Arab American University
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Screening for Potential Genetic Factors that Cause Polycystic Ovary Syndrome in a Cohort of Palestinian Women

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Declaration

I declare that, except where explicit reference is made to the contribution of others, this thesis is substantially my own work and has not been submitted for any other degree at the Arab American University or any other institution.

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Dedication

I wholeheartedly dedicate this study to my role model, my father Rawhi Fattouh, who always taught me to never give up, to my mother, Adila Al-Khalidi, my eternal source of inspiration, thanks to whom I am here, and to my brothers for their endless love.

To the one I learn from daily and the constant source of my joy and God's grace, my son Mohammed Hamdan.

To my beloved ones and those close to my heart who are always supportive in every step I take in my life journey.

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To my beloved homeland and its righteous martyrs and brave detainees.

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Abstract

Polycystic ovary syndrome (PCOS) is the most common endocrinological disorder that affects women during their reproductive age. It is the leading cause of hyperandrogenism and anovulatory infertility in approximately 90% of cases. The complexity of PCOS is that it is highly heterogeneous, polygenic, and multifactorial, and it is accompanied by several comorbidities, including metabolic disorders, insulin resistance, type 2 diabetes mellitus, glucose intolerance, hypertension, and cardiovascular disorders. The prevalence of PCOS is constantly increasing, depending on ethnicity, and its etiology is believed to be rooted in environmental and genetic factors. However, its inheritance pattern is difficult to explain due to various relevant genes. Increasing PCOS cases are recorded among Palestinian females with a severe lack of genetic information. This study aimed to identify potentially pathogenic genetic variants associated with PCOS and to measure the frequency of the disease among Palestinian women who are attending IVF gynecological clinics for assisted reproductive techniques. Five women diagnosed with PCOS, according to Rotterdam Criteria, were recruited for the study. Whole-exome sequencing was conducted on five probands to identify relevant genetic variants linked to familial PCOS, followed by familial segregation analysis. In-silico-predicted tools were then employed to assess the potential deleterious effects of the identified variants. PCOS frequency was evaluated among a cohort of 200 patients from different regions of Palestine who attended IVF centers following specified diagnostic criteria. The data revealed from the five indicated families revealed 13 heterozygous variants in different genes, namely CYP21A2, LDLR, MCM6, IKBKB, STOX1, MC4R, IRS1, IRS2, PON1, and FBN1. In-silico analysis revealed four uncertain significance variants and seven pathogenic variants. The results suggested a possible association between LDLD (Arg595Trp), FBN1 (Gly1058Ser), PON1 (Met127Arg), STOX1 (Ile186Thr), MC4R (Glu308Lys), IRS1 (Ser564Asn, Arg1221Pro, Gly669Cys) and IRS2 (Ser66fs*27) polymorphisms and PCOS pathogenesis. Several studies reported the association of genetic factors with PCOS pathogenesis. However, these results are still controversial.

Keywords: Polycystic ovary syndrome, comorbidities, Insulin receptor substrate, polymorphisms, follicular atresia.

Table of Contents

List of Tables

List of Figures

List of Definitions of Abbreviations

Abbreviations	Title
17-OHP	17-Hydroxyprogesterone
5'-UTR	5'-Untranslated region
5α-DHT	Dihydrotestosterone
ACMG	American College of Medical Genetics
AE-PCOS	Androgen Excess and PCOS Society
AHD	Atherosclerotic heart disease
AMH	Anti-Mullerian Hormone
AR	Androgen Receptor
ART	Assisted Reproductive Technologies
ASRM	the American Society for Reproductive Medicine
BDRR	Big Dye Terminator
BMI	Body Mass Index
bp	Base pair
C4	Complement component 4 protein
CAH	Congenital Adrenal Hyperplasia
cAMP	Cyclic Adenosine Monophosphate
CAT	Catalase
Chr	Chromosome
COBALT	Constraint-based Multiple Alignment Tool
CRE WHiRL	the Center for Research Excellence in Women's Health in Reproductive Life
CRP	C-reactive protein
CVDs	Cardiovascular Diseases
CYP17A1	Cytochrome P450 family 17 subfamily A member 1
CYP21A2	21-Hydroxylase (cytochrome P450 family 21 subfamily A member 2
DENND1A	DENN domain-containing protein 1A
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone Sulfate
DNA	Deoxyribonucleic acid
DNAJC27	DnaJ Heat Shock Protein Family (Hsp40) Member C27
EDCs	Endocrine-disrupting chemicals
EDGC	Eone Diagnomics Genome Center
ESHRE	the European Society of Human Reproduction and Radiology
F	Family
FATHMM	Functional Analysis through Hidden Markov Models
FBN1	Fibrillin-1
FFA	Free Fatty Acids
FSH	Follicle-stimulating Hormone
FSHR	Follicle-stimulating Hormone Receptor
FT	Free Testosterone
FTO	alpha-ketoglutarate-dependent dioxygenase
G, P, A, E	Gravida, Para, Abortion, Ectopic pregnancy

GATA	GATA Binding protein 4
GAWS	Genome-wide association Studies
GBD	Global Burden of Disease
Gn-RHR	Gonadotropin-Releasing Hormone Receptor
GPA	Gravida Para Abortus terminology
GVGD	Grantham Variation, Grantham Difference
HA	Hyperandrogenism
Hcy	Hyperhomocysteinemia
HDL	High-Density Lipoprotein
Het	Heterozygous
HNF-4α	Hepatocyte Nuclear Factor-4 alpha
HPO	Human Phenotype Ontology
IGT	Impaired Glucose Tolerance
IHME	the Institute for Health Metrics and Evaluation
IKBKB	Inhibitor of nuclear Kappa B Kinase Subunit Beta
IL6	Interleukin 6
INSR	Insulin Receptor
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
IVF	In-Vitro Fertilization
LDL	Low-Density Lipoprotein
LDLR	Low-density lipoprotein receptor
LH	Luteinizing Hormone
LHCGR	Luteinizing hormone/Choriogonadotropin Receptor
LTBP1	Latent transforming growth factor beta binding protein 1
MC4R	Melanocortin-4 Receptor
MCM6	Minichromosome Maintenance Complex Component 6
MENA	Middle East and North Africa
MetS	Metabolic Syndrome
MNCs	Mononuclear Cells
mRNA	messenger Ribonucleic acid
N/A	Not available
NCAH	Non-Congenital Adrenal Hyperplasia
NCBI	National Center for Biotechnology Information
NCEP	the National Cholesterol Education Program
NEIL2	Nie Like DNA Glycosylase2
NGS	Next-generation sequencing
NIH	National Institute of Health
NMD	Nonsense-mediated decay
non-HDL-C	non_High Density Lipoprotein Cholesterol
non-LDL-C	non_Low Density Lipoprotein Cholesterol
NTC	No-template Control

OMIM	Online Mendelian Inheritance in Man
OS	Oxidative Stress
OSA	Obstructive Sleeping Apnea
PCOM	Polycystic Ovarian Morphology
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type9
PolyPhen	Polymorphism Phenotyping
PON1	Paraoxonase-1
PRL	Prolactin
PROVEAN	Protein Variation Effect Analyzer
QD	Qual score normalized by allele depth (AD)
ROS	Reactive Oxygen Species
SHBG	Sex Hormone Binding Globulin
SIFT	Sorting Intolerant from Tolerant
SLE	Systemic Lupus Erythematosus
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variation
SORBS1	Sorbin and SH3 domain containing 1
STOX1	Storkhead box 1
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TGFβ	Transforming-growth factor Beta
TNFα	Tumor Necrosis Factor-alpha
TNXA	Tenascin-X, A
TNXB	Tenascin-X, B
UTRs	Untranslated Regions
VAT	Visceral Adipose Tissue
VCF	Virtual Contact File
VDR	Vitamin-D Receptor
WES	Whole Exome Sequencing
WGS	Whole Genome sequencing
WHO	the World Health Organization
YAP1	Yes-associated protein 1
YLDs	Years lived with disability

Chapter One: Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrinological disorder that affects reproductive-aged women, with a global prevalence between 5.6%-21.3% in adult reproductive women and 6% among teenagers, depending on the studied population and the used definitions. (Hossain et al., 2022; H. J. Teede et al., 2018). PCOS is recognized as a heterogenic reproductive and metabolic endocrine disorder and one of the leading causes of infertility. It is considered a complex and multifactorial disease that has many adverse effects on reproductive, metabolic, and psychological health in women. (Adam et al., 2022).

The main characteristics of PCOS are hyperandrogenism, oligomenorrhoea, anovulation, and accumulation of small antral follicles. (Hossain et al., 2022). Morphological changes on ultrasound are accompanied in 90% of women with PCOS, in which the ovaries appear enlarged with increased cysts on the surface accompanied by polycystic ovarian morphology (PCOM). The excess biosynthesis of androgens leads to increased number of follicles, theca interna, and an increase in the proliferation of granulosa cells. In addition, a thickened capsule layer and drastic elevation in the number of small and immature antral follicles are arrested at the cell cycle stages of folliculogenesis, particularly at the pre-antral or early antral stages of development are observed (Jonard & Dewailly, 2004; Kumariya et al., 2021). In contrast, few dominant follicles undergo selection processes. (Bhimwal et al., 2023; Gu et al., 2022; Jones & Goodarzi, 2016; Saud et al., 2020; Yen et al., 2004). These features lead to infertility in PCOS patients because they usually have poor-quality oocytes and embryos, especially when synchronized with metabolic syndrome. (Alhilali et al., 2022). About three-quarters of all anovulatory infertility cases are diagnosed as PCOS, which also causes 90% of hirsutism. (Franks et al., n.d.).

Most patients with PCOS suffer from many symptoms, like hirsutism and acne, due to hormonal imbalance and high levels of free testosterone (FT) (Jones & Goodarzi, 2016), sleep apnea, systemic chronic inflammation, and metabolic disorders, including obesity, dyslipidemia, impaired glucose tolerance, and insulin resistance (IR), which increase the risk of type two diabetes mellitus (T2DM), hypertension, and abdominal fats (Crespo et al., 2022). Psychological conditions, such as depression, negative body image, poor self-esteem, and decreased life quality complement these changes. Moreover, PCOS women are more susceptible to developing cardiovascular diseases (CVD) since they have

decreased antioxidants and increased oxidative stress (OS), which plays a crucial role in pathogenesis of CVDs (Mallappa Bannigida et al., n.d.). Since PCOS is a pre-oxidant state and stress mediators contribute to the development of IR, HA, and dyslipidemic tendencies, OS may have a role as a co-mediator in exacerbating inflammatory milieu in PCOS patients (Jeelani, Ganie, Masood, et al., 2019).

1.1 History

Polycystic ovary syndrome (PCOS) is also called Stein-Leventhal syndrome, according to two American scientists and gynecologists, Irving F Stein and Michael L Leventhal, whose names have been attached to the disease condition since 1935, when they defined the disease in a small group of women with problems in anovulation, infertility, obesity, hirsutism, and oligomenorrhoea. (Mohamed-Hussein & Harun, 2009).

Most of those studied cases had polycystic ovaries utilizing ultrasonic devices as diagnostic tools. Diagnosis was confirmed depending on the number of follicles in one ovary (12 or more follicles with 2-9mm in diameter) or on the ovarian size, which was at least 75% of the uterine size. At the same time, they excluded sizes that were less than 25% of the uterus. (Atoum et al., 2022; Nicolaides et al., 2020).

Cooper *et* al. demonstrated the genetic basis of PCOS in 1968 when early studies on several families reported an increased prevalence of PCOS among the proband's siblings, which ranges between 51% and 66% in first-degree relatives. (Jones & Goodarzi, 2016).

1.2 Complexity

The complexity of PCOS lies in the fact that it is a multifactorial and heterogeneous disease with a wide range of symptoms and comorbidities that vary from one patient to another. Several factors tend to influence the complexity of the disease, including genetic, hormonal, and environmental factors. (Adam et al., 2022).

1.3 Diagnosis

The diagnosis of PCOS was controversial, with many patients have been misdiagnosed. In order to diagnose PCOS properly, gynecologists should exclude any other endocrinopathies that share the same symptoms as PCOS. In addition, they should rule out ovulatory dysfunction from other causes, like pregnancy, hyperprolactinemia, and hyperthyroidism. Some diseases that could be misdiagnosed with PCOS are Cushing's

syndrome, androgen-producing tumors, nonclassical adrenal hyperplasia, and drug-induced androgen excess (Dumesic et al., 2015).

1.4 Differential Diagnosis and Diagnostic Criteria

Because PCOS is severely heterogeneous, it was crucial to establish specific criteria to help in diagnosis and treatment. PCOS diagnosis is based on the presence of two or more of the three main criteria: ovulatory dysfunction, PCOM, and clinical/biochemical hyperandrogenism (Revised 2003 Consensus on Diagnostic Criteria and Long-Term Health Risks Related to Polycystic Ovary Syndrome the Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, n.d.). These criteria helped classify the disease into different phenotypes, as described in Table 1.

It is worth mentioning that the Rotterdam criterion, proposed in 2003 by the European Society of Human Reproduction and Radiology (ESHRE) and the American Society for Reproductive Medicine (ASRM), is widely utilized by most gynecologists and other healthcare personnel. The revised NIH workshop also suggested it in 2012 (H. Islam et al., 2022). These criteria excluded other endocrine disorders that exhibit similar symptoms as in PCOS, which may lead to misdiagnosis, like thyroid dysfunctions, hyperprolactinemia, hypogonadotropic hypogonadism, androgen-producing tumors, Cushing's syndrome, and non-congenital adrenal hyperplasia (NCAH) (H. Islam et al., 2022).

Table 1.1 The Main Four Diagnostic Guidelines for PCOS Diagnosis in Adult Women.

