenism are, biochemical hyperandrogenism is likely more helpful in determining the diagnosis of PCOS **(Joham *et al.*, 2022).** Blood testosterone levels are directly correlated with higher serum LH levels **(Mariani *et al.*, 2022).**

Hyperprolactinemia and polycystic ovary syndrome (PCOS) are on the list of the most frequent causes of female infertility **(Dehghan *et al.*, 2021)**. Opinions on the relationship of PCOS and hyperprolactinemia widely vary: from the recognition of elevated levels of Prolactin as a disorder characteristic of PCOS on one hand, to the obligatory exclusion of hyperprolactinemia **(Zafar *et al.*, 2021).**

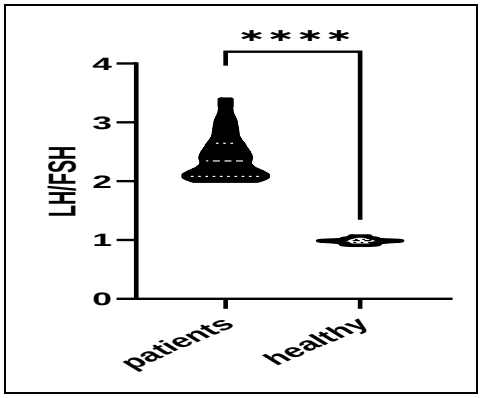
There have been several possible mechanisms increasing Prolactin secretion that may occur in PCOS. The effect of estrogens, which stimulate the synthesis and secretion of prolactin, as well as cell proliferation of lacto tropic pituitary cells. elevated estradiol levels in PCOS could result in increased Prolactin

concentrations **(Szosland *et al.*, 2015).** The role of Prolactin in promoting insulin secretion in islets is well-demonstrated. According to research, prolactin can influence the target organs' essential enzymes and transporters involved in the metabolism of glucose and lipids as well as metabolism balance **(Ramirez and de Sá, 2021)**. Therefore, It has been hypothesized that prolactin is intimately linked to insulin resistance, hypertension, thromboembolic stroke, and coronary syndrome **(Chen *et al.*, 2022)**.

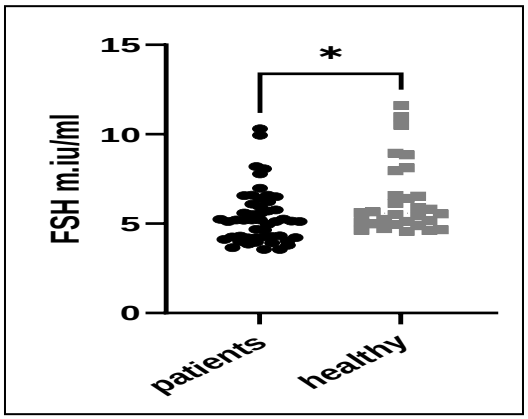
A relatively significant subset of infertile women includes those with PCOS. Hyper inflammation, insulin resistance, decreased glucose tolerance, metabolic syndrome, hyperlipidemia, hypertension, and an elevated risk of cardiovascular disease are all intimately related to PCOS. According to research by Haiyan Yang, PRL is considerably lower in PCOS patients than in controls **(Yang *et al.*, 2020)**. While others showed that the serum Prolactin upper reference limit was almost 1.5 times higher than in controls. The route behind PCOS-related Prolactin elevation may be explained by a decrease in central dopaminergic tone that causes both Prolactin and LH levels to rise **(Mahboobifard *et al.*, 2022).** Another prior study have been reported cases of twin sisters presenting with features of PCOS associated with idiopathic hyperprolactinemia **(Goyal and Ganie, 2018).**



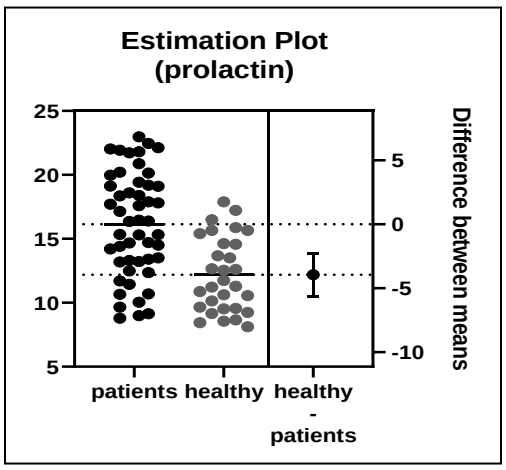
A. LH



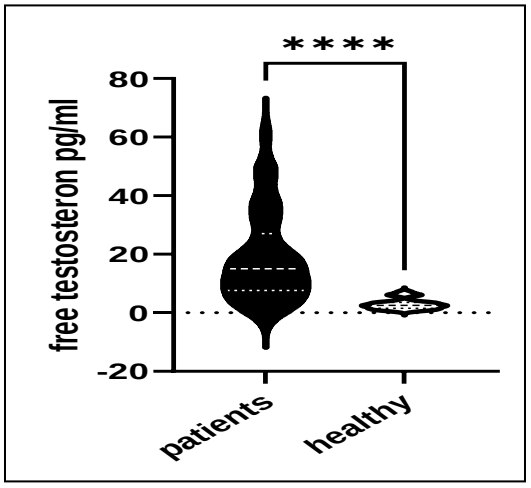
B. LH/FSH



C.FSH



D. Prolactin



E. Free Testosterone

Fig. (3.2): Difference between levels of (A) LH, (B) LH/ FSH ratio, (C) FSH, (D) Prolactin and (E) Free Testosterone between PCOS patients and healthy control group

3.1.3. Interleukin-10 Level in PCOS Patients and Healthy Control Group

Comparison between IL-10 level in the study groups revealed in **Table (3.3) and Figure (3.3).** the result demonstrates that IL-10 level significantly lower in PCOS patients group (P <0.0001) when compared with healthy control group.

Table (3.3): Comparison of serum interleukin 10 level between PCOS patients group and healthy control group

Parameter	Control group Mean ± SD	Patients group Mean ± SD	P- value
IL-10 (pg/mL)	4.53 ± 0.75	2.19 ± 0.47	<0.0001

The data represented as mean ± SD, SD: standard deviation, IL-10: interleukin 10

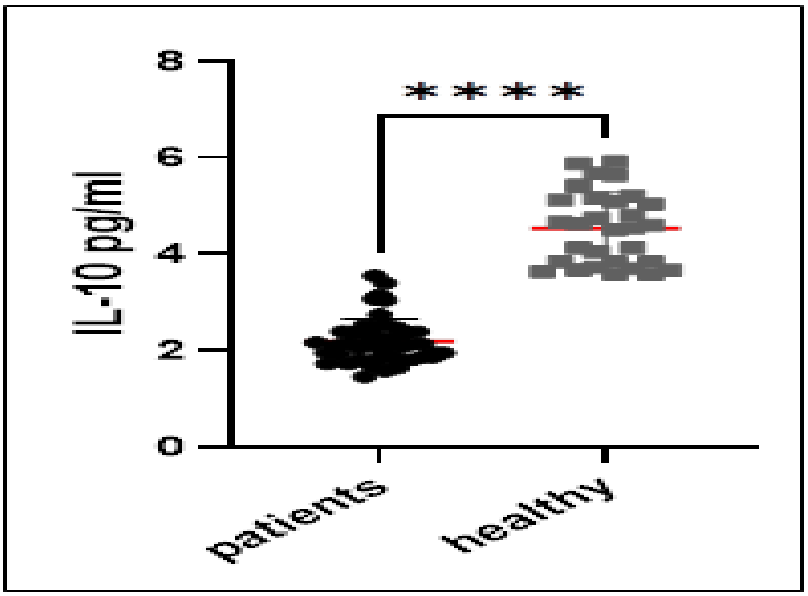


Fig. (3.3): difference in mean levels of IL-10 between PCOS patients with healthy control group

The current result revealed a significant lower level of IL-10 in women with PCOS when compared with healthy control women may be related to chronic low-grade inflammation which may be suggested as a key contributor of the

pathogenesis and development of polycystic ovary syndrome (PCOS). Th1 cells, macrophages, dendritic cells (DC), B cells, and different CD4+ and CD8+ T cell subsets all express pro-inflammatory cytokines, which is suppressed by the anti-inflammatory cytokine IL-10. In addition to stimulating the functions of innate and Th2-related immunity and suppressing Th1-related immune responses, the main function of IL-10 is to down-regulate the synthesis of chemokines and cytokines **(Jafarzadeh *et al.*, 2021).** The decline in IL10 secretion was substantially linked with PCOS illness **(Talaat *et al.*, 2016).**

Ovarian dysfunction, delayed follicular maturation, and altered steroidogenesis are all caused by an imbalance of pro- and anti-inflammatory cytokines **(Artimani *et al.*, 2018).** Some studies have hypothesized that IL-10 may be crucial for maintaining pregnancy by supporting the formation of Th2 cytokine environment, progesterone production, and corpus luteum maturation **(Ghandehari *et al.*, 2021 and Shepherd *et al.*, 2021)**.

The main characteristics of the metabolic abnormalities typical of PCOS include insulin resistance and hyperinsulinemia. There is growing proof that IL-6, IL-10, and TNF- are crucial in triggering insulin resistance, however; low IL-10 levels have been reported to be associated with obesity and metabolic syndrome **(Vural *et al.*, 2010).** Abraham Gnanadass et al., found that a low level of IL-10 is related to metabolic syndrome and obesity. Reduction in plasma IL-10 was seen in PCOS patients **(Abraham Gnanadass *et al.*, 2021).**

In prior study by Karadeniz et al., revealed that Interleukin IL-10 is a major anti-inflammatory cytokine that has been associated with obesity and type 2 diabetes and IL-10 regulates downwards the production of these pro-inflammatory cytokines Low IL-10 levels have been detected in the patients with

high BMI and insulin levels **(Karadeniz *et al.*, 2008)**. In the last decade, Interleukin IL-10, an anti-inflammatory cytokine, has been the subject of extensive research because of its role in mediating the immunosuppressive and anti-inflammatory effects. **(Hyvärinen *et al.*, 2018)**. Given that persistent low-grade inflammation has been significantly linked to the pathogenesis of PCOS **(Morong and González, 2022).**

Anti-inflammatory and immune-suppressive properties in a variety of diseases, including PCOS. In line with the current study, a recent investigation found that women with PCOS had considerably decreased serum levels of IL-10 **(Chugh *et al.*, 2021).** Another research by Sylus *et al.*, also showed that inflammation is frequently linked to PCOS, and in these individuals, it affects ovarian folliculogenesis, abnormal steroidogenesis in the ovary, and hyperinsulinemia. An anti-inflammatory cytokine called interleukin-10 (IL-10) controls how pro-inflammatory cytokines behave when there is inflammation. Reduced IL-10 levels have been observed in women with PCOS and IL-10 gene variation has been associated with PCOS **(Sylus *et al.*, 2020).**

3.1.4. Programmed Cell Death Protein 1 of PCOS Patients and Healthy Control Group

Comparison between PD-1 protein level in the study groups revealed in **Table (3.4) and Figure (3.4).** the result demonstrate that PD-1 level significantly elevated in PCOS patients group (P=0.01) when compared with healthy control group.

