HLA-B*0702 Class-I Allele, Anti-FSH, Anti-LH, and Vitamin D3: Potential Links with Polycystic Ovary Syndrome in Women of Erbil City, Iraq

A Thesis

Submitted to the Council of the Erbil Technical Health and Medical College at Erbil Polytechnic University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Laboratory Technology

By

Rand Maurice Aziz

B.Sc. Biology Department - College of Science - Baghdad University

Supervised by:

Assistant Professor Dr. Najat Jabbar Ahmed Berwary

Erbil, Kurdistan

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DECLARATION

I declare that the Master of Science (M.Sc.) thesis entitled: "**HLA-B*0702 Class-I Allele, Anti-FSH, Anti-LH, and Vitamin D3: Potential Links with Polycystic Ovary Syndrome in Women of Erbil City, Iraq**" is my own original work, and hereby I certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due to acknowledgment is made in the text.

Signature:

Student Name: Rand Maurice Aziz

Date:

SUPERVISOR CERTIFICATE

This thesis has been written under my supervision and has been submitted for the award of the degree of Master of Science in Medical Laboratory Technology with my approval as a supervisor.

Assistant Professor Dr. Najat Jabbar Ahmed

Signature Name

Date

I confirm that all requirements have been fulfilled

Signature:

Name: Assistant Professor Dr. Najat Jabbar Ahmed

Head of the Department of Medical Laboratory Technology

Date:

I confirm that all requirements have been fulfilled

Postgraduate Office

Signature:

Name:

Date:

Examining Committee Certification

We certify that we have read this thesis: **HLA-B*0702 Class-I Allele, Anti-FSH, Anti-LH, and Vitamin D3: Potential Links with Polycystic Ovary Syndrome in Women of Erbil City, Iraq** and as an examining committee examined the student (Rand Maurice Aziz) in its content and what related to it. We approve that it meets the standards of a thesis for the degree of Master of Science in Medical Laboratory Technology.

Signature:
Name: Dr. Zahra Abdulqader Amin
Assistant Professor
Member
Date:

Signature:
Name: Dr. Twana Ahmed Mustafa
Assistant Professor
Member
Date:

Signature:
Name: Dr. Najat Jabbar Ahmed
Assistant Professor
Supervisor/ Member
Date:

Signature:
Name: Dr. Zakariya Abdullah Yassen
Assistant Professor
Chairman
Date:

Signature:
Name: Dr. Jawdat Jaafar Khattab
Professor
Dean of the Erbil Technical Health and Medical College
Date:

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SUMMARY

Polycystic Ovary Syndrome (PCOS) is regarded as a global health problem since its causes and diagnosis have confused many researchers to this day. This study aims to investigate the impact of four important parameters on PCOS in women of Erbil city, including Human Leukocyte Antigen (HLA) represented by the HLA-B*0702 allele, Anti-Follicle Stimulating Hormone (anti-FSH) antibodies, Anti-Luteinizing Hormone (anti-LH) antibodies, and vitamin D3. It is regarded as novel in this field. For this purpose, one hundred blood samples, including EDTA for molecular characterization and serum samples for immunological analysis were collected from (60 PCOS patients and 40 healthy controls) who attended the Maternity Teaching Hospital and some private clinics and hospitals in Erbil City between October 2021 and January 2022. After DNA extraction, all of the extracted DNA samples were genotyped using a PCR-based approach with specific sequence primers. Anti-FSH and anti-LH antibodies serum levels were assessed by Enzyme-Linked Immunosorbent Assays (ELISA), whereas vitamin D3 assessment was done using an Electrochemiluminescence (ECL) technology on a Cobas e411 immunoassay analyzer.

The outcomes of the study, with an odds ratio (OR) of 2.167 at a 95% confidence interval (CI) of 0.8167 to 6.330, signifies a higher risk and suggesting that the syndrome is more common with the HLA-B*0702 allele which is harmful in this case. The serum levels of anti-FSH and anti-LH antibodies in primary infertile PCOS patients were significantly higher than in the control group and secondary infertile PCOS patients. A significant positive linear relationship was also discovered between these antibodies (P-value < 0.0001). Furthermore, anti-FSH antibodies showed a significant positive correlation with FSH, just as anti-LH antibodies did with LH (P-value < 0.01). However, both antibodies demonstrated non-significant

positive correlations with the LH/FSH ratio and non-significant negative relationships with vitamin D3 (P-value ≥ 0.05). Hypovitaminosis D3 was observed in most PCOS patients and healthy controls with a significant difference (P-value < 0.01). According to the findings, the HLA-B*0702 allele is linked to PCOS susceptibility and could be employed as an immunogenetic marker. The results also supported the idea that antibodies against follicle-stimulating hormone and luteinizing hormone are naturally found antibodies in PCOS patients rather than signs of autoimmune disease. Women with PCOS are additionally more inclined to develop vitamin D3 deficiency.

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List of Abbreviations

Abbreviations	Meaning
A4	Pro-androgens Androstenedione
Abs	Antibodies
AE-PCOS	Androgen Excess and PCOS Society
AFC	Antral Follicle Count
AGD	Anogenital Distance
AMH	Anti-Müllerian Hormone
ANA	Anti-Nuclear Antibodies
AOA	Anti-Ovarian Antibodies
ATPO	Anti-Thyroid Peroxidase Antibodies
AUC	Area under the curve
BMI	Body Mass Index
CD8	Cluster of Differentiation 8
CI	Confidence Interval
CVD	Cadiovascular Disease
DHEAS	Dehydroepiandrosterone Sulphate
dNTP	Deoxyribonucleotide Triphosphate
EC	Endometrial Cancer
ECL	Electro-ChemiLuminescence
ELFA	Enzyme-Linked Fluorescent Immunoassay
ELISA	Enzyme-Linked ImmunoSorbant Assay
ERs	Estrogen receptors
FIGO	International Federation of Gynecology and Obstetrics
FSH	Follicle Stimulating Hormone
GAD	Glutamic Acid Decarboxylase
GCs	Granulosa Cells
GnRH	Gonadotropin Releasing Hormone
GWAS	Genome-Wide Association Study
HA	Hyperandrogenemia
HLA	Human Leukocyte Antigen
HRP	Horseradish Peroxidase
HRQoL	Health-Related Quality of Life
IR	Insulin Resistant
IVF	In Vitro Fertilization
LH	Luteinizing Hormone

Abbreviations	Meaning
LH/FSH	Luteinizing Hormone / Follicle-Stimulating Hormone ratio
MHC	Major Histocompatibility Complex
NICHD	National Institute of Child Health and Human Development
NK	Natural Killer
O.D	Optical Density
OCPs	Oral Contraceptive Pills
OGTT	Oral Glucose Tolerance Test
OR	Odds Ratio
PCOM	Polycystic Ovary Morphology
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
POF	Premature Ovarian Failure
ROC	Receiver operating characteristic
SHBG	Sex Hormone Binding Globulin
SPR	Solid Phase Receptacle
T	Testosterone
T2DM	Type 2 Diabetes
T3	Triiodothyronine
T4	Thyroxine
TBE	Tris-borate-EDTA
TC	Theca Cells
TCR	T-cell receptor
TE Buffer	Tris and EDTA Buffer
TMB	(3,3',5,5'-Tetramethylbenzidine)
TRAbs	Thyrotrophic Receptor Antibodies
TSH	Thyroid-stimulating hormone
uNK	Uterine Natural Killer
VDBP	Vitamin D Binding Protein
VDD	Vitamin D3 Deficiency
VDRs	Vitamin D Receptors
VIDAS	VITEK Immuno-Diagnostic Assay System
VITD	Vitamin D
WHO	World Health Organization
(mFG) Score	Modified Ferriman–Gallwey Score
25-OH-D	25-Hydroxyvitamin-D
λ DNA	Lambda DNA
3β-HSD	3β-Hydroxysteroid Dehydrogenase

CHAPTER ONE
INTRODUCTION

1. INTRODUCTION

Polycystic ovary syndrome (PCOS) is a widespread endocrine-reproductive-metabolic condition in women that contributes significantly to infertility (Dennett and Simon, 2015). Stein and Leventhal were the first to describe it in 1935 (Escobar-Morreale, 2018). PCOS is a condition that affects females of childbearing age and provokes an irregularity in a female's reproductive hormones and resulting in ovarian problems. Unfortunately, the molecular pathogenesis of PCOS remains a mystery, and the phenotypes are highly variable, making a quick and unambiguous diagnosis difficult. PCOS is a multifactorial disorder in which genetic tendency, socioeconomic factors, ethnicity, style of life, and metabolic factors all play a role and interact, as do inflammatory and immunological interactions (Sattler et al., 2021).

According to Rotterdam Criteria, the occurrence of at least two of the following three criteria is required for PCOS diagnosis: menstrual irregularities, excess androgen production (clinical and/ or biochemical), and polycystic ovaries in ultrasonographic image. Perhaps the most frequent symptom is the excess production of androgens, which can induce hirsutism, acne, and alopecia. Women with PCOS experience a variety of chronic issues linked to excess androgen which not only have a detrimental influence on women’s physically and psychologically health, but are also connected to major pregnancy complications such as early delivery and preeclampsia (Naver et al., 2014).

Polycystic ovary syndrome is related with a variety of morbidities, such as infertility, metabolic disease, obesity, type 2 diabetes mellitus, an increased

risk of heart disease and endometrial dysplasia (Hussein et al., 2018, Bulsara et al., 2021). Because PCOS is the most common ovarian illness, it presents a major obstacle to both physicians and academic researchers. Despite high frequency of PCOS and its detrimental effects on women's reproductive health, the precise underlying cause is unknown (El Hayek et al., 2016).

Although the exact cause of PCOS is not clear, several studies have confirmed the involvement of both environmental and genetic variants in the progression of PCOS. Human Leukocyte Antigen (HLA) is one of the implicated genetic factors in PCOS (Aajil, 2018). The HLA system is a critical part of the human immune system that is ruled by a chromosome 6 gene. It encodes molecules of cell surfaces which designed to transmit peptides to T-cell receptors (TCRs). They are highly polymorphic, which indicates that they have several distinct alleles, enabling them to fine-tune the immune system (Sanchez-Mazas, 2020). Such variation may highlight the significance of the HLA in conferring an immune-genetic predisposition to develop polycystic ovaries. Some HLA antigens raise the likelihood of acquiring certain autoimmune illnesses (Aajil, 2018). As a result, HLA alleles are regarded as immune-genetic indicators (Ad'hiah, 2018).

Female reproduction may be impacted by disruptions of the genital tract, endocrine, and immunological systems. Thus, in a variety of circumstances, the female's ovary can become the victim of an autoimmune response, going to release autoantibodies that damage healthy tissue and molecules (Haller-Kikkatalo et al., 2012). In the case of PCOS, progesterone production is insufficient and unable to control the frequency of GnRH/LH pulses; as a result, excessive estrogen formation may result in autoantibodies in these patients (Mobeen et al., 2016). Accordingly, the detection of particular antibodies remains the best scientific and medical marker of autoimmunity. This syndrome is among the most likely reasons of females' infertility. Previously, increased levels of antibodies against FSH were

observed in infertile women, a research shows that such antibodies are associated to dysregulation of immune system response (Haller et al., 2007).

Multiple targets seem to be implicated in the instance of ovarian autoimmunity, anti-follicle stimulating hormone antibodies, for example, may interact and inhibit the FSH action to produce immunological complexes that cause its removal. Furthermore, such antibodies may prevent FSH from interacting with its individual receptors (Kara et al., 2019). There have been few individual studies on the level of anti-LH antibodies in PCOS patients. Hussein's and Abood & Hathal's findings for anti-LH antibodies in PCOS were similar. When compared to controls, PCOS women had higher serum levels of anti-LH antibodies (Hussein et al., 2018, Abood and Hathal, 2021).

Vitamin D, on the other hand, is motivating a huge amount of research nowadays because it has been associated to a list of ailments, such as autoimmune disorders and PCOS (Arslan et al., 2019). Many people of various ages are complaining of vitamin D-inadequacy which considered as a worldwide concern Nonclassical targeted organs for vitamin D, a crucial regulator of calcium phosphorus balance and bones health, have been identified.These include reproductive organs.Vitamin D-receptors (VDRs) on the other hand were found to be in a variety of body tissues, such as reproductive, immunological, and endocrine tissues, implying that vitamin D may be associated in female reproductive tasks (Arslan and Akdevelioğlu, 2018). Recently, many researches have been conducted to study the association between polycystic ovarian syndrome and vitamin D3. To this day, the role of vitamin D in this syndrome is unclear. However, some studies have linked vitamin D3 to the metabolic and hormonal irregularities seen in PCOS patients by influencing insulin secretion and activity. Therefore, it is very likely to be involved in PCOS (Hamdi et al., 2018, Eftekhar et al., 2020).

1.1. The Aims of This Study

For of the significance of PCOS as a public medical condition that affects women's quality of life and makes them more vulnerable to serious health issues, this research sought to:

1. Investigate the impact of four essential parameters on PCOS in women of Erbil city including (HLA Class I, anti-FSH antibodies, anti-LH antibodies and vitamin D3) and assess their potential for use in diagnosis.
2. Highlight the role of HLA represented by (HLA-B*07:02 allele) in the possibility of conveying immunogenetic susceptibility to develop PCOS which considered as a novel study in this domain.
3. Detection and estimation the level of anti-FSH and anti-LH antibodies in sera of both PCOS and healthy individuals using ELISA kits.
4. Assess the effect of family history in developing PCOS.
5. Estimation of vitamin D3 levels in sera of PCOS patients and healthy control group using an electrochemiluminescence immunoassay and search for a possible relationship with anti-FSH and anti-LH antibodies.
6. Assess and compare the demographic and clinical manifestations of women with and without PCOS.

CHAPTER TWO
LITERATURE REVIEW

2. LITERATURE REVIEW AND THEORETICAL BACKGROUND

2.1. Polycystic Ovary Syndrome (PCOS)

Polycystic ovary syndrome (PCOS) is a multifaceted illness that affects reproductive-age women (Bharathi et al., 2017). The development of this condition is hypothesized to be influenced by genetic and environmental factors, hormone fluctuations and lifestyle (Bulsara et al., 2021).Women with PCOS may face short and long term potential health risks. They experience a number of related symptomatology such as menstrual irregularities and androgen excess, which in turn have a significant influence on quality of life. They are possibly more predisposed to obesity, metabolic syndrome, and diabetes type 2 as well as an increased risk of heart disease, fertility problems, cancers, and psychological issues. Usually women with PCOS are depressed as a consequence of androgenic symptoms such as hirsutism and alopesia that challenge their feminine identity in addition to overweight and low reproductive performance (Kaczmarek et al., 2016).

Polycystic ovary syndrome remains one of the most misunderstood health conditions among patients and physicians. Possible causes of PCOS misconceptions include its heterogeneous nature and several unanswered questions concerning its etiology (Dokras et al., 2017). Treatment must be symptoms-focused and adapting to the patient's circumstances and individual needs (Escobar-Morreale, 2018).

The World Health Organization (WHO) listed PCOS in the international classification of diseases in 1990 as one of the most significant

diseases of ovarian dysfunction (Escobar-Morreale, 2018). It confirmed that accurate detection of the pattern of secretion of LH is indicative of subtle abnormalities, such as the repetitive high-amplitude pulsing that occurs in PCOS and contributes to menstrual flow disturbances. Anovulation in PCOS decreases fertility only when associated with obesity, and the cause is unknown. Moreover, gestational diabetes and pregnancy-induced high blood pressure seem to be more prevalent in PCOS pregnancies, but more thorough future data are required to determine the scope of the problem. The WHO also emphasized the importance of focusing on the long-term health effects of PCOS. Endometrial cancer is more likely, and type-2 diabetes is three to four times more common in females with a history of PCOS than in the overall public. Cardiovascular risk indicators are also more prevalent in PCOS than in control subjects (Birch Petersen et al., 2016).

2.1.1. Brief Insight into Female Reproductive Endocrinology

Woman's reproductive physiology is a complicated combination of neuroendocrine and endocrine signals influencing the hypothalamus, pituitary gland, and ovaries. At the hypothalamic level, the principal signal from the neurological system is gonadotropin releasing hormone (GnRH), which influences the function of anterior pituitary gland and regulates the production of both gonadotropins, FSH and LH. Both gonadotropins then drive follicular development in the ovaries and ovulation; FSH promotes ovarian follicle maturation and regulates ovum production in women, whereas LH boosts the ovaries' synthesis of estrogen and progesterone, increases corpus luteum growth, and ultimately initiates ovulation (Goldsammler et al., 2018, Knudtson and McLaughlin, 2019).

Estrogen encourages the growth and releasing of an ovum each menstrual cycle. The ovary's corpus luteum secretes progesterone, which

prepares the endometrium for the reception and advancement of the fertilized egg. It also impedes estrogen formation after ovulation (Christensen et al., 2012). Since ovulation is the consequence of a complex balance and interplay of hormones, any change in these systems may have an impact on its physiology. Ovulation failure is most commonly caused by PCOS, one of the leading causes of females' infertility (Cunha and Póvoa, 2021).

2.2. The Epidemiology of Polycystic Ovary Syndrome

The first research to determine the incidence of PCOS in a random population was conducted in the southern USA and published in 1998. Several studies since then have found that PCOS affects between 5-20% of reproductive-aged women, depending on the criterion employed (Azziz, 2018). Ethnic disparities in PCOS prevalence and phenotype have long been recognized. Assessing the prevalence of this syndrome is a special challenge due to the high great variability and inconsistency across the different diagnostic criteria. Even after visiting several physicians, a huge proportion of individuals remain undiagnosed. Many studies undertaken throughout the world are plagued by limited sample size, selection bias, and a lack of cross-study comparability (Wolf et al., 2018). According to the World Health Organization (WHO), around 3.4% of women worldwide suffer from PCOS (Bharathi et al., 2017).

Although evidence on the PCOS incidence in the overall South Asian community is scarce, the disease was found in 52% of South Asian women, with serious symptoms and a high rate of metabolic disruption. These symptoms appear to be more extreme in PCOS women from diverse ethnic origins. Using Rotterdam criteria, a study of 2270 infertile Indian women revealed a 46.5% frequency of PCOS (Kudesia et al., 2017). A meta-analysis study found that PCOS was significantly higher among Chinese women,

posing a serious public health issue with a prevalence of 6.05% in general public and 13.69% in infertile patients. Patients appeared to exhibit more serious phenotypes than a decade before, which could be attributed to an increased rate of obesity, hyperandrogenism, and fertility problems (Wu et al., 2021, Yang et al., 2022).

Few research have been undertaken in our country to establish the prevalence and features of PCOS. A study to evaluate the prevalence of PCOS in Al-Hilla City found a frequency of 28.9%. About 19.9% of them were adolescents and a lower prevalence above 40 years (3.2%) (Witwit, 2019). In Kurdistan region of Iraq, particularly in the city of Erbil, a study was conducted to determine the incidence of PCOS in infertile Kurdish patients.The following conclusions were made by the researchers in this study: PCOS was found in 33% of the studied population, and menstrual irregularities were observed in 75-85%. Furthermore, when compared to the control group, patients had a higher hirsutism profile but a lower acne characteristics (Hussein and Alalaf, 2013). Jamal and Ismael, on the other hand, revealed through a research in 2019, that (18.8%) of the investigated sample had PCOS. Furthermore, patients with a history of amenorrhea and oligomenorrhoea had the greatest polycystic ovaries prevalence, 92.3% and 75.2%, respectively (Jamal and Ismael, 2019). According to Jabbar Ahmed et al. the majority of women with PCOS have irregular menstrual periods and 75% have hirsutism. Acne was seen in 48.4% of the afflicted women (Jabbar Ahmed et al., 2020). In the city of Duhok, 51.9% have a family history of PCOS. Menstrual cycle irregularities were found in 97.2% of the women, while infertility history was found in 27.8%. The most prevalent result 86.1% was hirsutism. On U/S examination, 75.3% of women had polycystic ovaries (MUHYADIN et al., 2020).

2.3. History of PCOS

Polycystic ovary syndrome was first documented by American gynecologists Stein and Leventhal, from which the term "Stein-Leventhal syndrome", was derived (Escobar-Morreale, 2018). Both scientists described a group of hirsute women with menstrual irregularities, and sizable ovaries with numerous tiny follicles in 1935. Nevertheless, shortly after the description of a technique for measuring testosterone levels in plasma in 1961, elevated circulating levels of androgens in women with PCOS were observed. Researchers were seeking for a diagnostic technique to replace roentgenography or reconnaissance laparotomy, which were previously employed to identify polycystic ovaries. Ultrasound imaging of the reproductive system was a significant advancement in clinical practice (Szydlarska et al., 2017).

In 1990, the National Institutes of Health (NIH) developed consensus criteria for diagnosis of PCOS, describing the disorder as the presence of androgen excess and persistent anovulation in the absence of other reasons of anovulatory infertility (Clark et al., 2014). Finally, the international evidence based guidelines cover a list of recommendations and priority concerns in order encourage standard evidence-based healthcare and enhance the experience and health outcomes of PCOS women (Teede et al., 2018).

2.4. Etiology and Complications

Pathogenesis of PCOS is complicated and multifactorial since it arises from interaction between neuro-endocrine; environmental conditions; obesity and genetics as shown in Figure (2-1). These factors fuel the roots of imbalanced hypothalamus-pituitary-ovarian axis signals, which foster ovarian

and adrenal hyperandrogenism which is the PCOS characteristic hallmark (Ibáñez et al., 2017, Bulsara et al., 2021).

Various Genome-Wide Association Studies (GWAS) have shown distinct loci and alleles that have a significant influence in PCOS phenotyping (Hayes et al., 2015, Dumesic et al., 2015). Other studies revealed that environmental factors such as lifestyle, dietary food, endocrine-disrupting toxins and glycotoxins can induce genetic variation, metabolic and reproductive disrupting, leading to PCOS phenotypes. Such variables contribute to hyperinsulinemia, hyperandrogenism, oxidative stress, as well as irregular menstruation (Rutkowska and Diamanti-Kandarakis, 2016).