Diagnostic guideline	**Clinical presentation**			**Practical notes**
	Clinical and biological hyperandrogenism	**Oligo-anovulation**	**Polymorphic ovarian morphology**	
National Institute of Health (NIH) 1990	+	+	N/A	Requires both traits and excludes other etiologies.
Rotterdam criteria 2003	+/-	+/-	+/-	Requires 1 out of 3 traits and excludes other etiologies

Androgen Excess and PCOS Society (AE-PCOS) 2006	+	+/-	+/-	It requires hyperandrogenism with one or both of the other and excludes other etiologies.
Revised NIH 2012	+/-	+/-	+/-	Requires 2 out of 3 traits and excludes other etiologies. Needs phenotypic characteristics: a) All three traits. b) Hyperandrogenism+ PCOM. c) Oligo-anovulation+ PCOM.

1.5 Phenotypes and Classification

PCOS classification into different phenotypes depends on the main features that appear in each PCOS woman. This classification helps establish a precise diagnosis and manage the proper treatment protocol. Ultrasound classifies it into four phenotypes (Table 2). Phenotypes A and B are the most severe, with the highest metabolic risks and more evidence of menstrual dysfunction (Moolhuijsen et al., 2022). Phenotype A is the most frequent among PCOS women (44%-65%), followed by phenotype B (8%-33%), phenotype C (3%-29%), and phenotype D (0%-23%) (Khan et al., 2019).

Table 1.2 PCOS Classification into Four Phenotypes

PCOS Phenotypes	Name	Traits
A	Classical PCOS	Hyperandrogenism + Ovulatory dysfunction + PCOM
B	Classical PCOS	Hyperandrogenism + Ovulatory dysfunction
C	Ovulatory PCOS	Hyperandrogenism + PCOM
D	Non-hyperandrogenic PCOS	Ovulatory dysfunction + PCOM

1.6 Heritability

PCOS appears to have a vital heritable component. Early family studies investigated the increasing prevalence of PCOS traits among the proband's siblings as an indication of the mode of inheritance as autosomal dominant. About 60% to 70% of daughters born to women with PCOS have 5-fold increasing risk of manifesting the disease in their later reproductive age (Mimouni et al., 2021). Some studies suggested the heritability of a single dominant gene with high penetrance of particular gene mutation (Urbanek et al., 1999).

Previous in vivo and in vitro studies estimated the heritability percentage of PCOS to be between 38% and 71%, highlighting the genetic pattern of PCOS and suggesting that it is a polygenic disorder since several genes and polymorphisms were explored as susceptible causes of PCOS. (X. Li et al., 2022; Zhang et al., 2023). Genome-wide association Studies (GAWS) helped identify the genetic loci associated with the development of PCOS. The first two gene loci were identified in Han Chinese women and replicated in European women. (Goodarzi et al., 2012). These studies suggested ancient evolutionary traits among ancestries.

1.7 Prevalence and Ethnicity of PCOS

PCOS is a common disorder in many ethnic groups. PCOS phenotypic expression recorded many ethnic variations in different populations, including Americans, Africans, Europeans, Latinas, Caribbean Hispanics, Asians, and Middle East women (Ladson et al., 2011). Prevalence and symptoms also vary significantly among women of different ethnicities. For example, Hispanic women are more prone to the risk of metabolic syndrome (MetS) and type 2 diabetes millets (T2DM), while African women are more prone to have CVD and hypertension. (Ehrmann et al., 2006). However, these variations are insignificant in the younger population, which requires more expanded investigations (Dumesic et al., 2015).

In the last few years, the prevalence of PCOS has increased globally. It is evaluated by the diagnostic criteria that have been applied and by the variability of ethics and the phenotypes of the disease among individuals (Douma et al., 2019). It varies between 4% to 20% according to various subpopulations under the influence of genetic and environmental variabilities (Heidarzadehpilehrood et al., 2022).

According to the National Institute Criteria (NIH) 1990 and revised NIH 2012, PCOS affects 6% to 10% of women worldwide, while according to the World Health

Organization (WHO), it affects 3.4% of women worldwide in 2012, from 12% to 15% according to AE-PCOS 2006 criteria, while ESHRE/ASRM 2003 criteria estimated the worldwide prevalence between 6% to 21%. Depending on a combination of different diagnostic criteria for PCOS, it was found that the prevalence of the disease in Caucasians, Chinese, African, and Middle Eastern women is 5.5%, 5.6%, 6.1%, and 6.1-16%, respectively. (Atoum et al., 2022; Dhar et al., 2022).

1.8 Family Segregation in Familial Polycystic Ovaries

Family studies are critical for understanding the phenotype spectrum and identifying genetic susceptibility to the disease by following narrower diagnostic criteria to diagnose the affected individuals. (Revised 2003 Consensus on Diagnostic Criteria and Long-Term Health Risks Related to Polycystic Ovary Syndrome the Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, n.d.). The genetic basis of PCOS is getting more apparent with the development of many family studies, although the mode of inheritance has yet to be promptly affirmed. Several studies have been carried out and reported many candidate genes that have a vital role in the etiology of the disease, including mutation-analysis studies, linkage studies, family segregation studies, and case-control studies. The data from these studies are not indecisive since they have not been exceptionally replicated. Complex diseases like PCOS usually face difficulties improving gene contribution due to other environmental factors, genetic heterogeneity, and multiple etiologies. (Urbanek et al., 1999).

Several studies on family members of PCOS probands elucidated familial clustering of the disease's reproductive traits consistent with genetic susceptibility driving such features. For example, premature balding was used to allocate the status of the affected males in the family of the probands, and they found a dominant mode of inheritance. (Carey' et al., 1993). The study reported a 51% rate for PCOM or male-pattern baldness in first-degree relatives, suggesting a single gene could be responsible for male-pattern baldness in male relatives and hirsutism and oligomenorrhea in PCOS women. (Carey' et al., 1993). High levels of androgens affect up to 40% of reproductive-aged sisters, and the brothers and sisters of PCOS probands have hyperandrogenism, suggesting that high levels of testosterone in PCOS women are monogenic traits within families, in addition to the metabolic features of the syndrome that appeared to be present in both females and males of the family. However, these studies need to be more comprehensive due to the

small sample size. (Dapas & Dunaif, 2022). A previous survey of 29 PCOS women with ten control women reported that 22% of male first-degree relatives had early onset male-pattern baldness. In contrast, 61% of female first-degree relatives were affected. (Piperi, n.d.). When PCOS diagnosis is absent while there is a single-component phenotype, it means that there is an apparent phenotypic heterogeneity among patients with solid evidence of the presence of a genetic basis, depending on the fact that the proband's sisters had inherited some of the genetic factors but not all traits of the disease acquiring a partial phenotype. (Jones & Goodarzi, 2016).

1.9 PCOS Comorbidities

There are strong associations between PCOS and many comorbid diseases like cardiovascular disorders (CVDs), impaired glucose tolerance (IGT), gestational diabetes, abortions, type 2 diabetes Mellitus (T2DM), and many others. Many of these metabolic and reproductive problems seem to be due to an intrinsic feature of PCOS-insulin resistance (IR) (H. Islam et al., 2022).

1.9.1 Cardiometabolic Events

The most distinguishable events in PCOS. It is believed that this event is linked with dysglycemia resulting from insulin resistance, which is characteristic of PCOS. Impaired glucose tolerance (IGT), dyslipidemia, hypertension, obesity, metabolic disorders, and a sedentary lifestyle are the most famous manifestations of cardiometabolic risk factors. (H. Islam et al., 2022).

1.9.2 CVDs

Several investigations revealed strong relationship between PCOS and CVDs. Early studies elucidated that dyslipidemia, chronic low-grade inflammations, and increased oxidative stress (OS) are the critical risk factors that affect CVDs risk in PCOS patients. However, the clinical manifestations of CVDs are insufficient, and more investigations are needed (Perovic Blagojevic et al., 2022).

1.9.3 Impaired Glucose Tolerance (IGT)/ Type2 Diabetes Miletus (T2DM)

Previous studies reported an independent prevalence of T2DM and IGT among PCOS patients, and it got worse with increased body mass index (BMI) (H. Islam et al., 2022).

1.9.4 Dyslipidemia and Obesity

Dyslipidemia is a CVD risk factor in PCOS women. 70% of PCOS women were known to have dyslipidemia according to the National Cholesterol Education Program (NCEP) report, since they recorded high levels of lipids, mainly low-density lipoprotein (LDL), high-density lipoprotein (HDL), non-HDL cholesterol (non-HDL-C), LDL-C, and triglycerides (TG). It was observed that HDL-C and TG were higher among obese PCOS women, indicating a relationship between PCOS and obesity (Wild et al., 2011). Evidently, it becomes crucial to suggest guidelines for diagnosing PCOS patients, including assessing the lipid profile (H. J. Teede et al., 2018).

Obesity has always been linked with the detection of PCOS, especially in severe cases, and it plays a vital role in CVD and infertility events. Measuring BMI in patients has always been essential for diagnosis and treatment processes. There is a conception that improved an opposite causality of obesity that PCOS itself increases the susceptibility to gain weight. Solid evidence of the causal relationship is unavailable. (Batarfi et al., 2019).

1.9.5 Obstructive Sleeping Apnea (OSA)

OSA is a chronic sleeping disorder accompanied by a disruption in the function of the upper airway, hypoxia, and an erratic sleeping pattern. Previous meta-analysis studies demonstrated a positive correlation between OSA and PCOS. (Kahal et al., 2020). It is linked to modified sympathetic activity, heart rates, and blood pressure, possibly leading to cardiovascular diseases. (Aul et al., 2000).

1.9.6 Endometrial Cancer

Although several factors and morbidities influence endometrial cancer, it was noticed by early investigations that it rises by 2-6 times in PCOS women. This is probably due to the anovulatory cycles and the endometrium being continuously exposed to excessive flux of Estrogen. (Charalampakis et al., 2016). Meanwhile, PCOS women have significant decrease in progesterone levels. Consequently, the high levels of Estrogen with the absence of progesterone contribute to the development of cancer. (Tian-Min et al., 2022).

1.9.7 Infertility

There is a distinguished link between PCOS and gestational complications, some of which include gestational diabetes, preeclampsia, and recurrent miscarriages. Part of

fertility problems in PCOS women are ramifications of already existing endocrinological and metabolic events, such as hyperandrogenism and high BMI. Several studies also showed atypical newborn anthropometrics in children of PCOS women, so they suggested particular pre-conception screening guidelines for PCOS women (H. Islam et al., 2022).
Moreover, PCOS is characterized by multiple cystic follicles, which have a decreased number of granulosa cells and an increased number of steroidogenic cells in theca interna. This means that in the PCOS case, there are proliferation-differentiation abnormalities in both granulosa layers and theca interna, leading to hormonal changes in the patient's ovary, which cause the development of many cysts in the ovarian stroma. (Bhimwal et al., 2023). Anovulation is also one of the most common features of PCOS, in which the oocyte is not released due to hormonal imbalance, where the decreased levels of Progesterone hormone and Estrogen hormones act as unopposed, leading to endometrial hyperplasia (an overgrowth of the uterus lining). In addition to infertility, some PCOS patients who can get pregnant suffer from complications. It increases the risk of infant mortality rates and recurrent abortions in the first trimester of pregnancy, which could reach 44% of PCOS cases. This is due to the shallow levels of Progesterone and the high levels of Androstenedione, Dehydroepiandrosterone (DHEA), and Testosterone. (Bhimwal et al., 2023; Glueck et al., n.d.; Jeelani, Ganie, Amin, et al., 2019; Saud et al., 2020).
A previous study on animal modeling that has been induced with a high-fat diet demonstrated that PCOS increases follicular wall thickness, and infertility is also associated with dyslipidemia. (Liu et al., 2019).

1.10 Pathogenesis of PCOS

PCOS pathogenesis needs to be better elucidated due to the comorbid diseases and the multiple factors influencing the disease, its severity, and the variety of its phenotypes. Therefore, more investigations are needed to understand the disease's molecular mechanisms.
Hyperandrogenism is one of the main clinical features of PCOS, which occurs due to elevated levels of testosterone, dehydroepiandrosterone sulfate (DHEAS), and androgen, leading to ovarian dysregulation and irregular adrenal steroidogenesis. The high levels of androgen in the follicular fluid block the development of these follicles, causing atresia (follicle growth arrest). The second feature is metabolic dysfunction due to insulin resistance and hyperinsulinemia. (Tian-Min et al., 2022). IR and hyperinsulinemia are

key factors that change metabolisms and function of androgen (Alkhuriji et al., 2021). Oxidative stress (OS) has been considered to be associated with PCOS pathogenesis. Hyperandrogenism, insulin resistance, and obesity features play an essential role in developing OS, which, in turn, worsen these metabolic problems. OS is characterized by excess reactive oxygen species (ROS) and an imbalance between oxidants and antioxidants. According to many researches, women with PCOS have far higher levels of oxidative circulating markers than healthy women, and these indicators are thought to be able to trigger PCOS pathophysiology. The risk of malignant transformation and DNA damage to ovarian epithelial cells is increased when OS occurs during multiple ovulations (Alkhuriji et al., 2021). ROS and free radicals are required in many stages of female reproduction. Still, elevated levels of them are linked with infertility and reproductive disorders like PCOS, and enzymatic and non-enzymatic antioxidants work together in the body to keep harmless levels of reactive oxygen species stable in the oocyte microenvironment, which is very important for reproduction health (Pérez-Ruiz et al., 2021). Several antioxidative defense mechanisms are established, where the antioxidative enzymes, such as paraoxonase-1 (PON 1) and catalase (CAT), have an essential role in the pathogenesis of infertility. Early studies elucidated that some circulating antioxidative biomarkers were decreased in PCOS women, particularly PON1 activity. (Herman et al., 2020). Dyslipidemia is another finding that underlies PCOS pathogenesis. It is the most common metabolic disturbance in 70% of PCOS patients. Previous studies demonstrated that PCOS patients had significantly elevated levels of LDL-C and suggested that hyperandrogenism, hyperinsulinemia, insulin resistance, and obesity contribute to developing hypertriglyceridemia. However, the pathogenic mechanisms still need to be fully understood and investigated in more significant subjects. (Hassan et al., n.d.).