Table (3.4): Comparison of serum programmed cell death protein 1 level between PCOS patients and control group

Parameter	Control group Mean ± SD	Patients group Mean ± SD	P- value
PD-1 (ng/mL)	0.28 ± 0.09	0.34 ± 0.1	0.01

The data represented by mean ± SD, SD: standard deviation, PD-1: programmed cell death protein 1

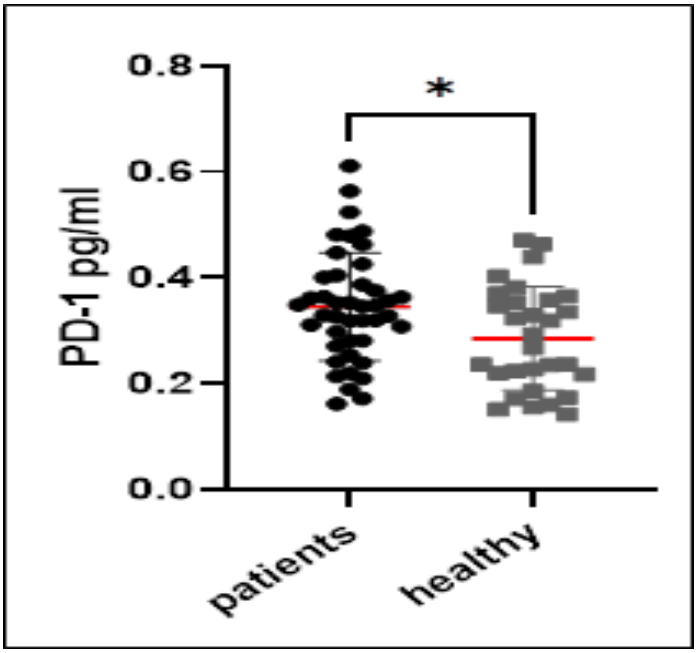


Fig. (3.4): difference in mean levels of PD-1 between PCOS patients with healthy control group

At the best of our knowledge the higher level of PD-1 in Iraqi women with PCOS is shown in the current findings as a first study to investigate whether inflammation and immune modulation may play significant roles in the development of PCOS. The PCOS group with infertility expressed considerably more PD-1 on CD4+ T cells than did the control group **(Li *et al*., 2019b).**

Programmed cell death or apoptosis is an essential component of human ovarian function and development. During early fetal life approximately 7×10^6 oocytes are formed in the human ovary **(Regan *et al.*, 2018)**. However, apoptotic cell death causes a significant reduction in the amount of oocytes even before delivery. Every menstrual cycle, a number of primordial follicles begin to form in the reproductive system **(He *et al.*, 2021)**. Typically, only one will ovulate, and the remaining follicles will undergo atresia through the process of apoptosis. Only about 400 follicles will ultimately ovulate throughout a woman's reproductive life **(Mehedintu *et al.*, 2021 and Secomandi *et al.*, 2022)**. After ovulation, the dominant follicle develops into the corpus luteum, a brand-new endocrine gland that is in charge of producing progesterone and keeping the endometrium in place during the first trimester of pregnancy **(Gingold *et al.*, 2022)**. To allow for the next menstrual cycle's growth of new follicles, corpus luteum activity must stop if pregnancy is not achieved **(Bishop *et al.*, 2022).** In the human ovary, corpus luteum regression is also caused by apoptosis **(De Pol *et al.*, 1997 and Wang *et al.*, 2017).**

PCOS women display more maturing and following atrophic follicles that not pass into dominant follicles, with abnormal apoptosis of granulosa cells in PCOS folliculogenesis. That lead to anovulation and infertility **(França and Mendonca, 2021).** Infection resistance, immunological tolerance, and cancer immunity are all mediated by PD-1 levels. It has been noted that this inducible receptor is expressed on peripheral T cells after activation **(Lu *et al.*, 2022)**. Programmed Cell Death-1 interacts with PD-L1 and PD-L2 to inhibit antiviral T cell responses. which showed that the expression of PD-1 was significantly higher in CD4+ or CD8+ T cells from the PCOS group with infertility than it was in cells from the control group **(Zhao *et al.*, 2022).** The immune pathogenesis in the ovary of

PCOS patients with infertility may be influenced by T cell malfunction, which may be an immunological characteristic **(Qin *et al.*, 2016 and Shamsi *et al.*, 2022)**. Li *et al.,* suggested that elevated PD-1 expression in PCOS patients with infertility may inhibit T cell survival and activation **(Li *et al.*, 2019b)**. These findings imply that one of the fundamental reasons for the pathophysiology of PCOS may involve chronic inflammation **(Wira *et al.*, 2013).**

Previous study by Han *et al.,* has disagreement with the present results showed that the level of PD-1 was lower in the serum of patients when compared with the control group. The pathophysiology of PCOS may be attributed to the low level of PD-1 in the serum of PCOS patients, who are unable to effectively block T and B cell activity and proliferation **(Han *et al.*, 2021).** Induced Cell Death Protein 1 modulates T-cell activity, activates antigen-specific T cell apoptosis, and inhibits regulatory T cell apoptosis, all of which are crucial in suppressing immunological responses and fostering self-tolerance. Cancer immune escape is caused by the PD-1/PD-L1 axis, which also has a significant impact on cancer therapy **(Han *et al.*, 2020).** In the current results concluded that the abnormalities of PD-1 in the serum of PCOS patients may be related to the onset of PCOS.

3.1.5. Correlations of BMI and WHR Data Base with Hormonal Parameters in PCOS patients

The anthropometric parameters represented by BMI and WHR data base correlations with hormonal level in PCOS patients group were showed in **Table (3.5).** The result showed that there is significant positive correlation between BMI and (LH r= 0.540, p= <0.0001), (LH /FSH r= 0.517, p= <0.0001) and (FT r= 0.350, p= 0.003). Also, there is significant positive correlations between WHR

and (LH r= 0.611, p= <0.0001), (LH /FSH r= 0.611, p= <0.0001), (prolactin r= 0.287, p= 0.009) and (FT r= 0.445, p= <0.0001).

Obesity and polycystic ovarian syndrome are factors in the metabolic problems that affect women who are of reproductive age. In PCOS women, abdominal obesity leads to further disturbances in metabolic and hormonal parameters. In women with PCOS, weight management should be suggested as the first line of treatment **(Milnerowicz and Madej, 2017).**

Table (3.5): Correlation of hormonal parameters with BMI and WHR data base in PCOS patients group

Parameters	Correlation parameters	BMI (Kg/m^2)	WHR
LH (m.IU/mL)	**r**	**0.540**	**0.611**
	P	**<0.0001**	**<0.0001**
FSH (m.IU/Ml)	**r**	**-0.065**	**-0.097**
	P	**0.564**	**0.388**
LH / FSH	**r**	**0.517**	**0.611**
	P	**<0.0001**	**<0.0001**
Prolactin (ng/Ml)	**r**	**0.206**	**0.287**
	P	**0.066**	**0.009**
FT(pg/Ml)	**r**	**0.350**	**0.445**
	P	**0.003**	**<0.0001**

r: spearman r, BMI: body mass index, WHR: waist hip ratio, LH: luteinizing hormone, FSH: follicular stimulating hormone and FT: free testosterone.

Adiposity plays a crucial role in the development and maintenance of PCOS and strongly influences the severity of both its clinical and endocrine features in many

women with the condition **(Barber and Franks, 2021)**. Even in women with normal ovaries, obesity most likely has a role in the characteristics of hyperandrogenism. The genes that control ovarian steroidogenesis as well as those that affect body mass index (BMI) and adiposity are candidates for PCOS **(Baptiste *et al.*, 2010)**.

The combined impact of a genetic predisposition to obesity in the context of an obesogenic environment is a possible explanation for the mechanisms behind the development of obesity in women with PCOS (poor diet and reduced exercise). Obesity and PCOS are frequently linked, and both affect reproductive health **(Barber et al., 2006 and Barber and Franks, 2021)**.

Despite the fact that a number of theories have been put out to explain the pathogenic mechanism of PCOS, it is widely acknowledged that insulin resistance (IR) plays a significant etiological role that is independent of obesity but enhanced by it. As a result of the hyperinsulinemia, increased ovarian androgen production is activated, which causes PCOS **(Calcaterra *et al.*, 2021)**. Additionally, the chronic inflammation brought on by obesity has an effect on ovarian physiology because it impairs insulin sensitivity. Adolescents with obesity and PCOS are first-line treated with nutritional treatments, lifestyle modifications, and, if necessary, weight loss **(Calcaterra *et al.*, 2021)**. Adipose tissue alters structurally and functionally with obesity, causing high blood sugar, high cholesterol, high leptin levels, and persistent low-grade inflammation **(Calcaterra *et al.*, 2022)**. In reproductive organs, excessive free fatty acids may have a toxic effect that results in long-lasting cell damage and a persistent low-grade inflammatory state. It is believed that lipotoxicity caused by obesity changes how the mitochondria operate in the oocyte **(Fabozzi *et al.*, 2022)**.