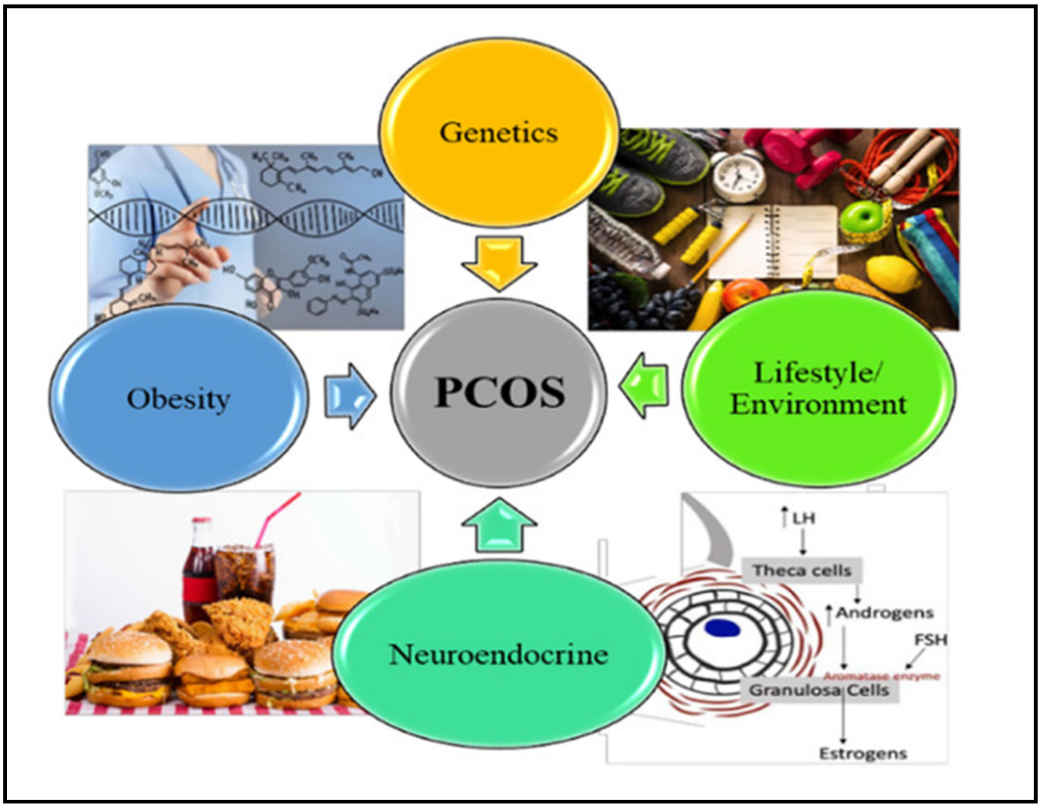


Figure (2-1): A diagram depicts the risk factors that contribute to development of PCOS (Bulsara et al., 2021).

2.4.1. Overproduction of Androgens

Hyperandrogenemia refers to a patient's higher productivity of androgens, a group of hormones that are essential in male physiology, whereas females produce them in little amounts. As a result, females suffering from hyperandrogenism face the problem of poor follicle formation (Walters et al., 2019). Surplus androgen enhance the growth of primordial follicles and boost the number of antral ovarian follicles during the initial gonadotropin phase (Rosenfield and Ehrmann, 2016). The releasing of both FSH and LH is activated by GnRH emission from the hypothalamus. LH stimulates the LH receptor in ovarian thecal cells for androgen biosynthesis, whereas FSH acts on the FSH receptor in ovarian granulosal cells to convert androgen to estrogen and encourage follicular development (Ashraf et al., 2019). Androgen exposure may disrupt hormonal balance by increasing GnRH pulse rate, resulting in a significant increase in LH to FSH ratio and follicle arrest (Dumesic et al., 2015, Walters et al., 2018).

2.4.2. Insulin Resistance (IR) and Type 2 Diabetes (T2DM)

Hyperinsulinemia is the chief reason of extra androgens since insulin mimics the effect of luteinizing hormone directly and raises GnRH indirectly (Puttabyatappa and Padmanabhan, 2018). Insulin on the other hand, reduces sex hormone binding globulin (SHBG), a major circulation protein that regulates testosterone levels. As a result, lower SHBG levels result in higher levels of free androgens, which cause skin related- manifestations (Rojas et al., 2014). Since, hyperinsulinemia associated with β cells disorders, it increases the risk of a number of disorders, such as T2DM, high blood pressure, dyslipidemia, and heart disease (Bednarska and Siejka, 2017, Rocha et al., 2019, Osibogun et al., 2020).

2.4.3. Obesity and PCOS

Obesity is linked to aberrant hypothalamic-pituitary-ovarian axis performance, which causes the onset of PCOS (Legro, 2012). It causes excess insulin production and also worsens the lipid metabolism profile and glucose intolerance in PCOS patients, as illustrated in Figure (2-2). It also stimulates androgen levels production by triggering luteinizing hormone, resulting in androgen excess (Glueck and Goldenberg, 2019). Otherwise, Leptin has such a direct influence both on neuro-hormonal as well as reproductive performance in overweighed PCOS patients. In addition, hyperleptinemia may impair ovarian follicular development. Accordingly, Rojas and his colleagues, asserted that reducing visceral fat would regulate hunger, blood glucose, and lipolysis, raise SHBG, and consequently controlling the ovarian androgen task (Rojas et al., 2014).

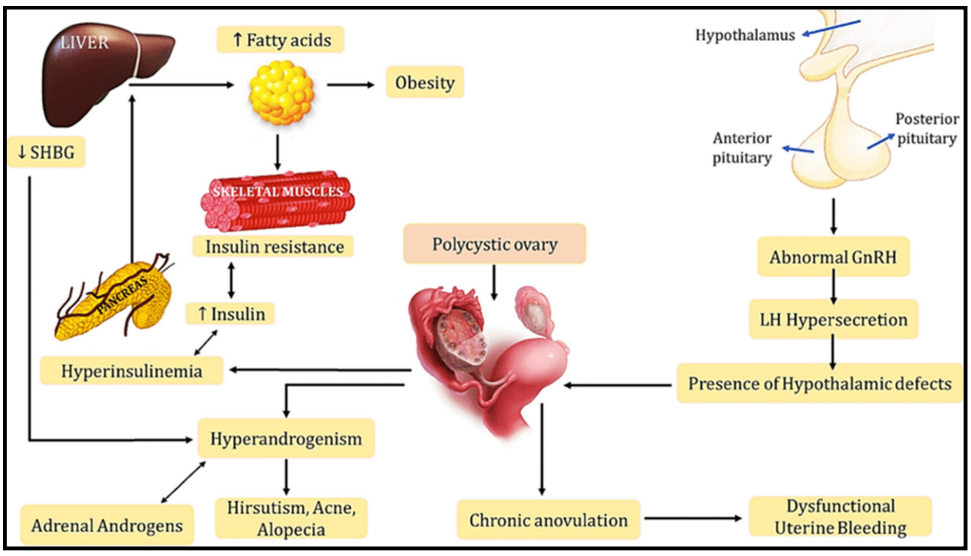


Figure (2-2): A diagram displays the complex relationships contributing underlying the etiopathogenesis of PCOS (Chaudhary et al., 2021).

2.4.4. Cancer Risk and PCOS

Endometrial cancer (EC) is the most common malignancy associated with PCOS, according to the International Federation of Gynecology and Obstetrics (FIGO) which also classified the menstrual irregularities. Extended periods of anovulation may increase exposure to androgens and estrogen, raising the risk of EC (Gibson et al., 2014, Hong et al., 2021).

2.5. The Role of Hormonal Factors in PCOS

Polycystic ovary syndrome is characterized by a hormonal imbalance that interrupts the menstruation periods, ovulation and, of course, conception. These hormones are like a tangled web, and the female reproductive system's performance is strongly reliant on their balance. The following hormones are involved in PCOS:

2.5.1. Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH)

Normal follicular development and ovulation is the result of the simultaneous activities of both FSH and LH through a complicated interplay including the hypothalamus, anterior pituitary gland, reproductive system, and oocytes (Bosch et al., 2021). Females having PCOS show persistent gonadotropins secretion anomalies. A neuroendocrine function associated with PCOS appears to be persistently fast LH/GnRH pulsatility, which favors LH over FSH, resulting in elevated (LH: FSH) ratios in PCOS. Inadequate FSH levels impede follicular growth, but high LH levels boost ovarian androgen production (Malini and Roy George, 2018, Mitrašinović-Brulić et al., 2021). An imbalance in LH and FSH levels drives incorrect testosterone production. According to the literature, high testosterone levels, along with

familial history, genetics, and environmental variables, may influence hormone levels including LH and FSH (Alsaadi and Mohamad, 2019, Shenta et al., 2020). High testosterone levels can disrupt follicular growth, leading to anovulation and irregular menstrual cycles (Rosenfield and Ehrmann, 2016).

2.5.2. Androgens

Androgens are male hormones, though females produce a small amount as well. Hyperandrogenism is a PCOS-critical feature since increased levels of androgen hormones represent the most persistent symptom in the majority of sufferers. It can cause feminine physical changes such as masculine-style hair growth, acne, and hair loss. Women of hyper- androgenic PCOS have increased levels of various androgens, such as testosterone (T), dehydroepiandrosterone sulfate (DHEAS), pro-androgens androstenedione (A4), and the 3β-hydroxysteroid dehydrogenase enzyme (3β-HSD) (Keefe et al., 2014, Rodriguez Paris and Bertoldo, 2019).

2.5.3. Estrogen and Progesterone

Estrogen, in addition to female characteristics, aids in the growth and maintenance of the female genital tract. Progesterone and estrogen are required to prepare the uterus for menstruation. LH stimulates thecal cells (TC), resulting in androgen secretion. Some androgens bind to sex-hormone-binding globulin (SHBG), while others travel to neighboring granulosal cells (GCs) and are converted to estrogen in response to FSH stimulation. This causes an increase in estrogen levels, which then provides positive feedback in terms of LH biosynthesis, resulting in anovulation. Women with PCOS have abnormal estrogenic hormone and estrogen receptor (ER) function (Xu et al., 2021).

2.5.4. Insulin

Insulin is the only hormone capable of reducing levels of blood glucose; hence it is essential for glucose homeostasis (Rorsman and Ashcroft, 2018). Importantly, Hyperinsulinemia seems to be critical in the hyperandrogenemia preservation, both functioning directly to trigger excess androgen by theca cells and as a co-gonadotropin, increasing the impact of the elevated LH stimulation found in the majority of PCOS women. Furthermore, elevated insulin may have further consequences since it has been linked to androgen central actions in lowering progesterone control of the GnRH pulse generator (Marshall and Dunaif, 2012). The insulin resistance is believed to start in womb and may be inherited (Macut et al., 2017). In PCOS patients, insulin is known to influence the levels of genes associated with steroidogenesis process while increasing testosterone levels (Abraham Gnanadass et al., 2021).

Despite the fact that several studies have demonstrated the link between insulin resistance and PCOS, the processes behind its underlying origin remain a mystery. IR and hyperinsulinemia can boost hormonal and reproductive functions in people with PCOS. An increase in androgens, on the other hand, might well impede insulin function either directly or indirectly through numerous alteration that occur in various tissues. The excess fat, which these women also have, may aggravate the overall image (Moghetti and Tosi, 2021).

2.5.5. Thyroid Stimulating Hormone (TSH)

Thyroid stimulating hormone is the major trigger for thyroid hormones synthesis by the thyroid gland. Based on the link between hypothyroidism, insulin resistance, and reproductive diseases, it is probable that a significant

imbalance in thyroid function may originate, sustain, or aggravate PCOS symptoms. This is why the link between hypothyroidism and PCOS has piqued the curiosity of numerous academics in recent years (Tagliaferri et al., 2016). Many researchers believe that the insulin resistance, which really is frequent in PCOS women, seems to be induced by hypothyroidism (Escobar-Morreale, 2018). TSH elevation contributes to its influence on FSH receptors. A rise in collagen deposition in the ovaries has also been linked to hypothyroidism (Singla et al., 2015). Furthermore, hypothyroidism and high TSH levels are frequent in PCOS patients, and their occurrence is related with an undesirable metabolism (Trummer et al., 2015).

2.6. Other Significant Health Implications of PCOS

2.6.1. The Impact of PCOS on Female Fertility

Polycystic ovary syndrome is one of the most common reasons that women have difficulty conceiving. A female's reproductive hormones imbalance may prevent mature eggs from developing and being released. Ovulation and pregnancy are impossible in the absence of a mature egg. PCOS's main pathological features are hormonal dysfunction and IR are frequently associated with BMI. Obesity and fertility problems are inextricably linked. So losing weight seems to be the most important factor that influences the outcomes of both fertility and pregnancy (Silvestris et al., 2018, Cena et al., 2020)

Obesity is found in about 60-80% of PCOS patients (Moran et al., 2012). Though several obese women are fertile, possessing a high body weight index raises the chance of reproductive issues. Accordingly, a link between Body fat percentage, hormone levels, and the LH/FSH ratios in PCOS patients was discovered (Khmil et al., 2020).

2.6.2. The Metabolic Consequences of PCOS

Metabolic syndrome refers to the PCOS clinical manifestations such as the insulin resistance, weight gain, and excess androgens, which are the most frequent risk considerations among PCOS patients as previously stated in this study. Furthermore, studies discovered that PCOS patients have a significant increase of dyslipidaemia, who had elevated levels of bad cholesterol including low and very low-density lipoproteins, triglycerides, and free fatty acids, but low levels of high-density lipoprotein cholesterol, usually known as "good cholesterol" (Chen and Pang, 2021).

PCOS's metabolic features can contribute to a variety of cardiovascular diseases (CVD), including high blood pressure, atherosclerosis, and coronary heart disorders. Furthermore, mitochondrial dysfunction contributes to CVD in PCOS women, since heart cells require a lot of energy provided by mitochondria (Ding et al., 2018b). Aside from the effects of IR and obesity, high androgen has been linked to CVD. Androgen overabundance may result in sympathetic nervous system stimulation and oxidative stress. However, it is unclear if these individuals are ultimately at risk of heart disease, as further more prospective studies are required (Gunning and Fauser, 2017).

2.6.3. The Impact of PCOS on Psychological Wellbeing

Several studies have found that PCOS patients are more likely to have psychiatric issues. These women have low self-esteem and physical satisfaction, which puts them at a higher risk of anxiety and depression (Böttcher et al., 2018, Sánchez-Ferrer et al., 2020). Obesity, hirsutism, irregular menstrual periods and infertility are all related with PCOS and certainly contribute to stress that women face due to the challenges to their feminine identity and self-image (El Hayek et al., 2016).

2.6.4. The Impact of Diet and Lifestyle on PCOS

Several academic societies have revealed aggravating factors in PCOS which are among the leading causes of infertility including: genetics & family history, as well as lifestyle & dietary variables (El Hayek et al., 2016). women with and without PCOS had similar dietary patterns and energy demands, including protein, carbohydrates, and fats consumption while others revealed that PCOS patients consumed more calories, despite no change in protein, carbs, or fats intake (Moran et al., 2013, Rodriguez Paris et al., 2020).

Exercise and calorie restricted dietary are recommended as important aspects of managing obesity in PCOS patients. In addition, lifestyle changes are regarded as a cost-effective 1st therapy and an essential supplement to medicine (Legro et al., 2013, El Hayek et al., 2016). Ultimately, differing health-related quality of life (HRQoL) outcomes among PCOS patients from various backgrounds demonstrate that both ethnicity and culture play a crucial role in assessing quality of life in such women (Alghadeer et al., 2020).

2.7. The Role of Genetic Variables and Familial Clustering

Further genetic information started to appear in early PCOS literatures, and it soon became clear that the disease has a multi-genetic foundation (Hoeger et al., 2021). Some genes are mostly associated with the pathway of insulin signaling and resistance while others linked with chronic inflammations, abnormalities of reproductive hormones as well as androgen receptors & vitamin D receptors (VDRs) (Zhao et al., 2016).

Over the last decade, GWASs have significantly increased understanding of PCOS pathogenesis and progression by identifying various critical genes associated in hypothalamic–pituitary paths, gonadotropin activities, inflammations and the action and secretion of insulin (Hiam et al.,

2019, Deswal et al., 2020) Despite the fact that such identified genetic susceptibility alleles account for less than 10% of PCOS inheritance, other causative variables must be taken into account (Hoeger et al., 2021). Due to variances among ethnic communities, the great majority of previous genetic research has not yielded consistent data indicating the potential genetic differences related with PCOS due to diverse genomic architectures underpinning certain PCOS phenotypes (Zeber-Lubecka and Hennig, 2021).

A trans-generational transmission of PCOS studies reveal that the syndrome has transitional roots across generations, with girls born to PCOS mothers having a 5-fold chance of acquiring the illness (Risal et al., 2019). One mechanism for example, the early exposure to androgen hormone may lead to increased risk to PCOS in neonate girls. Maternal testosterone levels in PCOS women were found to be a predictor of neonates with longer anogenital distance (AGD) born to PCOS moms and these newborns are at increased metabolic - androgenic risks (Wu et al., 2017, Barrett et al., 2018, Risal et al., 2019). However, the mechanism by which the daughters are susceptible to androgen excess exposure is yet unclear; anti-müllerian hormone (AMH) may be one implicated factor (Glintborg et al., 2019).

According to studies, mice exposed to large amounts of AMH in the delayed stages of their pregnancy had PCOS progeny with increased LH pulsatility and androgen levels (Tata et al., 2018, Piltonen et al., 2019).

2.7.1. The Clinical Role of HLA in PCOS

The major histocompatibility complex (MHC) is a large genetic complex found in all vertebrate species which also contains approximately 200 genes and plays a vital role in immunity. MHC class I and II are cell-surface glycoproteins that bind both intracellular and external peptides. It is

named after its role in organ rejection and tissue compatibility. It also enables the immune system to identify any invading disease (Mosaad, 2015).

The human MHC is located on chromosome 6 and is known as human leukocyte antigen (HLA), which is skilled in introducing short peptides to T-cells and function a significant role in human immunological defensive strategy (Holoshitz, 2013). The HLA system is a multiple-gene family that is quite complex and spans approximately 3,600 kilo bases of DNA. They are extremely polymorphic and are divided into three classes: Class I (A, B, and C) genes are found on all nucleated cells, while class II (DP, DQ, and DR) genes are found on antigen-presenting cells (APC) (Neuchel et al., 2021). The class III genes, in turn, are located between the two classes and comprise genes with immuno-regulatory roles including components of the complement system and the tumor necrosis factor family and heat shock proteins (Cruz-Tapias et al., 2013b). Such nomenclature originated from historical HLA discovery sequences: they were designated using Roman numbers and English characters when they were identified, as illustrated in Figure (2-3).

HLA class I and II alleles as well as their haplotypes were shown to be positively or negatively associated with a variety of disease, suggesting that HLA alleles their haplotypes have both disease susceptibility and protective properties (Cruz-Tapias et al., 2013a). HLA class I are made up of one trans-membranous heavy chain with 3 extracellular domains (a1–3) as well as a light chain made up of b2-microglobulin (b2m).Class I molecules possess an immunoglobulin-like tertiary structure, to the most extra-cellular regions containing virtually all amino acid allelic diversity (Dyer et al., 2013).

The usual function of HLA class I is to deliver peptides from exhausted or faulty intracellular peptides and proteins from invading viruses inside the cell to the T-cell receptor (TCR) onto CD8+ cytotoxic T-cells, resulting in immunological mechanisms that damage the cell (Kaseke et al., 2021). HLA class II, on the other hand, are expressed constitutively only in

immunological active cells such as B-cells and other antigen presenting cells. Each protein has an alpha- and beta-chain, which are both encoded within HLA-DRA and HLA-DRB1. Class II proteins and the attached peptides interact with CD4+ helper T- cells and their receptors (Mosaad, 2015). Significant advances in DNA-based technologies and advanced understanding of the MHC's complexities lead to the discovery of HLA disease correlations (Al Naqbi et al., 2021).

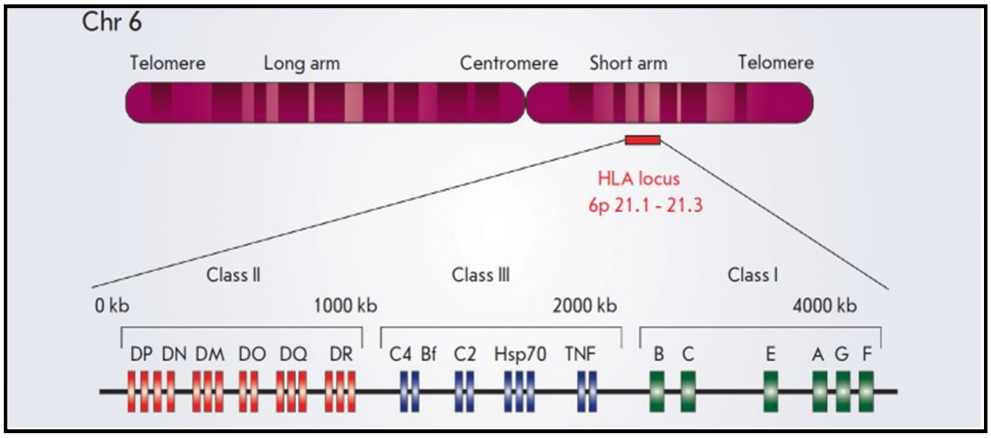


Figure (2-3): A schematic representation of the HLA gene on the chromosome six short arm ranging from (6p21.1 to p21.3) loci (Zakharova et al., 2019).

2.7.2. The HLA Disease Association

The HLA disease association is described as a statistically higher frequency of the HLA alleles in people with the disease relative to people who do not have the disease (Holoshitz, 2013). Since the discovery of HLA, scientists have been studying the function of MHC gene families in a variety of disorders. The links between autoimmune illnesses and allelic variations in potential MHC genes were also well recorded. Even after several decades of research, understanding of MHC correlations with diseases vulnerability has

been limited to Europeans, with little knowledge in other nations and ethnicities (Al Naqbi et al., 2021).

The mechanism behind the HLA-disease relationship is unknown; nevertheless. Several hypotheses have been advanced, broadly divided into (1) those who criticise "mistaken identity," in which an HLA allele seems to to be connected with the illness despite the fact that the true guilty relates to a distinct haplotpic locus or associates via linkage disequilibrium; (2) those who blame immunological reaction to self-antigens due to abnormal T-cell supply (Mosaad, 2015).