The origin of diseases in adults has been emphasized in gametogenesis and embryo development. Mothers who were exposed to metabolic disturbances of PCOS before or during pregnancy will increase the risk in their offspring of PCOS. Nevertheless, PCOS pathogenesis must be better understood due to its complexity and heterogeneity. This is the most prominent challenge clinicians face regarding understanding the etiology of the disease to provide proper diagnosis and treatments.

1.11 Etiology of PCOS

Both hereditary and environmental etiological factors play essential roles in PCOS. In addition to genetic intervention, genetic and environmental factors combine with other

factors contributing to the progression and severity of the disease, like lifestyle, unhealthy diets, obesity, hormonal imbalance, infectious agents, and many others (H. Teede et al., 2010).

The main etiological factor is hyperandrogenism, which was approximately reported in 60%—80% of PCOS women. According to diagnosis based on the National Institutes of Health (NIH) criteria, insulin resistance is the second contributor, found in 50 %-80 % of PCOS women. This is unlike the European Society for Human Reproduction (ESHRE)/ American Society of Reproductive Medicine (ASRM) criteria, where diagnosed women appear to have less insulin resistance and insulin levels (H. Teede et al., 2010).

Several studies revealed multiple genes involved in several pathways implicated in the etiology of PCOS, such as steroidogenesis, metabolic, gonadotropin-regulatory, bodyweight-regulating, and insulin-signaling pathways. Multiple single nucleotide polymorphisms (SNPs) were reported as crucial factors in abnormal transcriptional activities of specific genes related to PCOS. These polymorphisms include specific variants in the follicle-stimulating hormone receptor (FSHR), alpha-ketoglutarate-dependent dioxygenase (FTO), insulin resistance (IR), and insulin receptor substrate (IRS), vitamin-D receptor (VDR), gonadotropin-releasing hormone receptor (Gn-RHR) genes, and other polymorphisms. Genetic defects interrupting these biochemical pathways, especially genes that encode hormonal receptors like luteinizing hormone (LH), follicle-stimulating Hormone (FSH), and androgen, as well as insulin and leptin receptors, can lead to ovarian dysfunction. (Urbanek et al., 1999). Ovaries are the primary sites for steroidogenesis, where granulosa and theca cells development play essential roles in development and differentiation of follicles (Chaudhary et al., 2021). Steroidogenic enzymes, including multiple cytochrome P450 enzymes, steroid reductase enzymes, and deoxysteroid dehydrogenases, produce several steroid hormones, including androgens and estrogens. PCOS women have elevated LH levels, which leads theca cells to increase activity of steroidogenesis and upregulate StAR, P450scc, 3-HSD, and CYP17, the enzyme that produces androstenedione, which is influenced by high insulin levels (Dadachanji et al., 2018). Synthesis of androgens is increased when SHBG levels are lowered by HA and IR, which are common among women with PCOS. On the other hand, hyperactive ovarian theca steroidogenesis in PCOS patients increases androgenic steroid production, leading to HA (Nelson et al., 1999). PCOS women also exhibit decreased aromatase activity and decreased FSH levels, which in turn impairs and stops follicular development. This can also lead to HA due to excess accumulation of androgens (Figure

1). Consequently, HA appears to be a critical factor in PCOS pathogenesis, influencing both metabolic and reproductive health in PCOS (Chaudhary et al., 2021).

In summary, the progression of the disease is regulated by various genetic and environmental factors that directly or indirectly influence the function of ovaries (Bhimwal et al., 2023).

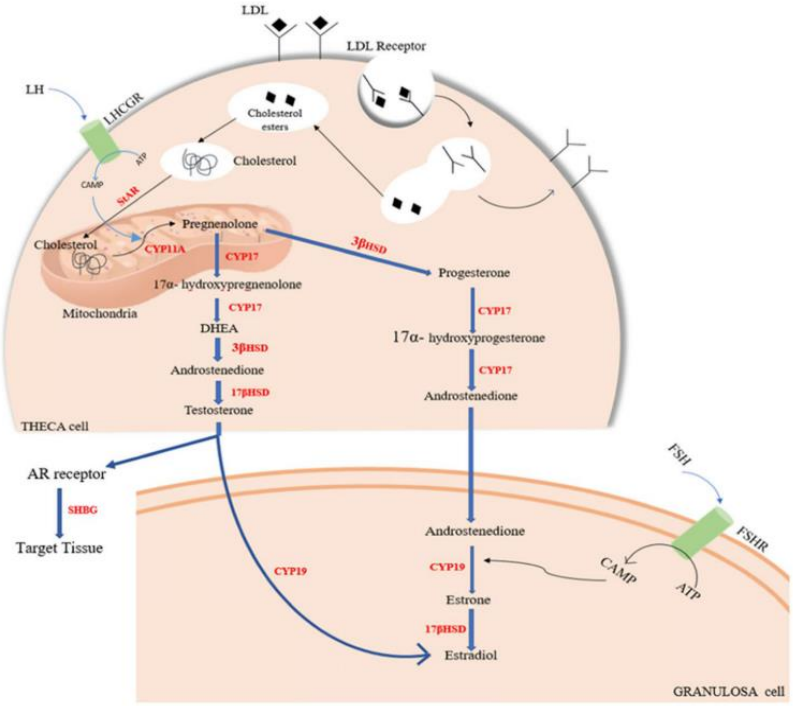


Figure 1.1 Schematic Diagram of Steroidogenesis Pathway and Enzymes Involved in the Biosynthesis (Chaudhary et al., 2021).

1.12 Risk Factors

Hormonal imbalance is one of the most common etiological factors of PCOS. Some factors, such as environmental and genetic factors, significantly affect hormonal imbalance in concordance with lifestyle, habits, and diet (Figure 2).

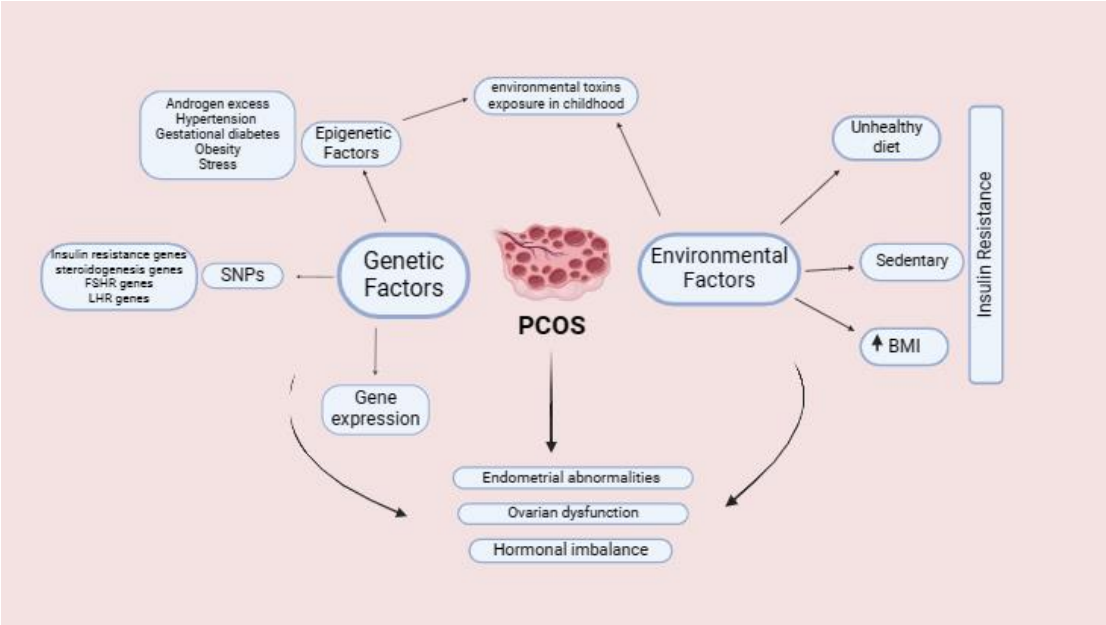


Figure 1.2 Flowchart of Associated Risk Factors in the Etiology of PCOS. Created by Biorender; http://www.biorender.com.

Therefore, genetics, epigenetics, environmental factors, low-grade inflammation, metabolic disorders, and oxidative stress all damage normal ovarian function and PCOS phenotype (Zhao et al., 2023).

HA and IR are fundamental pathophysiological foundations of PCOS that can be induced by OS imbalance. Glucose diet in PCOS can increase levels of OS and activate nuclear transcription factor-kB, which increases chronic low-grade inflammatory state and the expression of many inflammatory factors, including IL-6 and TNFα, which can promote theca-interstitial cells in the ovary (González et al., 2006). In inflammatory conditions, cytochrome P450-17α hydroxylase (CYP17) expression is elevated, which increases androgen synthesis. In OS state, hepatocyte nuclear factor-4α (HNF-4α) expression is downregulated, increasing androgen activity by inhibiting SHBG expression, resulting in HA, which can enhance mononuclear cells (MNCs) sensitivity to glucose and raise the generation of ROS and inflammatory markers (Sun et al., 2021). Meanwhile, TNF-α, as IR mediator, interferes with insulin activity, prevents muscle and adipose tissues from absorbing glucose, and triggers inflammatory pathways in insulin target cells, resulting in Serine phosphorylation of IRS1 and insulin signaling pathway impairment. In the case of IR, tissue's sensitivity to insulin decreases, and high insulin levels promote theca-interstitial cell growth, lower SHBG levels, increase testosterone secretion, and exacerbate HA. Additionally, IR can stimulate mobilization of adipose tissues and liver glycogen synthesis, resulting in increased free fatty acids (FFA) levels in serum. Elevated

levels of FFA and glucose encourage ROS production, which increases OS imbalance, where OS interacts with IR and HA, creating vicious cycle of mutual promotion and contributing to PCOS development (Figure 3) (W. Li et al., 2022).

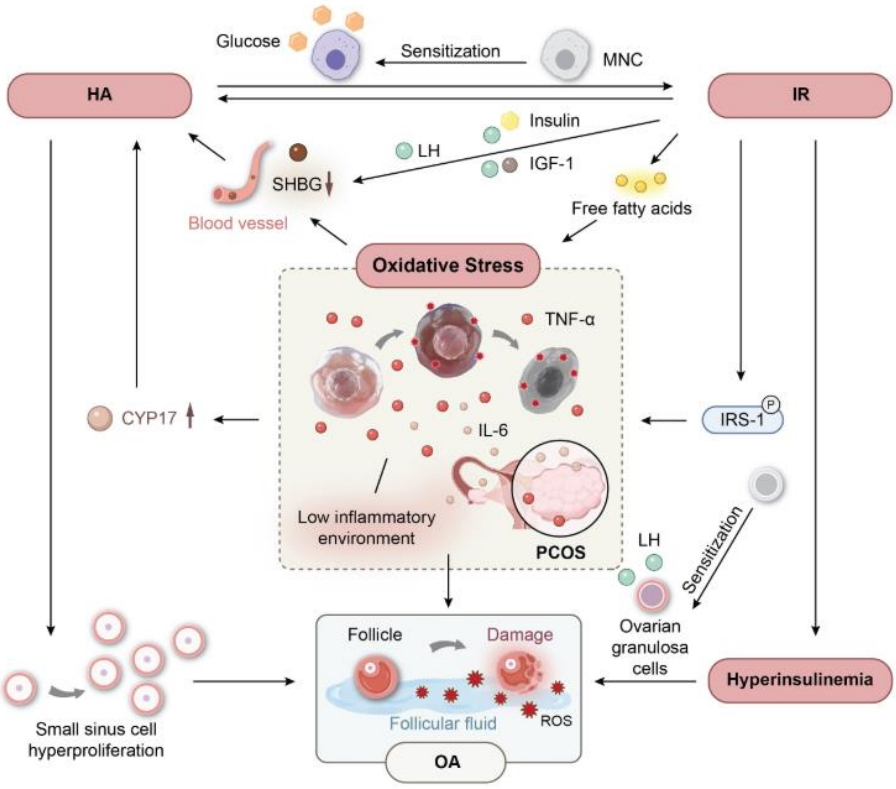


Figure 1.3 Vicious Circle Between Oxidative Stress (OS) and Hyperandrogenism (HA) Insulin Resistance (IR) Leading to Ovulation Disorder in PCOS (W. Li et al., 2022).

1.12.1 Hormonal Factors

Hormones are crucial in regulating menstrual cycles and the ovary's function, and any disturbances in these hormones affect normal ovarian function ovary. This leads to cystic development inside the sac of the ovary. Hormonal pathway defects cause the classical characteristics of PCOS. The most common hormonal features in PCOS patients include hormonal imbalance in GnRH, LH, FSH, Prolactin, and androgen. It has been noticed that LH and FSH levels were higher in PCOS patients in different early studies. Elevated LH levels stimulate ovaries to release excessive amounts of androgens, mainly testosterone, which is one of the most common characteristics of PCOS (Alkhuriji et al., 2021). In addition, hyperinsulinemia and hyperandrogenism are the most critical factors that increase the severity of the disease. High levels of insulin impact theca cells, increasing androgen levels that trigger visceral adipose tissue (VAT), which produces free fatty acids (FFA), leading to insulin resistance. (Shaikh et al., 2014). Consequently, androgen levels

increase due to insulin resistance and high insulin levels, leading to anovulation. (Ajmal et al., 2019).