The assessment of visceral obesity is a better evaluation, compared to the assessment of overall obesity, Abdominal obesity plays crucial role in metabolic disorders and it was shown that obesity worsens the presentation of PCOS **(De Silva *et al.*, 2022)**. there are disorders in hormonal parameters in serum of women with higher WHR value. female with abdominal obesity as the WHR value increased, the concentrations of fasting glucose, insulin and free testosterone increase significantly **(Milnerowicz and Madej, 2017)**. Because the resulting insulin resistance can produce hyperandrogenism in the ovary and, in some cases, the adrenal glands, it appears that abdominal obesity may be a significant factor in the development of PCOS **(Ding *et al.*, 2021)**. Resultant androgen excess may then also lead to more and more accumulation of fat in the abdominal site. This vicious cycle can result in the development of PCOS complications. PCOS pathogenesis may be significantly impacted by the development of oxidative stress by visceral buildup of adipose tissue **(Chen *et al.*, 2014)**. In respect to serum testosterone, lipid and lipoprotein profiles differed in PCOS women with abdominal obesity. In women with PCOS, there is a substantial interaction between obesity, testosterone, and dyslipidemia, suggesting that the syndrome itself is a major contributor to the abnormalities in lipoproteins in addition to the effects of obesity **(Couto Alves *et al.*, 2017).**

3.1.6. Correlation of Interleukin–10 level with Anthropometric and Biochemical Parameters in PCOS Patients

Interleukin–10 correlations with anthropometric and biochemical parameters in PCOS patients group are showed in **Table (3.6) and Figures (3.5, 3.6, 3.7, 3.8, 3.9 and 3.10).** The result showed that there are significant negative correlations between level of IL-10 and (BMI $p<0.0001$, $r= -0.66$), (WHR $p<0.0001$, $r= -$

0.66), (LH p<0.0001, r= -0.63), (LH / FSH ratio p<0.0001, r= -0.72), (Prolactin p=0.008, r= -029), (FT p< 0.0001, r= 0.534) and there are non-significant positive correlations with age and FSH revealed with IL-10 level in PCOS patients group. The most typical cause of female infertility is thought to be polycystic ovarian syndrome (PCOS), an endocrine condition marked by hyperandrogenemia, anovulation or infrequent ovulation, and polycystic ovary morphology. Reduced T regulatory cells caused a reduction in the release of anti-inflammatory factors such interleukin-10 **(He *et al.*, 2020).**

Table (3.6) Correlation of serum IL-10 level with anthropometric and biochemical parameters in registered PCOS patients group

Parameters	r	P- value
Age (Years)	**0.133**	**0.237**
BMI (kg/m²)	**-0.682**	**<0.0001**
WHR	**-0.664**	**<0.0001**
LH (m.IU/mL)	**-0.639**	**<0.0001**
FSH (m.IU/mL)	**0.136**	**0.232**
LH / FSH	**-0.721**	**<0.0001**
Prolactin (ng/mL)	**-0.290**	**0.008**
FT (pg/mL)	**-0.534**	**<0.0001**

r: spearman r, BMI: body mass index, WHR: waist hip ratio, LH: luteinizing hormone, FSH: follicular stimulating hormone and FT: free testosterone.

Hyperinsulinemia stimulates the pituitary's reaction to gonadotropin-releasing hormone (GnRH), which increases the release of luteinizing hormone and androgen. This influences how well the hypothalamus-pituitary-ovarian gonadal axis works (HPO axis) **(Geraghty, 2022)**. Hyperandrogenism may prevent the growth of follicles, cause follicular atresia, and encourage insulin resistance as a result of feedback. These changes are related to the pathogenesis of PCOS and this inflammatory condition could damage

insulin sensitivity and promote the development of PCOS **(Wang *et al.*, 2019a)**. Low-grade chronic inflammation and imbalance between pro- and anti-inflammatory cytokines has been proposed to play a role in the pathogenesis of PCOS. Interleukin-10 (IL-10) is an anti-inflammatory cytokine considered as a down regulator of cytokine production by Th1 cells and macrophages **(Yang *et al.*, 2021b)**.

The current results shown a negative correlation between serum IL-10 level and BMI, WHR, LH, LH/FSH ratio, prolactin and Free Testosterone levels. While, there is no correlation with age or FSH level in PCOS patients group.

Prior study by Yang et al., found that obesity has been reported to have a modulatory effect on the ovulatory functions of patients with PCOS. Obesity may disrupt normal folliculogenesis through increased production of IL-10 in visceral fats **(Yang et al., 2021a)**.

Another study by Artimani et al., found that the serum level of IL-10 in PCOS patients group revealed a significant correlations with visceral adiposity which represented by WHR **(Artimani *et al.*, 2018).**

The anti-inflammatory cytokines such as interleukin 10 are produced by T regulatory cells. A significant positive correlation was observed between the ratio of T regulatory cells and LH levels in PCOS patients while other hormones such as FSH will not affect the ratio of T regulatory cells. T regulatory cell-related to IL-10 decreased in PCOS patients **(Yang *et al.*, 2021b).**

Study by Cabus et al., shown that the women with PCOS had considerably higher LH/FSH ratio and a significant positive correlation with IL-10 level in PCOS subjects than in controls **(Cabus *et al.*, 2020).**

Low levels of IL-10 were reported in PCOS, and it was discovered that these levels had an impact on pregnancy rates and outcomes. Our findings showed that infertile women with PCOS who had a high BMI had significantly lower serum IL-10 levels. Additionally, IL-10 was found to be negatively correlated with BMI, indicating that IL-10 levels in PCOS patients decrease as BMI rises. These results corroborated other studies showing a decrease in IL-10 levels in PCOS and obesity. Anti-inflammatory cytokines like IL-10 are decreased because inflammation and weight gain are linked **(Sylus *et al.*, 2020 ; Zhang *et al.*, 2017 and Jung *et al.*, 2008)**.

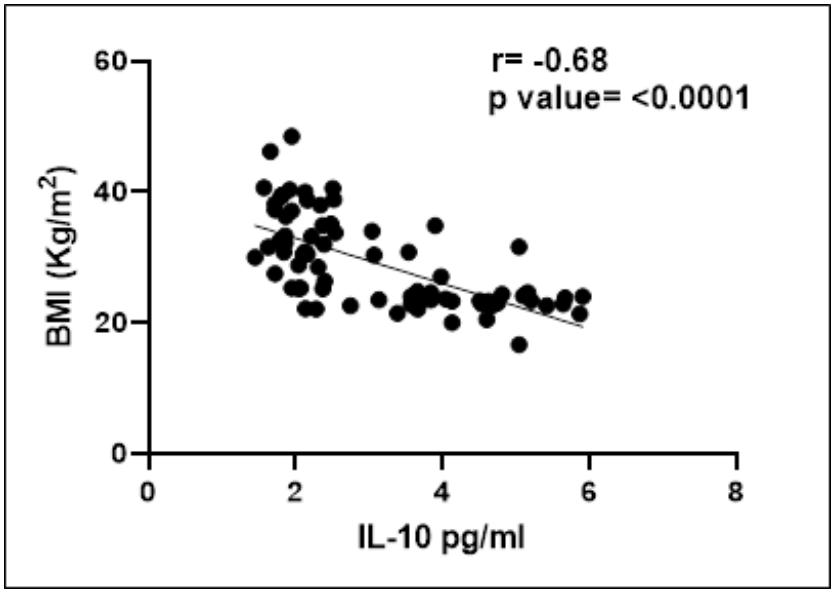


Fig. (3.5): Correlation between data of BMI and IL-10 level in PCOS patients group

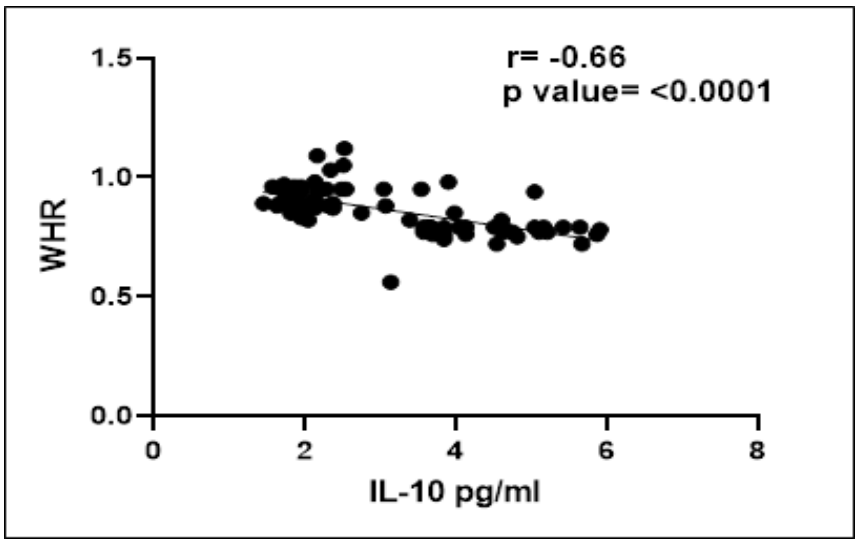


Fig. (3.6): Correlation between data of WHR and IL-10 level in PCOS patients group