2.7.3. The Relationship between HLA-Class I and PCOS

HLA antigens and/or alleles have been linked to a variety of disorders, including PCOS, and the data suggested that HLA is important in giving an immunogenetic susceptibility to acquire such diseases (Aajil, 2018). Several studies have been done to investigate the association between immunity and fertility difficulties in PCOS patients. Due to the HLA system's vast polymorphic and haplotypic inheritance pattern, ethnic groups vary greatly. Furthermore, HLA systems have been linked to autoimmune, infectious & endocrine illnesses in terms of immunological regulation, suggesting that HLA systems may have an influence on PCOS groups as well. However, only a few studies in different communities all over the world have found a link between PCOS and the HLA system, with contradictory findings, and a number of them shed light on the connection between HLA-class I antigens and PCOS (Kaibe et al., 2006, Ahn et al., 2011, Kim et al., 2011, Aajil, 2018)

Importantly, females with PCOS seem to be more likely to develop certain forms of cancers due to altered genetic, hormonal, and metabolic conditions (Dumesic and Lobo, 2013, Ding et al., 2018a). A study discovered a link with HLA-B7. When compared to controls, the rate of the HLA-B7

allele was higher in breast cancer patients (Lavado et al., 2005). Both Harris and Terry stated that PCOS traits have already been connected to both elevated and lowered breast cancer risk (Harris and Terry, 2016), which was considered the most common cause of mortality in a long-term PCOS patient follow-up (Ding et al., 2018a).

According to one study, having HLA B*07 and DQB1*0302 raises the chance of developing cervical cancer in a racially diverse Costa Rican population (Wang et al., 2001). Asian Indians also seem to be at greater risk of developing cervical cancer because they carry the HLA-B*07 allele (Bhattacharya and Sengupta, 2007). Qiu and colleagues discovered many high-risky alleles in females from cervical neoplasia families. HLA-B*07 showed the most promise as a molecular biomarker (Qiu et al., 2011).

2.8. Autoimmunity in PCOS and its Effect on Female Fertility

Autoimmunity is synonymous with malfunction of different immune system products result in production of autoantibodies directed toward typical body antigens. The hypothalamic-pituitary-ovarian system controls female reproductive system activities through a sequence of key events and regulated interactions. Female reproduction may thus be influenced by diseases or disturbances of the genital tract, neuroendocrine and immune system or even by some stressful general illnesses. In a variety of settings, including multiple organ-specific or systemic autoimmune illnesses, the female ovary can become the target of an autoimmune attack (Haller-Kikkatalo et al., 2012).

In PCOS, a low progesterone level stimulates the immune system, causing it to create more estrogen, resulting in a variety of autoantibodies. PCOS has been linked to a variety of autoantibodies (Mobeen et al., 2016). PCOS-related infertility is linked with both a change in folliculogenesis and the primary follicle selecting, resulting in anovulation. However, many

critical issues and questions remain unanswered, including how autoimmunity is related to an imbalance of the different components of the female's immune response and how to form auto-antibodies targeted directly against antigens of the body's components (Haller-Kikkatalo et al., 2012).

2.8.1. Generation of Antibodies against FSH in PCOS

Women were shown to have anti-FSH antibodies. FSH antibodies, both naturally occurring and produced by exogenous gonadotropins, were discovered. Natural FSH antibodies were discovered in endometriosis and PCOS patients who had not undergone ovarian stimulation by IVF. These antibodies were also found in healthy, non-pregnant women, albeit at a lower frequency (Haller et al., 2005). A shift has occurred in the immune system, and the antigen responsible for its production has either been circulating FSH in the female body or FSH in the sperm. Anti-FSH antibodies were found to be elevated in infertile women who had previously received IVF treatment, and previous studies have linked such antibodies to impaired ovarian responses to IVF stimulation. Such antibodies may thus inhibit FSH by preventing it from binding to its own receptor or by trapping it within immunological complexes (Haller et al., 2008, Kara et al., 2019). According to the literature, anti-FSH levels in PCOS patients seem to be greater than in healthy subjects (Hussein et al., 2018, Jabbar Ahmed et al., 2020).

2.8.2. Generation of Antibodies against LH in PCOS

Regarding anti-LH antibodies, there have been relatively few previous research into the role of anti-LH antibodies in this syndrome. An earlier study found that both FSH and LH are important in ovarian development because they stimulate granulosa cells, increase intrafollicular spacing, antral

production, maintenance of thecal surface, and development of circulatory system. However, it was also discovered that LH anti-sera, but not FSH anti-sera may inhibit ovulation (Kara et al., 2019). Hussein's and Abood & Hathal's results for anti-LH antibody levels in PCOS were identical in two separate investigations. PCOS women had significantly greater serum anti-LH antibodies levels than controls. Since FSH and LH are related in function and site of secretion, antibodies against both are correlated (Hussein et al., 2018, Abood and Hathal, 2021).

2.8.3. PCOS Related-Autoantibodies

In PCOS, the levels of progesterone are low which cannot reduce GnRH/LH pulse frequency; thus excessive estrogen may result in autoantibodies (Mobeen et al., 2016), the following are some of them:

- Anti-Nuclear Antibody (ANA)

  A link between PCOS and ANA was observed. Inflammations, immunological hyperstimulation, and tissue damage reveal intracellular antigens leading to the formation of ANA, a hallmark of autoimmune diseases (Makled et al., 2015). Patients with PCOS had higher ANAs than healthy participants and its production may be due to self-reaction responses against intracellular antigens, implying an autoimmune mechanism in the pathogenesis of PCOS (Samsami Dehaghani et al., 2013).
- Anti-Thyroid Antibody

  In autoimmune thyroiditis, autoantibodies against one or even more thyroid gland components are formed, including anti-thyroid peroxidase (ATPO), thyrotrophic receptor antibodies (TRAbs), and thyroglobulin antibodies. Several studies have found a substantial

correlation between anti-thyroid antibodies and PCOS and it has been highly suggested that all PCOS patients should be evaluated for their thyroid hormones profile even if they do not have overt thyroid failure (Kachuei et al., 2012, Menon and Ramachandran, 2017).

- Anti-Islet Cell Antibody

  Autoantibodies against islet cell react with islet cell antigens in a complicated pathway that begins with interactions with insulin or glutamic acid decarboxylase (GAD). Such pattern of autoimmune response to islet cell proteins shows that insulin-producing cells are degrading in a specific sequence. These auto-antibodies can be used to predict a person's likelihood of getting type I diabetes (Mobeen et al., 2016, Hlail et al., 2021).

- Anti-Ovarian Antibody (AOA)

  Anti-ovarian antibodies were detected in higher concentrations in PCOS patients than in control subjects, but their existence did not correspond with clinical symptoms of PCOS. Despite the presence of these antibodies, their pathogenic role is highly debated (Hlail et al., 2021). AOA induces ovarian autoimmunity and is regarded as an indicator of ovarian damage (Al-Naffakh and Risan, 2020).

2.9. The Potential Nutritional Deficiencies in PCOS

Minerals and vitamins are known to be vital for female reproductive function. Minerals, in particular, can be linked to ovulation, metabolic activity, and hormonal regulation. The significance of macro and micronutrients in the development of PCOS is almost unknown. Only individual studies were conducted to investigate the composition of micro and macro-elements in PCOS women (Pokorska-Niewiada et al., 2021).

Vitamin B6 is known to impact the maintenance of normal progesterone levels in the blood. Vitamin B12 deficiency might contribute to ovulation and embryo implantation problems. This vitamin is required for the formation of RBCs &WBCs. As a result, women with PCOS should take supplements containing group B vitamins owing to the possibility of deficiency. In contrast, folic acid (vitamin B9) deficiency is linked to a greater risk of hypertension and cardiovascular disease, both of which have been closely associated with PCOS. It has been demonstrated that a higher homocysteine content in the blood reduces reproductive capacity and contributes to pregnancy complications (Szczuko et al., 2016). Also, PCOS has been shown to be related with oxidative stress and inflammation, as well as IR independent of obesity and abnormal follicular development.

Vitamin E, which is commonly utilized in reproductive medication, can effectively repair the negative effects of oxidative stress on both reproductive and endocrine systems. A deficiency of vitamin E can result in female infertility, miscarriage, and other pregnancy-related illnesses (Chen et al., 2020). Noteworthy, the medical community is divided on which vitamin is the most necessary for the body. The general agreement is that it is either vitamin B12 or vitamin D. While both deserve to be first, vitamin D is leading a lot of research and has been related to many conditions, including autoimmune disorders and PCOS, simply because many individuals are deficient nowadays (Arslan et al., 2019).

2.9.1. The Role of Vitamin D3 in PCOS

Vitamin D3 is an essential nutrient for female's reproductive health (Nair and Maseeh, 2012). VITD is a true steroid hormone and the only vitamin that the human body can synthesize and produce in the skin after direct exposure to sunshine (Aziz et al., 2020). It has an undeniable role in

both innate and adaptive immunity and this helps greatly in understanding its role on women's health (Khadilkar, 2013). It is commonly established that vitamin D3 maintains bone health by regulating calcium and phosphorus balance. However, because of the wide occurrence of the vitamin D receptors (VDRs) throughout the body, vitamin D3 effects extend to a variety of tissues, including women's reproductive system. Females with no VDRs are infertile and have impaired folliculogenesis (Irani and Merhi, 2014).

It is worth noting that VITD is thought to have a role in cell differentiation, proliferation, cardiac health, and protection against some chronic and autoimmune diseases. In addition, it also plays an important role in the prevention as well as the management of certain kinds of cancer (Khadilkar, 2013). Both insufficiency and deficiency of vitamin D3 have also been connected to a variety of reproductive illnesses, such like PCOS, endometriosis, premature ovarian failure (POF), and ovarian tumors (Dabrowski et al., 2015, Colonese et al., 2015).

Vitamin D3 has garnered considerable attention lately since there is a lot of evidence that it is important for reproductive function along with PCOS (Demer et al., 2018). It has also been demonstrated to play a significant part in the gene regulation related to glucose and lipid metabolism. Data from observational studies show a strong link between vitamin D3 deficiency (VDD) and many of the endocrine, metabolic, and clinical features of PCOS such as ovulatory dysfunction, hyperandroginism, higher BMI, IR and diabetes risk resulting in chance of developing cardiovascular diseases (Irani and Merhi, 2014, Issa, 2017, Grzesiak et al., 2021). Obese polycystic patients had lower blood levels of 25-hydroxyvitamin-D (25-OH-D) than non-obese polycystic females because females with PCOS are more prone to metabolic abnormalities (De Leo et al., 2016, Davis et al., 2019).

2.10. Diagnosis of Polycystic Ovary Syndrome

Considering the complexities of this syndrome, different sets of PCOS diagnostic criteria have been established, as illustrated in Figure (2-4).

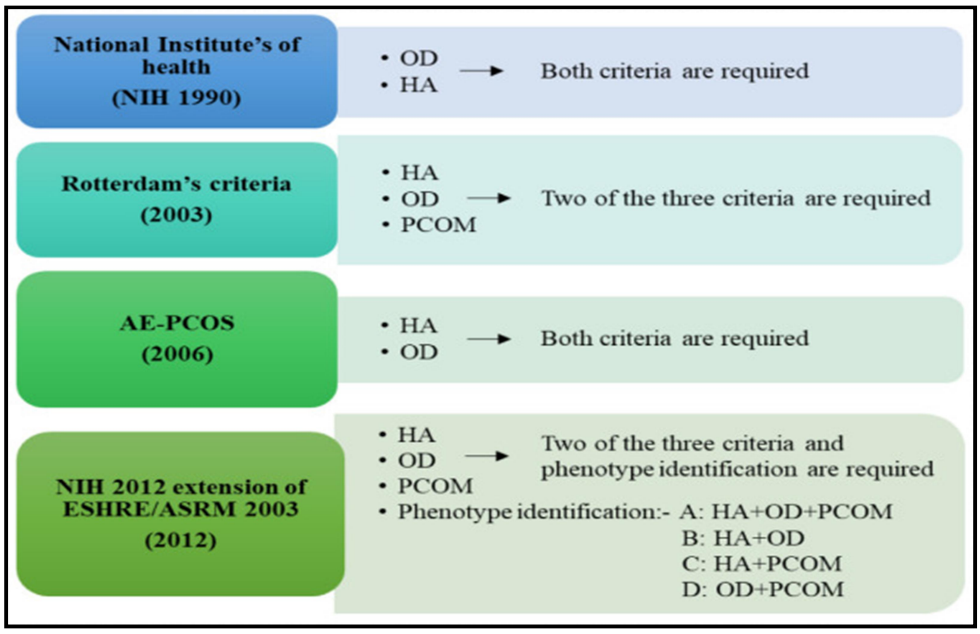


Figure (2-4): PCOS diagnostic criteria. NIH/NICHD: National Institute of Health/National Institute of Child Health and Human Disease; ESHRE/ASRM (Rotterdam): European Society for Human Reproduction and Embryology/ American Society of Reproductive Medicine; AE-PCOS: Androgen Excess and PCOS Society (Bulsara et al., 2021, Kalyanaraman and Pal, 2021).

2.10.1. Clinical Diagnosis

The clinical examination should include a modified Ferriman- Gallwey (mFG) score grading of hirsutism, location of acne and kind of lesions, seborrhea, and the presence of alopecia. BMI, waist measurement, and blood pressure should all be taken into account. In addition, the history should contain the duration of menstruation, infertility, diet, and family history (Gainder and Sharma, 2019).

Three scientific organizations' expertise was combined to develop current recommendations for PCOS diagnosis and treatment. The Rotterdam definition in 2003 is the most commonly used PCOS categorization, and it is now endorsed by the majority of scientific organizations and health officials. According to the criteria, PCOS can be diagnosed in a woman whom has at least two of the following symptoms: clinical and/or biochemical excess androgens, ovulatory disruption, and polycystic ovary morphology (PCOM). Clinically, hyperandrogenism is defined as the occurrence of acne, androgenic alopecia, and hirsutism. Chemically, by increased serum concentration of total or free testosterone or DHEA sulfate (Legro et al., 2013, Milewicz et al., 2018, Witchel et al., 2019).

The 2006 Androgen Excess and PCOS Society (AE-PCOS) necessitates the existence of androgen excess that must be supported by evidence of ovarian disturbances and/or PCOM (Dewailly et al., 2014). Additionally, a previous definition used by the National Institute of Child Health and Human Development requires the existence of both androgen excess and ovulatory disruption but does not take ovarian morphology into account. This definition includes both the Rotterdam and AE-PCOS categories, and it reflects the most severe phenotype in the PCOS range (Kalyanaraman and Pal, 2021).

Hirsutism, or the appearance of an extra terminal hair in a male-like pattern, is the most prevalent clinical manifestation of hyperandrogenism. The (mFG) score is used in clinical settings to determine the degree of terminal hair development in male-like regions. This score is calculated for 9 body regions (e.g. upper lip, chin & neck, and upper chest area) by assigning a score ranging from zero (without visible terminals hair) to four (terminal hair development), then summing the scores. The majority of observers reported a cutoff value for (mFG) score of 6, 7, 8, and even 10 (Azziz, 2018).

Acne is another skin manifestation of PCOS. The majority of PCOS patients have facial acne, but near to 50% of women also have acne on their neck, chest, and upper back. Androgen hormones enhance production of sebum, promoting aberrant desquamation of follicular epithelium and the creation of comedones. In addition, *Propionibacterium acnes* colonization causes inflammation and the production of papules and scarring.

Alopecia is a condition that causes male pattern baldness due to progressive hair loss. In this case, hyperandrogenism raises 5-alpha reductase and androgen receptor levels while decreasing cytochrome p450 enzyme levels, causing a drop in terminal hairs and conversion to vellus hairs (Gainder and Sharma, 2019, Wang et al., 2019). Ovulatory disturbance is a term used to describe either oligomenorrhea (periods ≥ 35 days apart but < 6 months apart) or amenorrhea (absence of menstruation for 6 to 12 months after a cyclic pattern establishment) (Jamal and Ismael, 2019).

2.10.2. Ultrasound Diagnosis

The goal of ultrasonography in PCOS is to correctly identify and record the existence of polycystic ovaries. However, since PCOS is a syndrome, the appearance of ovarian cysts alone is inadequate for diagnosis (Lee and Rausch, 2012). According to Dewailly et al., an increase in ovarian volume, in addition to follicle number per ovary, may be regarded accurate indicators of PCOM. As a result, a polycystic ovary is one with 12 or more cysts ranging in diameter from 2 to 9mm, or one with volume more than 10mL. A single ovary that meets one or both of these characteristics is sufficient to identify polycystic ovary (Dewailly et al., 2014).

2.10.3. Laboratory Diagnosis

There is no specific test for PCOS. Clinical manifestations such as skin related symptoms and ovulatory dysfunction are among the most important diagnostic tools used by physician (Williams et al., 2016). Anumber of lab tests are also used to make the final diagnosis:

- To confirm biochemical hyperandrogeniemia, levels of common androgen hormones to be evaluated include testosterone (total/ free), androstenedione, and DHEA.
- Increased LH production, increased GnRH/LH pulsatility, and normal to low FSH levels are the gonadotropin irregularities found in PCOS. The increased LH/FSH ratio during follicular phase has been identified as a good PCOS marker (Gainder and Sharma, 2019).
- Blood testing for co-occurring conditions, such as the oral glucose tolerance test (OGTT), which is regarded to be the most effective approach to assess both insulin and glucose yield (Ortiz-Flores et al., 2019). Furthermore, the lipid panel assesses all of the critical parameters linked with elevated cholesterols (Kim and Choi, 2013).
- Prolactin is a hormone released by pituitary gland that promotes breastfeeding in women. Hyperprolactinemia (one of the exclusion conditions), can induce irregular menstruation similar to that seen in PCOS ladies (Szosland et al., 2015).
- Thyroid-stimulating hormone (TSH) is released by the pituitary gland as well and controls the release of both triiodothyronine (T3) and thyroxine (T4). This blood test set can be used to exclude out thyroid disease as a causative agent of menstrual irregularities. Low amounts of either might cause menstrual alterations similar to that seen in PCOS people (Kamrul-Hasan et al., 2020).

2.11. Management of PCOS

Because there is no universal therapy for PCOS, therapies must be customized to the specific requirements of an individual patient. In fact, pharmaceutical therapy goals may include androgen overabundance, oligo-ovulation, and IR. However, a healthy lifestyle is always recommended in order to avoid or treat obesity (Escobar-Morreale, 2018).

2.11.1. Lifestyle Improvements

It is the first treatment step, which involves diet restrictions, exercises, and losing weight. Even a 5% reduction in total body mass improves IR and testosterone levels, resulting in significant improvements in fat mass and heart disease risk markers (Papakonstantinou et al., 2022).

2.11.2. Medical Treatment

2.11.2.1. Targeting Oligo-ovulation

Menstrual disruption and sub-fertility of varying degree are clinical implications of oligo-ovulation in PCOS patients. Notably, severe menstruation disruption puts women at risk of endometriosis and cancer, as well as infertility. The use of oral contraceptive tablets to maintain the endometrium, which may potentially improve hyperandrogenism, is one treatment option (Azziz et al., 2016). Oral contraceptive pills (OCPs) include estrogen, which suppresses LH, raises SHBG, and lowers ovarian androgen synthesis. Such effects include lower free testosterone, which also reduces the known skin-related symptoms (Gainder and Sharma, 2019).

2.11.2.2. Insulin Sensitizers

The most frequent strategy for treating IR in PCOS is to employ insulin- sensitizer medications, specifically Metformin which has benefits to lifestyle therapies in terms of weight loss; however it is greater in terms of androgen reduction (Sharma et al., 2019). Lifestyle improvements combined with metformin are related with decreased BMI, subcutaneous fat tissue, and improved menstruation compared to lifestyle modifications alone. Metformin combined with oral contraceptive tablets may prevent a certain decrease in metabolic activity, especially in females of non-hyperandrogenic PCOS (Escobar-Morreale, 2018).

2.11.2.3. Targeting Androgen Overabundance

In women with PCOS, the cutaneous manifestations of hyperandrogenism typically necessitate a mix of cosmetic and/or oral medications. Anti-androgens, such as Spironolactone, Cyproterone acetate, and Flutamide, primarily operate through inhibiting the activity of androgen binding receptors or by inhibiting the 5-alpha reductase, which lowers androgen synthesis (Luque-Ramirez et al., 2018).

2.11.2.4. Permanent Hair Removal and Cosmetic Therapy

Electroepilation and laser photothermolysis are two methods for permanently destroying hair follicles. Electrolysis can cause pigmentation after inflammation, whereas laser device is more expensive albeit less painful, faster and destroys the hair follicle without harming neighboring tissues. Along with destruction, it causes the shrinkage of course hair to vellus hair. Other medical therapies for facial hair growth includes Eflornithine

hydrochloride (13.9%) and Fluridil gel (2%) for favorable safety profile (Gainder and Sharma, 2019). Additionally, Retinoids and oral antibiotics can be used to treat acne. Retinoids can unclog pores and minimize acne breakouts by preventing dead cells from clogging pores (Leyden et al., 2017),whilst Minoxidil, 2% and 5% solutions, can be used to treat androgen-related alopecia, by increasing hair growth and preventing future hair loss in addition to hair transplantation (van Zuuren et al., 2016, Azziz, 2018).

2.11.2.5. Psychiatric Support

Due to the chronic nature of PCOS, women are more likely to experience behavioral and psychological problems. Every PCOS patient should receive proper consultation as well as suitable therapy. Usually women with PCOS are depressed as a consequence of androgenic symptoms that challenge their feminine identity, overweight, and low reproductive performance (Kaczmarek et al., 2016).

CHAPTER THREE
MATERIALS AND METHODS

3.1. Materials

The laboratory instruments and equipment, chemicals and commercial kits employed in this study are depicted in tables (3-1), (3-2) and (3-3).