1.12.2 Environmental Factors

Environmental factors and lifestyle lead to deterioration in the reproductive cycle through loss of physical activities, irregular or impaired menstrual cycle, and many other actions that contribute to PCOS prevalence (Bhimwal et al., 2023).

Some environmental factors, like physical and chemical factors, diet, and lifestyle, contribute to PCOS pathogenicity and play an essential role in gene expression related to the disease. There is evidence that environmental toxins strongly impact human reproductive health. Endocrine-disrupting chemicals (EDCs) are a specific group known as endocrine disruptors that can disrupt the hormonal system, causing hormonal imbalance in PCOS patients. (H. Islam et al., 2022).

1.12.3 Genetic Factors

Although PCOS etiological causation is unclear and still under investigation, there is much evidence about familial genetic predisposition factors interacting with environmental factors in utero and life before puberty. Genetic-environmental interaction plays a significant role in PCOS pathogenesis. Epigenetic factors affect gene expression without changing the underlying genomic sequence, significantly impacting PCOS phenotypes. (Nicolaides et al., 2020). Investigating single nucleotide polymorphisms (SNPs) is essential in detecting heritability events causing diseases, and Genome-wide association studies (GWAS) helped scientists to identify the disease's associated genes, which revealed more than 90% of disease-linked single nucleotide polymorphisms (SNPs) reside in the non-coding region, contributing to complex disorders. (Prabhu et al., 2021).

1.13 Guidelines for the Assessment and Management of PCOS

Due to clinical practice gaps and discrepancies, PCOS patients suffer from poor care and delayed diagnosis or even misdiagnosis. Thus, it was necessary to create guidelines based on medical information and clinical evidence that are easily accessible to scientific researchers, students, clinicians, and patients alike. For this purpose, “International Evidence-based Guideline for the Assessment and Management of Polycystic Ovary Syndrome 2023” was recently published by the Center for Research Excellence in

Women's Health in Reproductive Life (CRE WHiRL) in partnership with the American Society of Reproductive Medicine (ASRM), the European Reproduction and Embryology (ESHRE), and the Endocrine Society, in cooperation of more than one thousand experts in healthcare domain, international patient/ consumer advocacy groups, clinical perspectives, researchers, and academic professionals. This guideline aims to provide transparent information related to PCOS, assisting healthcare professionals and consumers to create effective care and appropriate management to improve the lifestyle of PCOS patients. (*International Evidence-Based Guideline for the Assessment and Management of Polycystic Ovary Syndrome 2023*, 1968).

1.14 Treatment

Treatment of PCOS is not precise enough due to the complexity of the disease, and its pathogenicity is not elucidated yet. Several treatments are available to assist women who are seeking to conceive, which vary from one patient to another, depending on the symptoms and disease severity. (Tian-Min et al., 2022).

Suggested treatments are listed before, but it is worth noticing that these suggestions are to tackle the symptoms, helping PCOS women to live better and healthier lifestyles. (Balen & Rutherford, 2007).

1.15 Genetic Predispositions of PCOS

Gene expressions in different loci support the strong relationship between PCOS and genetic predisposition. Several genes and proteins are either unexpressed or overexpressed. These expression patterns lead to structural and functional changes at the protein level, resulting in PCOS's phenotypic traits.

Several genes were found to be essential in manifesting the disease. These genes play an important role in blocking and regulating the activities of hormonal and metabolic pathways. In the development of the disease at the genetic level, abnormal gene regulation takes place, causing many post-translational modifications in the protein products. (Bhimwal et al., 2023). Several genes were reported in family studies to contribute directly or indirectly to PCOS progression, elucidating that it is a polygenic disease. The development of the disease involves gene-gene interactions, gene-environment interactions, or single genes. (H. Islam et al., 2022; Jones & Goodarzi, 2016).

Using Mendelian random analysis, it has been reported that SNPs associated with PCOS had causation in insulin resistance, decreased sex hormone binding globulin (SHBG), and increased BMI in PCOS women. (Tian-Min et al., 2022).

Twin studies, family-based studies, and genome-wide association studies (GWAS) revealed 20 loci located near putative PCOS genes, among other ones, like DENND1A, LHCGR, FSHR, YAP1, INSR, etc. (Kulkarni et al., 2019).

1.16 Genes Relevant to PCOS

A list of genes involved in the pathogenesis of PCOS is summarized in Figure 4. However, the prevalence of these genes varies in different populations and regions, which is attributed to the high heterogeneity of the disease associated with various ethnicities.

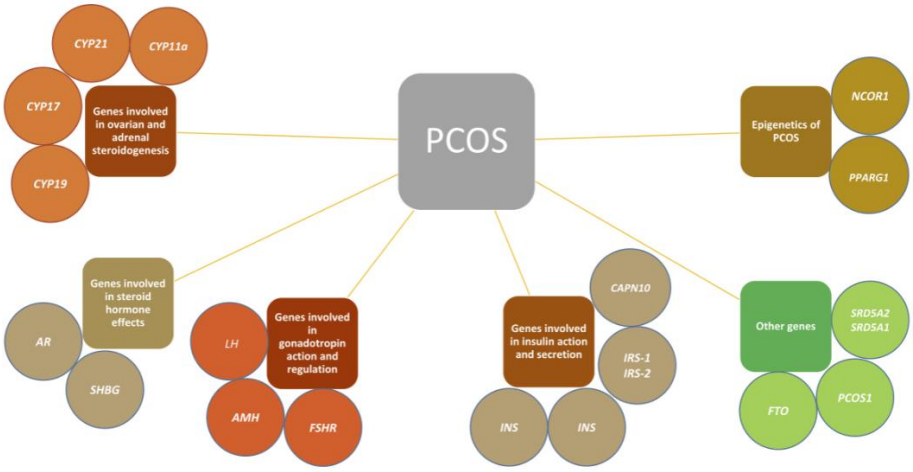
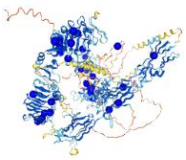
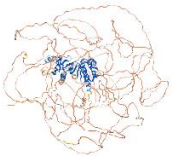
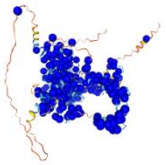
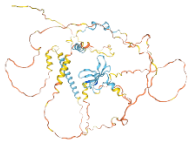
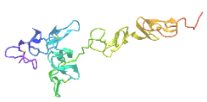
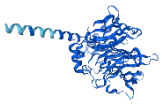
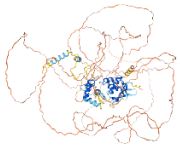
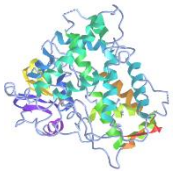
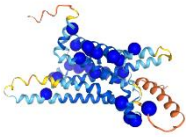


Figure 1.4 A summary of Some Genes Involved in PCOS Highlights the Complexity of the Disease.(Khan et al., 2019).

Recent studies were conducted on different ethnic groups to identify candidate genes and their association with the prevalence of PCOS worldwide. Some candidate genes relevant to PCOS are illustrated in Table 3. Data was collected from many studies made available to researchers and clinicians to understand the etiology of the disease and provide proper diagnosis and treatment management. PCOS databases currently available online are PCOSDB, PCOSKB, and PCOSBase. These databases for PCOS, particularly PCOSKBR2, are comprehensive sources that provide a one-stop online portal for updated and curated information on gene and pathway correlations in PCOS and its comorbidities. (Afiqah-Aleng et al., 2017; Sharma et al., 2020).

Table 2.1 A summary of Candidate Genes Associated with PCOS

Name of the gene	Location	Function	Phenotype	Structure with clinical variants
Insulin-Receptor (INSR)	chr 19p13.2	Insulin metabolic action (Receptor tyrosine kinase mediates the pleiotropic actions of insulin).	PCOS with Hyperinsulinemia+ T2DM	 (38 clinical variants)
Insulin Receptor Substrate-1 (IRS-1)	chr 2q36.3	Mediate the control of various cellular processes by insulin.	PCOS with IR + T2DM	 (2 clinical variants)
Insulin Receptor Substrate-2 (IRS-2)	chr 13q34	Mediate the control of various cellular processes by insulin.	PCOS with IR + T2DM	 (0 Clinical variants)
Low-density lipoprotein (LDLR)	chr 19p13.2	Binds LDL, the primary cholesterol-carrier lipoprotein of plasma, and transports it into cells by endocytosis.	PCOS with dyslipidemia (Hypercholesterolemia)+ CVDs	 (483 clinical variants)
Yes-Associated Protein-1 (YAP1)	chr 11q22.1	Transcriptional regulator in Hippo signaling pathway. Regulates cell proliferation, differentiation, and apoptosis.	PCOS with metabolic diseases (increases BMI)	 (0 Clinical variants)
DENN Domain Containing 1A (DENND1A)	chr 9q33.3	Guanine-nucleotide exchange factor (GEF) mediates the endocytic recycling of selective cargos involved in cell signaling during embryonic development.	PCOS with increased androgen biosynthesis_ T2DM	 (0 Clinical variants)
Fat Mass and Obesity gene (FTO)	Chr 16q12.2	Regulates fat mass, energy homeostasis, and adipogenesis; controls adipocyte differentiation into white or brown fat cells; and controls body mass.	PCOS with Metabolic disorders (Obesity + CVDs)	 (4 Clinical variants)
Fibrillin-1 (FBN1)	chr 15q21.1	For tissue homeostasis. Provides force-bearing structural support in elastic and nonelastic connective tissues such as blood vessels and	PCOS with Metabolic diseases+ CVDs+ hormonal imbalance+	 (0 Clinical variants)

		skin. Binds heparin (essential for assembly of microfibrils).		
Paraoxonase-1 (PON1)	chr 7q21.3	Mediated enzymatic protection of low-density lipoproteins against oxidative modification and consequent events that lead to atheroma formation.	PCOS with obesity_ CVDs+ Oxidative-stress-related diseases	 (0 Clinical variants)
Storkhead box-1 (STOX1)	chr 10q22.1	It protects the placenta, regulates mitochondrial homeostasis, and regulates the level of reactive oxidative species and reactive nitrogen species.	PCOS with Preeclampsia	 (0 Clinical variants)
21-Hydroxylase (cytochrome family21 subfamily member2) (CYP21A2)	chr 6p21.33	Important in lipid metabolism and adrenal steroidogenesis, it catalyzes the hydroxylation at C-21 of progesterone and 17-α-hydroxyprogesterone to form intermediate metabolites in the biosynthetic pathway of mineralocorticoids and glucocorticoids.	PCOS with HA	 (0 Clinical variants)
Melanocortin-4 Receptor (MC4R)	chr 18q21.32	Important regulator for energy homeostasis and somatic growth. Mediates hormonal actions. Regulates body weight.	PCOS with Obesity	 (30Clinical variants)

1.17 Genotype-Phenotype of PCOS

The phenotypic heterogeneity of PCOS cases is the central dilemma for researchers and has made it difficult to make definitive conclusions regarding PCOS etiology and pathogenicity. Several studies on families and populations demonstrated the association of many genetic variants in PCOS pathogenesis. They focused on the variants implicated in PCOS pathogenesis, including insulin homeostasis, androgen levels, lipid metabolisms, steroidogenesis, folliculogenesis, oxidative stress, and inflammations. Genome-wide association Studies (GWAS), Whole Genome sequencing (WGS), and Whole Exome Sequencing (WES) were conducted to identify genotypic variations correlated with phenotypes to establish genotype-phenotype causality. However, few studies succeeded in yielding statistically significant associations. Over 200 genes have been proposed as candidate genes in PCOS. In the Han Chinese population, eleven susceptible genes were reported, including LHCGR, DENND1A, FSHR, YAP1, and

TOX3. While in the Caucasian population, two novel genes were reported, FSHβ and GATA4/NEIL2 (Hong et al., 2020). This explains how ethnicity is a crucial factor in determining the frequency of genetic variation. The studies also revealed many gene variants in PCOS unrelated to its phenotypic characteristics.

Due to the various phenotypes in PCOS, it is essential to identify the single nucleotide polymorphisms (SNPs) and their association with phenotypic features in PCOS. An early study on 12 genes found that FSHβ was associated with LH and free testosterone levels in PCOS women, indicating that FSHβ is a candidate gene in PCOS pathogenesis. (Hong et al., 2020). Additional studies revealed that the YAP1 gene, a transcriptional co-activator in the Hippo pathway, is involved in regulating cell proliferation and apoptosis in addition to its role in regulating the organ's size, as in ovarian enlargement, as a candidate gene in PCOS women. It was observed that in some PCOS women, methylation of the YAP1 promoter was decreased in granulosa cells, and the degree of this event depends on the levels of testosterone in those patients. Moreover, it was found that interrupting this pathway in PCOS women leads to metabolic disorders leading to T2DM. (Alhilali et al., 2022; Hong et al., 2020). Another investigation reported that genes associated with insulin homeostasis, like IRS1 and INSR genes, correlated with PCOS pathogenesis, where some SNPs in these genes were found in PCOS women. (Adam et al., 2022).

Numerous genetic variants were reported in PCOS cases, which led to controversial opinions regarding mode of inheritance. There is evidence that PCOS is an autosomal dominant inherited disorder. However, most studies reported that PCOS is most likely an oligogenic/polygenic multifactorial disorder influenced by genetic and environmental factors. Nevertheless, the underlying mode of inheritance for PCOS is not well illustrated due to several conditions, including epigenetic modifications, incomplete penetrance, and environmental factors, influencing the incidence of various observed phenotypes. (Hong et al., 2020; McAllister et al., 2015; Zhou et al., 2021).