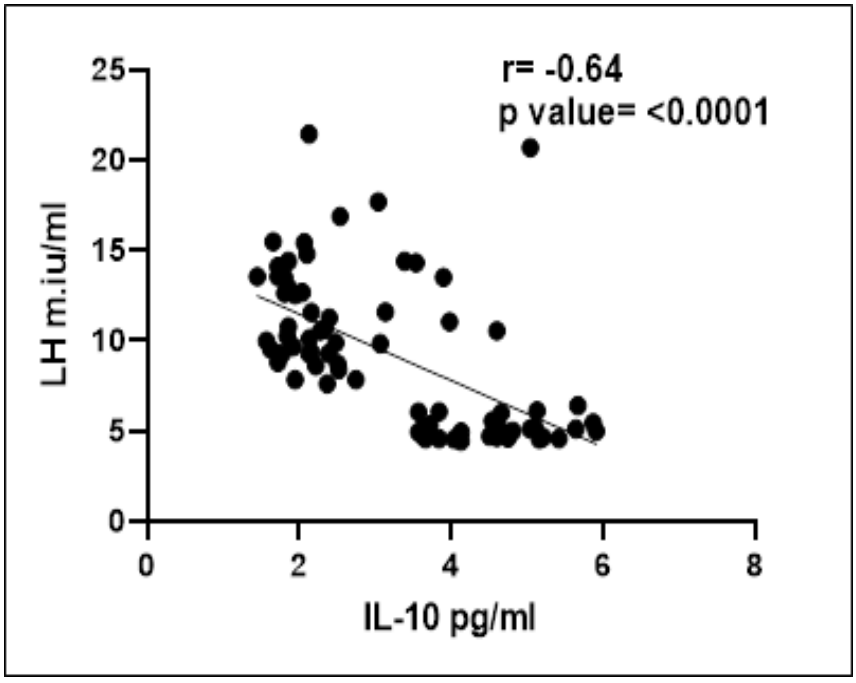


Fig. (3.7): Correlation between LH and IL-10 levels in PCOS patients group

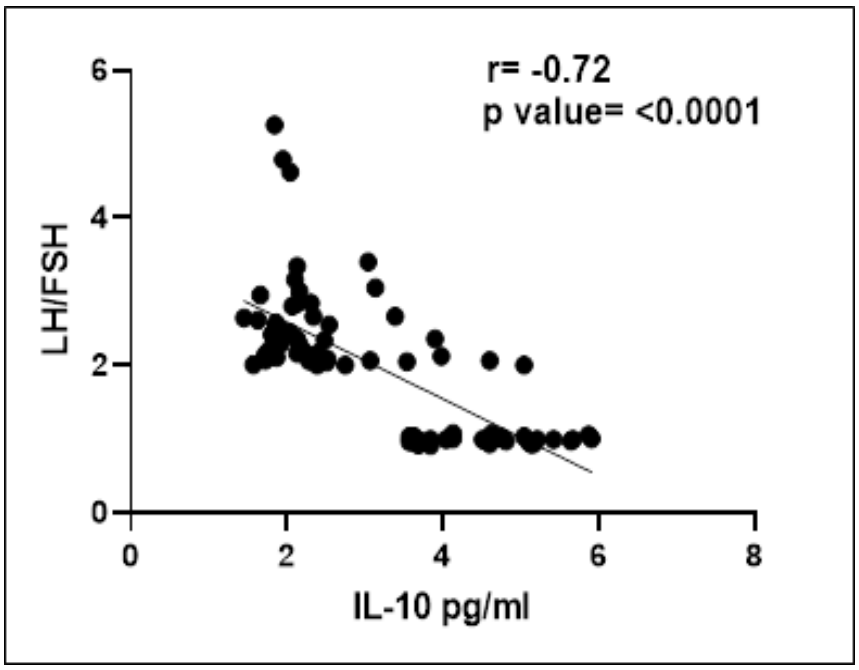


Fig. (3.8): Correlation between data of LH/FSH ratio and IL-10 level in PCOS patients group

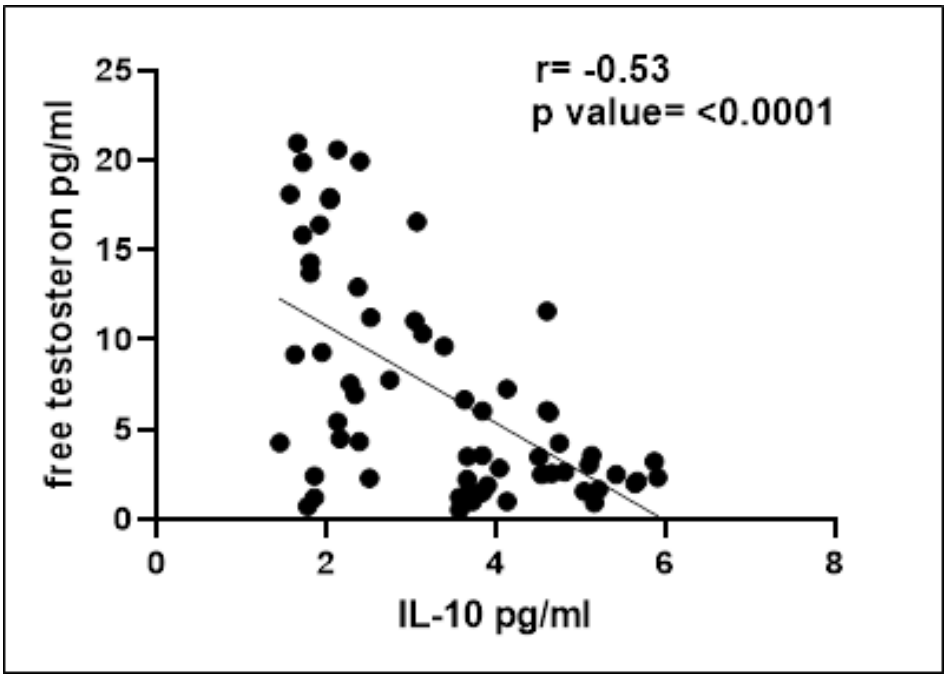


Fig. (3.9): Correlation between Free Testosterone hormone level and IL-10 cytokine level in PCOS patients group

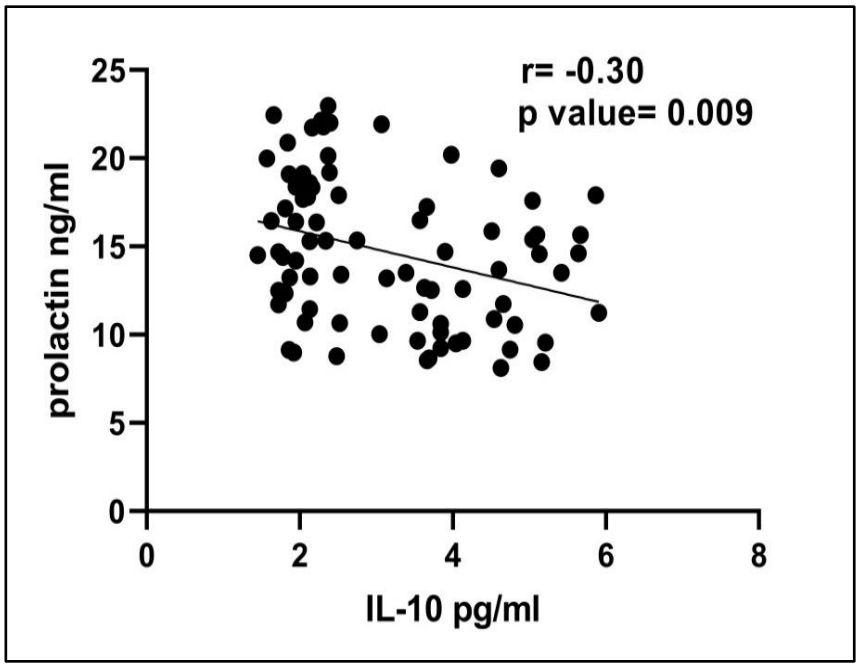


Fig. (3.10): Correlation between prolactin hormone level and IL-10 cytokine in PCOS patients group

3.1.7. Correlation of Programmed Cell Death – 1 Protein Level with Anthropometric and Biochemical Parameters in PCOS Patients Group

Programmed Cell Death – 1 protein level correlation with anthropometric and biochemical parameters in PCOS patients group was shown in **Table (3.7) and Figures (3.11, 3.12, 3.13 and 3.14).** The result demonstrates that there are significant positive correlations between serum PD-1 level and (BMI p=0.019, r=0.260), (WHR p=0.022, r=0.254), (LH p=0.011, r=0.283), (Free Testosterone=0.004, r=0.343), and there are non-significant correlations with age, FSH, LH/ FSH ratio, Prolactin and IL-10 revealed with PD-1 protein.

Table (3.7) Correlation of serum PD-1 level with anthropometric and biochemical parameters in the registered PCOS patients group

Parameters	r	P- value
Age (Years)	-0.063	0.577
BMI (Kg/m²)	0.260	0.019
WHR	0.254	0.022
LH (m.IU/mL)	0.283	0.011
FSH (m.IU/mL)	0.018	0.870
LH / FSH	0.185	0.100
Prolactin (ng/mL)	0.117	0.297
FT (pg/mL)	0.343	0.004
IL- 10 (pg/mL)	-0.110	0.330

r: spearman r, BMI: body mass index, WHR: waist hip ratio, LH: luteinizing hormone, FSH: follicular stimulating hormone, FT: free testosterone and IL-10: interleukin 10.

The current result of PCOS patient's hormones and other parameters has been shown to different association with PD-1 protein. Also, the number of studies about PD-1 level in PCOS patients is very little studies. The current results showed that the serum level of PD-1 protein was not correlated with age, FSH, LH/FSH ratio, Prolactin and IL-10 level. While, there is significant positive correlation with the data of BMI, WHR, LH and Free Testosterone hormone in PCOS patients group.

Immune checkpoints are regulatory molecules of the immune system and play an important role in maintaining immune homeostasis and self-tolerance. The

first immune checkpoints that were identified include programmed cell death protein-1 (PD-1) **(Marin-Acevedo *et al.*, 2021)**.

Higher BMI has been linked to better clinical outcomes for cancer patients taking checkpoint inhibitors for programmed cell death protein-1 (PD-1)/programmed cell death-ligand 1 (PD-L1), according to several research. Obesity accelerates tumor growth, immunological aging, and PD-1-mediated T cell failure **(Wang et al., 2019b, Cortellini et al., 2020 and Angeli *et al.*, 2022).** There was strict correlation between increased PD-1 expression and weight gain **(Oldenhove *et al.*, 2018).**

Central obesity represented by Waist Hip Ratio (WHR) high level has positive correlation with PD-1 protein level in lung cancer patients group **(Barbi *et al.*, 2021)** and in PCOS patients group of the present study

So, Luteinizing Hormone level which increased significantly in PCOS patients has positive correlation relation with PD-1 protein level. Free Testosterone Hormone level increase as diagnostic factor of PCOS patients also has positive correlation relation with PD-1 protein level.

That made a suggestion that PCOS patients' serum PD-1 abnormalities may be connected to the development of PCOS and to considered of PCOS as low grade inflammation and dysfunction of T cells as one of the underlying mechanisms for PCOS pathogenesis.