Table (3-1): Instruments and equipment used in the study

Instruments and Equipment	Supplier	Origin
Vidas Multiparametric Immunoassay Analyzer	BIOMÉRIEUX	France
Cobas e 411 Analyser	Roche	Germany
ELISA Microplate Reader	BioTek ELx800	USA
Centrifuge	MMN	Sweden
Tube Roller Mixer	KJMR-II	China
Digital Deep Freezer (-20)	Hisense	China
Heratherm Compact Incubators	Thermo Fisher Scientific	USA
Digital Balance	Floria	Turkey
Laboratory Hood	Pars Azma	Iran
Refrigerated Centrifuge	Nüve	Turkey
Thermo-Shaker Incubator	Thermo Fisher Scientific	USA
Mini Vortexer	Neuation iSwix Jr. VT	India
Nanodrop UV Spectrophotometer	Thermo Fisher Scientific	USA
Microwave Oven	Hisense	China
Agarose Gel Electophoresis Unit	NOGEN	Iran
DNA/Rna Gel Electrophoresis UV Transilluminator	UVP	USA
Veriti ™ 96-Well Fast Thermal Cycler	Thermo Fisher Scientific	USA

Instruments and Equipment	Supplier	Origin
Single Channel Micropipettes (0.5-10, 20-200, 100-1000µL)	Accumax Fab	Germany
Microcentrifuge tubes (0.2 and 1.5ml)	Biotech Concern	Bangladesh
Tips (10, 200, 1000µL)	Biotech Concern	Bangladesh
K3 EDTA Tubes	Alfa-Med	India
Gel & Clot Activator Tubes	Alfa-Med	India

Table (3-2): Commercial kits and reagents used for the immunological study

Immunological kits and materials	Supplier	Origin
VIDAS® LH kit	BIOMÉRIEUX	France
VIDAS® FSH kit	BIOMÉRIEUX	France
Human Anti-FSH ELISA kit For Research use only	AL- Shkairate Establishment	Imported from USA by Jordanian company
Human Anti-LH ELISA kit For Research use only	AL- Shkairate Establishment	Imported from USA by Jordanian company
Elecsys Vitamin D Reagents	Roche	Germany

Table (3-3): Commercial kits and chemicals used for the molecular study

Molecular kits and materials	Supplier	Origin
Blood DNA Preparation- solution kit	Jena Bioscience	Germany
GoTaq® G2 Green Master Mix	Promega	USA
PCR Sizer 100bp DNA Ladder	Norgen Biotek	Canada
Lambda DNA	Promega	USA
Agarose Powder	Norgen Biotek	Canada
10x TBE Buffer Dry Pack	Apex Bioresearch	USA
GoldView I Nuclear Staining Dyes	Solarbio Science & Technology	China

Molecular kits and materials	Supplier	Origin
DNA Gel Loading Dye	Norgen Biotek	Canada
Nuclease- Free Water	Norgen Biotek	Canada
Forward and Reverse Primers	Integrated DNA Technologies (IDT)	Canada
99.8+% Isopropanol	ThermoFisher Scientific	USA
99.9% Ethanol	SLC- Delhi Chemicals	India

3.2. Methods

3.2.1. Participants

This case-control study included 100 women aged 15 to 37 years who attended the Maternity Teaching Hospital and some private hospitals/laboratories in Erbil City, Kurdistan region of Iraq from October 9th, 2021 to July 1st, 2022. Sixty women were clinically diagnosed with PCOS by the clinics and hospital consultant medical staff using the Rotterdam criteria, which included two of the three following features: clinical and/or biochemical hyperandrogenism, ovulatory disruption (oligo/or anovulation), and PCOM (an increased ovarian volume >10 cm3 and/or an increased antral follicle count (AFC) ≥12 in one ovary) in ultrasound figure (Birch Petersen et al., 2016). The remaining 40 participants were healthy subjects with regular menstrual cycles with no other signs or symptoms of PCOS who visited gynaecology clinics for check-ups or worked in medical clinics.

3.2.1.1. Screening Questionnaire

Participants who met the study's inclusion criteria were asked to fill out a questionnaire (prepared under the supervision of a gynaecologist) that asked about the most common symptoms of PCOS (Appendix A_1). The gynaecologist assessed hirsutism using the modified Ferriman and Gallwey (mF-G) score. Mild acne on the cheeks, jaw line, chin, and rarely upper neck was observed in several PCOS individuals. Acne, as described by patients, involves more sensitive nodes beneath the skin that tend to flare up before menstruation, which is usually irregular. Weight and height were determined and the body mass index (BMI) was calculated. Weight was measured using digital scales to the closest 0.1 kg, while a stadiometer was used to measure height to the closest centimetre. BMI was simply calculated using the formula: (BMI = weight in kg/height in m^2). In addition, age, marital status, ethnic background, PCOS family history, underlying chronic health conditions, medication and supplement intake, and some other basic information were all documented.

3.2.1.2. Exclusion Criteria

1. Disorders that mimic the clinical characteristics of PCOS were ruled out during data collection. These included: thyroid disease, hyperprolactinemia, and non-classical congenital adrenal hyperplasia (Legro et al., 2013).
2. Since a section of this study involves a molecular study including HLA as one of the genetic factors implicated in PCOS, and in order to acquire more specific results, all selected patients were Kurdish from Erbil city.

3.2.1.3. Ethical Approval

The Medical Ethics Committee of Erbil Technical Health and Medical College/ Erbil Polytechnic University approved and authorized the current study as per the order no. (154), dated 11/11/2021. Verbal consent to participate in the research was obtained through interviews with research participants, and their anonymity and privacy have been kept confidential.

3.2.2. Samples Collection and Processing

Venous blood sample (8ml) was collected aseptically by disposable syringe from each participant between the first and fifth day of their menstrual cycle, the early follicular phase. Five millilitres of collected blood sample was transferred into gel and clot activator tube and allowed the blood to clot at room temperature for (15–30) minutes. After centrifugation at 5000rpm/10 minutes, the resulting supernatant was the serum obtained for immunological based methods including: (1) enzyme-linked fluorescence immunoassay (ELFA) on VIDAS family device, (2) enzyme-linked immunosorbant assay (ELISA) and (3) electrochemiluminescence (ECL) test on a Roche Diagnostic Cobas e411 immunoassay system. The remaining (3ml) of whole blood transferred into EDTA tube for molecular-based technique. To avoid repeated freezing and thawing, the serum was separated into two Eppendorf tubes (1.5ml) and stored frozen at (−20°C) along with EDTA samples till time of analysis. Figure (3-1) illustrates a flow chart of the present study methodology.

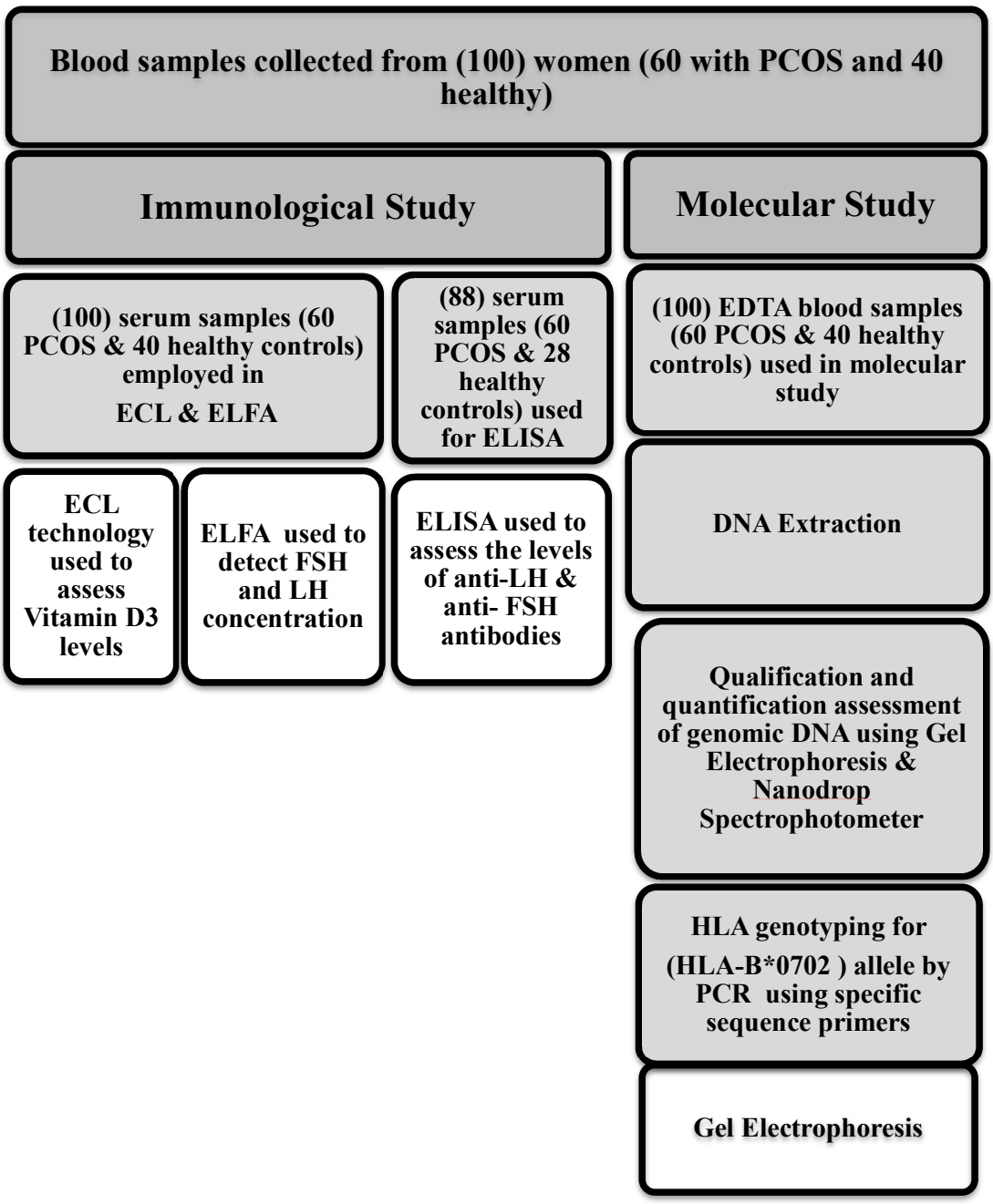


Figure (3-1): A flow chart summarizes the methodology of the present study

3.2.3. Molecular Characterization

HLA typing was performed in this study using a molecular approach to evaluate if there is an association between PCOS and (HLA-Class I) representing by HLA-B*0702 allele in women of Erbil City.

3.2.3.1. Preparation of Tris-borate-EDTA Buffer (10x TBE)

According to the manufacturer's instructions, stock solution of 10x TBE concentrate buffer was prepared using 10x TBE buffer dry packs from (Apex Bioresearch, USA), (Catalogue No.20-131), by dissolving the contents of the dry pack in (1 liter) distilled water.

3.2.3.2. DNA Markers

The DNA markers used in the current study include the DNA Ladder and Lambda DNA.

DNA Ladder (100bp):

The Norgen PCR Sizer 100bp DNA Ladder (Canada) has 10 different fragments ranging in size from 100 to 1000bp. This Ladder was designed for PCR product size validation by estimating the size of the certain fragment.

Lambda DNA (50kb):

The undigested λ DNA from (Promega, USA) was used as a molecular weight size marker in gel analysis of genomic DNA to assess the quality of DNA by gel electrophoresis.

3.2.3.3. Genomic DNA Extraction

For the molecular study, genomic DNA was extracted from 100 whole blood samples, collected from 60 women with PCOS and 40 healthy women, using Blood DNA Preparation-solution kit from (Jena Bioscience, Germany), (Catalogue No.PP-205S). This kit is designed for the simple and rapid isolation of genomic DNA from whole blood samples. About (30-50µg) purified genomic DNA was expected per preparation of (300µL) whole blood sample.The solution-based technique reduces DNA fragmentation, which can be an issue in spin-column / filtration-based approaches. In addition, it was safe and produced no hazardous waste, because neither phenol nor chloroform was employed. The DNA extraction steps will be mentioned in the following sections.

3.2.3.3.1. Samples Preparation and DNA Extraction

Before the extraction, the frozen EDTA blood samples were thawed at room temperature and gently mixed to ensure uniformity in accordance with the manufacturer's instructions. In addition, (48ml) of 99.9% ethanol was added to the washing buffer bottle.

Cell Lysis:

RBC lysis solution (900µL) was mixed with (300µL) whole blood sample in a (1.5 ml) microtube, which was then inverted 10 times and incubated at room temperature for 3 minutes before being centrifuged for 3 minutes at 14,000 rpm in a refrigerated centrifuge (Nüve, Turkey). With a pipette, a part of the supernatant was removed, leaving the visible cell pellet behind. The tube was then vortexed vigorously for about 10 seconds to ensure that it was thoroughly re-suspended. The re-suspended cells were then pipette

up and down with (300µL) of cell lysis solution until no clumps were seen. The samples were then incubated in a thermo-shaker incubator (Thermo Fisher Scientific, USA) for 10 minutes at 65°C to facilitate lysis.

Protein Precipitation:

One hundred micro-liters protein precipitation solution was added to the cell lysate and vigorously vortexed for 20 seconds to fully mix and no visible clumps. The precipitated proteins formed a tight and dark pellet after being centrifuged at 14,000 rpm for 3 minutes.

DNA Precipitation:

A clean (1.5 ml) microtube, (300µL) of 99.8+% Isopropanol was added and mixed with the supernatant by inverting the microtube gently for 1 minute before centrifuging at 14,000 rpm for 3 minutes. Significantly, DNA was visible with the naked eye as a little white pellet. The supernatant was then discarded, and the tube was briefly drained on clean absorbent paper. To wash the DNA pellet, 500µL of washing buffer was added and the tube was inverted several times. After centrifugation at 14,000 rpm for 3 minutes, the ethanol was carefully discarded and the DNA pellet dried at room temperature for about 10-15 minutes.

DNA Hydration:

In this step, a 100µL of DNA hydration solution was added and vortexed for 5 seconds to mix. The sample was then incubated at 65°C for 30 minutes to speed up rehydration before being frozen at (-20°C) until the qualitative and quantitative evaluation of genomic DNA.

3.2.3.4. Qualification of Genomic DNA Concentration

Agarose gel electrophoresis was used to determine the quality concentration of genomic DNA. The assay was carried out by preparing agarose gel (1%), then inserting a gel comb into an agarose gel casting tray and pouring the gel into the tray after adding nuclear staining dye and allowing it to solidify completely while avoiding overflow to adjacent wells. Following that, five microliters of undigested Lambda DNA (λ) marker was mixed with 3µL loading dye and loaded into the first lane, on the left side of the gel wells, and the DNA samples were mixed with loading dye in a ratio of 1:5 (2µL loading dye: 10µL DNA) before being loaded into the gel wells. The power supply was then used to turn on the electrophoresis device which was run for 45 minutes at 3-5 volts per cm (Russell and Sambrook, 2001).

3.2.3.5. Quantification of Genomic DNA Concentration and Purity

The Nanodrop spectrophotometer technology is intended for measuring a nucleic acid concentration in (1µL) sample's volume. The innovative sample retention technique of this spectrophotometer eliminates the need for cuvettes while collecting measurements. In this study, the NanoDrop UV spectrophotometer from (Thermo Fisher Scientific, USA) was used to determine the concentration and purity of the extracted DNA. The concentration of DNA in the solution was determined for DNA using the DNA conc. (ug/µl) = (OD_{260} *100 (dilution factor) * 50µg/ml)/1000.

Theoretically, OD_{260} of one corresponds to approximately (50µg/ml) for double strand DNA. The optical density, on the other hand, was measured by determining the absorbance at (OD_{260}nm and OD_{280} nm) and the (A_{260}/A_{280}) ratio estimated the purity of DNA (Russell and Sambrook, 2001). A good, purified DNA had an absorbance ratio ~1.8 to 2.0. A ratio lower than 1.6,

indicates protein contamination, while a ratio greater than 2.0 indicates RNA contamination (Ghatak et al., 2013, Lucena-Aguilar et al., 2016).

The quantitative measurement technique began with the operating of the Nanodrop and its software by clicking the nucleic acid button, preceded by cleaning the surface of the measuring pedestal with deionized distilled water or nuclease free water. By clicking the blank button, one microliter of the blank, which included DNA hydration solution (TE Buffer), was read. The surface of the NanoDrop spectrophotometer was then wiped clean with deionized distilled water before adding (1µl) of the first DNA sample. The reading was then completed by clicking the measurement button, and both DNA concentration and purity were recorded. After the spectrometry procedure was finished, the sample was wiped off the measurement base using a soft tissue, and the process was repeated for each DNA sample. The DNA was stored at -20°C until it was used in the PCR.

3.2.3.6. HLA-B*0702 Allele Genotyping

For the molecular study, HLA-typing test was performed on (100) DNA samples, 60 samples for women with PCOS and 40 samples for healthy controls. The HLA-B*0702 allele was identified using a PCR-based approach with specific sequence primers that allowed for accurate, sensitive, repeatable with better flexibility and resolution genotyping of such allele (Law et al., 2019).

3.2.3.6.1. Primers

The primers were provided in lyophilized form by (Integrated DNA Technologies IDT, Canada), (Catalogue No.323009337and 323009338) for Forward and Reverse primers, respectively. To obtain (100µM) concentration,

the primers were dissolved in nuclease- free water (163µL and 168µL for the forward and reverse primers, respectively) in compliance with the assembly specifications. Table (3-4) lists the primers employed in this study.

Table (3-4): HLA-B*0702 allele primers sequences used in this study

Primer	Recognition sequence (5'- 3')	Product size (bp)	Reference
B0702-492 **F**	**ACTCCATGAGGTATTTCTACACCT**	**210bp**	(Law et al., 2019)
B0702-701 **R**	**TCTGTGCCTGGGCCTTGT**		

F: Forward primer; R: Reverse primer

3.2.3.6.2. PCR Amplification

For the purpose of PCR-amplification of DNA, GoTaq®G2 Green Master Mix kit from (Promega, USA), (Catalogue No.REFM782A) was used. This kit was a ready-to-use premixed solution including appropriate quantities of GoTaq® G2 DNA Polymerase, dNTPs, $MgCl_2$, and reaction buffers for effective PCR amplification. Furthermore, GoTaq® G2 Green Master Mix contained two dyes (blue and yellow) that enabled for progress monitoring during electrophoresis. Its reactions showed dense enough for direct loading onto agarose gels, and the GoTaq® G2 DNA Polymerase displayed 5′→3′ exonuclease activity. Table (3-5a) shows the PCR solution ingredients required for master mix reaction of one sample in paired primers (25µL as a final volume) for PCR running according to the manufacturer's instructions.

Table (3-5a): Reaction mixes for a (25µL) volume for PCR per/sample

Components of PCR reaction mixture	Volume	Final concentration
GoTaq® G2 Green Master Mix, 2X	12.5µL	1X
Upstream primer, 10µM	0.25-2.5µL	0.1-1.0µM
Downstream primer, 10µM	0.25-2.5µL	0.1-1.0µM
DNA template	1- 5µL	< 250ng
Nuclease free water	To a final volume of 25µL	N.A

To get the best amplification outcomes, common PCR issues associated especially with the final concentration of DNA and (upstream and downstream) primers in a 25µL final volume PCR runs were resolved. Table (3-5b) displays the PCR- reaction master mixes required for one sample in paired primers (25µL as a final volume) for PCR running after optimization.

Table (3-5b): Reaction mixes for a (25µL) volume for PCR/one sample after optimization

Components of PCR reaction mixture	Volume	Final concentration
GoTaq® G2 Green Master Mix, 2X	12.5µL	1X
Upstream primer, 10µM	0.75µL	0.3µM
Downstream primer, 10µM	0.75µL	0.3µM
DNA template	2µL	< 250ng
Nuclease free water	9µL	N.A

The entire PCR cycle procedure was automated and lasted nearly an hour and 45 minutes. It was controlled by a Veriti™ 96-Well Fast Thermal Cycler (Thermo Fisher Scientific, USA), which was programmed to change the reaction temperature every several minutes to allow for DNA denaturing and synthesizing resulting in effective amplification.

The cycler's procedure included the following steps:

1. Initial DNA Denaturation: The reaction temperature was raised to 95°C for 5 minutes, which melted the double stranded DNA into single-stranded DNA.
2. DNA Denaturation: lasted 30 seconds to finish the denaturation.
3. Primer Annealing: Once the strands were separated, the temperature was reduced to 63°C for 30 seconds to allow the primers to anneal to the complementary areas of the template. The annealing temperature was related to the melting temperature (T_m) of the primers, and also the time and temperature required for primer annealing were dependent on the base composition (G-C) concentration.
4. Extension: By adding dNTPs, the DNA polymerase created a new DNA strand complementary to the DNA template strand. The temperature for thermo-stable DNA polymerase was 72°C for 1 minute.
5. Final Extension: This step was conducted 5 minutes after the final PCR cycle at 72°C to guarantee that any remaining single-stranded DNA was completely extended. Table (3-6) summarized the PCR steps and conditions followed in this study.

Table (3-6): Setting up thermo-cycling conditions (Lorenz, 2012).

PCR process steps	Temperature (°C)	Required time	Number of cycles
Initial DNA Denaturation	95	5 minutes	1
Denaturation	95	30 seconds	35
Primer Annealing	63	30 seconds	
Extension	72	1 minute	
Final Extension	72	5 minutes	1

Once the PCR was finished, the thermal cycler was adjusted to 4°C to keep the PCR products intact until the tubes could be withdrawn from the machine and move on to the next step which is agarose gel electrophoresis.

3.2.3.7. Agarose Gel Electrophoresis for DNA Products

Electrophoresis in (2%) agarose gel stained with safe stain was used to assess the PCR products.

3.2.3.7.1. Preparation and Casting an Agarose gel

- A gel electrophoresis casting tray with the appropriate dimensions was prepared. The board's sides were surrounded by sealed edges which a specific comb was attached to produce wells in one side of the gel.
- For the casting tray, 1% agarose gel (Norgen Biotek, Canada) was prepared to qualify genomic DNA by dissolving (1gm) of agarose in (10ml) of 10x TBE buffer and the volume was completed to (100ml) of distilled water. Similarly, agarose gel (2%) was prepared to run the amplified DNA, microwaving for about one minute, and cooling at 50-55°C for 1 minute. Following that, and in accordance with the manufacturer's guidelines, (10µL) of Gold View I Nuclear Staining Dye (Solarbio Science & Technology, China) was added to guarantee that DNA fragments fluoresce in the gel.
- One liter of 1x TBE was prepared for electrophoretic chamber (tank) by mixing (100ml) of 10x TBE buffer with (900ml) distilled water.
- After inserting a gel comb into a casting tray, the gel was poured into the tray and allowed to solidify completely while preventing overflow to adjacent wells.

- Five microlitres of DNA ladder were placed into the first lane, left side of the gel wells. The DNA samples were mixed with a ratio of 1:5 loading dye (2µL loading dye: 10µL DNA) before being loaded into the gel wells.