Chapter Two: Literature Review

2.1 Literature Reviews on PCOS

Although there is still a lack of data on the accurate definition and causation of PCOS, particularly the genetic aspect, numerous studies have been conducted and have been able to identify several SNPs that play important roles in causing PCOS among women in their reproductive age. Large-scale studies in European and American populations demonstrated that the prevalence of PCOS ranges between 4% and 8%. In contrast, few studies have shown the prevalence of PCOS in the Middle East, and much fewer among Arab population (Musmar et al., 2013).

The rapid development of bioinformatics and its widespread dissemination has contributed to enhancing genetic knowledge and building robust databases, which aided researchers in studying gene variants, allele frequency, and genotype-phenotype correlation at family, societal, and global levels. Next-generation sequencing (NGS) and Genome-wide association Studies (GWAS) from different populations have identified many genetic loci correlated to PCOS phenotypes. Different findings were documented from studies conducted on populations from various regions. For example, a Dutch twin study was carried out to identify associated genes with hyperandrogenism, the most common feature in PCOS, where they investigated 37 candidate genes involved in the androgen synthesis pathways, and showed a significant linkage with follistatin and CYP11A. (Vink et al., 2006).

Since PCOS is characterized by increased number of antral follicles and early growth of the preantral follicles, associated with abnormalities in insulin receptor binding, post-receptor signaling, and primary abnormality in insulin secretions, several genes were found to have an impact on early folliculogenesis, including follistatin genes implicated in the androgen metabolism pathway. The candidate genes include LHR, CYP11A, CYP17, CYP19. The genes involved in insulin secretions and actions are INSR, IRS, and Calpin10, and genes involved in obesity, such as PON1 and SORBS1. (Franks et al., n.d.; Wood et al., 2003).

Another study demonstrated that genetic predisposition factors like androgen receptor gene (AR), follicle-stimulating hormone gene (FSHR), fat mass obesity genes like aromatase genes (CYP11A1, CYP17A1, CYP1A1, CYP21A1, and CYP19A1) were related to PCOS cases. (Ajmal et al., 2019).

In a different study, mutations in the AMH hormone were associated with the pathogenesis of PCOS by disturbing AMH transcriptional inhibition of CYP17A1, leading to increased androgen synthesis. (Crespo et al., 2018).

The various studies on different communities, with the exclusion of Mena regions, resulted in a noticeable gap in understanding genetic variations regarding PCOS as they play an essential role in evolution, disease etiologies, genotype-phenotype relationship, and disease management and treatment. One of the few studies that was conducted in Mena, include a case-control study conducted in Jordan, determined the association between LH/CG hormone receptor (LHCGR) polymorphism (rs 2293275) with many symptoms in Jordanian women with PCOS like hirsutism, acne, amenorrhea, infertility, LH/FSH ratio, and increased body mass index (BMI). It was found that BMI was higher among PCOS women compared with control women. About 90% of patients had ovulatory dysfunction and selected genotypes of the LHCGR gene showed statistically significant higher LH and LH/FSH values. As a result, LHCGR (rs 2293275) alleles could modulate the hormonal levels in women with PCOS. This polymorphism could influence a specific population due to specific interactions between clinical and environmental factors. (Alfaqih et al., 2018).

A cross-sectional study was also conducted in Saudi Arabia on PCOS patients diagnosed according to Rotterdam Criteria showed that MC4R variants (rs 12970134 and rs 17782313) were significantly associated with increased BMI levels and obesity in PCOS Saudi women (Batarfi et al., 2019).

Minimal studies regarding the prevalence of PCOS were performed on the Palestinian population. A cross-sectional study was conducted at al-Najah National University in West Bank, which estimated PCOS prevalence among female students based on NIH criteria as 7.3% in their study group. Conclusively, PCOS prevalence among Palestinian females is considered high, similar to Mediterranean and Caucasian populations. However, according to Rotterdam criteria, the prevalence percentage should be doubled. (Musmar et al., 2013). Another cross-sectional study was performed in the Gaza Strip, Palestine, aimed to evaluate the inflammatory status among PCOS women, depending on previous investigations that insulin resistance plays an essential role in the inflammation process. The results showed the development of chronic low-grade inflammation among PCOS women in the Gaza Strip through the elevation of some inflammatory markers, including proinflammatory cytokines and C-reactive protein (CRP) (Taha, 2022).

2.2 The Aim of the Study

PCOS is characterized as a multigenic disorder, and many single nucleotide polymorphisms (SNPs) have been identified as genetic factors that lead to comorbid diseases. The current study aims to investigate potentially pathogenic genetic variants that are associated with PCOS among a cohort of Palestinian women who are diagnosed with this condition and visiting IVF clinics seeking conception. Furthermore, it is essential to estimate the frequency of PCOS in different regions of Palestinian territories and compare it with the prevalence percentages in different neighborhoods and other selected populations.

2.3 Study Significance

In the past few years, PCOS has increased at a steady pace among Palestinian women, which has called for intensive scientific research to provide appropriate diagnosis and thus find effective treatments. PCOS complexity lies in the fact that it is a multifactorial disorder, and it is evident that genetic factors play a crucial role in the etiology of the disease, which stimulated the importance of performing genetic screening and analysis to explore the candidate genes linked to PCOS among Palestinian patients.

Providing genetic data regarding PCOS in Palestine is necessary to avoid misdiagnoses and improper treatments. In the present study, the aim is focused on elucidating the contribution of specific genetic variants in the etiology of PCOS among PCOS patients who are undergoing assisted reproductive technologies (ART) that could be utilized to solve the problems they face regarding infertility. As an initial step to identify the candidate genes implicated in PCOS etiology, five probands from unrelated families were included in the study who were diagnosed as PCOS patients according to Rotterdam Criteria and underwent genetic analysis, using NGS for the extracted DNA, and familial segregation for all family members of the probands. We also tested the association between alleles of the candidate gene markers. The data indicated the presence of various SNPs in different genes that are considered relevant genes to PCOS. These variants seem essential to diagnose other comorbidities known to be accompanied by the disease.

To the best of our knowledge, this is the first study conducted to identify potential genes that contribute to PCOS pathogenesis among the Palestinian population. It employed affordable genetic screening tools and techniques that are easily performed locally with high accuracy. In addition, the data provided added information to understand the molecular basis of the disease and the importance of building solid genetic data for PCOS

and constructing specific guidelines to help clinicians and searchers follow strategic therapies and management for PCOS.

2.4 Study Limitations.

Many PCOS cases can be misdiagnosed with other diseases like congenital adrenal hyperplasia (CAH), Cushing's syndrome, hyperprolactinemia, and other disorders. For this reason, very selective study subjects are needed that are appropriately diagnosed. Since PCOS is a complex disease because it is multifactorial, where several genetic and epigenetic factors have been identified to play essential roles in developing the disease. The sample size was small indicating the need to expand the investigation to include more study subjects to carry out a complete genome screening. In addition, one of the most challenging problems in complex diseases is the polygenic nature of the disease. This indicates many genetic variants may contribute a small susceptibility risk for the disease. Thus, a fairly larger sample size is needed to detect the influence of individual SNPs. Moreover, the heterogeneity of the disease could lead to significant differences between individuals in the same population and even in the same family members, making it more challenging to get a clear definition of the disease. However, the presented results seem applicable more broadly within the population.

Chapter Three: Methodology

3.1 Study Subjects

The present study included a cohort of unrelated families with one or more affected subjects who were diagnosed with polycystic ovary syndrome (PCOS) according to the 2003 Rotterdam Criteria. They were recruited from obstetrics and gynecology clinics and IVF centers in Palestine. All patients received specific questionnaires, which were filled with the aid of the researcher. Inclusion and exclusion criteria were determined based on the critical data in the questionnaires. After that, all family members, including male and female first-line relatives of the five probands, were asked to sign the consent forms to participate in the study. Finally, PCOS frequency was calculated among a certain number of women who were diagnosed as polycystic patients according to Rotterdam Criteria and suffering from infertility. Around 200 women were chosen independently from different IVF centers in various regions of Palestine, including the West Bank, Gaza Strip, and Jerusalem. Inclusion criteria were PCOS patients diagnosed according to the Rotterdam Criteria. PCOS diagnosis was confirmed when two of the three symptoms were met: polycystic ovaries morphology on ultrasound examination, oligo/ anovulation, and hyperandrogenism. Exclusion criteria were based on clinical diagnosis. The excluded patients were those who had other diseases that imitate PCOS and display similar symptoms, such as 21-hydroxylase deficiency, Cushing's syndrome, non-classical adrenal hyperplasia (NCAH), hypothyroidism, hypercortisolism, androgen-secreting tumors, hyperprolactinemia, premature ovarian failure, and ovarian neoplasm, women who are under medication influencing hypothalamic-pituitary-gonadal axis, and PCOS women experience infertility due to male factor infertility.

Ethical committee approval was received by the Palestinian Health Research Council (PHRC) (approval number: PHRC/HC/1337/23). Written consent forms were provided and assigned by all participants before collecting samples. The study was conducted following the Declaration of Helsinki.

3.2 Parameters Analysis

The hormonal analyses, including Free testosterone (FT), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and LH/FSH ratio, were measured on day two or day three of the follicular phase by chemiluminescence immunoassay. Polycystic ovarian morphologies were confirmed by utilizing ultrasound assessment when $\geq$ 12 follicles

were detected in each ovary measuring 2-9mm in diameter and increased ovarian volume >10cm³. Other hormone tests, including thyroid stimulating hormone (TSH), prolactin (PRL), and cortisol, were previously conducted by clinicians to rule out misdiagnosis with other diseases rather than PCOS.

3.3 Genomic DNA Extraction

Genomic DNA was extracted from peripheral blood samples of the five probands with their family members according to the manufacturer's instructions, using Wizard® genomic DNA purification kit (Promega, USA) (Corporation, n.d.). DNA purity was assessed by NanoDrop 200 (Thermo Scientific), and DNA integrity was assessed by 1.5% agarose gel electrophoresis. All samples were stored at -20°c for later analysis.

3.4 Next Generation Sequencing

DNA samples of the five probands were subjected to Whole-Exome Sequencing (WES) according to the manufacturer's instructions of Illumina® DNA Prep with Enrichment. Sequencing data were analyzed according to the EDGC analysis pipeline. Workflow is summarized in figure 5 according to manufacturer's instructions (Illumina, 2021).

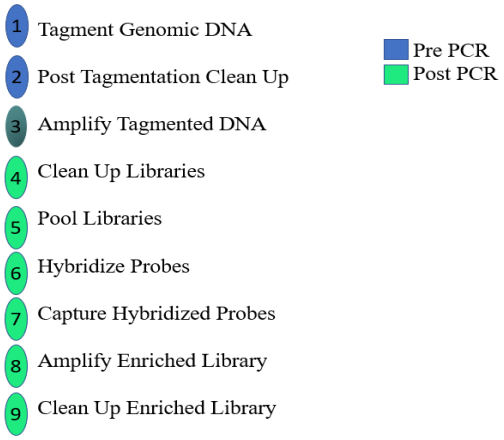


Figure 2.1 Illumina DNA Pre with Enrichment Reference Guide (Illumina, 2021)

3.4.1 Genomic DNA Tagmentation

DNA fragmenting and tagging with adaptor sequences were processed by adding 30µL of each sample into separate wells of a 96-well PCR plate, resulting in a final concentration of 500ng. Tagmentation master mix was then added to each well, consisting of eBLT and TB1 buffers. Then, the plates were sealed with Microseal B and

placed in a thermal cycler, which was set to preheat the lid to 100°C with a reaction volume of 50µL at 55°C for 5 minutes and held at 10°C.

3.4.2 Post Tagmentation Cleanup

To wash DNA tagged with adaptors, 10µL of Stop Tagment Buffer 2 (ST2) was added to Tagmentation reaction. The mixture was centrifuged at 1600 rpm for one minute and set on a magnetic stand. 100µL of Tagment Wash Buffer (TWB) was then added to the beads, and samples were replaced in the magnetic stand. The supernatant was removed, and this step was repeated two times.

3.4.3 Tagmented DNA Amplification

For amplifying Tagmented DNA, 40µL of PCT master mix (EPM) was added into each well. Afterward, pre-paired index-1 and index-2 adaptors, each comprising 10 base pairs, were added from index adaptor plate in each well. Amplification was conducted using eBLT PCR Program on thermal cycler as follows:

Rection Volume 50µL Preheat Lid 100°C		
72°C	3min	
98°C	3min	
98°C	20sec	9 cycles
60°C	30sec	
72°C	1min	
72°C	3min	
10°C	Hold	

3.4.4 Cleanup Libraries

To ensure the purity of the amplified libraries, 45µL of each well's supernatant was gently transferred to the corresponding wells of a new MIDI plate. 77µL of nuclease-free water and 88µL of AMPure XP beads were added. Then, they were washed using 200µL of 80% Ethanol before adding 17µL of resuspension buffer (RSB). Then, the quality of each library was evaluated using an Agilent Technology 2100 Bioanalyzer (DNA 1000 kit).

3.4.5 Pool Libraries

For pooling pre-enriched libraries, specific indexes were assigned to each library, allowing DNA libraries to be merged into a single pool. Volume needed from each sample was determined based on its concentration to a achieve a final volume of 30µL (150ng of DNA), and these volumes were then combined in PCR tube.

3.4.6 Probes Hybridization

Targeted DNA regions were captured using specific probes. 50µL of NHB2, 10µL EHB2, and 10µL of enriched probe panel were mixed with 30µL of sample. The tube was then placed in a thermal cycler on NF-HYB program, which was as follows: preheat the lid to 100°C, total reaction volume 50µL, heat to 95°C for 5 min, 16 cycles of 1mon each, starting at 94°C and decreasing temperature by 2°C per cycle, and finally, hold the program at 62°C for 24 hours.