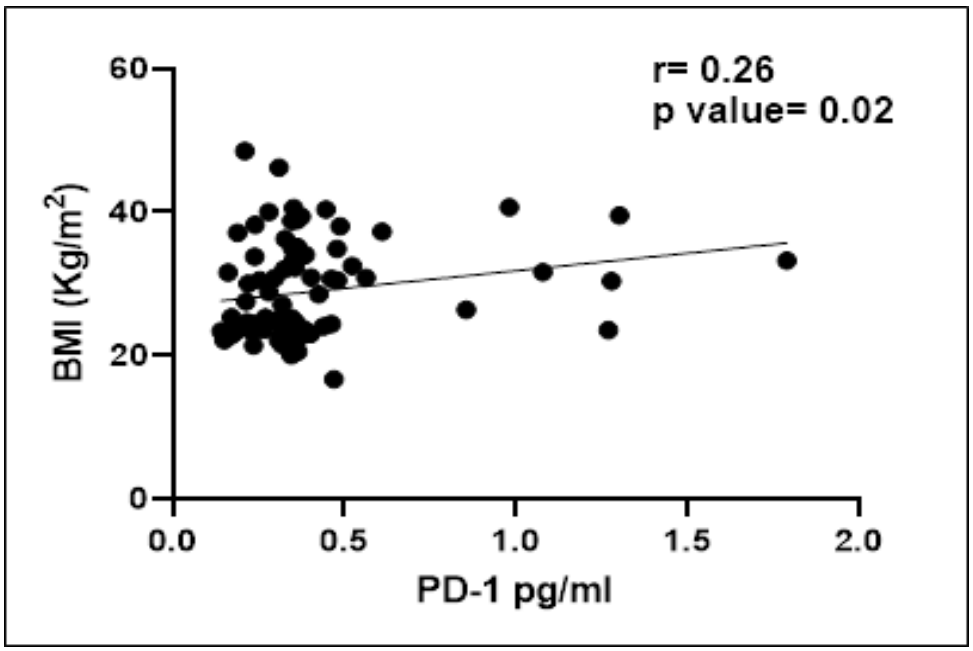


Fig. (3.11): Correlation between data of BMI and serum PD-1 protein level in PCOS patients group

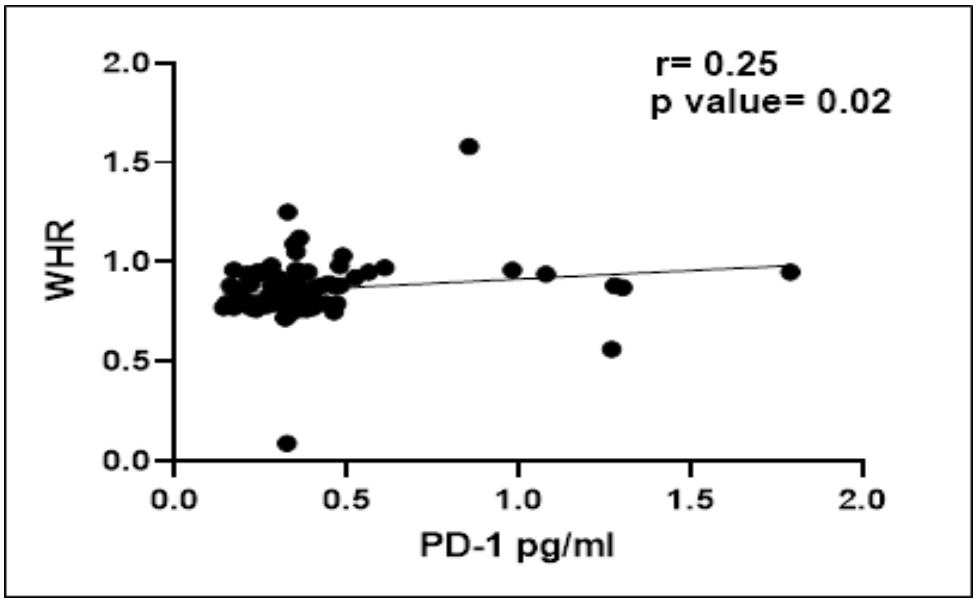


Fig. (3.12): Correlation between data of WHR and serum PD-1 protein level in PCOS patients group

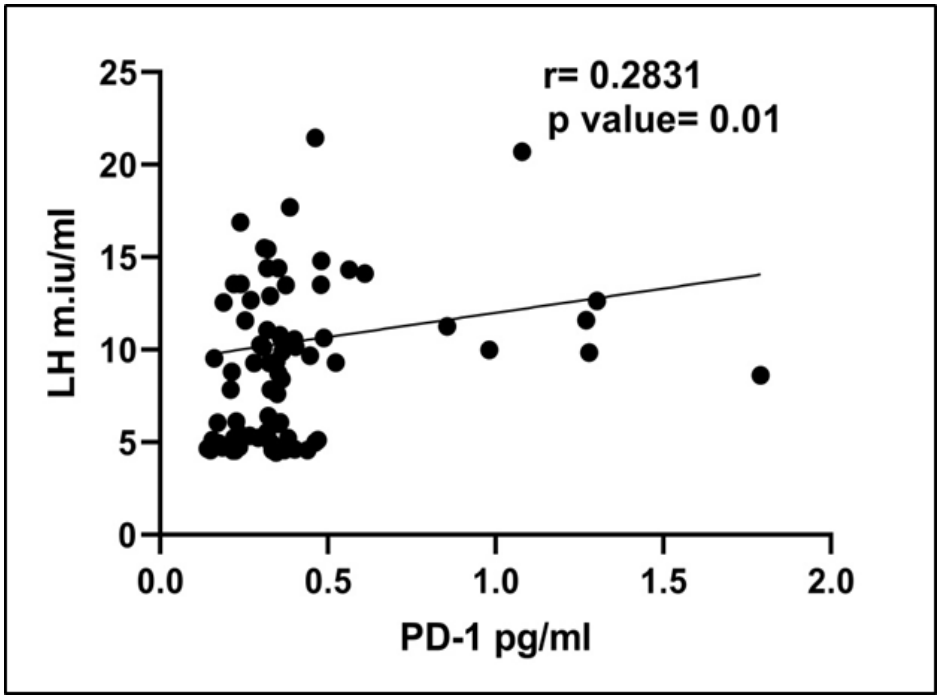


Fig. (3.13): Correlation between LH level and PD-1 protein level in PCOS patients group

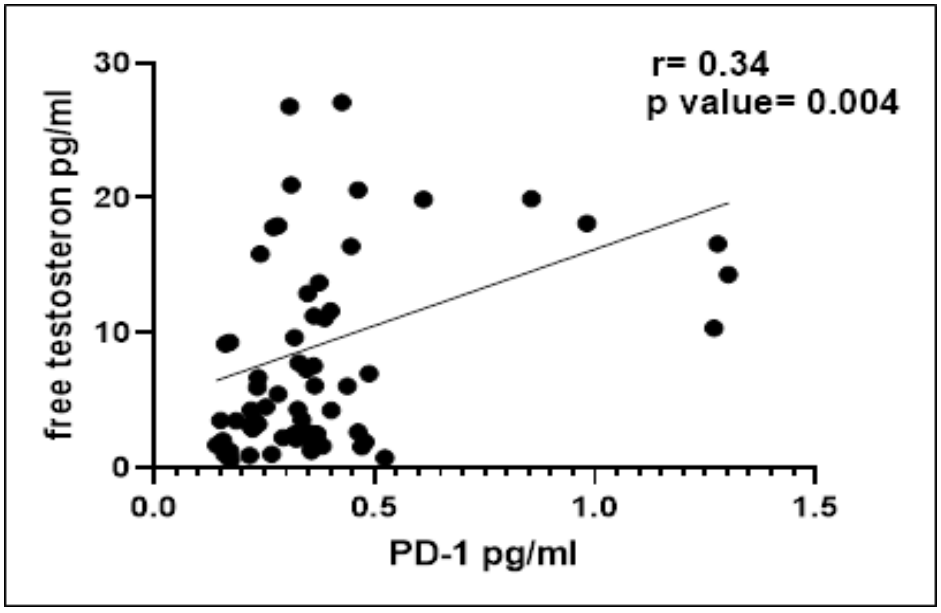


Fig. (3.14): Correlation between FT hormone level and PD-1 protein level in PCOS patients group

3.1.8. Receiver Operating Characteristics for Programmed Cell Death Protein-1 and Interleukin 10 in the PCOS Patients and Control Group

To investigate the effectiveness of PD-1 and IL-10 levels as biomarkers for predicting PCOS, in this performed ROC analyses in PCOS patients and healthy controls. IL-10 biomarker exhibited excellent diagnostic efficacy (sensitivity: 96.8%, specificity: 91.8%), (AUC 0.931) and best cut-off values was determined by the AUC was 3.555 pg/mL. Among PCOS subjects the (AUCs (95%CI) for IL-10: 0.860 – 1.000), (P value<0.0001), **Figure (3.15)**. While, PD-1 cytokine exhibit poor diagnostic efficacy (sensitivity: 63.3%, specificity: 60.0%), (AUC 0.695) and best cut-off values was determined by the AUC 0.3245, the (AUCs (95%CI) for PD-1: 0.577 – 0.813), (P value<0.004), **Figure (3.16)**. Sensitivity and specificity of PD-1 and IL-10 markers for diagnosing PCOS were showed in **Table (3.8).**

Table (3.8): Receiver operating characteristic-area under curve analysis of the IL-10 and PD-1 for the diagnosis of Polycystic Ovary Syndrome

Variable	Cut-off concentration	Sensitivity %	Specificity %	AUC	95% CI of AUC	p-value
IL-10 (pg/ml)	**3.555**	**96.8**	**91.8**	**0.931**	**0.860-1.000**	**<0.0001**
PD-1 (pg/ml)	**0.3245**	**63.3**	**60.0**	**0.695**	**0.577-0.813**	**<0.004**

AUC: Area Under the Curve, CI: Confidence Interval.

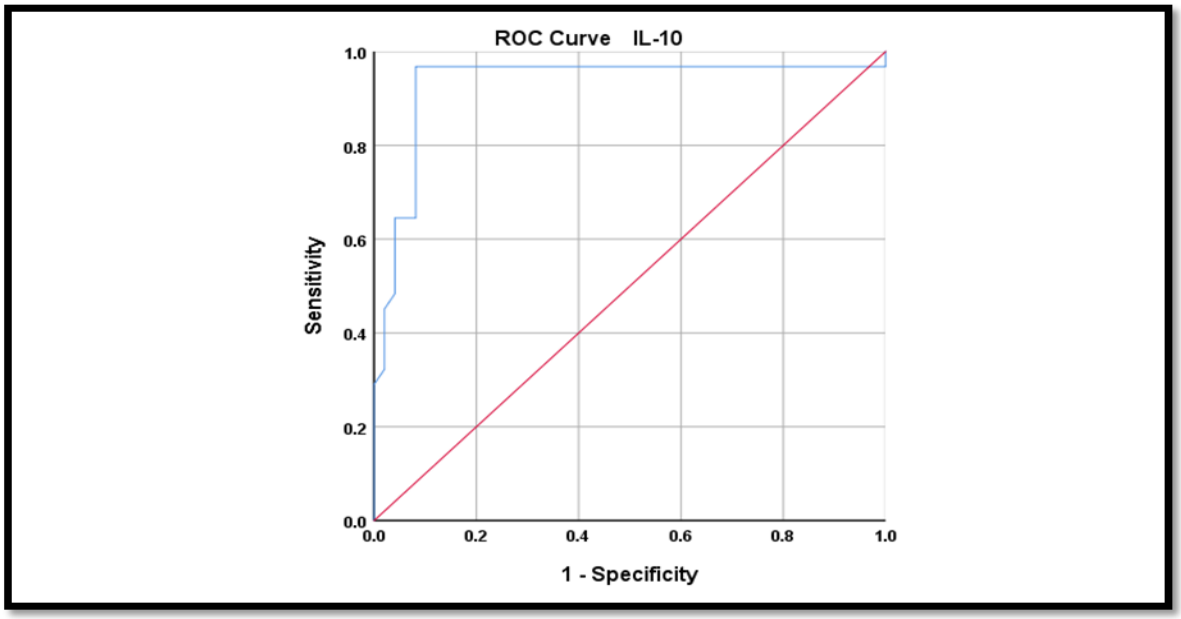


Fig. (3.15): Receiver Operating Characteristic curve of IL-10 level for diagnosis of PCOS