3.2.3.7.2. Running the Agarose gel

The gel device lid and its neighboring electrodes were attached with the negative electrode on the same side as the wells found. The gel was run for 45 minutes at (3-5 volts) per centimeter for genomic DNA. For amplified products, the gel was first run at (45-volts) per centimeter for 15 minutes to achieve adequate resolution, until the DNA migrated over 0.5 cm toward the positive electrode. The voltage was then increased to (135 volts) and the electrophoresis was conducted for approximately 1 hour (Russell and Sambrook, 2001).

3.2.3.7.3. Visualization of Amplified Products

UV Transillumination (UVP, USA) at (240, 366nm) wave length was used to visualize the DNA banding pattern (Russell and Sambrook, 2001). The gel was lit from below by placing it on the transilluminator glass and the 100bp DNA Ladder (Norgen Biotek, Canada) was used as a molecular marker and photographs were captured with a digital photography camera (Canon G12).

3.2.4. Immunological Laboratory Assays

3.2.4.1. Estimation of Follicle Stimulating Hormone and Luteinizing Hormone

VIDAS® Kits for Follicle Stimulating Hormone (FSH) (BIOMÉRIEUX, France), (Catalogue No.REF 30407) and Luteinizing Hormone (LH) (BIOMÉRIEUX, France), (Catalogue No.REF 30406) were applied to estimate FSH and LH concentration in sera of 100 participated women including 60 PCOS patients and 40 healthy women. The procedure principle combines a one-step immunoassay sandwich approach with a final fluorescent detection (ELFA) which offered an easy, reliable, and ultrasensitive approach for quick detection of hormones concentration in human serum (Proverbio et al., 2013).

3.2.4.1.1. Principle of the Assay

This test is designed to be used on VIDAS family devices as a quantitative enzyme-linked immunoassay (ELFA) to determine the concentrations of FSH and LH in serum samples separately. The equipment was already in control of all assay steps and the temperature. The solid phase receptacle (SPR), a pipette tip-like disposable tool, acted as both a solid phase and a pipetting tool for this test. The interior of SPR were coated with mouse monoclonal anti-FSH or anti-LH antibodies during manufacturing, and the reagents for the experiment were kept in sealed reagent strips. Serum sample was placed into a well containing antibodies linked with alkaline phosphatase and the mixture was cycled into or out of the SPR, and the hormone bound to antibodies coated on the SPR and to the conjugate, producing a “sandwich.”

Unbound conjugate was eliminated during the wash stages. The SPR was used to cycle a luminous substrate (4-methylumbelliferyl phosphate). The change of the substrate to the fluorescent product was catalyzed by the enzyme that remained on the SPR wall (4-methylumbelliferone). Finally, the fluorescence emission was measured using the optical scanner in the VIDAS equipment; which was related to the FSH or LH concentrations in the sample. When the experiment was finished, the results were automatically assessed by the device and printed for each sample.

3.2.4.1.2. Assay Procedure

For this test, each sample received one "FSH" or "LH" strip and one "FSH" or "LH" SPR. Following calibration, (200µl) of each serum sample was placed into a strip well. The reagent strips and SPRs were then placed into the appropriate position on the instrument, and the color labels with the assay code on the SPRs and reagent strips were examined to ensure that they matched. The equipment performed all of the assay steps automatically, and the experiment was completed in about 40 minutes. The SPRs and strips were removed from the device once the test was done.

3.2.4.2. Quantitative Detection of Anti-FSH and Anti- LH Antibodies

This procedure was done using commercially available ELISA kits, Human Anti- FSH ELISA kit (Catalogue No.RDEEH4473) and Human Anti-LH ELISA kit (Catalogue No.RDEEH4472) from (USA). Anti-FSH and anti-LH antibodies were investigated in the serum of (88) participants including (60 PCOS patients and 28 healthy controls).

3.2.4.2.1. Principle of the Assay

These kits were based on sandwich enzyme-linked immunosorbent assay technology. Antigen was pre-coated onto 96-well plates and the horseradish peroxidises (HRP) conjugated antigen was used as detection antibodies. After that, the standards, test samples, and HRP conjugated detection antigen were added to the wells and rinsed with wash buffer. TMB (3, 3', 5, 5'-tetramethylbenzidine) substrates were used to visualize the HRP enzymatic reaction. TMB was catalyzed by HRP to create a blue product that became yellow with the addition of stop solution. The yellow density is related to the amount of sample collected in the plate. The O.D. absorbance was measured at (450nm) using a (BioTek ELx800, USA) Microplate Reader.

3.2.4.2.2. Reagents Preparation

All reagents and serum samples were brought to room temperature for about 20 minutes before being used. The following were among the preparation steps:

1) Wash Buffer:

   Distilled water was used to dilute (30ml) of concentrated washing buffer in (750ml).

2) Standards:

One standard vial (lyophilized human anti-FSH or anti-LH) was filled and mixed with (1ml) of sample dilution buffer, labelled as zero tube, and kept at room temperature for 10 minutes. Seven Eppendorf tubes (EP tubes) were labelled with 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th}, and blank tubes, respectively. Sample dilution buffer (0.3ml) was added into each tube and (0.3ml) of the standard solution from zero tube was transferred to 1^{st} tube and mixed, then (0.3ml) from 1^{st} tube transferred to 2^{nd} tube and mixed and so on. The sample

dilution buffer was used as blank control. Figure (3-2) depicts the process of preparing standard serial dilutions of a human anti-FSH and anti-LH ELISA kits according to the manufacturer's instructions.

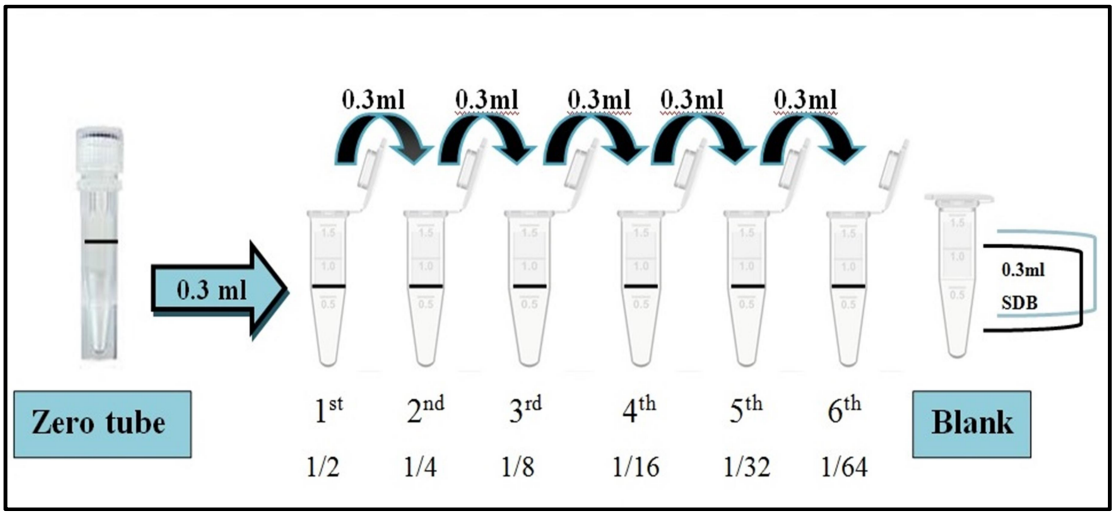


Figure (3-2): Preparation of standard serial dilutions of human anti-FSH and anti-LH antibodies ELISA kits. SDB: Sample dilution buffer.

3) Preparation of HRP-Labelled Antigen Working Solution:

The HRP- detection antigen was diluted with antigen dilution buffer at a ratio of (1:100), which was (0.11ml) of HRP- detection antigen with (10.89ml) antigen dilution buffer.

3.2.4.2.3. Assay Procedure

First, the ELISA microplate was rinsed twice before adding the standard, samples, and blank. Following that, (100µl) of zero,1st, 2nd, 3rd, 4th, 5th, 6th, and blank tubes were added to the standard wells (the first column of microplate) and their locations were recorded. Next, (100µl) of properly diluted samples (diluted at ½ with sample dilution buffer) were added to test sample wells. Following a 90 minutes incubation period at 37°C after sealing

the plate with a cover, the plate was washed three times with washing buffer. HRP-labelled antigen working solution (100µl) was added to the above-mentioned standards, test samples, and blank wells and the plate was covered and incubated at 37°C for 30 minutes before being washed 5 times with washing buffer. TMB substrate solution (90µl) was added to each well and incubated for 10-20 minutes at 37°C in the dark. Finally, (50µl) of stop solution was then added to each well, and the blue colour quickly became yellow and the result was read at 450 nanometre by microplate reader.

3.2.4.2.4. The Optical Density (O.D.) Measurement

The optical densities of antibodies to both FSH and LH were measured at 450nm employing a Microplate Reader depending on the relative O.D. The (relative O.D.450) = (O.D.450 of each well) – (O.D.450 of blank well)

The ELISA standard curves were displayed as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The standard curves were used to interpolate the target concentration of the samples. The concentration was calculated by multiplying the interpolated concentration by the dilution factor. The dilution factor was calculated using the equation (Final volume/ Original volume), which equalled two. The ELISA standard curves of the human (a) anti-follicle stimulating hormone and (b) anti-luteinizing hormone antibodies used in the current study are shown in Figure (3-3).

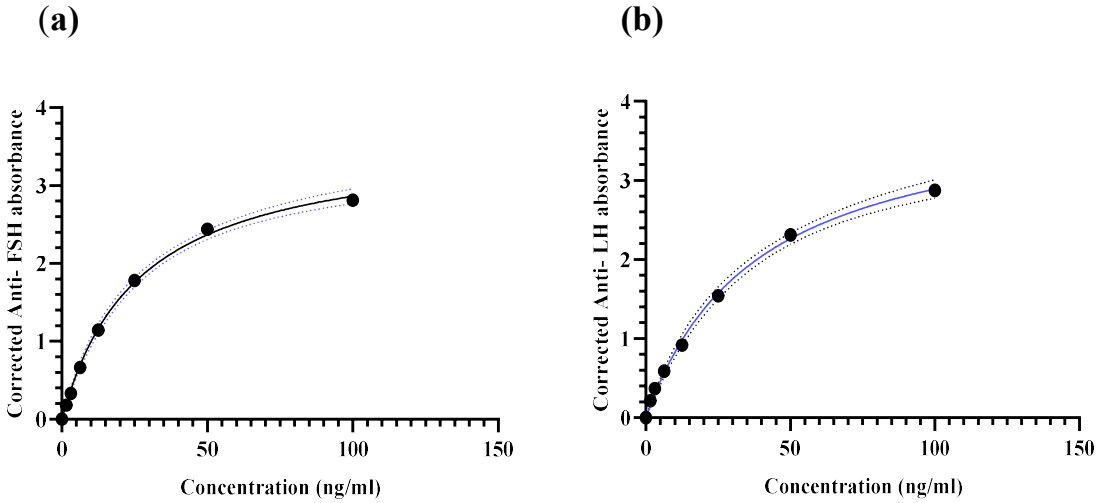


Figure (3-3): ELISA standard curves for human (a) anti-follicle stimulating hormone and (b) anti-luteinizing hormone antibodies.

3.2.4.3. Assessment of Vitamin D Levels

Serum 25(OH)D was measured for (100) samples including (60 PCOS patients and 40 healthy controls) using an electrochemiluminescence (ECL) test on a Roche Diagnostic Cobas e411 immunoassay system with a vitamin D kit from (Germany, Catalogue No.REF05894913190). ECL is a process that generates highly reactive molecules from stable precursors at the top of an electrode. Light is produced when these highly reactive species combine with others. This assay offered precise, excellent sensitivity, simple and easy to use technique (Martins-Costa et al., 2013, Qasemi et al., 2021) as well as the entire reaction was precisely automated and software-controlled.

3.2.4.3.1. Assay Principle and Procedure

Cooled reagents (Elecsys Vitamin D Reagents, Roche, Germany) were brought to nearly (20°C) and placed on the analyzer's reagent disk. The

system automatically controlled the temperature of the reagents as well as the opening and shutting of the bottles, and the test took a total of 27 minutes to complete.

- 1st incubation: After incubating the sample (20µL) with pretreatment reagents 1 and 2, bound 25-hydroxyvitamin D becomes released from the vitamin D binding protein (VDBP).
- 2nd incubation: Incubating the pretreatment sample with the ruthenium labeled vitamin D binding protein results in the formation of a complex between the 25-hydroxyvitamin D and the ruthenylated VDBP. A unique unlabeled antibody binds to the presence of 24, 25-dihydroxyvitamin D in the sample and suppresses cross-reactivity to this vitamin D metabolite.
- 3rd incubation: Unbound ruthenylated labeled vitamin D binding proteins become occupied with the addition of streptavidincoated microparticles and 25-hydroxyvitamin D tagged with biotin. The combination of biotin with streptavidin results in the formation of a complex consisting of the ruthenylated vitamin D binding protein and the biotinylated 25-hydroxyvitamin D, which binds to the solid phase.
- Reaction mixture was then aspirated into the measurement cell, where the microparticles were magnetically trapped on the electrode's surface. The unbound substances subsequently extracted using ProCell/ ProCell M. When a voltage applied to the electrode, chemiluminescent emission occurs and detected by a photomultiplier.
- The results were calculated using a calibration curve obtained by 2-point calibration on the instrument and a master curve given by the reagent barcode.

3.2.5. Statistical Analysis

All statistical analysis was carried out using GraphPad Prism 8.0.1 (244) software (San Diego, California, USA). All data fulfilled the requirements for performing normality tests including (Shapiro-Wilk, and the D'Agostino). The independent t-test (parametric, two-tailed) and one-way analysis of variance (ANOVA) test were used to compare PCOS patients with healthy controls in terms of demographic and clinical parameters. Fisher's exact test was used to assess and compare the HLA-B*0702 allele and its frequency in both groups. To demonstrate the relationship of HLA-B*0702 allele with the risk of PCOS, the OR and its 95% CI were computed.

The receiver operating characteristic (ROC) curve and its corresponding area under the curve (AUC) were used to illustrate the relationship between clinical sensitivity and specificity for possible cut-off values of anti-FSH antibodies ELISA test and anti-LH antibodies ELISA test in a graphical way. For this purpose, Youden's index was used to determine the most appropriate cut-off value for each ROC curve. Pearson's correlation (P.C) was used to assess the relationships between the immunological parameters investigated. The results are presented as mean ± SD (Mean with 95% CI bars) and the significance level is denoted as * $P < 0.05$; ** $P < 0.01$; or **** $P < 0.0001$.

CHAPTER FOUR
RESULTS

4. OBTAINED RESULTS

4.1. The Demographic Characteristics of the Study Participants

Table (4-1) illustrates the demographic characteristics of the 100 women who participated in this study, ranging in age from 15 to 37 years, including 60 PCOS patients and 40 healthy women serving as a control group. The majority of the PCOS patients (n =43; 71.67%) were between the ages of 18 and 26, indicating a higher prevalence among younger females. The age mean±SD for patients and healthy controls were (25.02±4.135) versus (25.8±5.239), indicating no significant differences between the two groups (P-value ≥ 0.05) as illustrated in Figure (4-1a). Eighty-four women (84%) had been married for ≥ 1 year, while the remaining sixteen were singles. Regarding PCOS patients, 30% had a family history of PCOS, and more than half (n=42; 70%) had high BMIs, which included both overweight and obese. The BMI mean values show a significant difference (P-value < 0.05) between two groups (27.44±4.302) versus (25.79±3.442), however 57.5% of the healthy control group showed high BMIs; Figure (4-1b).

Fertility status was classified into two categories in the current study, according to the WHO: primary infertility, which accounting for approximately 47% of PCOS women, and secondary infertility, which accounting for about 33% of patients with the exception of 12 (20%) single ladies. In regard of the healthy controls, 36 out of 40 were fertile and had children, with the remaining four being single ladies.

Table (4-1): Demographic characteristics-based distribution of the study participants

Characteristics	PCOS (n=60)	Controls (n=40)	P-value
	n (%)	n (%)	
Age (years) Mean±SD	25.02±4.135	25.8±5.239	0.4068 ns
Age < 18	1 (1.67)	2 (5)	
18 - 26	43 (71.67)	19 (47.5)	
27 - 37	16 (26.67)	19 (47.5)	
Marital status			
Married	48 (80)	36 (90)	
Single	12 (20)	4 (10)	
Family history of PCOS			
Yes	18 (30)	0 (0)	
No	42 (70)	40 (100)	
BMI (kg/m^2) Mean±SD	27.44±4.302	25.79±3.442	0.0461 *
Underweight < 18.50	0 (0)	0 (0)	
Normal range 18.50-24.99	18 (30)	17 (42.5)	
Overweight 25-29.99	24 (40)	18 (45)	
Obese ≥ 30	18 (30)	5 (12.5)	
Fertility			
Fertile	0 (0)	36 (90)	
Primary infertility	28 (46.67)	0 (0)	
Secondary infertility	20 (33.33)	0 (0)	
Single	12 (20)	4 (10)	

P- value ≥ 0.05 not significant (ns)

P-value P- value < 0.05 significant *

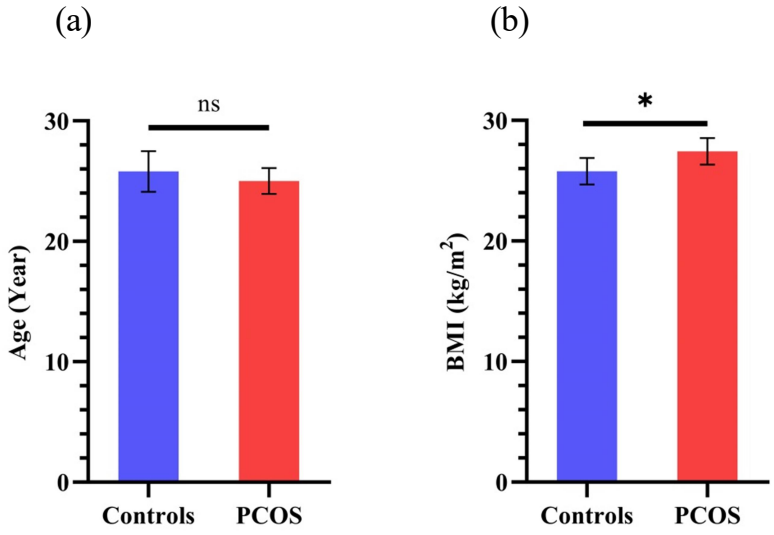


Figure (4-1): Differences in (a) age and (b) BMI mean values between controls and PCOS patients. Mean with 95% CI bars; n=100 (60 PCOS, 40 controls).

4.2. The Clinical Characteristics of the Study Participants

Table (4-2) displays the clinical features of women who participated in the study, including PCOS patients and healthy controls. Menstrual irregularities were reported by 57 PCOS patients (95%), with amenorrhea (n=8; 13.33%) and oligomenorrhea (n=49; 81.67%) accounting for the majority of patients. In contrast, no menstrual irregularities were seen in healthy women. Concerning the dermatological signs of androgen excess, such as hirsutism and acne, which are considered to be key markers of PCOS, the majority of PCOS patients (n=56; 93.33%) met the criterion for mild hirsutism from the upper lip, chin, and lower abdomen, as assessed by gynecologists. Mild acne was found in (n=37; 61.67%) of the patients, while the control group had no history of hirsutism, but a few of them (n=3; 7.5%) had mild acne. According to trans-abdominal ultrasound findings, the majority of patients (76.67%) had PCOM.

Table (4-2): Women's clinical characteristics-based distribution

Characteristics	PCOS (n=60)	Controls (n=40)
	n (%)	n (%)
Menstruation		
Regular	3 (5)	40 (100)
Amenorrhea	8 (13.33)	0 (0)
Oligomenorrhea	49 (81.67)	0 (0)
Hirsutism*		
Mild 8-16	56 (93.33)	0 (0)
No hirsutism <8	4 (6.67)	40 (100)
Mild acne		
Yes	37 (61.67)	3 (7.5)
No	23 (38.33)	37 (92.5)
Ultrasound findings		
Positive	46 (76.67)	0 (0)
Negative	14 (23.33)	40 (100)

*As per the modified Ferriman-Gallway (mF-G) score (Mahajan et al., 2021).

4.3. Differences in FSH, LH and LH/FSH in PCOS Patients and Controls

Regarding hormonal indices, Table (4-3) displays the mean values of FSH and LH along with their ratios in both groups. FSH mean ±SD was normal (3.514±1.272) versus (3.846±1.081) for control group indicating no significant difference Figure (4-2a), while LH mean±SD was elevated in cases compared to controls (9.189±4.262) versus (5.807±2.015) with a significant difference as shown in Figure (4-2b). Accordingly, the LH/FSH mean±SD in patients (2.529±0.457) was higher than in controls (1.543±0.439), indicating a significant difference between the two groups, as shown in Figure (4-2c). The outcomes demonstrated that 86.67% of patients had LH/FSH >2 LH/FSH which is frequently requested to assist in the diagnosis of PCOS.

Table (4-3): Mean serum levels of FSH, LH and their ratios in PCOS patients and controls

Characteristics	PCOS (n=60)	Controls (n=40)	P-value
	n (%)	n (%)	
FSH levels (IU/L) Mean±SD	3.514±1.272	3.846±1.081	0.1788 ns
LH levels (IU/L) Mean±SD	9.189±4.262	5.807±2.015	<0.0001 ****
LH/FSH ratio Mean±SD	2.529±0.457	1.543±0.439	<0.0001 ****
LH/FSH > 2	52 (86.67)	6 (15)	
LH/FSH ≤ 2	8 (13.33)	34 (85)	

P-value ≥ 0.05 not significant (ns)
P-value < 0.0001 significant ****

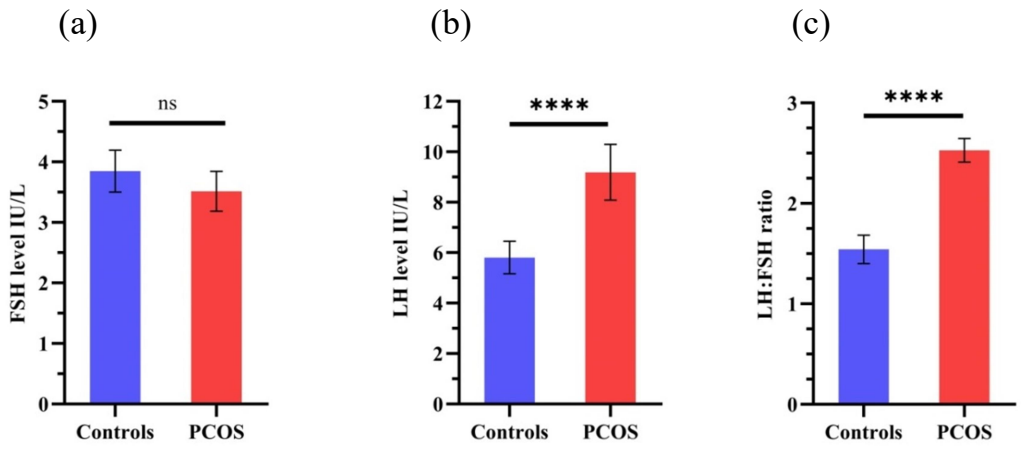


Figure (4-2): Differences in (a) FSH, (b) LH and (c) LH/FSH mean values between control participants and PCOS patients. Mean with 95% CI bars, n=100 (60 PCOS, 40 controls).