3.4.7 Hybridized Probes Capture

The samples were centrifuged at 280g for 30 seconds. In separate tube, 250µL of Streptavidin Magnetic Beads (SMB3) were added to 100µL of sample in order to capture hybridized probes located in targeted regions. Then 200µL of Enhanced Enrichment Wash (EEW) was added for washing, followed by preparing a mixture with 342µL of Enriched Elution Buffer 1 (EE1) and 18µL of 2N NaOH (HP3) for elution. Then, 23µL of the prepared elusion mixture was added to sample and incubated for two minutes at room temperature, and centrifuged at 280g for 30 seconds, then it was replaced on magnetic stand for two minutes. 21µL of supernatant was then combined with 4µL of Elute Target Buffer-2 (ET2) in new tube.

3.4.8 Enriched Library Amplification

For amplifying the enriched library, 5µL of PCR primer Cocktail (PPC) and 20µL of Enhanced PCR Mixture (EPM) were added. The sample was then centrifuged and placed on thermal cycler using the following program:

Rection Volume 50µL Preheat Lid 100°C	
98°C	60sec
98°C	20sec
60°C	30sec
72°C	30sec
72°C	5min
10°C	Hold

10 cycles

3.4.9 Amplified Enriched Library Cleanup

45µL of AMPure XP Beads were added to the sample tube to purify the library; then, the sample was washed twice by adding 200µL of 80% Ethanol and 32µL of RSB. Finally, the library concentration was measured using Qubit dsDNA HS assay (kit no. Q23850). The mean fragment size was determined using a High-sensitivity DNA kit (kit no. 5067).

3.5 Data Analysis and SNP Selection

FastQ paired-end reads were mapped to the reference human genome version GRCh38 using BWA-MEM sequence alignment software, which produced the mapped reads in Bam format. Mapped reads were then filtered on two criteria: firstly, we only retained the paired reads for which both forward and reverse reads had been mapped to the reference successfully by using Samtools, and secondly, all PCR duplicates were removed using RmDup tool. Subsequently, all filtered mapped reds were used to call the variants using FreeBayes variant detector to identify all indels and single nucleotide polymorphisms (SNPs).

After that, all variants were filtered to prioritize the more likely ones contributing to PCOS phenotypes in probands, where the list of variants that were produced in VCF format was filtered and prioritized concerning their potential relevance to PCOS. SnpEff tool, which annotates the variants with their calculated efficiency on known genomic features, used and produced an annotated VCF file that includes the variant effects annotations. VCF file contains chromosome number, genes position, genes names, reference and variant nucleotides, genomic context; variant location whether on exon, intron, splice site, or elsewhere, amino acid substitution, synonym; whether the variant is

synonymous or not; the clinical significance of the variant; whether it is pathogenic or not, phenotypes according to OMIM and HPO, phenotype correlation score using Exomiser tool, predicted pathogenicity scores according to SIFT tool, PolyPhen22 tools and others, the averaged frequency of the variant; whether the frequency occurs in the population or not. All variants that were unlikely to have a pathogenic effect, such as deep intronic variants (but still keeping those that affect splice sites), variants in UTRs, upstream and downstream of the gene, and variants with QD less than three or low scores of phenotypic correlations were filtered out.

Thirteen variants were detected and checked up to prioritize likely causative variants based on genotype-phenotype correlation and gene-disease relationship. As parents' samples were not subjected to WES, we were unable to identify a single candidate variant. Consequently, we chose the most candidate variants in each family, determined by the highest correlation score to carry out family segregation analysis.

3.6 Primer Designs

Primers for PCR amplification and genotyping of the indicated variants were designed using Primer3 Plus (http://www.primer3plus.com/index.html/).

The list of predicted gene variants and designed primers is in Table 4.

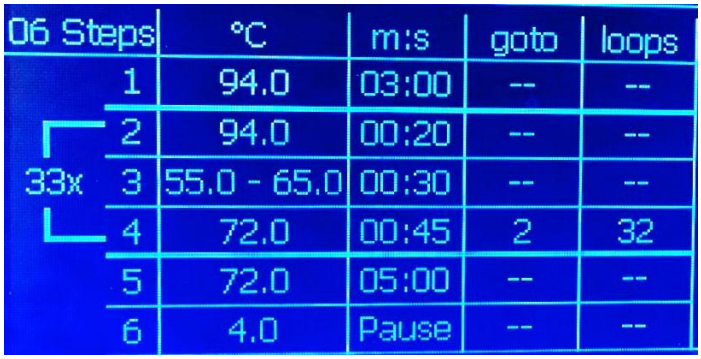
Table 2.2 Primer Designs of the Variants Found in Five PCOS Probands.

Gene	cDNA change	Amino acid change	Primer Sequence	Product Size
CYP21A2	c.*2405_*2406delGTinsTC		F: 5'_CTGAACAAGTCCCCTCCAGA R: 5'-GGAGGCACGGAGTAGAGAGA	216 bp
LDLR	c.1783C>T	p. Arg595Trp	F: 5'- CAGCACGTGACCTCTCCTTA R: 5'- AGTCTGTGTCTATCCGCCAC	229 bp
MCM6	c.1917+323t>c		F: 5'-GGACGCTTGAATGCCCTTT R:5'- TGAGTGTAGTTGTTAGACGGAGAC	308 bp
IKBKB	c.2081C>T	p. Ala69Val	F: 5'-CTAGAGCAAGGTCCGTGGT R: 5'-CAGTGACAAACGCCTCCATC	206 bp
LDLR	c.-18C>T		F: CCCCTGCTAGAAACCTCACA R: 5'-GGGCTCCCTCTCAACCTATT	246 bp
IRS1	c.3662G>C	p. Arg1221Pro	F: 5'- GGACTTCAAACAGTGCCCTC R: 5'- TTGTCACCATGAAACGCACC	212 bp
IRS2	c.195delG	p. Ser66fs*27	F: 5'- AACAACAACAACCACAGCGT R: 5'- GATGTTCAGGCAGCAGTCG	231 bp

FBN1	c.3172G>A	p. Gly1058Ser	F: 5'- TGCCCCACATTTTCTTATTCTTG R: 5'- GGACAGCCTTAATTCTTGCGA	241 bp
PON1	c.380T>G	p. Met127Arg	F: 5'- GGAGGTGGGTTGAAATTGGT R: 5'- CCTACTCTGGCCAAAAGGAA	300 bp
IRS1	c.2005G>T	p. Gly669Cys	F: 5'- ACTATATGCCCATGAGCCCC R: 5'- CGTTTGTCCACAGCTTTCCA	213 bp
STOX1	c.557T>C	p. Ile186Thr	F: 5'- AGGCATTGCAATTCCATCGG R: 5'- GGCGACTTTCATCTGATGGC	176 bp
MC4R	c.922G>A	p. Glu308Lys	F: 5'- GCCCCATTCTTCCTCCACTT R: 5'- CGTGCTCTGTCCCCATTTAA	241 bp
IRS1	c.1691G>A	p. Ser564Asn	F: 5'- AACAACAACAACCACAGCGT R: 5'- GATGTTCAGGCAGCAGTCG	248 bp

3.7 Primer Testing

The efficiency and specificity of the primers were validated through PCR amplification using genomic DNA to optimize PCR conditions and assess primer performance. PCR reactions were performed using forward and reverse primers for each variant using Go Tag®, Promega, USA, and were run on a programmed thermocycler (Flex Cycler) using the following amplification program. The optimal PCR amplification program for the primers was the following: Initial denaturation at 94°c for 3 minutes, followed by 33 cycles of denaturation at 94°c for 20 seconds, annealing at 55-65°c for 30 seconds, and at 72°c for 45 seconds, followed by the extension step at 72°c for 5 minutes. PCR amplification was conducted for all individuals of the proband's families. PCR products were visualized in gel electrophoresis with NTC and DNA ladder (50 or/and 100 bp).



06 Steps	°C	m:s	goto	loops
1	94.0	03:00	--	--
2	94.0	00:20	--	--
33x 3	55.0 - 65.0	00:30	--	--
4	72.0	00:45	2	32
5	72.0	05:00	--	--
6	4.0	Pause	--	--

3.8 Family Segregation

Thirteen variants were identified in the five probands as the most correlated to PCOS. Thirteen primers were designed to target detected variants, as shown in Table 4, followed by PCR amplification and Sanger sequencing.

3.8.1 PCR Amplification

All related genetic variants were conducted on each member of each family. The specific PCR reaction was prepared in a total volume of 20µl, as illustrated in Table 5. All PCR mixtures were run on a thermocycler (Flex Cycler) using the previously outlined amplification program.

Table 2.3 PCR Reaction Mixture

Reagent	Volume
PCR master mix (2X)	10 µl
Forward primer (10 picomoles)	0.5 µl
Reverse primer (10 picomoles)	0.5 µl
DNA sample (100ng)	1.0 µl
Nuclease free water	8.0 µl

3.8.2 Sanger Sequencing

All samples were sequenced using BigDye™ Terminator v3.1 Cycle Sequencing Kit using Applied Biosystems Genetic Analyzer per the manufacturer's instructions -Thermo Fisher (Fisher Scientific, n.d.).

At first, 5µl of PCR product was treated with 1µl of the reagent EPPIC-FAST (Catalog £1021-100F A&A Biotechnology) to perform PCR cleanup. Then, the products were placed at 37°C on the thermocycler for 10 minutes, followed by 1 minute at 80°C. Subsequently, the cycle sequencing was initiated by mixing 2µl of the cleaned PCR with 18µl of BigDye Terminator mix (BDRR), and the resulting mixture was then placed in the thermocycler and run on a program as described in Table 2.3.

Table 2.4 The Reagents Used for BDRR Mix Preparation and the BDRR-PCR Program.

Reagent	Volume	BDRR-PCR Protocol		
		Temperature	Time	Number of cycles
Sequencing buffer (5X)	3.5µl	96°C	20 sec	1
BDRR	1.0µl	96°C	10 sec	25
Sequencing primer	2.0µl	50°C	5 sec	25
H2O	11.5µl	60°C	4 min	25
Total volume	18µl	4°C	Hold	∞

3.9 In-Silico Analysis

All variants were analyzed in silico, and several bioinformatics prediction tools were used to predict the potential impact of amino acid substitutions or indels on the structure and

function of the protein and to predict the pathogenicity of these variants. The utilized tools included the following:

- SIFT-Indel (Sorting Intolerant from Tolerant) (https://www.sift.bii.a-star.edu.sg/).
- PolyPhen v2 (http://www.genetics.bwh.harvard.edu/pph2/).
- Clinvar (http://www.ncbi.nlm.nih.gov/clinvar/)
- Mutation Taster (https://www.mutationtaster.org/).
- PROVEAN (Protein Variation Effect Analyzer) (http://provean.jcvi.org/index.php)
- FATHMM V2.3 (Functional Analysis through Hidden Markov Models) (http://fathmm.biocompute.org.uk/).
- GVGD (Grantham Variation, Grantham Difference) (http://agvgd.hci.utah.edu/index.php).
- COBALT: The alignment tool to detect the conservation of the variant locus (https://www.ncbi.nlm.nih.gov/tools/cobalt).

3.10 Study Population Characteristics

PCOS frequency was tracked across various regions within Palestine. Data were collected through medical history records about PCOS women who are visiting IVF centers looking forward to conceiving and were already diagnosed as polycystic patients by clinicians according to Rotterdam Criteria. Pooled data was collected from West Bank, Jerusalem, and Gaza Strip IVF clinics. Around 200 PCOS women, including the study subjects, were selected randomly from patient's medical files in each IVF clinic. PCOS frequency was then calculated by determining the percentage of PCOS cases to unaffected cases using the formula (Total number of PCOS women/ Total number of affected and unaffected women × 100). The prevalence of PCOS in Palestine was then compared with other regions in the MENA region using data tools of the Institute for Health Metrics and Evaluation (IHME) (https://vizhub.healthdata.org/gbd-compare/).

Chapter Four: Results

4.1 Study Subject

The study subjects included five women who were diagnosed with polycystic ovary syndrome (PCOS) according to Rotterdam Criteria by gynecologists in different IVF clinics. One of the women participating was an adolescent and had not been married yet. The baseline clinical characteristics of the participants are listed in Table 8. All patients were asked to complete a questionnaire with all the necessary information about their medical history. The subjects share similar gynecological age and BMI. The median age of participants was 28 years, and they shared similar symptoms and complications. Most participants had metabolic health problems due to high BMI. The median BMI of participants was 25.0, which falls within overweight. Their menstrual cycles are irregular, and they failed to conceive spontaneously and had to be induced in all participants. The obstetric history of the married participants was significantly similar, in which Gravida Para Abortus terminology (GPA) is used to describe the obstetric history of the patients (G: Garvida, P: Para, A: Abortion, E: Ectopic pregnancy). Patients one and three had G1P1A0E0, which means they got pregnant once and had one livebirth child. However, their pregnancies were induced after undergoing repeated failure IVF trials, while patient number 4 did not get pregnant at all, even after many IVF attempts (G0P0A0E0).

4.2 Family Description and Clinical Characteristics

Hormonal parameters, including Free Testosterone (FT), Luteinizing hormone (LH), follicle-stimulating hormone (FSH), and LH/FSH ratio, were tested for all patients on day two or three of the follicular phase. Patients 4 and 2 had slightly high LH levels, while free testosterone and FSH levels were normal. Patient number five had high testosterone levels, which indicated severe hyperandrogenism.

LH/FSH ratio was recorded as 1:1 in the first patient, while in patients 2 and 3, the ratios were more than 1:1, and in patient 5, the ratio was less than 1:1, which is a significant feature for PCOS (Table 7).