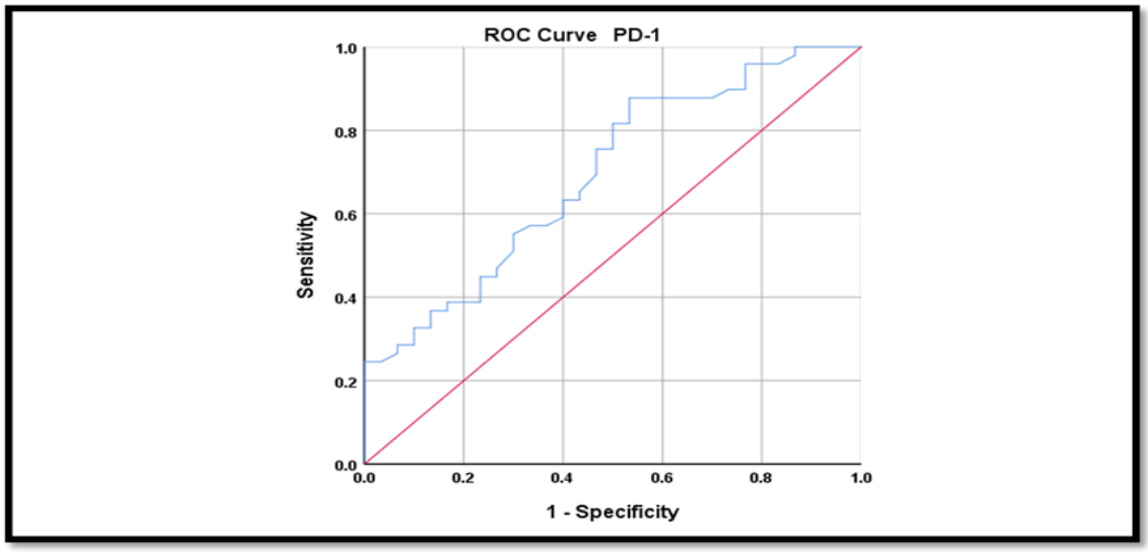


Fig. (3.16): Receiver Operating Characteristic curve of PD-1 level for diagnosis of PCOS

For all potential cut-off values for the diagnostic test, receiver operating characteristic curves are created by displaying the sensitivity (true-positive fraction) on the ordinate as a function of the complement of specificity (false-positive fraction). Therefore, the better the sensitivity and specificity the

diagnostic test has for all conceivable cutoff values, or more specifically, the higher the suitability of the diagnostic test for illness detection, the more the curve deviates towards the left upper corner. Sensitivity (also known as the 1- "false negative fraction") and specificity (sometimes known as the 1- "false positive fraction") are not affected by the prevalence of the disease in the sample, in contrast to accuracy. As a result, ROC curve analysis offers a description of disease detectability that is unaffected by decision threshold effects or disease prevalence. ROC curves are defined by the area under the ROC curve (AURC). The likelihood of successfully differentiating between people who are afflicted and those who are not is represented by the AURC. As a result, the ideal diagnostic test would have an AURC of 1, and tests with AURCs below 0.5 would not be able to distinguish between people who are affected and those who are not affected **(Escobar-Morreale *et al.*, 2001)**.

The current study further evaluated ROC curves and calculated the AUC to assess the sensitivity and specificity of blood PD-1 and IL-10 in PCOS and non-PCOS patients. Additionally, the AUC model has shown that IL-10 can be a reliable predictive biomarker for PCOS diagnosis. In PCOS, the threshold levels for IL-10 and PD-1 were determined. The cutoff values were computed as 3.555 percent for IL-0 and 0.3245 percent for PD-1, and these percentages were used to create a ROC curve for the two markers. In order to effectively recall clinicians, the threshold values of IL-10 percent and PD-1 percent were determined. In order to clarify the specificity and sensitivity of these cytokines in the PCOS diagnosis, Therefore, a ROC curve analysis was done for the current findings. The ROC analysis's findings show that IL-10 is typically useful for detecting PCOS. The ROC curve's AUC was greater than 90%. As a result, the measurement of the IL-10 cytokine greatly increased the sensitivity and specificity of PCOS diagnosis.

While PD-1's AUC is 69% percent. Thus, PCOS diagnosis has a low sensitivity and specificity.

3.2. Genotype Study Results

Hereditary and hormonal factors assumed to play a central role in the etiology of PCOS. This study is the first study that investigate the relationship of PD-1 with PCOS in two orientations: genetical level by study rs2227982 SNP of PD-1 gene, and clinical level at PCOS Iraqi patients.

3.2.1. Real-Time PCR for Programmed Cell Death-1 Gene (rs2227982) Polymorphism

The subjects that enrolled in present study were classified into two genotypes, for PD-1gene (G>A) (rs2227982): one homozygous for the G allele (GG) wild type and one heterozygous (GA) **Figure (3.17)** and **Table (3.9)** summarize genotyping of study subjects according to polymorphism of (G>A) (rs2227982) of the PD-1gene.

Table (3.9): polymorphism of PD-1 gene (rs2227982)

Dye	Allele	Genotype
Allele 1 (FAM) Wild type	**Allele G**	**Genotype GG**
Heterozygote (FAM + HEX)	**Allele G + Allele A**	**Genotype GA**
Allele 2 (HEX) Pure mutant	**Allele A**	**Genotype AA**

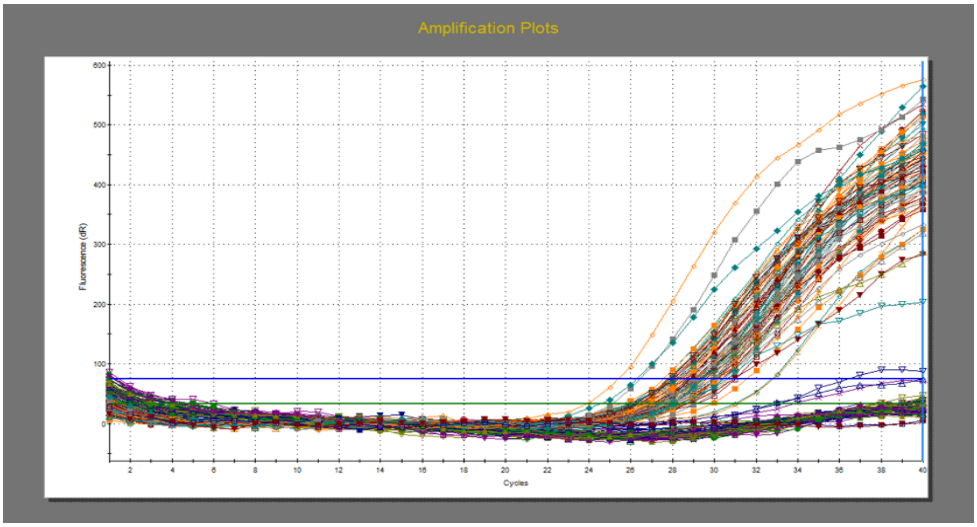


Fig. (3.17): Amplification plots for detection of PD-1gene (G>A) (rs2227982) gene polymorphism by RT-PCR technique. PCR products with two possible genotypes (GA or GG). Each colored line represented a sample. The horizontal blue line represents the threshold point of FAM emission. On the other hand, the horizontal green line represents the threshold point of HEX emission. An NTC (No Template Control) reactions were used as a control to eliminate the possibility of contaminated reactions.

Out of 80 patients, there were 26 heterozygous (GA) genotypes (32.5%) and 54(GG) genotypes (67.5%). While, in 80 control group there was 12 heterozygous (GA) genotypes (15%) and 68 (GG) genotypes (85%) of SNP rs2227982 in the PD-1gene

Allele frequencies for 160 women that involved in the present study of PD-1gene (rs2227982) in patient group G allele134 (84%) and A allele 26 (16%).while in control grope G allele 148 (93%) and A allele 12 (7%) of SNP rs2227982 in the PD-1gene. The genotyping results of current study were displayed in **Table (3.10) and Figure (3.18).** The allele frequency results of current study were displayed in **Table (3.11) and Figure (3.19).**

The results demonstrated that the patients with PCOS had a seriously higher frequency of PD-1 GA/GG genotypes (rs2227982) compared with the controls. PD-1 (rs2227982) GA genotype was related significantly with a higher frequency of PCOS p-value (0.0151), OR (2.728), CI 95% 1.230 to 5.977. Nevertheless, the GA heterozygous genotype carriers have two-fold risk to develop PCOS in comparisons with the wild type carriers. Also, PD-1gene allele frequencies (rs2227982) A allele had significance p-value (0.0237), OR (2.393), CI 95% 1.197 to 4.815 with PCOS patient.

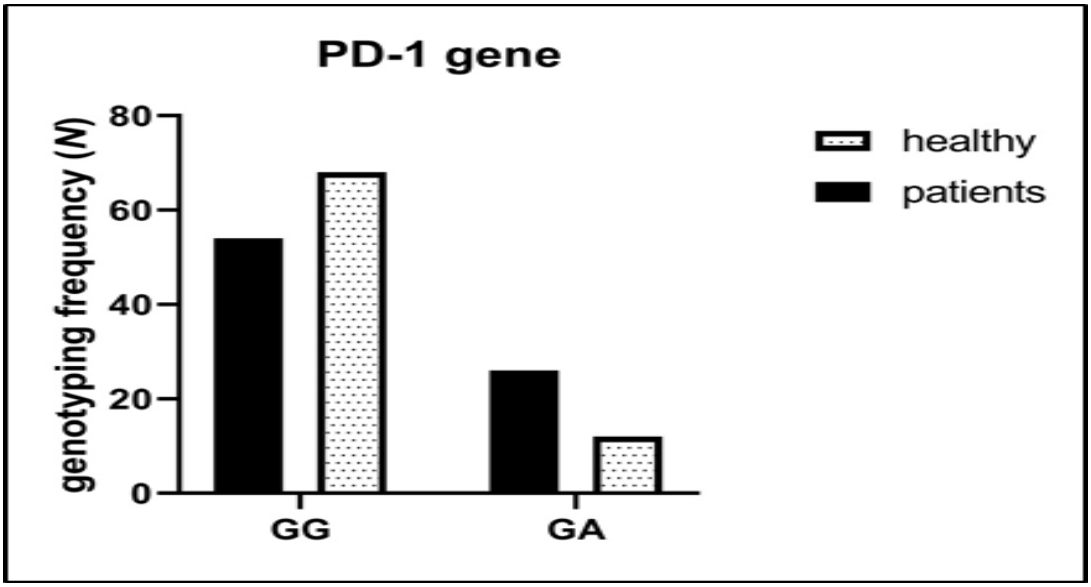


Fig. (3.18): Genotyping Frequency of PD-1 gene(rs2227982) with two possible genotypes (GA or GG).