4.4. Molecular Study Results

4.4.1. Qualification and Quantification of Genomic DNA

Figures (4-3) and (4-4) show the genomic DNA of 100 samples extracted from blood cells, including 60 polycystic ovary syndrome patients

and 40 healthy control subjects. Agarose gel electrophoresis (1.0%) which was run for 45 minutes at 3-5 volts per cm. was used to assess the quality concentration of genomic DNA while the Nano-Drop spectrophotometer was used to quantify the purity and concentration of genomic DNA.

4.4.2. HLA Typing

HLA typing test for the HLA-B*0702 allele was performed for all participants using extracted genomic DNA and a PCR-based technique with specific sequence primers. Figure (4-5) and (4-6) show agarose gel electrophoresis (2.0%) revealing 210bp Uniplex PCR products matching to HLA-B*07:02 allele genotyping for PCOS patients and healthy controls, respectively, following PCR amplification.

4.4.3. Calculation of Odds Ratio with a 95% Confidence Interval

In this study, the OR was calculated using the findings of HLA genotyping for 100 samples (patients and controls). The goal of detecting and calculating the OR is to determine whether the HLA-B*07:02 allele is a risk factor associated with PCOS. Table (4-4) demonstrates the OR findings in which 60 out of 100 women had PCOS. In this group, 52 women (86.67%) carried the HLA-B*0702 allele, while 8 (13.33%) had not. The remaining 40 samples, on the other hand, were for healthy controls, and of this group, 30 women (75%) carried the HLA-B*0702 allele, while 10 women (25%) had not, indicating a positive association of the HLA-B*0702 allele with PCOS (OR: 2.167; CI: 0.8167 to 6.330), as shown in Figure (4-7).

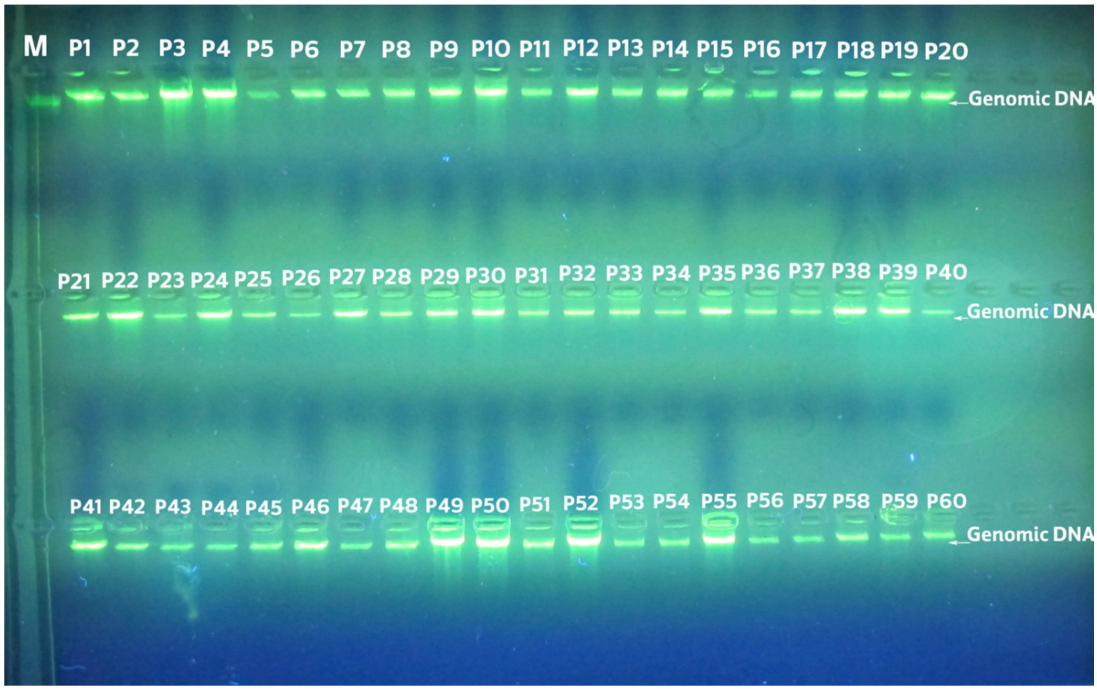


Figure (4-3), illustrates genomic DNA specimens isolated from blood cell samples of patients with polycystic ovary syndrome (PCOS). Electrophoresis was performed on a 1 percent agarose gel which was run for 45 minutes at 3-5 volts per cm. The lanes from P1 to P60 represent the patients with PCOS. Undigested lambda (λ) DNA, which has a standard molecular weight marker ~ 50 kb, is represented by lane M.

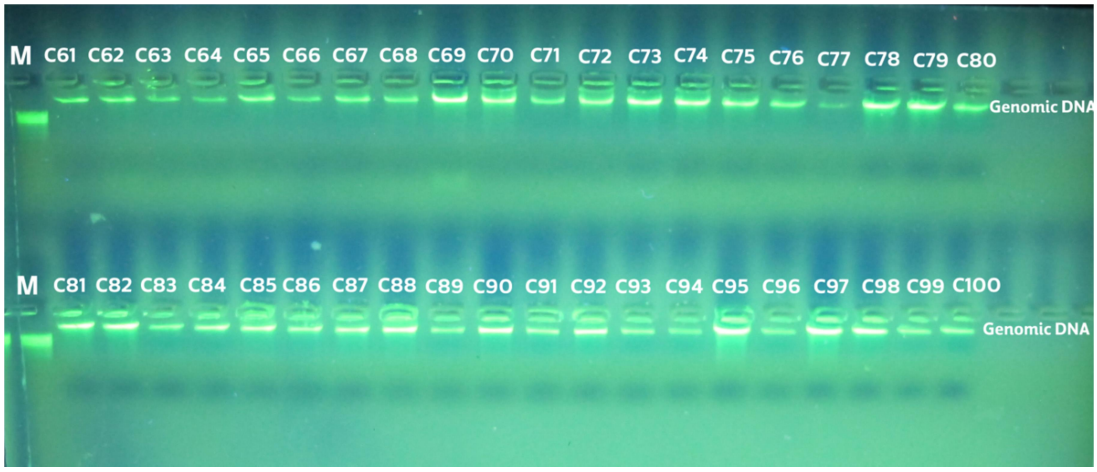


Figure (4-4), shows genomic DNA extracts from blood cell samples of healthy control women. On a 1 percent agarose gel, electrophoresis was carried out for 45 minutes at 3-5 volts per cm. The participants that are typically healthy are shown in the lanes from C61 to C80 and C81 to C100. The Lane M represents undigested lambda (λ) DNA with a molecular weight marker of approximately 50 kb.

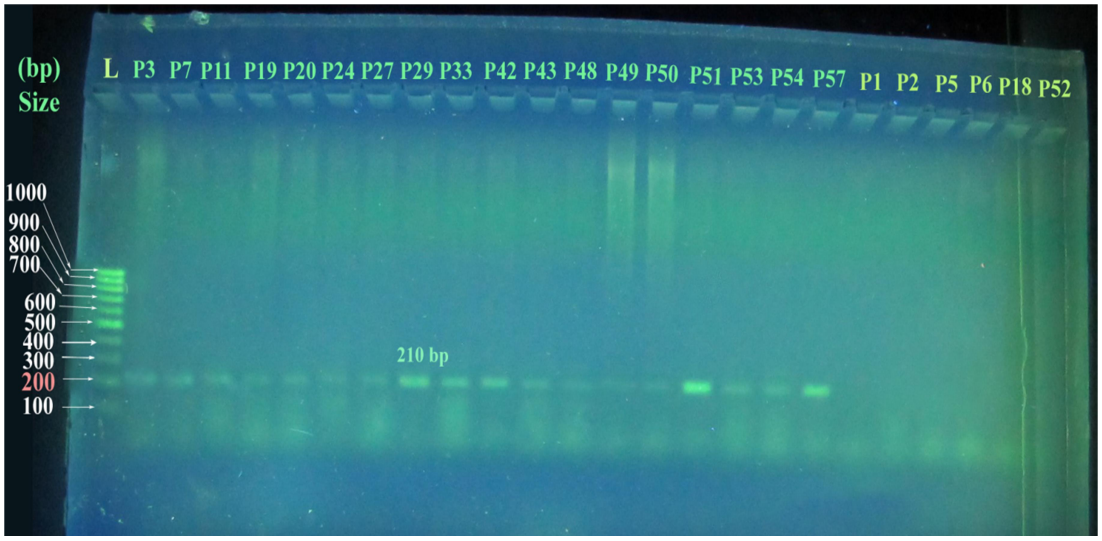


Figure (4-5): Agarose gel electrophoresis (2%) demonstrated 210bp Uniplex PCR products corresponding to amplification of HLA-B*07:02 allele genotyping. Lane L: 100bp DNA ladder. Lanes P3, P7, P11, P19, P20, P24, P27, P29, P33, P42, P43, P48, P49, P50, P51, P53, and Lanes P54 and P57 exhibit positive PCR DNA amplicons sizes (210bp), which correlate to individuals with PCOS when used with the designated HLA-B*07:02 specific primers. While the lanes P1, P2, P5, P6, P18, and P52 correspond to PCOS patients and represent negative PCR results (no amplification).

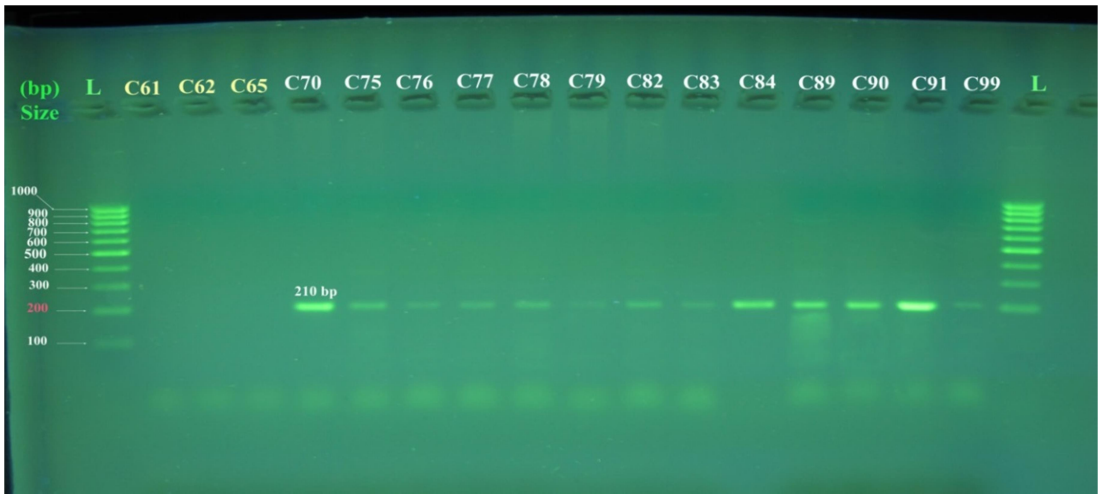


Figure (4-6): Agarose gel electrophoresis (2%) exhibited 210bp Uniplex PCR products associated with genotyping of the HLA-B*07:02 allele. Lane L: 100bp DNA ladder. When the prescribed HLA-B*07:02 specific primers are employed, lanes C61, C62, and lane C65 reveal negative PCR DNA amplicons sizes (210bp) correspond to healthy control women. While the lanes C70, C75, C76, C77, C78, C79, C82, C83, C84, C89, C90, C91, and C99 represent positive PCR result products and correspond to healthy women.

Table (4-4): Odds Ratio for HLA-B*0702 allele shows the allele association with PCOS

Exposure		PCOS patients	Controls	OR (95% CI)
		n (%)	n (%)	
With HLA-B*0702 allele	Yes	52 (86.67)	30 (75)	2.167 (0.8167 to 6.330)
Without HLA-B*0702 allele	No	8 (13.33)	10 (25)	
Significance level (P-value)				0.1847^{ns}

HLA: Human Leukocyte Antigen; OR: Odds Ratio; CI: Confidence Interval; P-value is for Fisher test; P-value ≥ 0.05 indicate not significant (ns). (Note: OR was calculated for 100 samples, including 60 PCOS patients and 40 controls).

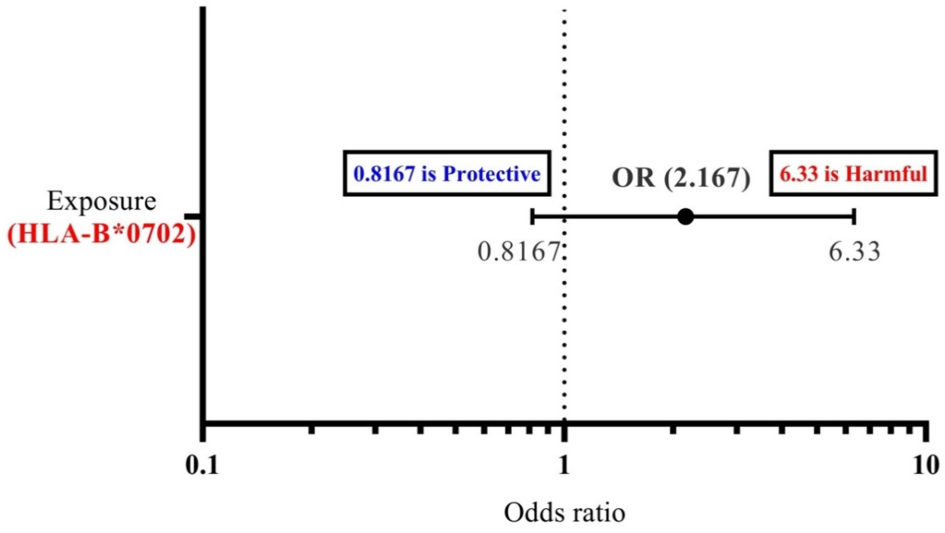


Figure (4-7): Odds Ratio for HLA-B*0702 allele indicates positive association with PCOS. An odds ratio (OR) >1 implies the disease is more frequent with exposure and thus the exposure may be harmful, suggesting a positive (Andrade, 2015).

4.5. Immunological Study Results

4.5.1. Quantitative Detection of Anti-FSH & Anti- LH Antibodies

Table (4-5) depicts the level of anti-FSH and anti-LH antibodies in the sera of 88 participants, including 60 PCOS patients and 28 healthy controls. As demonstrated in Figure (4-8a), there was no statistically significant difference in mean±SD of anti-FSH between patients (1.305±0.875) and controls (0.971±0.620). Figure (4-8b) shows a significant difference in mean±SD of anti-LH between patients (2.368±1.479) and controls (1.538±0.965).

Table (4-5): Mean serum levels of anti-FSH & anti-LH Abs in PCOS patients and controls

Characteristics	PCOS patients	Controls	P-value
	n (%)	n (%)	
Anti-FSH Abs (ng/ml) Mean±SD	1.305±0.875	0.971±0.620	0.0727^{ns}
Anti-LH Abs (ng/ml) Mean±SD	2.368±1.479	1.538±0.965	0.0082^{**}

P-value ≥ 0.05 not significant (ns)
P-value < 0.01 significant **

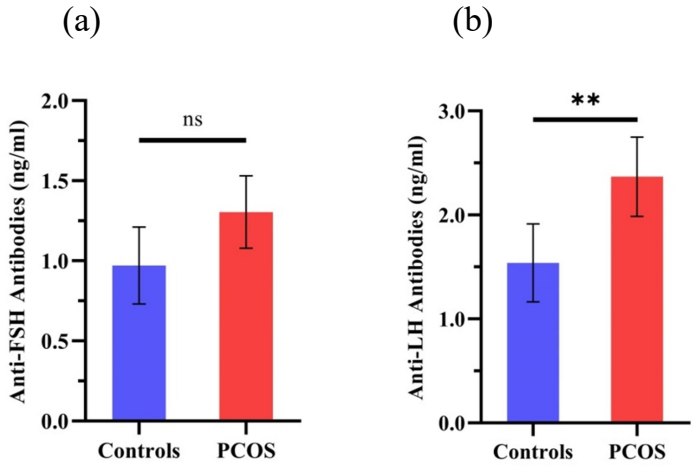


Figure (4-8): Differences in mean serum levels of (a) anti-FSH and (b) anti-LH antibodies between controls and PCOS patients. Mean with 95% CI bars, n=88 (60 PCOS, 28 controls).

After classifying PCOS patients based on their fertility status, anti-FSH antibodies level were higher in primary infertile PCOS cases (2.062±0.5724) showed a significant difference (P-value < 0.0001) when compared to control group (0.9708±0.6200). The similar significant difference was recorded when anti-FSH antibodies level in primary infertile PCOS cases (2.062±0.5724) was compared to that in secondary infertile PCOS cases (0.6680±0.4460) as shown in Figure (4-9). In addition, there was no significant difference between the control and secondary infertility groups (0.9708±0.6200 versus 0.6680±0.4460; P-value ≥ 0.05) as shown in Table (4-6) which compares anti-FSH antibodies mean ± SD in PCOS patients with primary and secondary infertility, as well as the control group, using one way ANOVA.

Table (4-6): One way ANOVA comparisons of anti-FSH antibodies in the sera of both PCOS cases with primary and secondary infertility and control group

Multiple comparisons	Mean[1]±SD	Mean[2]±SD	Mean Diff.	P-value
Controls[1] vs. Cases[2] with primary infertility	0.9708±0.6200	2.062±0.5724	-1.091	<0.0001****
Controls[1] vs. Cases[2] with secondary infertility	0.9708±0.6200	0.6680±0.4460	0.3028	0.1631 ns
Cases[1] (Primary Infertility) vs. Cases[2] (Secondary Infertility)	2.062±0.5724	0.6680±0.4460	1.394	<0.0001****

P-value ≥ 0.05 not significant (ns)
P-value < 0.0001 significant ****

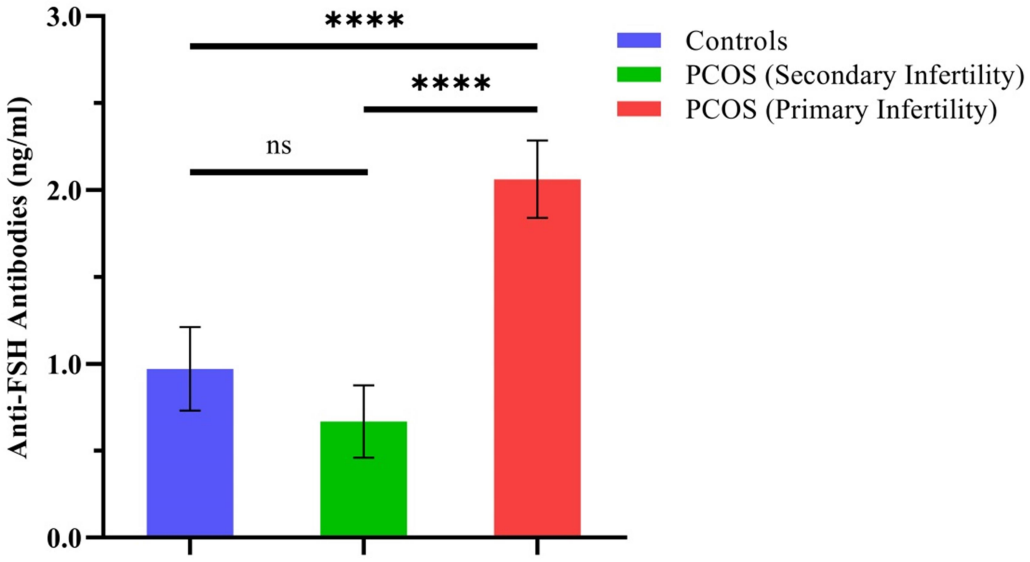


Figure (4-9): One way ANOVA comparisons of anti-FSH antibodies mean levels in the sera of PCOS patients with primary and secondary infertility versus healthy controls. Mean with 95% CI bars. Controls number = 28 fertile women; PCOS cases number with primary infertility =28 women; PCOS cases number with secondary infertility =20 women; (Note: 12 PCOS single ladies were excluded).

4.5.2. ROC Curve for Anti-FSH and Anti-LH Antibodies

Table (4-7) summarizes the ROC curve analysis for both human anti-FSH and anti-LH antibodies ELISA assays. Receiver Operating Characteristic (ROC) shows in a graphical way the relationship between the clinical sensitivity and specificity for potential cut-off values for both ELISA tests. The area under the curve (AUC) was calculated for both anti-FSH and anti-LH antibodies ELISA tests using their optical density (OD) readings and the most appropriate cut-off value for each curve was determined using Youden's index, which computes the maximum vertical distance from the line of identity. At this point, the maximum Youden's index was chosen, which equals (Sensitivity + Specificity - 1), as shown in Figure (4-10).