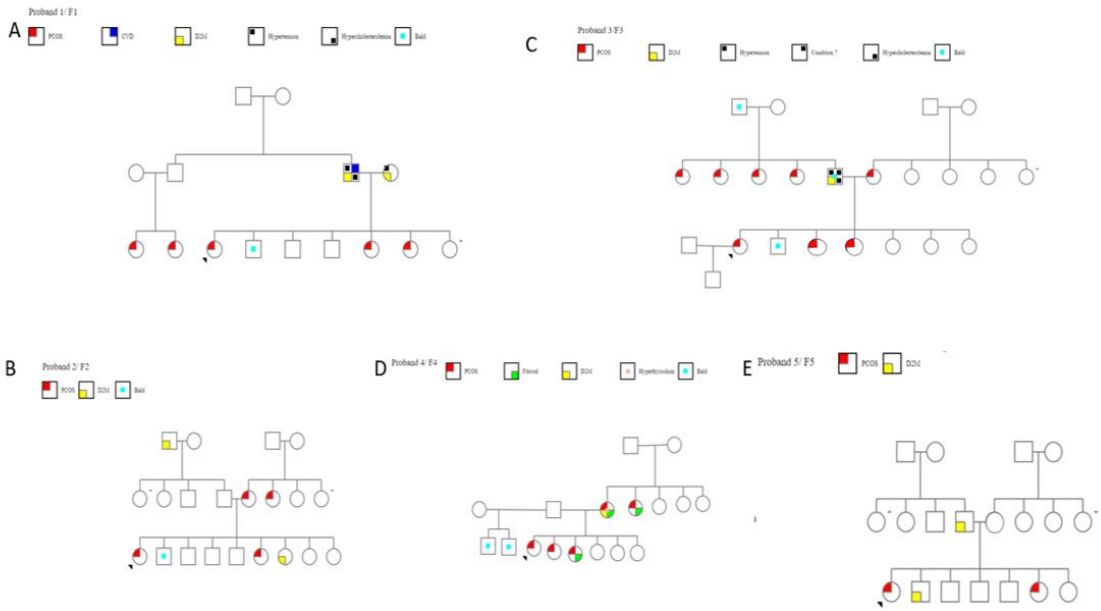


Figure 3.1 The family pedigree with the probands showing the phenotypes according to the data collected from the questionnaire. The red symbol resembles PCOS, the yellow symbol indicates T2DM, the green symbol is for fibroids, and the blue symbol indicates CVDs. The blue dot symbol indicates baldness, and the black symbols resemble hypercholesterolemia, hypertension, and high triglyceride levels. A) In Family 1 (F1), the black arrow indicates proband-1, who has two sisters, two cousins with PCOS, and one bald brother. T2DM was diagnosed in both paternal and maternal lines; B) Family 2 pedigree (F2) shows that the black arrow refers to proband-2. The proband and one sister inherited the disease from their mother, as they have a maternal aunt affected with the disease. In addition, the proband has one bald brother; C) Family 3 (F3) pedigree, where the black arrow refers to proband-3. PCOS was detected in the mother and paternal aunts in this family. In addition, one brother inherited baldness from his father and paternal grandfather; D) Family 4 (F4) pedigree shows the proband-4 with a black arrow. PCOS was diagnosed in the proband's mother, one maternal aunt, and two sisters. In addition, the proband has two paternal uncles with masculine baldness; E) Family 5 (F5) pedigree shows proband-3nwith the black arrow, who has one sister with PCOS and one brother with T2DM inherited from the paternal line.

Table 3.1 Demographic, Anthropometric, Clinical History, and Clinical Parameters of Probands According to Questionnaires

Proband/ Family no.	**1/ F1**	**2/ F2**	**3/ F3**	**4/ F4**	**5/ F5**
Age	28yrs	28yrs	34yrs	25yrs	18yrs
BMI	25	24.2	30	21.5	35
Obstetric history/ pregnancy status	G1P1A0E0/ Induced	G1P0A1E0/ Induced	G1P1A0E0/ Induced	G0P0A0E0/ Induced	Not married
Menstrual cycle	Irregular	Irregular	Irregular	Irregular	Irregular
Symptoms	HA, PCO, acne, abdominal fats, oligomenorrhoea, hirsutism, weight gain.	HA, PCO, acne abdominal fats, oligomenorrhoea, hirsutism, weight gain, OSA.	HA, PCO, acne, abdominal fats, hirsutism, weight gain, OSA.	HA, PCO, hirsutism, oligomenorrhoea, OSA.	HA, PCO, abdominal fats, acne, hirsutism, oligomenorrhoea, weight gain, OSA, IR.
Hormonal analysis	**FT:** 2.71 pg/ml **LH:** 6.49 IU/L **FSH:** 6.32 IU/L. **LH/FSH:** 1.0	**FT:** 2.46 pg/ml **LH:**8.9 IU/L **FSH:** 6.9 IU/L **LH/FSH:** 1.3	**FT:** 1.74 pg/ml **LH:** 5.8 IU/L **FSH:**3.24 IU/L **LH/FSH:** 1.8	**FT:** 1.69 pg/ml **LH:** 11.8 IU/L **FSH:** 6.0 IU/L **LH/FSH:** 1.9	**FT:** 882 pg/ml **LH:** 4.4 IU/L **FSH:** 6.0 IU/L **LH/FSH:** 0.7
PCOS in family members	Two sisters and two cousins	One paternal aunt, mother, and two sisters	Two sisters, all paternal aunt, and one maternal aunt	Two sisters and one maternal aunt	One sister
Family diseases	DM, CVDs, hypertension, high cholesterol and triglycerides	Maternal DM, hypertension.	Hyperandrogenism, DM, hypertension	NR	DM, Hypertension
Male balding	One brother	One brother	All paternal family members	Two brothers	N/A

4.3 Identification and Association Analysis of SNPs-Related PCOS

In order to identify single nucleotide polymorphisms (SNPs) related to PCOS, whole exome sequencing was performed for five PCOS Proband patients who participated in the study. Different SNPs were identified; thirteen variants were identified and filtered in all tested patients.

Patient 1

Two variants were identified as described in Table 8. The identified variants include.

1. **CYP21A2 (21-Hydroxylase):**

The heterozygous indel variant at chr6: 32043539 led to a missense mutation, in which two nucleotides (GT) were deleted, and nucleotides TC were inserted at positions 2405 and 2406 within the 3'UTR.

2. **LDLR (Low-Density Lipoprotein Receptor):**

The LDLR gene was identified as a missense mutation and classified as likely pathogenic. The heterozygous C>T substitution at chr19: 11116936 resulted in the abnormal translation of the LDLR protein at position 595 of the protein sequence, where Arginine was substituted into Tryptophan.

Table 3.2WES Results of Proband One Show Two Variants in the CYP21A2 and LDLR Genes.

Gene	Position	Reference Sequence	cDNA Change	Amino Acid Change	dpSNP rs ID	Zygosity	ACMG Significance
CYP21A2	Chr6:32043539	NM_000500.9	c.*2405_*2406del GTinsTC	_	rs 1562773221	Het	Uncertain Significance
LDLR	Chr19:11116936	NM_000527.5	c.1783C>T	p. Arg595Trp	rs 373371572	Het	Likely pathogenic

Patient 2

The second patient was found to have three variants (as summarized in Table 9):

1. **MCM6 (Minichromosome Maintenance Complex Component 6):**

A heterozygous T>C substitution was identified as a point mutation 323 nucleotides downstream of nucleotide 1917 within the intron region of the MCM6 gene. This variant has uncertain significance. However, it might impact the splicing or other regulatory mechanisms and thus result in abnormal translation of the MCM6 protein.

2. **IKBKB (Inhibitor of nuclear Kappa B Kinase Subunit Beta):**

A heterozygous C>T substitution at position chr8: 42326064 was identified in the IKBKB gene. This missense variant has uncertain significant results in abnormal translation of the IKBKB protein at amino acid position 694 of the protein sequence, where Alanine amino acid is substituted into valine.

3. **LDLR (Low-Density Lipoprotein Receptor):**

An uncertain significance variant in the LDLR gene was also detected for this patient on chr19: 11089531. A heterozygous C>T substitution was detected at position 18

nucleotides upstream of the coding sequence, most likely in the promoter region. It does not impact the protein sequence but affects gene expression.

Table 3.3 WES Results of Proband Two Show Three Variants in the MCM6, IKBKB, and LDLR Genes.

Gene	Position	Reference Sequence	cDNA Change	Amino Acid Change	dpSNP rs ID	Zygosity	ACMG Significance
MCM6	chr2:135851079	NM_005915.6	c.1917+323T> C	_	rs41456145	Het	Uncertain Significance
IKBKB	chr8:42326064	NM_001556.3	c.2081C>T	p. Ala694Val		Het	Uncertain Significance
LDLR	chr19:11089531	NM_000527.5	c.-18C>T	_		Het	Uncertain Significance

Patient 3

The third PCOS patient was found to have three uncertain significant variants (Table 10).

1. **IRS1 (Insulin Receptor Substrate-1):**

The heterozygous G>T substitution was identified at chr2: 226796734, which leads to the substitution of Glycine into Cysteine at amino acid position 669 of the IRS1 protein.

2. **STOX1 (Storkhead Box-1):**

The heterozygous T>C substitution at chr10: 68884353 was identified, in which Isoleucine is substituted into Threonine at amino acid position 186 of the protein sequence. This can alter the structure or function of the STOX-1 protein or alter both.

3. **MC4R (Melanocortin-4-Receptor):**

A heterozygous G>A substitution was detected at chr18: 60371428 of the MC4R gene. Glutamic acid changes into Lysine at amino acid position 308 of the protein sequence.

Table 3.4 WES Results of the Proband-3 Show Three Variants in the IRS1, STOX1, and MC4R Genes.

Gene	Position	Reference Sequence	cDNA Change	Amino Acid Change	dpSNP rs ID	Zygosity	ACMG Significance
IRS1	chr2:226796734	NM_005544.3	c.2005G>T	p. Gly669Cys	rs557319201	Het	Uncertain Significance
STOX1	chr10:68884353	NM_152709.5	c.557T>C	p. Ile186Thr	rs778194886	Het	Uncertain Significance
MC4R	chr18:60371428	NM_005912.3	c.922G>A	p. Glu308Lys	rs375095163	Het	Uncertain Significance

Patient 4

Four variants were found in the fourth patient. One of these identified variants was classified as a likely pathogenic variant (Table 11).

1. **IRS1 (Insulin Receptor Substrate-1):**

   The heterozygous G>C substitution was detected at chromosome two (chr2: 226795077). It causes a substitution of the Arginine amino acid with Proline amino acid at position 1221, consecutively causing abnormal protein translation of the IRS1 protein.

2. **IRS2 (Insulin Receptor Substrate-2):**

   The heterozygous deletion of nucleotide G was detected at chromosome 13 (Chr13:109785858). It leads to a frameshift mutation at the Serine residue 66 protein sequence position. This variant leads to a premature stop codon after 27 nucleotides, which may result in a truncated nonfunctional protein or no protein.

3. **FBN1 (Fibrillin-1):**

A likely pathogenic heterogenous G>A substitution variant was detected in the FBN1 gene at chr15:48488404. This substitution replaces the Glycine amino acid with Serine at position 1058 of the protein sequence.

4. **PON1 (Paraoxanase-1):**

A heterozygous T>G substitution was detected at chr7: 95311568. In the start codon (ATG) of exon 5, the amino acid Methionine was replaced with Arginine at position 127 of the protein sequence. This mutation affects translation initiation, causing significant changes in protein structure and function.

Table 3.5 WES Results of the Proband-4 Show Four Variants in the IRS1, IRS2, FBN1, and PON1 Genes.

Gene	Position	Reference Sequence	cDNA Change	Amino Acid Change	dpSNP rs ID	Zygosity	ACMG Significance
IRS1	Chr2:226795077	NM_005544.3	c.3662G>C	p. Arg1221Pro	_	Het	Uncertain Significance
IRS2	Chr13:109785858	NM_003749.3	c.195delG	p. Ser66fs*27	_	Het	Uncertain Significance
FBN1	Chr15:48488404	NM_000138.5	c.3172G>A	p. Gly1058Ser	rs886039208	Het	Likely Pathogenic
PON1	Chr7:95311568	NM_000446.7	c.380T>G	p. Met127Arg	rs144390653	Het	Uncertain Significance

Patient 5

The 18-year-old patient was diagnosed with severe PCOS with IR comorbid disease. She was found to have one uncertain significant variant (Table 12).

1. **IRS1 (Insulin Receptor Substrate-1):**

An uncertain significance variation was detected in the IRS1 gene, in which a heterozygous G>A substitution at position chr2: 226797048 was detected. This variant led to amino acid substitution, where the Serine amino acid at position 564 of the protein sequence was changed to Asparagine.

Table 3.6 WES Results of the Proband-5 Show One Variant in the IRS1 Gene.

Gene	Position	Reference Sequence	cDNA Change	Amino Acid Change	dpSNP rs ID	Zygosity	ACMG Significance
IRS1	chr2:226797048	NM_005544.3	c.1691G>A	p. Ser564Asn	rs1187676556	Het	Uncertain Significance

3.4 Sanger Sequencing: Genotype Segregation

All family members' samples were Sanger sequenced to validate the variants, as well as the genetic segregation of indicated variants using NGS. Noticeably, none of the participants carried any variant in a homozygous state.