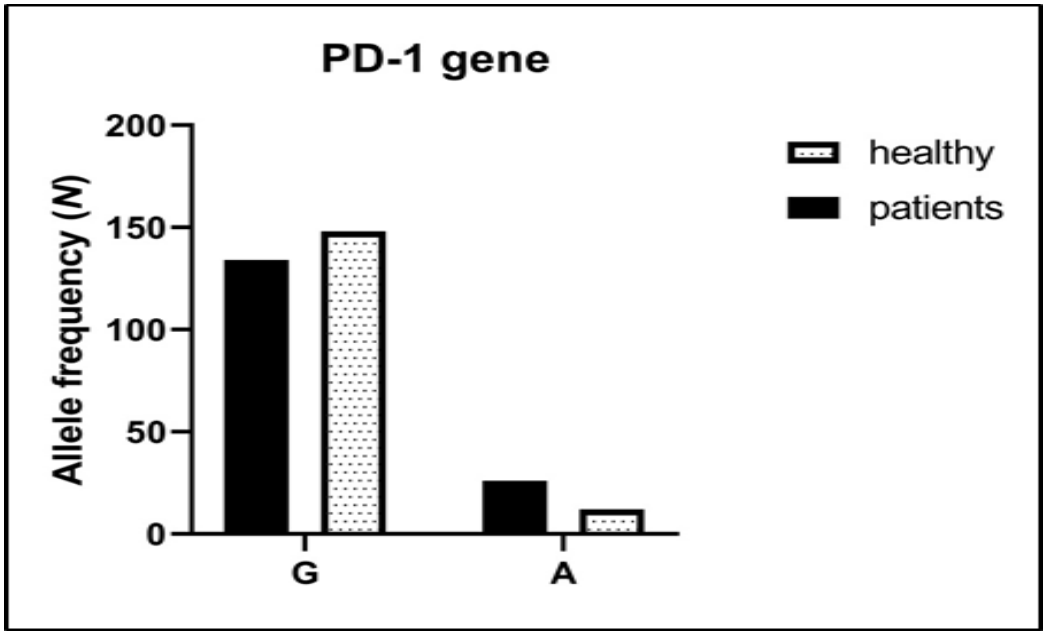


Fig. (3.19): Allele Frequency of PD-1 gene(rs2227982) with alleles A and G

Table (3.10): PD-1(rs2227982) (G/A) genotypes frequency distribution in PCOS patients and Healthy control groups.

rs2227982 (G/A)		Study groups		OR	CI 95%	*P-value*
		Patients, *N*(%)	Healthy, *N*(%)			
Genotypes	GG	54(67.5)	68(85)	References group		
	GA	26(32.5)	12(15)	2.728	1.230 to 5.977	0.0151*
	AA	0(0)	0(0)			
Total		80(100)	80(100)			

Table (3.11): PD-1 (rs2227982) (G/A) allele frequency distribution in PCOS patients and Healthy control groups

Allele frequencies					
Rs2227982(G/A)	Patients, N(%)	Healthy, N(%)	OR	CI95%	P-value
G allele	134(84)	148(93)	References group		
A allele	26(16)	12(7)	2.393	1.197 to 4.815	0.0237*
Total	160(100)	160(100)			

Attention and focus on the genetic side in all diseases has become one of the most important fields now. The clustering of PCOS within families provides evidence for its strong genetic component or PCOS like features among the relatives of the affected individual woman **(Dapas and Dunaif, 2022)**. Individuals with PCOS face multiple social and stress-induced issues; therefore, various aspects of PCOS were studied to reach a definite conclusion. Various clinical and hormonal biomarkers and genetic polymorphisms of different single nucleotide polymorphism (SNPS) have been studied in PCOS Iraqi women **(Alwan and Al-Heety, 2021).**

The present study is a novel study that identified PD-1 gene polymorphisms (rs2227982) correlation with progression and pathogenesis of PCOS disease. PD-1gene (rs2227982) were detected in heterozygous genotypes (GA) in PCOS patients, this demonstrates the positive association with PCOS risk and

pathogenesis in Iraqi population and could be used as possible biomarkers to prognosticate the risk of PCOS. The current study showed that allele A has significant linking with PCOS and conceder a risk factor and involved in pathogenesis of PCOS. When the PD-1 gene is knocked out, its initial negative regulatory function is eliminated, leading to higher levels of inflammatory factors.

This imbalance of proinflammatory and anti-inflammatory cytokines increases the development of associated disorders. Immune system dysfunction and PD-1 are connected **(Wu *et al.*, 2019)**.

In addition, one of the clinical characteristics of PCOS women is inflammation **(Pawelczak *et al.*, 2014).** Therefore, the current study suggests that the pathogenicity of PCOS is connected to the immune response involving PD-1 gene polymorphism. While, many studies results detected that PD-1 gene (rs2227982) was act as a risk factor in various cancers for examples, leukemia, gastric adenocarcinoma **(Karami *et al.*, 2020)**, esophageal squamous cell carcinoma **(Tang *et al.*, 2017)** and ovarian cancer **(Tan *et al.*, 2018)**. Clinical studies using PD-1 inhibition to treat various advanced human malignancies have shown sustained effectiveness. Additionally, by targeting the programmed cell death pathway, the success of PD-1 immune checkpoint inhibition therapy creates a platform for the development of novel cancer immunotherapy, though recent research has shown that different patients with the same type of cancer may react differently to PD-1 inhibition immunotherapy **(Berger and Pu, 2018).**

Rs2227982 locates in the PD-1gene on exon-5 and its polymorphism lead to a nonsynonymous mutation which produce an amino acid exchange (Alanine to Valine) through protein biosynthesis, which affect the PD-1cytokine function

(Huang *et al.*, 2019). The immune system is consisting of numerous of biological structures that protect the body from disease so it is a defense system. when the immune system is disabled, it can produce several diseases. New studies have revealed that immune system mechanisms are included in polycystic ovary syndrome **(Abbas *et al.*, 2019)**. PCOS patients were established to be immersed in a chronic low-grade inflammation condition, which include increase leukocytes, dysfunction of endothelium, and the proinflammatory cytokines disturbance. Large numbers of immunocompetent cells, including T and B cells, dendritic and macrophages cells, have been present in human preovulatory follicles **(Li *et al.*, 2019b)**.

The current study findings also demonstrated that PD-1 rs2227982 was significantly associated with pathogenesis and progression of Polycystic Ovary Syndrome patients group.

3.2.2. Association between Programmed Cell Death-1 and Interleukin-10 with Programmed Cell Death-1 Gene rs2227982 SNP in the PCOS Patients and Control Group

Programmed Cell Death -1 gene (rs2227982) association with IL-10 and PD-1 levels in PCOS patients and control group showed in **Figure (3.20 and 3.21).** The result showed that there is non-significant association between IL-10 level and PD-1 gene(rs2227982). While, the result showed that there is a significant association between PD-1 level and PD-1 gene(rs2227982) (GA) genotype.

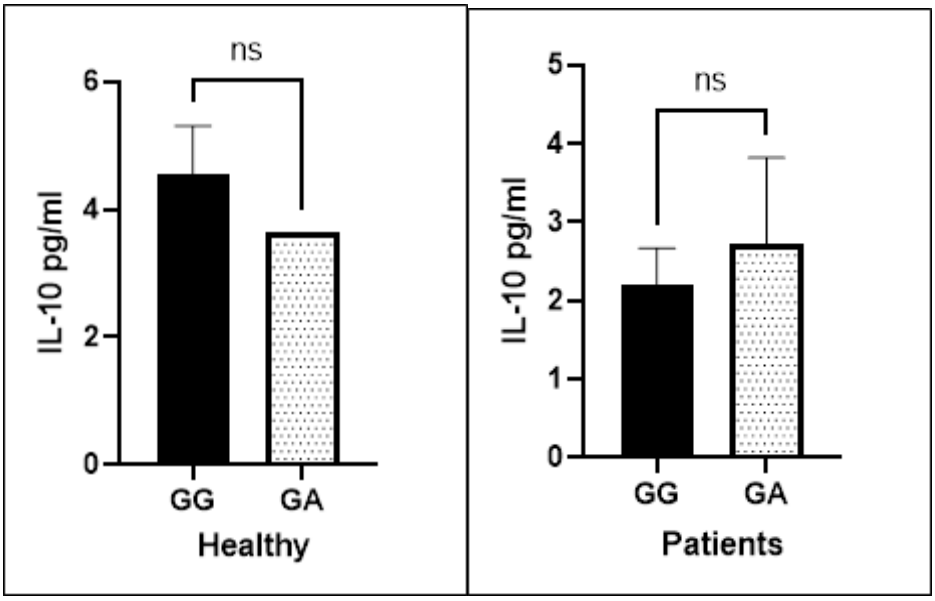


Fig. (3.20): Association of IL-10 level with PD-1 gene (rs2227982) in the registered PCOS patients and control group