Table (4-7): AUC for ROC curve of human anti-FSH and anti-LH antibodies ELISA assays

Characteristics	Analysis of OD values	
	Anti-FSH antibodies	Anti-LH antibodies
Area under the ROC curve (AUC)	0.6167	0.6673
Standard Error (SE)	0.06125	0.05864
95% Confidence interval	0.4966 to 0.7367	0.5523 to 0.7822
Significance level (P- value)	0.0791 ^{ns}	0.0118*
Cutoff value	> 0.2006	> 0.2252

OD × 2 (DF) = cut off value in the table; DF: Dilution Factor
P-value ≥ 0.05 not significant (ns)
P-value < 0.05 significant *

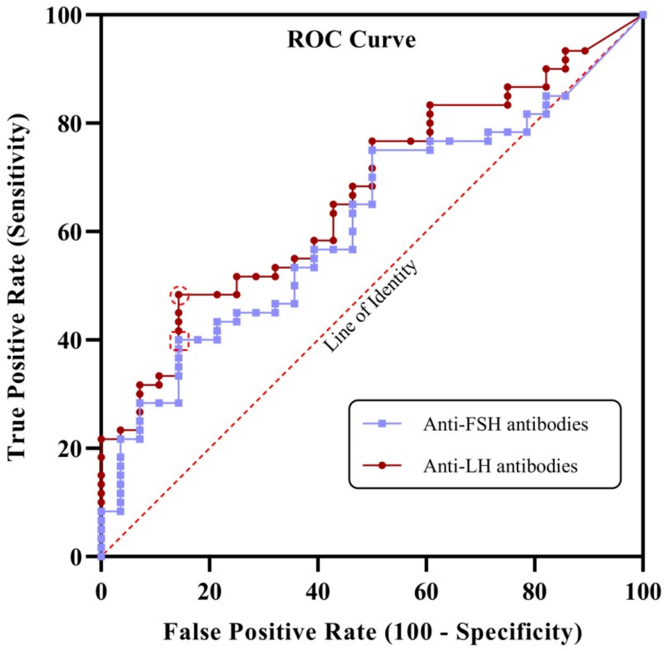


Figure (4-10): ROC curve for human anti-FSH and anti-LH antibodies tests. ROC: Receiver Operating Characteristic curve; AUC: Area under the ROC curve; the ROC curve and AUC can be used to estimate a test's discriminative power. The more closely the curve follows the upper left corner and the greater the area beneath the curve, the better the test distinguishes between individuals with and without disease (Doi and Williams, 2013, Habibzadeh et al., 2016).

4.5.3. Evaluation of Serum Vitamin D3

Table (4-8) illustrates vitamin 25(OH)D clusters and their outcomes in 100 participants. A serum level of >30ng/ml was considered sufficient, 20-30ng/ml as insufficient, 10- < 20 as deficient and less than 10 as severe vitamin 25(OH)D deficiency (Ringe and Kipshoven, 2012). Only 12% of all 100 women had sufficient values, 28% had insufficient values, 36% had deficient values, and 24% had severe vitamin 25(OH)D deficiency. When comparing patients to healthy subjects, the results revealed that 40% of patients were deficient, 28.33% were severely deficient, and 28.33% were insufficient, compared to 30%, 17.5%, and 27.5% in the control group,

respectively. As a result, mean±SD for vitamin D3 in patients and controls was (16.33±7.722) and (22.62±11.47), respectively, indicating a significant difference between the two groups, as shown in Figure (4-11), with the majority (n=41; 68.33%) of PCOS patients experiencing a deficient to severe vitamin D3 deficiency.

Table (4-8): Lab results of vitamin D3 clusters in PCOS patients and control

Characteristics	PCOS patients	Controls	P-value
	n (%)	n (%)	
Vitamin D_3 (ng/ml) Mean±SD	16.33±7.722	22.62±11.47	0.0014**
Sever deficiency <10	17 (28.33)	7 (17.5)	
Deficient 10- < 20	24 (40)	12 (30)	
Insufficient 20-30	17 (28.33)	11 (27.5)	
Sufficient > 30	2 (3.33)	10 (25)	
Potential intoxication >80	0 (0)	0 (0)	

P-value < 0.01 significant **

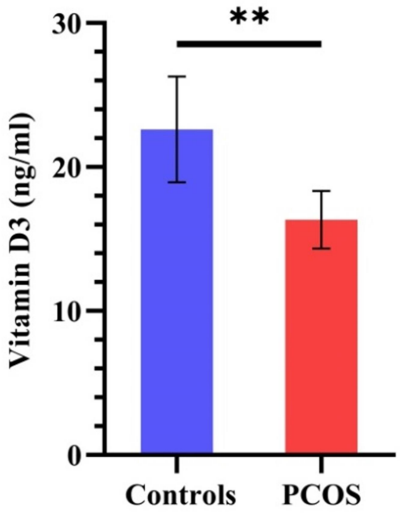


Figure (4-11): Differences in serum vitamin D3 levels between control group and PCOS patients. Mean with 95% CI bars, n=100 (60 PCOS, 40 controls).

4.5.4. Pearson's Correlation Analysis

Figure (4-12) depicts scatter plots with varying Pearson's correlations between 2 variables in PCOS patients. Pearson's correlation coefficient was used to assess the strength and direction of a linear relationship between two normally distributed factors in PCOS patients, as listed below:

(A) Correlation between anti-FSH and anti- LH antibodies

The results show a significant perfect positive linear correlation (P.C value =0.9863) between anti-FSH and anti-LH antibodies with (P-value < 0.0001).

B) Correlation between anti-FSH antibodies and FSH

Anti-FSH antibodies and FSH were shown to have a significant positive correlation (P.C value =0.3364) with (P-value < 0.01).

(C) Correlation between anti-LH antibodies and LH

There is a significant positive correlation between anti-LH antibodies and LH. (P.C value =0.2922) with (P-value < 0.05).

(D) Correlation between anti-FSH antibodies and LH/FSH

There is no significant positive correlation between anti-FSH and LH/FSH ratio (P.C value =0.05175) with (P-value ≥ 0.05).

(E) Correlation between anti-LH antibodies and LH/FSH

There is no significant positive correlation between anti-LH and LH/FSH ratio (P.C value =0.08564) with (P-value ≥ 0.05).

(F) Correlation between anti-FSH antibodies and vitamin D3

Anti-FSH antibodies have a non-significant inverse correlation with vitamin D3 levels (P.C value = - 0.04621) with (P-value ≥ 0.05).

(G) Correlation between anti-LH antibodies and vitamin D3

The same thing was seen with anti-LH antibodies, which revealed a non-significant negative correlation with vitamin D3. (P.C value = - 0.02185) (P-value ≥ 0.05)

(H) Correlation between vitamin D3 and FSH

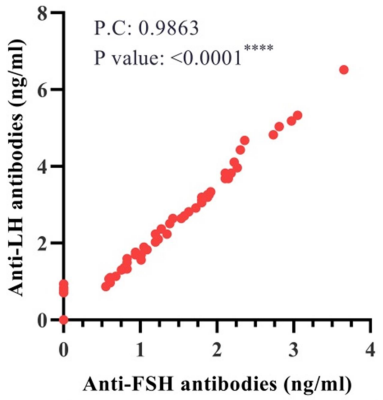
There was no significant positive correlation between vitamin D3 and FSH (P.C value =0.04156) with (P-value ≥ 0.05).

(I) Correlation between vitamin D3 and LH

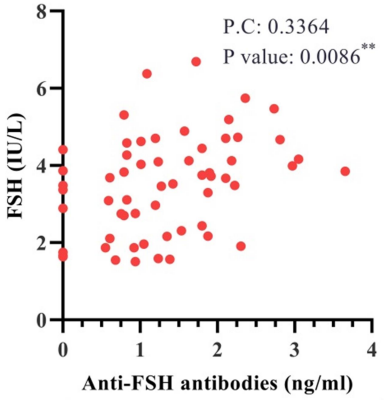
There was no significant positive correlation between vitamin D3 and LH (P.C value =0.08722) with (P-value ≥ 0.05).

(J) Correlation between vitamin D3 and BMI

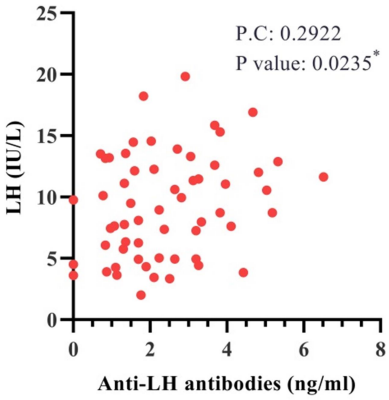
Finally, there is a non-significant negative correlation between vitamin D3 and the BMI (P.C value = - 0.07167) with (P-value ≥ 0.05).



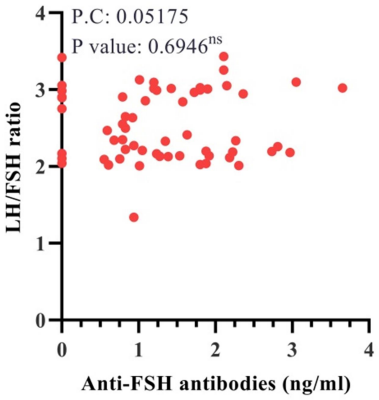
(A) Correlation between Anti-FSH and Anti-LH antibodies



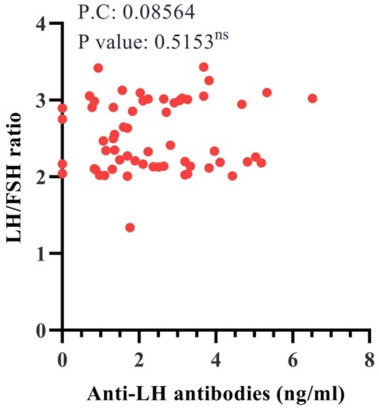
(B) Correlation between Anti-FSH antibodies and FSH



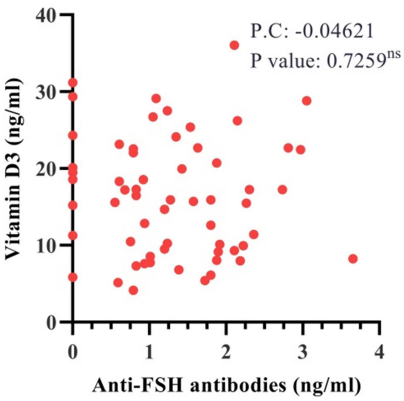
(C) Correlation between Anti-LH antibodies and LH



(D) Correlation between Anti-FSH antibodies and LH/FSH



(E) Correlation between Anti-LH antibodies and LH/FSH



(F) Correlation between Anti-FSH antibodies and Vitamin D3

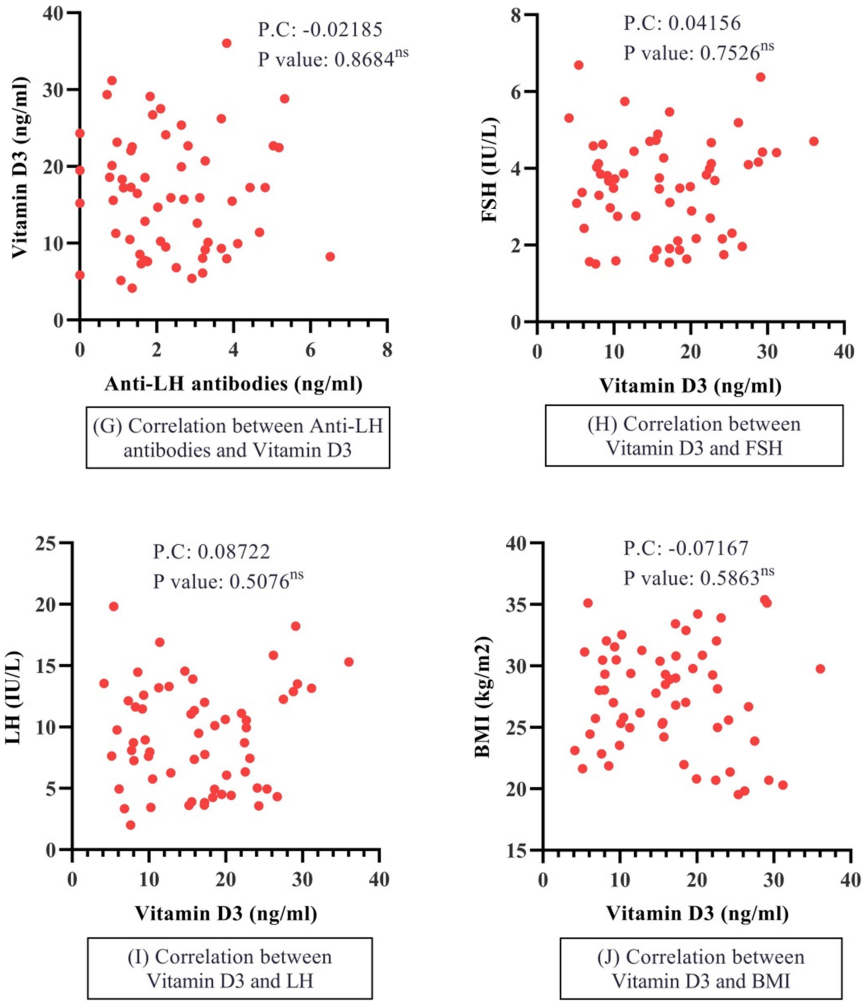


Figure (4-12): Scatter diagrams with varied Pearson's coefficients between two parameters in the sera of PCOS patients. The correlation between two parameters is one in which either as, one value increases, the other tends to increase as well (positive relationship), as shown in (A, B, C, D, E, H&I); or as one value increases, the other value drops (negative relationship), as shown in (F, G &J). The Pearson's correlation offers a value between (−1 and 1) with 0 representing no correlation, in which 1 representing perfect positive correlation, and −1 representing perfect negative correlation (Nettleton, 2014).

CHAPTER FIVE
DISCUSSION

5.1. Discussion

Searching for answers to PCOS-related questions frequently leads to additional queries. Even PCOS experts, many of whom consider this disorder as a medical mystery, believe this. Women face challenges both before and during the diagnosis and treatment. Furthermore, physicians face additional challenges due to the absence of an identifiable cause of the condition, a lack of standardized diagnosis and information on long-term consequences, as well as the multiple phenotypes of PCOS.

For the great significance and diversity of clinical effects of PCOS as a reproductive, metabolic, and psychological disorder that affects a woman's life quality, the present study was undertaken to investigate the role of four important parameters on PCOS including HLA- Class I represented by HLA-B*0702 allele using a molecular approach and also anti-follicle-stimulating hormone antibodies, anti-luteinizing hormone antibodies, and vitamin D3 serum levels using immunoassay methods. In the current study, about 84% of all participants were married for ≥ 1 year. The patients' and healthy controls' mean ages were (25.02±4.135) and (25.8±5.239), respectively, indicating no significant differences between the two groups (P-value > 0.05).The results also show that the majority of the PCOS patients (n =43; 71.67%) was between the ages of 18 and 26, indicating a higher prevalence among younger females which concurs with past research (Ganie et al., 2020, Tabassum et al., 2021).

As previously stated, women with PCOS belong to a younger age group, indicating a higher incidence of PCOS (Rowlands et al., 2016). PCOS has age-related criteria for diagnosis and possible consequences; as female's

ovaries get older, their own androgen may decline. The prevalence of hirsutism and acne, ovarian size and follicles count may decrease with age as well (Hsu et al., 2013). Younger women, on the other hand, experience ovarian failure and hyperandrogenism, whereas metabolic disorders predominate as women get older (Falcetta et al., 2021). Family history, as a reflection of genetic risk, may be seen as a significant factor in assessing the risk of developing PCOS within families. As a result, thirty percent of the patients in this study had a family history of PCOS, while the rest of the patients did not. This figure is lower than the 52% reported by (MUHYADIN et al., 2020).

More than half of the PCOS patients in this study (n=42; 70%) were overweighed or obese. Such a finding is somewhat consistent with that of Sachdeva and others, who revealed that nearly 76% of patients with PCOS seemed to be overweighed or obese (Sachdeva et al., 2019b). The findings were, however, higher than those published by Jabbar Ahmed *et al*, and Saadia, who found that about 62% and 53% of patients, respectively, had high BMIs (Jabbar Ahmed et al., 2020, Saadia, 2020). The mean±SD BMI (27.44±4.302) for patients and (25.79±3.442) for control group differ significantly (P-value < 0.05). Several Iraqi studies found that PCOS women had a higher BMI than the control individuals (Al-Shattawi et al., 2018, Al-Juaifari and Al-Jumaili, 2020). Besides that, (57.5%) of the control group, which included overweight and obese individuals, had high BMIs.

Negative unhealthy lifestyle choices are implicated in our society. Obese women, in particular, are predisposed to PCOS. Obesity is also more common in PCOS women. As a result, it is unclear whether obesity causes PCOS or whether PCOS leads to obesity (Khmil et al., 2020). Furthermore, in this study, primary infertility state was nearly (47%) more usual than secondary infertility status, which comprised approximately (33%). Previous studies demonstrated that the frequency of primary infertility is higher than

secondary infertility and the ovulation problems are among the causes of female infertility, with PCOS being the most frequent cause. In addition, the impacts of lifestyle, age and BMI, nutrition, physical exercise, and stressful employment have recently attracted a lot of attention as contributing factors to infertility in women (Moridi et al., 2019, Deshpande and Gupta, 2019).

In terms of menstrual cyclic pattern, the study found that (95%) of patients experienced irregular periods, including (81.67%) with infrequent periods and (13.33%) with absent periods. Likewise, 93% of PCOS women had irregular menstrual cycles according to a previous study (Kamrul-Hasan et al., 2020) with 75-85% having oligomenorrhea (Harris et al., 2018) and 13.75% of had amenorrhea (Alsaadi and Mohamad, 2019). One of the most obvious symptoms of PCOS is irregular menstruation, which affects some but not all patients. Accordingly, only 5% of the patients in this study had regular menstrual cycles, which is lower than the 28.2% recorded in a previous study (Sidra et al., 2019). Further to that, approximately (93%) of patients in this study met the criterion of mild hirsutism from the top lip, jaw line, and lower abdomen. and around (62%) experienced mild acne. Healthy controls, on the other hand, had no history of hirsutism, but (7.5%) showed mild acne. Previous studies found that 91% of such patients had hirsutism (Najem et al., 2008) and 67.3% had mild acne (Sidra et al., 2019). Hirsutism state exacerbated in a PCOS individual who was overweight or obese (Neubronner et al., 2021).

Hirsutism and acne are both common signs of hyperandrogenism in PCOS, and their incidence is increased due to related hormonal alterations. According to the findings, approximately (77%) of the patients had PCOM on trans-abdominal ultrasound, which is fairly similar to 74% but less than the 89.1% found in other research findings (Najem et al., 2008, Esmaeilzadeh et al., 2014a). PCOS is marked by variations in clinical and hormonal manifestations among different phenotypes (Sachdeva et al., 2019a).

In terms of FSH and LH hormone testing evaluation, FSH mean± SD in both patients and controls in this study was within normal limits recording (3.514±1.272) and (3.846±1.081), respectively, implying no noticeable difference (P-value ≥ 0.05). In contrast, LH mean± SD, varied greatly (P-value < 0.0001) between patients (9.189±4.262) and controls (5.807±2.015). As a result, a substantial increase in LH/FSH ratios was observed in approximately (87%) of patients, which was generally higher than two and deemed to be one of the notable characteristics of PCOS and frequently recommended to support the diagnosis of PCOS (Malini and Roy George, 2018, Mitrašinović-Brulić et al., 2021). This ratio may have hampered follicular development in favor of LH, resulting in follicular atresia and the formation of immature follicles seen in ultrasound figure (Inan and Karadag, 2016). This syndrome is marked by high luteinizing hormone and unusual GnRH pulsatility, whereas FSH stays relatively regular (De Leo et al., 2016).

According to the literature, PCOS women seemed to have a significantly greater LH/FSH ratio than healthy subjects (Lerchbaum et al., 2021). Malini and Roy George discovered that approximately (55-75%) of PCOS women have elevated LH/FSH ratios, whereas Nath and colleagues found an elevated LH/FSH ratio in about 71%, but Esmaeilzadeh et al. discovered no relationship with this ratio (Esmaeilzadeh et al., 2014b, Malini and Roy George, 2018, Nath et al., 2019). Disparities could be explained by variations in sample's size and characteristics, in addition to the techniques used to identify each variable.

Several decades of research have revealed a number of diseases in humans that are markedly more common among those who carry specific HLA alleles, such as autoimmune, inflammation, & cancerous diseases. This phenomenon is also known as HLA-disease association, and the mechanism behind it is still being debated. The HLA system exhibits a high polymorphic and haplotypic inheritance pattern, leading to various ethnic distributions

(Mosaad, 2015). Previous individual studies on the relationship between HLA and PCOS have yielded conflicting results.

The polymorphism of the HLA-B*0702 allele in women with PCOS is one of the study's most significant findings. Sixty out of 100 samples were from women with PCOS. In this group, 52 (86.67%) women carried the HLA-B*0702 allele, while 8 (13.33%) did not. The remaining 40 samples were from healthy control women, 30 (75%) of whom had the mentioned allele and 10 (25%) did not. From this standpoint, the odds ratio (OR: 2.167; CI: 0.8167 to 6.330; p= 0.1847) demonstrates a positive association between the HLA-B*0702 allele and PCOS.

According to the literature, an odds ratio higher than one signifies a higher risk, suggesting that the disorder is more prevalent with an exposure that is harmful in this case, indicating a positive correlation; whereas an odds ratio less than one indicates that the disorder is less common with exposure and hence a protective effect, implying an inverse correlation. Furthermore, an odds ratio of (1) signifies that the exposure has no effect on the outcome probability, implying that no association exists (Szumilas, 2010, Andrade, 2015).

Importantly, previous research confirmed that the presence of the HLA-B*0702 allele in a haplotypes increases the risk of cancer and endometriosis in a significant number of PCOS women (Qiu et al., 2011, Harris and Terry, 2016, Ding et al., 2018a). As a result, this allele was chosen to be assessed in this study since an association between the aforementioned allele and PCOS has not been studied in other parts of the world, and it is likely unique to our geographic region, and thus the HLA-B*0702 genotyping results are a study groundbreaking in the field. Although the association did not reach statistical significance due to the small number of samples, these outcomes will provide insight into the immungenetics bases of PCOS progress and related clinical

features. Additional research with a larger sample size may be required in the future to confirm the association of this allele with PCOS.

A previous study linked PCOS to HLA Class I (HLA-B7 allele) antigens. Aajil's study included a group of Iraqi Arab women with PCOS, and the HLA serological typing method known as “microlymphocytotoxicity” was performed using commercially available kits (Aajil, 2018). The present study focused on women of Erbil city and employed a molecular approach for HLA-typing as more accurate, sensitive, and repeatable method with greater flexibility and precision, however it is an expensive method.