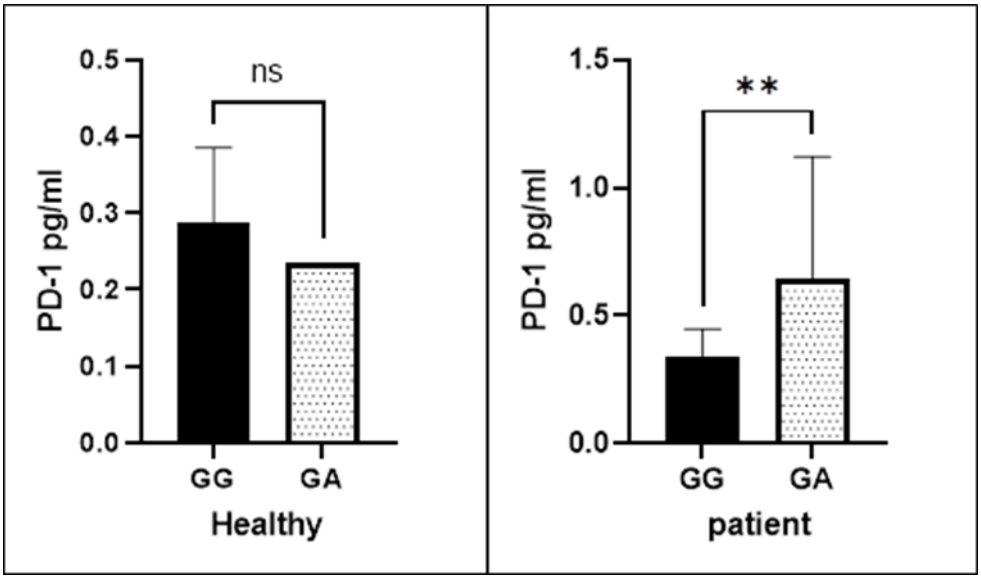


Fig. (3.21): Association of PD-1 level with PD-1 gene (rs2227982) in the registered PCOS patients and control group

By transmitting immunosuppressive signals to control T cell response, PD-1 maintained self-tolerance **(Togashi *et al.*, 2019)**. Although PD-1 was not listed as a candidate gene for complex diseases **(Wu, 2021)**, numerous studies have shown that PD-1 gene polymorphisms are associated with a number of

autoimmune diseases, including type 1 diabetes, subacute sclerosing panencephalitis, Graves' disease, Multiple Sclerosis, and rheumatoid arthritis **(Gu et al., 2018 and Okazaki and Okazaki, 2019)**. The current study demonstrated that rs2227982 Single Nuclear Polymorphism (SNP) of Programmed Cell Death – 1 was reported significantly associated with GA heterozygous genotype in PCOS Iraqi patients group in the current study. While there is non-significant association between PD-1(rs2227982) and GA genotype in healthy control group.

Also revealed no association between the investigated genotypes with IL-10 level in PCOS patients and control group.

4. Conclusions and Recommendations

4.1. Conclusions

According to the observed data by various bio statistical methods, we can conclude the following:

1. In women with PCOS of this study suffering from primary infertility, hirsutism, obesity and hormonal changes shown by increased levels of LH, LH/FSH, Prolactin and FT. While, decreased level of FSH in PCOS patients compared with healthy women.

2. There is a high level of PD-1 protein in PCOS patients that may be related to the pathogenesis of PCOS. There is a significant positive correlation between PD-1 level and BMI, WHR, LH and Free Testosterone.

3. There is a significant negative correlation between IL-10 level and BMI, WHR, LH, LH: FSH ratio and Prolactin. Interleukin -10 exhibit excellent diagnostic efficacy according to ROC analysis while PD-1 exhibit poor diagnostic efficacy.

4. Inflammatory markers have a significant role in regulating the ovary. Any disturbances in this can lead to ovarian dysfunction. The current result found a decrease in IL-10 level in patients with PCOS.

5. The current study identified novel association between PD-1 gene (rs2227982) heterozygous genotype GA of PCOS in Iraqi population and could be used as a possible biomarker of PCOS.

6. There is significant association between PD-1 gene (rs2227982) GA genotype and PD-1 level in PCOS patients and control group. while there is no association between our gene and IL-10 level.

4.2. Recommendations

1. Additional studies can be done on different SNPs of PD-1 gene in Iraq. To determine which SNPs are more public in the Iraqi population with PCOS.
2. Studying the effect of PDL-1 and its association with PD-1 gene in PCOS patients.
3. Studying the correlation between IL-10 gene with occurrence and pathogenesis of PCOS in Iraqi population.
4. Studying the role of insulin resistance in PD-1 genes on PCOS susceptibility and pathogenesis.

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الخلاصة

تعد متلازمة تكيس المبايض (PCOS) واحدة من أكثر اضطرابات الغدد الصماء والأيض شيوعًا. وهو اضطراب غير متجانس يتميز بخلل التبويض المزمن وفرط الأندروجين. إن التسبب في مرض متلازمة تكيس المبايض معقد له عدة اسباب منها التفاعل الجيني والبيئي ونمط الحياة.
تم ربط مؤشرات الالتهاب والتهابات منخفضة الدرجة بمتلازمة تكيس المبايض. لأن الإباضة عملية شبه التهابية ، يمكن للأنسجة الدهنية البطانية أن تحفز الاستجابة الالتهابية وتحافظ على الالتهاب في الخلايا الشحمية من خلال التأثير على إطلاق السيتوكينات الالتهابية. من هذا الالتهاب المنظم ، يمكن أن تنشأ متلازمة تكيس المبايض. يعد انترلوكين 10 (IL-10) أحد أفراد عائلة الخلايا التائية المساعدة (TH2) الذي يثبط وظيفة الخلايا .(TH1) للحفاظ على عمل المبيض بشكل صحيح ، يجب أن تكون مستويات علامات الالتهاب في حالة اتزان. قد تنجم التعديلات في تكوين الستيرويد وتأخر النضج الجريبي ومشاكل المبيض عن عدم التوازن بين السيتوكينات الداعمة للالتهابات والمضادة للالتهاب. اما بالنسبة لموت الخلايا المبرمج في الخلية الحبيبية فهو السبب الرئيسي للضمور الجريبي. تظهر نساء متلازمة تكيس المبايض (PCOS) أكثرعدد من الجريبات الناضجة والتي تضمردون الانتقال إلى جريبات سائدة ، مع موت الخلايا المبرمج غير الطبيعي للخلايا الحبيبية يساهم في تكوّن الجريبات المتكيّسة. يؤدي ذلك إلى عدم الإباضة والعقم. يلعب موت الخلية المبرمج(سيتوكين1-PD) وظائف مثبطة مهمة جدًا في الالتهابات وأمراض المناعة الذاتية وكذلك الأورام. مع العلم من أنه لم يتم تسجيل الارتباط بين تعدد الأشكال الجيني (rs2229782) 1-PD مع حدوث متلازمة تكيس المبايض حتى الان.

هدفت هذه الدراسة إلى استكشاف ما إذا كانت النساء المصابات بمتلازمة تكيس المبايض لديهن أي اختلاف في مستويات 1-PD و IL-10 مقارنة بالنساء غير المصابات بمتلازمة تكيس المبايض. أيضًا ، للتركيز على تحديد أي ارتباط بين مستويات 1-PD و IL-10 والمعلمات الهرمونية لدى النساء المصابات بالـ .PCOS ودراسة تعدد الأشكال الوراثي لجين 1-PD في النساء من متلازمة تكيس المبايض كتشخيص مبكر للمضاعفات ولتحديد الارتباط بين علامات الالتهاب والنتيجة من الأنماط الجينية.

تصميم دراسة مرضى واصحاء لـ 160 امرأة تتراوح أعمارهم بين (18-40) عامًا شملت 80 مريضًا من متلازمة تكيس المبايض و 80 امرأة غير مصابة بمتلازمة تكيس المبايض كمجموعة

اصحاء جميعهم في سن الإنجاب في استشارية الخصوبة الإنجابية بمستشفى أمراض النسائية والتوليد التعليمي بدائرة صحة كربلاء العراق وكلية الطب ، جامعة كربلاء ، العراق خلال الفترة من كانون الأول (ديسمبر) 2021 إلى آب (أغسطس) 2022. تم افتراض معايير روتردام - 2003 للإناث من متلازمة تكيس المبايض. تم قياس المستويات الهرمونية لكل من الهرمون اللوتيني LH وهرمون التحفيز الجريبي FSH والبرولاكتين بواسطة نظام المقايسة المناعية الآلية كيميائيًا. وتم قياس مستوى هرمون التستوستيرون الحر FT بواسطة مقايسة مناعية إنزيمية تنافسية باستخدام مجموعة ELISA كما تم استخدام مبدأ sandwich-ELISA لتحديد مستوى PD-1 ومستوى IL-10 .لقد تم قياس مؤشر كتلة الجسم BMI ونسبة الخصر إلى الورك WHR في هذه الدراسة. تم استخلاص الحمض النووي لكل عينة دم باستخدام مجموعة استخراج الحمض النووي الجينومي المحضّر .ثم تم تخزين الحمض النووي الجيني في درجة حرارة -20 درجة مئوية حتى الاستخدام. تم التنميط الجيني SNP لـ PD-1 (rs2227982) باستخدام التنميط الجيني التقليدي في الوقت الحقيقي GoTaq Qpcr PCR .تم استخدام لوحة الرسم البياني Prism 9.0.0 و SPSS 25 لجميع الحسابات الإحصائية

أظهرت نتيجة هذه الدراسة ارتفاعات كبيرة في تركيزات LH (p <0.001) ، ونسبة LH / FSH (p <0.001)ومستوى هرمون التستوستيرون الحر (p <0.001) ، ومستوى البرولاكتين (p <0.0001)ومستوى PD-1 (p. = 0.01) كانت سائدة في مجموعة مرضى متلازمة تكيس المبايض عند مقارنتها مع مجموعة التحكم. بينما انخفض بشكل ملحوظ في مستوى FSH (P = 0.01)ومستوى IL-10 (P <0.0001) خلال تقييم مشابه بين مرضى متلازمة تكيس المبايض ومجموعة التحكم (rs2227982) PDCD-1 .ارتبط النمط الجيني GA بشكل كبير مع تكرار أعلى للقيمة PCOS (p=0.0151) ، OR (2.728)، (95 CI ٪ 1.230 إلى 5.977)و PDCD-1 ترددات أليل جين (rs2227982) كان للأليل أهمية القيمة (p=0.0237) ، OR (2.393)، 1.197إلى 954.815 CI ٪ مع مريض متلازمة تكيس المبايض.

وفقًا للبيانات المسجلة ، خلصت الدراسة الحالية إلى أن مستوى بروتين موت الخلية المبرمج PD-1 زاد في مرضى متلازمة تكيس المبايض بينما انخفض مستوى IL-10 مقارنةً بالاصحاء. أيضًا ، يرتبط النمط الجيني GA لجين (rs2227982) PD-1 بشكل كبير مع القابلية للإصابة.

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الأساس الجزيئي لموت الخلايا المبرمج -1 وارتباطهما بالبروتين المشفر والإنترلوكين -10 في مرضى متلازمة تكيس المبايض في محافظة كربلاء

رسالة

مقدمة الى مجلس كلية الطب في جامعة كربلاء

كجزء من متطلبات نيل درجة الماجستير في الكيمياء السريرية

من قبل

هبة عقيل مسلم القريشي

بكالوريوس طب وجراحه عامة

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