The screening of HLA antigen in diverse communities around the world yielded a variety of outcomes. HLA-A*11, A*31, and B*54 were found to be linked with phenotypic frequencies in Korean women with PCOS when compared to control women (OR: 2.79, 95% CI; 1.07–7.30, OR: 6.05, 95% CI; 1.23–29.85 and OR: 6.40, 95% CI; 1.70–24.09), respectively (Kim et al., 2011, Ahn et al., 2011). In Japanese women, the risk alleles for PCOS were HLA-A*11 and DRB1*0403 (OR: 2.16, 95% CI; 1.09–4.26 and OR: 2.63, 95% CI; 1.15–5.98), respectively (Kaibe et al., 2006). A popular explanation is the distinct HLA distributions among ethnicities, or the different phenotypic features of PCOS, as with many other multi-factorial ailments.

The frequency of HLA gene allelic variants differs by region and is strongly influenced by environmental factors. This variation can be attributed to the function of the linked HLA proteins, whose basic task is to present bacterial and viral antigens to cells of the immune system, triggering the necessary immune response towards the pathogen. This helps to explain why alleles linked to disease susceptibility or protection in one geographical area do not confer a same susceptibility or protection in another (Lavado et al., 2005). The precise mechanisms underlying the relationship between PCOS and HLA alleles are uncertain and remain a source of contention. A theory proposes a direct link between a particular HLA allele and pathogenicity,

whereas another proposes linkage disequilibrium between the accountable gene and the sensitive HLA- allele (Kim et al., 2011). However, more effort is needed to better understand the HLA system and its association with diseases in Middle Eastern populations.

Other important parameters investigated in this study include anti-FSH and anti-LH antibodies. The serum concentrations of antibodies to follicle - stimulating hormone were not markedly different between patients and control subjects, except when patients were classified into primary infertile PCOS patients and secondary infertile PCOS patients, the results revealed significantly greater anti-FSH levels, particularly in primary infertile PCOS patients, compared to secondary infertile PCOS and also healthy subjects (P-value < 0.0001). Antibodies to luteinizing hormone were also significantly greater in PCOS than control subjects (P-value < 0.01).

An earlier study verified the presence of significant concentrations of anti-FSH in women suffering from infertility (Haller et al., 2008). Antibodies to FSH, both naturally occurring and generated by exogenous gonadotropins, have already been identified. It is possible that the immune system has shifted and the antigen involved in their initiation is either blood circulating FSH or seminal fluid in female's body (Morte et al., 2017). It may also be due to a genetic fault in female's genital tract mucosal tolerance that further causes anti-FSH IgG and IgA antibodies to accumulate in the early follicular follicles and prevent its development (Haller-Kikkatalo et al., 2012). Anti-FSH antibodies may block FSH by preventing it from binding with its receptor or by trapping it within immunological complexes (Haller et al., 2008, Kara et al., 2019).

Few studies have shed light on antibodies to both FSH and LH and their connection to PCOS, with noticeable differences when compared to a control group (Hussein et al., 2018, Jabbar Ahmed et al., 2020, Abood and Hathal, 2021). Auto-antibodies against hormones form once their levels

increase above their normal physiological concentrations (Thomas, 2001). In this study, the mean±SD of FSH (3.514±1.272) and LH (9.189±4.262) in PCOS patients were not exceed the critical threshold. Thereby also, the findings support the notion that such antibodies appear naturally in PCOS patients (Haller et al., 2005). Ludwig *et al*. conclude that auto-antibodies are also common in healthy persons and are often not pathogenic in this case (Ludwig et al., 2017). Natural antibodies to FSH were notably observed in patients with endometriosis and PCOS who had never had stimulation via IVF. Anti-FSH (IgG, IgM, and IgA) were also observed in healthy non-pregnant women, though at a lower frequency. Hormonal levels remain below a critical threshold for the activation of autoimmune reactions (Haller-Kikkatalo et al., 2012).

In the current study, the ROC curve and its corresponding AUC were also computed. This was included to demonstrate the link between clinical sensitivity and specificity for possible cut-off values for both ELISA tests. The area under the ROC curve is within the (0.5-1.0) range, with the minimum value indicating a random classifier's efficiency and the maximum value indicating the efficiency of a perfect classifier. Therefore, the greater the area underneath the curve, the better the test distinguishes between healthy and diseased individuals (Doi and Williams, 2013, Habibzadeh et al., 2016). Accordingly, the outcomes of the present study show that anti-LH antibodies, as an immunological diagnostic value (AUC=0.67), can distinguish PCOS patients from healthy subjects (P-value < 0.05). Anti-FSH antibodies, on the other hand, had poor immunodiagnostic value in this case (AUC=0.6167, P-value ≥ 0.05).

Vitamin D3 was another essential factor investigated. Hypovitaminosis D3 was observed in approximately 97% of patients and 75% of healthy controls, with mean serum 25OHD levels of 16.33±7.722 and 22.62±11.47ng/ml, respectively, revealing a significant difference (P-value <

0.01) with the majority of PCOS patients having a deficient to severe vitamin D3 deficiency (n=41; 68.33%). A similar conclusion was reached by previous studies (Elkholy et al., 2018, Khalifa et al., 2021). Thomson *et al*. found that between 67-85% of PCOS patients have vitamin D٣ levels below 20ng/ml (Thomson et al., 2012).

Other studies, observed no difference in 25(OH)D levels between PCOS and healthy individuals (He et al., 2015, Arslan et al., 2019). Vitamin D deficiency is highly frequent in PCOS women and is linked to a variety of symptoms such as insulin resistance, cardiovascular disease risk factors, fertility issues, obesity (Lin and Wu, 2015). Thomson and colleagues also confirmed that serum 25OHD is positively correlated with sex hormone binding globulin (SHBG) and negatively correlated with the degree of hirsutism in PCOS women. Furthermore, low vitamin D3 levels have been linked to calcium dysregulation, which results in follicular arrest in PCOS patients, resulting in menstrual and fertility issues (Thomson et al., 2012). Besides, sunlight exposure, dietary intake, and seasonal fluctuations, are all lifestyle-associated factors (de Santana et al., 2022).

In PCOS patients, Pearson's correlation analysis revealed non-significant negative correlations (P-value ≥ 0.05) between antibodies against FSH and LH and vitamin D3. Vitamin D3 is well-known for its power to regulate the immune system, and its deficiency is thought to be linked to B-cell hyperactivity (Trummer et al., 2018, Miao et al., 2020, Khalifa et al., 2021). Thus, it is regarded as a risk factor in the development of auto – antibodies (Colaris et al., 2017).

The results also indicate that vitamin D3 have no significant correlations with FSH, LH, and BMI. Previous research observed no significant relationship between serum 25(OH)D and both hormonal and the obesity-related factors. This association is most likely caused by volumetric dilution into greater volume of serum, fat, muscular and hepatic tissues.

Obese individuals are less inclined to be exposed to sunlight, resulting in inadequate biosynthesis of 25(OH)D made by the skin. Since obesity is more prevalent in PCOS women, low serum vitamin D concentrations may be the result of obesity rather than the cause. Obesity also has been linked to low 25(OH)D and genes associated with low 25(OH)D (Kim et al., 2014, Kumar et al., 2017, Arslan et al., 2019).

Pearson's correlation analysis also revealed that anti-FSH antibodies and follicle-stimulating hormone was found to have a significant positive correlation (P-value < 0.01), as well as anti-LH antibodies and luteinizing hormone (P-value < 0.05). Furthermore, antibodies against FSH were found to be significantly correlated to those against LH (P-value < 0.0001), indicating a perfect positive linear correlation since the P.C value (0.9863) is close to one (Nettleton, 2014).

It is difficult to explain such results, but it is known that luteinizing hormone, in conjunction with FSH, stimulates follicular growth and ovulation in female and both are secreted at the same site (anterior pituitary gland). In addition, both have very similar structures and their alpha subunits are nearly similar and comprise approximately 96 amino acids, whereas the β-subunits are special per each hormone. Follicle stimulating hormone is made up of 111 amino acids, whereas LH is made up of 120 amino acid subunits that give the specific biologic actions. Because FSH and LH are structurally similar homologous hormones, antibodies targeted directly against one might also cross-react with antibodies directed against the other (Morte et al., 2017). Previously, a strong relationship between anti-follicle stimulating hormone and anti-luteinizing hormone antibodies in PCOS patients was found (Abood and Hathal, 2021).

Anti-FSH and anti-LH antibodies also showed non-significant positive correlations (P value ≥ 0.05) with LH/FSH ratio. Two Iraqi studies discovered the same significant correlation (Hussein et al., 2018, Jabbar Ahmed et al.,

2020). As discussed, this is due to the fact that the increased LH production caused by hypothalamic-pituitary disruption, which causes GnRH disturbance and incorrect LH/FSH ratios, is a key characteristic of all PCOS patients (Nath et al., 2019). In PCOS, estrogen dominance is responsible for an unusual feedback mechanism resulting in increased luteinizing hormone secretion and low progesterone levels that are unable to suppress the GnRH/LH pulse frequency; consequently, autoantibodies production (Mobeen et al., 2016, Lerchbaum et al., 2021).

CHAPTER SIX
CONCLUSIONS, RECOMMENDATIONS AND FUTURE WORKS

6.1. Conclusions

This study came to the following conclusions:

- The HLA-B*0702 allele is considered to be an immunogenetic signifier for PCOS in women. Such findings would provide more information on the immunogenetic basics and clinical characteristics of PCOS in our geographical region, possibly opening the way for large population studies to confirm the association between the HLA-B*0702 allele and PCOS.
- Anti - FSH and anti - LH antibodies were higher in PCOS patients with primary infertility compared to healthy controls and patients with secondary infertility
- Family history of PCOS is considered as a significant factor in assessing the risk of developing PCOS.
- Although patients and healthy individuals in this study experienced a hypovitaminosis D3, PCOS patients were more inclined to develop vitamin D deficiency.
- Hirsutism, menstrual irregularities, and infertility are all common in women with PCOS, and they all pose challenges to the feminin identity. High BMI was also common among patients and the majority of them had elevated LH and LH/SFH ratios with normal FSH levels.

6.2. Recommendations and Future Works

The following points are recommended based on the study's findings:

- Anti-LH antibodies can assist in diagnosis since they showed an acceptable immunodiagnostic value and can distinguish between PCOS patients and healthy individuals, whereas anti-FSH antibodies showed a poor immunodiagnostic value in this study. Although, further studies involving large samples are required to fully comprehend the role of both anti-FSH and anti-LH in this syndrome.
- Public awareness of PCOS is required to help people understand what the disorder is and how it can be treated, as well as to provide support for women diagnosed with the syndrome in efforts to overcome the symptom and limit the effects of its related complications.
- Vitamin D3 detection should be included in PCOS-related tests list.
- Further molecular research on HLAB7-associated alleles is required to determine the risk of PCOS in our geographical region.
- Additional molecular studies on the HLA-B*0702 allele is necessary to determine DNA nucleotide polymorphism using the Restriction enzyme analysis method or genome sequencing using Next-generation sequencing (NGS).
- Future research is required to reveal the history of disease prevalence and to determine the impact of environmental and/or genetic factors.

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APPENDIX I

Screening Questions on the Most Frequent PCOS Symptoms and Other Relevant Information

Age (years): ☐

Height (cm): ☐

Weight (Kg): ☐ BMI (kg/m^2) = ☐

Duration with PCOS Symptoms (years):

Ethnicity:		☐ Kurdish	☐ Others	
Marital Status:		☐ Married	☐ Single	☐ Others
Education:	☐ Illiterate	☐ Primary	☐ Secondary	☐ College
Fertility:	☐ Fertile	☐ Primary Infertility	☐ Secondary Infertility	☐ Single
Menstrual Cycle:		☐ Irregular	☐ Regular	
Ultrasound Findings (PCOM):		☐ Yes	☐ No	
Family History of PCOS:		☐ Yes	☐ No	
Family History of cancer:		☐ Yes	☐ No	
(Endometrial, Breast, Ovarian and Cervical)		☐ Yes	☐ No	
Diabetes:		☐ Yes	☐ No	
High Blood Pressure:		☐ Yes	☐ No	
CVD:		☐ Yes	☐ No	
Unhealthy Cholesterol:		☐ Yes	☐ No	
Endocrine Disease (Thyroid, Adrenal, Cushing):		☐ Yes	☐ No	
Chronic Drug Use:		☐ Yes	☐ No	
PCOS Medication/ VITD supplementation		☐ Yes	☐ No	
Depression and Anxiety:		☐ Yes	☐ No	
Thinning hair (Baldness/Alopecia):		☐ Local	☐ Generalized	
Acne:	☐ Absent	☐ Mild	☐ Moderate	☐ Severe
Body Hairsuitism:	☐ Local	☐ Generalized		
Facial Hairsuitism:	☐ Local	☐ Generalized		
Did you infect with COVID-19:		☐ Yes	☐ No	

APPENDIX II

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ORIGINAL ARTICLE

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HLA-B*0702 class-I allele, anti-FSH, anti-LH, and vitamin D3: Potential links with polycystic ovary syndrome in women of Erbil city, Iraq

Najat Jabbar Ahmed Berwary[1], **Rand Maurice Aziz**[1, *]

[1]Department of Medical Laboratory Technology, Erbil Technical Health and Medical College, Erbil Polytechnic University, Erbil, Iraq

[*]**Corresponding author**
Rand Maurice Aziz
Department of Medical Laboratory Technology, Erbil Technical Health and Medical College, Erbil Polytechnic University, Erbil, Iraq
e-mail: rand.aziz@epu.edu.iq

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ABSTRACT

Polycystic ovary syndrome (PCOS) is a widespread endocrine-reproductive-metabolic condition with severe implications for females' health. The role of four essential parameters on PCOS, including human leukocyte antigens (HLA) represented by the HLA-B*0702 allele, anti-follicle stimulating hormone (anti-FSH) antibodies, anti-luteinizing hormone (anti-LH) antibodies, and vitamin D3 was investigated. A total of 100 samples were collected from Kurdish women attended the maternity teaching hospital and some private hospitals/laboratories in Erbil City from October 2021 to January 2022. The samples were genotyped using a PCR-based technique with specific sequence primers. The levels of anti-FSH and anti-LH antibodies were determined using an enzyme-linked immunosorbent assay (ELISA), while vitamin D3 levels were measured by an electrochemiluminescence (ECL) test on Cobas e411 immunoassay system. The odds ratio (OR) of 2.167 at a 95% confidence interval (CI) of 0.8167 to 6.330, indicated an essential link between the HLA-B*0702 allele and a risk of PCOS. Anti-FSH and anti-LH antibodies were significantly greater in PCOS patients, notably infertile women, than in healthy controls. A significant positive linear correlation was observed between antibodies against FSH and LH in patients. Most patients had hypovitaminosis D3 with a significant difference compared to controls. The results indicate that the HLA-B*0702 allele is associated with PCOS susceptibility and could be used as an immunogenetic marker. Besides, it supports the idea that anti-FSH, and anti-LH antibodies are naturally presence antibodies in PCOS patients instead of signs for autoimmunity. It also suggests that women suffer from PCOS are more prone to develop vitamin D3 deficiency.

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is a quite multifactorial disease in which genetic susceptibility, socio-economic status, ethnicity, metabolic and lifestyle, besides inflammatory and immunological responses, are all implicated and interact [1]. PCOS affects females of childbearing age and causes an abnormality in a female's reproductive hormones, which can lead to ovarian issues. Despite the widespread prevalence of PCOS and its negative effect on women's health, the exact cause is unknown [2]. One of the implicated immunogenetic factors is the major histocompatibility complex (MHC), also known as HLA system in human. The HLA is a vital component of the human immune system which is controlled by a gene on chromosome 6. They are highly polymorphic, with several distinct alleles that allow them to fine-tune the immune system [3]. Some of HLA alleles raise the likelihood of contracting certain diseases and their variance may emphasize the importance of HLA in imparting an immunogenetic proclivity to produce ovarian cysts [4, 5].

In a variety of situations, the ovaries may become the victim of an autoimmune attack, releasing autoantibodies that destroy healthy cells and molecules due to insufficient progesterone levels and an inability to manage the frequency of GnRH/LH pulses, resulting in excess estrogen production [6, 7]. Anti-FSH antibodies were found in high concentrations in infertile women [8]. Such antibodies may interfere with FSH activity

پوختە

فرەکیسی هێلکەدان (PCOS) وەک کێشەیەکی تەندروستی جیهانی سەیر دەکرێت چونکە هۆکار و دەستنیشانکردنی تا ئەمڕۆ زۆرێک لە توێژەرانی سەرلێشێواو کردووە. تێگەیشتن لە دابەشبوونی جیهانی و تایبەتمەندییە فینۆتایپییەکانی PCOS ئاڵۆز و چالاکە لەبەرئەوەی هۆکارە ژینگەییەکان و گۆڕانکارییە نەتەوەییەکان دەتوانن کاریگەری زیاتریان لەسەر ئەم حاڵەتە کلینیکییە هەبێت. ئەم توێژینەوەیە ئامانجی لێکۆڵینەوەیە لە کاریگەری چوار پارامێتەری گرنگ لەسەر PCOS لە ژنانی شاری هەولێر، لەوانە دژە جەستەی لیکۆسایتی مرۆڤ (HLA) کە بە ئالیلی HLA-B*0702 نوێنەرایەتی دەکرێت، دژەتەنی هۆرمۆنی هاندەری دژە فۆلیکۆل (دژە-FSH)، هۆرمۆنی دژە شلکردنەوە دژەتەنی (دژە-LH)، و ڤیتامین D3 لەم بوارەدا وەک ڕۆمان سەیر دەکرێت. بۆ ئەم مەبەستە سەد نمونەی خوێن بۆ تایبەتمەندکردنی گەردیلەیی و نمونەی سیرۆم بۆ شیکاری پارامیتەرەکانی بەرگریزانی (٦٠ نەخۆشی PCOS و ٤٠ کۆنترۆڵی تەندروست) وەرگیرا کە لە نەخۆشخانەی ئافرەتان و لەدایکبوونی فێرکاری هەولێر و هەندێک کلینیک و نەخۆشخانەی تایبەت لە شاری هەولێر لەنێوان ئۆکتۆبەری ٢٠٢١ و کانوونی دووەم ٢٠٢٢. دوای دەرهێنانی DNA، هەموو نموونەکانی DNA ی دەرهێنراو بە بەکارهێنانی ڕێگەیەکی بنەمادار بە PCR لەگەڵ پرایمەری تایبەت جینۆتایپ کران، هەروەها ئاستی سیرۆمی دژەتەنی (دژە-FSH) و (دژە-LH) بە بەکارهێنانی (ELISA) دیاری کرا، لە کاتێکدا هەڵسەنگاندنی ڤیتامین D3 بە بەکارهێنانی ئامێری Cobas e411 ئەنجامدرا.

دەرئەنجامەکانی توێژینەوەکە، بە ڕێژەی ئەگەرەکان 2.167 (OR) لە نێوان 0.8167 بۆ 6.330 متمانەی (CI) 95%، پەیوەندییەکی بنەڕەتی لە نێوان ئالیلی HLA-B*0702 و مەترسی تووشبوون بە PCOS نیشان دا. دژەتەنەکان دژی ئاستی سیرۆمی FSH و LH لە نەخۆشانی PCOS بە شێوەیەکی بەرچاو بەرزتر بوون، بە تایبەتی لە ژنانی نەزۆکدا، لە چاو کەسانی کۆنترۆڵ. ئەم جۆرە دژەتەنانە پەیوەندییەکی هێڵی ئەرێنی بەرچاویان هەیە (P-value < 0.0001) .

سەرەڕای ئەوە، دژەتەنی هۆرمۆنی هاندەری دژە فۆلیکۆل پەیوەندییەکی ئەرێنی بەرچاویان لەگەڵ FSH نیشان دا، هەروەک چۆن دژەتەنی هۆرمۆنی دژە لوتینیزکردن لەگەڵ LH کردیان (P-value < 0.01) بەڵام، هەردووکیان پەیوەندی ئەرێنی نابەرچاویان لەگەڵ ڕێژەی LH/FSH و هەروەها پەیوەندی نەرێنی نابەرچاو لەگەڵ ڤیتامین D3 نیشان دا (P-value ≥ 0.05).

کەمبوونەوەی ڤیتامین D3 لە زۆربەی نەخۆشانی PCOS و کۆنترۆڵە تەندروستەکاندا بە جیاوازییەکی بەرچاوەوە بینرا (P-value < 0.01). بەپێی دۆزینەوەکان، ئالیلی HLA-B*0702 پەیوەندی بە ئامادەیی PCOS هەیە و دەتوانرێت وەک نیشاندەری بەرگری جیناتی بەکاربهێنرێت. هەروەها ئەنجامەکان پشتگیریان لەو بیرۆکەیە کرد کە دژەتەنەکانی دژی هۆرمۆنی هاندەری فۆلیکۆل FSH و هۆرمۆنی لوتینیزەکردن LH بە شێوەیەکی سروشتی بوونیان هەیە لە نەخۆشانی PCOS دا دۆزراونەتەوە نەک نیشانەکانی نەخۆشی بەرگری خۆکار. سەرەڕای ئەوەش ئەو ژنانەی کە تووشی PCOS بوون زیاتر مەیلیان بۆ تووشبوون بە کەمی ڤیتامین D3 هەیە.

ئەلیلی HLA-B*0702 Class-I وەژە تەنی Anti-FSH و Anti-LH و ڤیتامینی D3: بەستەرە شاراوەکان لەگەڵ نەخۆشی ڤرەکیسی هێلکەدان PCOS لە ژنانی شاری هەولێر لە عێراق

نامەیەکە

پێشکەشی ئەنجومەنی کۆلێژی تەکنیکی تەندروستی و پزیشکی هەولێر کراوە لە زانکۆی پۆلیتەکنیکی هەولێر وەکو بەشێك لە پێداویستیەکانی بەدەست هێنانی پلەی ماستەر لە شیکاری نەخۆشییەکان

لە لایەن

رند موریس عزیز

بەکالۆریۆس لە زانستی بایۆلۆجی-زانکۆی بغداد

بە سەرپەرشتیاری

پ.ی.د. نجاة جبار احمد بەرواری

هەولێر-کوردستان

شوبات ٢٠٢٣