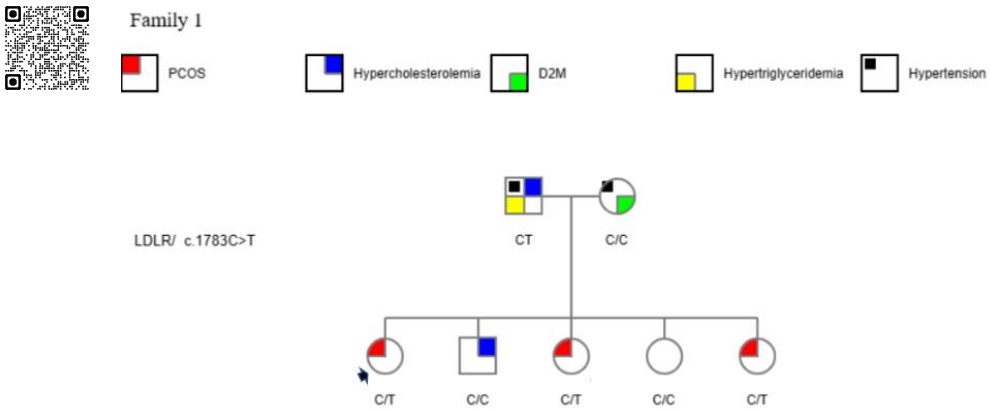
Family 1

Two variants were detected in the first family in two genes: CYP21A2 gene (c.*2405_*2406delGTinsTC) and LDLR gene (c.1783C>T).

The allele segregation in family number one for CYP21A2 gene is well segregated within the family, and the family pedigree shows autosomal dominant, in which the allele is expressed in all affected individuals; two PCOS sisters and the brother are carriers for GT/CT genotype in CYP21A2 which was inherited from the father who is a heterozygous carrier for the gene. However, the single nucleotide polymorphism (SNP) rs1562773221 on NCBI gives information about the TNXB gene but not CYP21A2. This gene is not relevant to PCOS. On NCBI, TNXB and TNXA lie on the opposite strand of DNA from the C4 and CYP21 genes. The last exon of TNXA and TNXB lies within the 3’ untranslated region of exon 10 in CYP21A1P and CYP21A2. Hence, the CYP21A2 gene was excluded from the presented study; further analysis and investigations will be done in the future for this patient.

The second variant identified in this family is in the LDLR gene, which was also identified in the father, who is diagnosed with hypercholesterolemia, and in his three

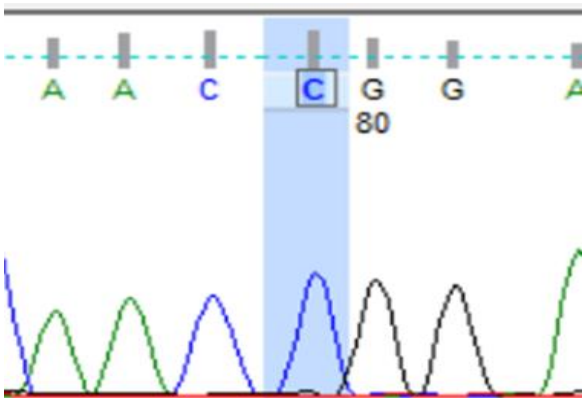
daughters, who were diagnosed with polycystic ovary syndrome. All these family members are heterozygous carriers for the C/T genotype, which might indicate that the variant has a potential pathogenic role in the etiology of the disease. This variant is appropriately segregated within the family members and shows a pattern consistent with autosomal dominant inheritance. Interestingly, unaffected family members are homozygous for the wild-type genotype (C/C) (Figures 7,8).



https://pedigree.progenygenetics.com/pedigree.png?X-XSRF-Cookie=1627677234&id=1717146816088

Figure 3.2 Family-1 Pedigree. The Pedigree Shows Genotyping Results Of LDLR Gene For Each Member Of Family 1. The Colored Symbols Indicate Phenotypes Within Family Members, As Shown In The Figure, And The Black Arrow Indicates The Proband.

CC Genotype



CT Genotype

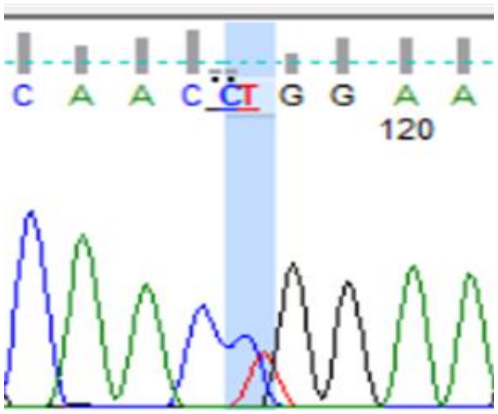
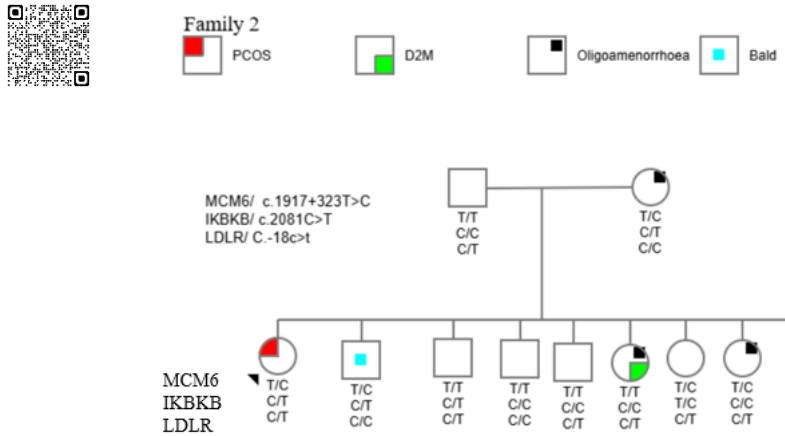


Figure 3.3 Sanger Sequencing Results of the LDLT (1783C.T) Variant, CC Genotype is the Wildtype, Double C/T. The Double Peaks Indicated the Heterozygous C/T Genotype.

Family 2

Three variants were identified in the second family are: MCM6 (c.1917+323T> C), IKBKB (c.2081C>T), and LDLR (c.-18C>T).

The proband is heterozygous for the MCM6 variant. She inherited the C allele from her mother, who suffers from oligomenorrhea. In addition to the proband, one brother with masculine baldness and two sisters, who have oligomenorrhea as their mother, are also heterozygous for this variant, which indicates that this variant may be related to oligomenorrhea, which is considered one of the symptoms of PCOS (Figures 9, 10). However, one sister who is affected with oligomenorrhea has the wildtype T/T genotype. In addition, one unaffected sister is heterozygous for this variant. These two findings indicate that although the C allele was detected in affected subjects in the family, this variant seems to be unrelated to PCOS or its symptoms, oligomenorrhea (Figures 9, 10). Regarding the IKBKB variant identified in the family, the T allele of the variant is expressed in the proband in a heterozygous manner. It is also expressed in one healthy sister and two brothers; one of them is bald, indicating that this variant is nonsignificant (Figures 9. 11). The T allele of the LDLR variant is expressed in the father, two healthy brothers, and all sisters, in addition to the proband, indicating that this variant is not significant in PCOS pathogenesis (Figures 9, 12). All together, it seems that none of the identified variants is significant to the disease.



https://pedigree.progenygenetics.com/pedigree.png?X-XSRF-Cookie=1832304021&id=1717148773689

Figure 3.4 Family-2 pedigree. The pedigree shows the genotyping results of the MCM6, IKBKB, and LDLR genes for each member of Family 2. The colored symbols indicate the phenotypes within the family members, as shown in the figure, and the black arrow indicates the proband.

TT Genotype

TC Genotype

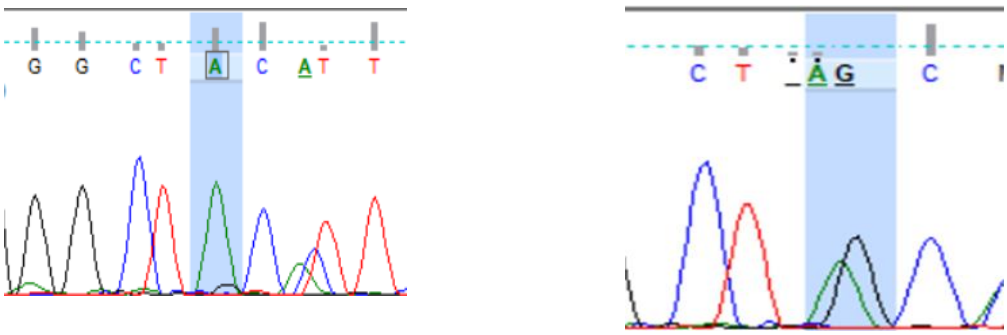


Figure 3.5 Sanger Sequencing Results Of MCM6 Gene, TT Genotype Is The Wildtype. Double Peaks Indicated A Heterozygous Genotype.

CC Genotype

CT Genotype

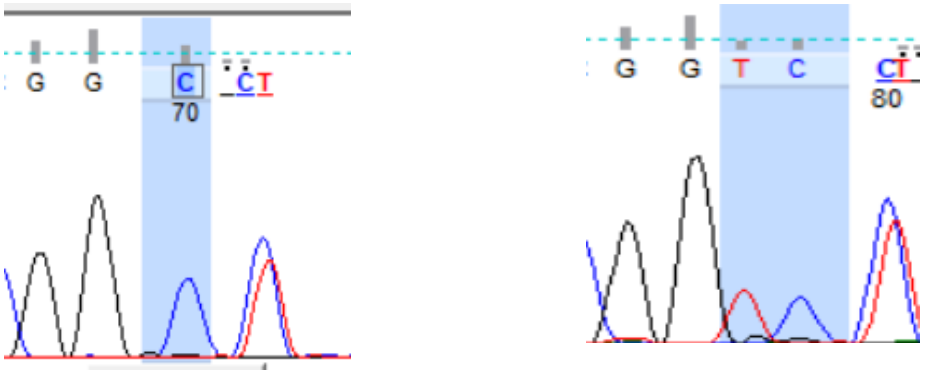


Figure 3.6 Sanger Sequencing Results of The IKBKB Gene Show That the CC Genotype Is The Wild Type. Double Peaks Indicated Heterozygous Genotype.

CC Genotype

CT Genotype

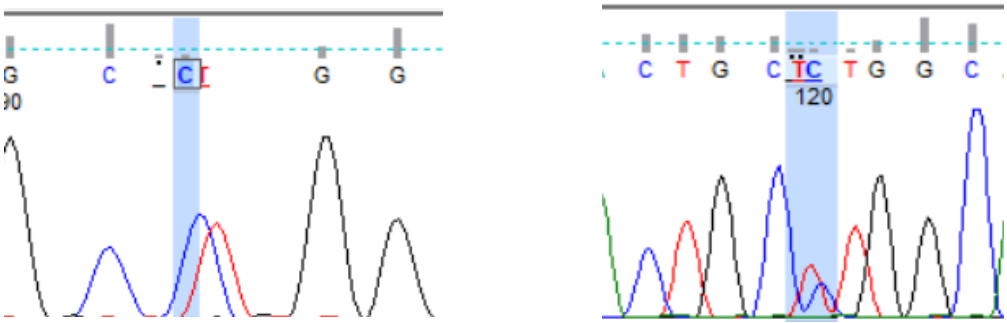
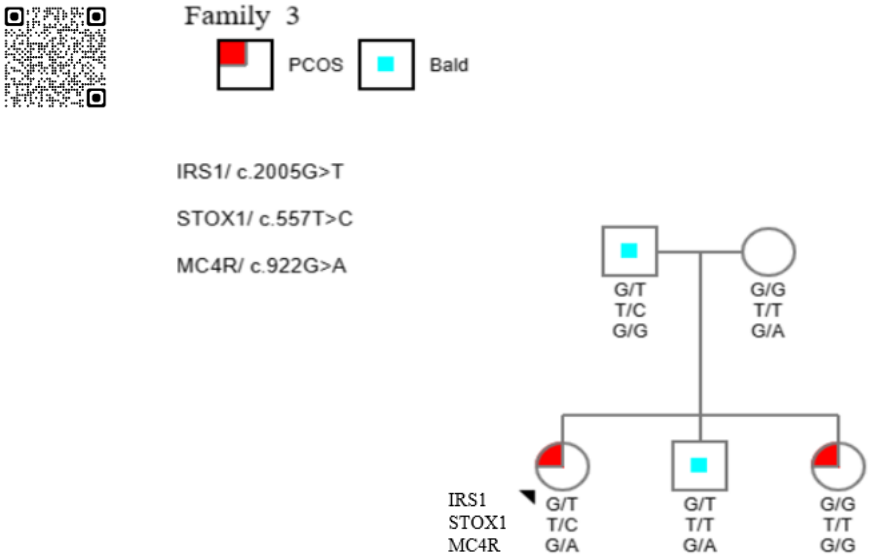


Figure 3.7 Sanger Sequencing Of The LDLR Gene, CC Genotype Is The Wildtype, And CT Genotype Is The Heterozygous Mutation.

Family 3

Three variants were detected in the third family: IRS1 (c.2005G>T), STOX1 (c.557T>C), and MC4R (c.922G>A).

The IRS1 variant is expressed in the proband and one brother with masculine baldness like the father, while the PCOS sister exhibits a wild-type genotype. This means that this variant did not segregate well within the family (Figure 13,14) and might be nonsignificant in relation to PCOS. The C allele of the STOX1 gene is expressed in both the proband and her father only. However, it failed to be segregated among the family members (Figures 13, 15), suggesting it is not significant to the etiology of the disease. The A allele of the MC4R gene is expressed in the mother, the proband, and her brother but not in the affected sister (Figures 13, 16). This also suggests that this variant could be nonsignificant in the etiology of PCOS since it failed to be segregated within the family.



https://pedigree.progenygenetics.com/pedigree.png?X-XSRF-Cookie=1832304021&id=1717149757186

Figure 3.8 The Pedigree Of Family 3. The Pedigree Shows The Genotyping Results Of The IRS1, STOX1, And MC4R Genes For Each Member Of Family 3. The Colored Symbols Indicate The Phenotypes Within The Family Members, As Shown In The Figure, And The Black Arrow Indicates The Proband.

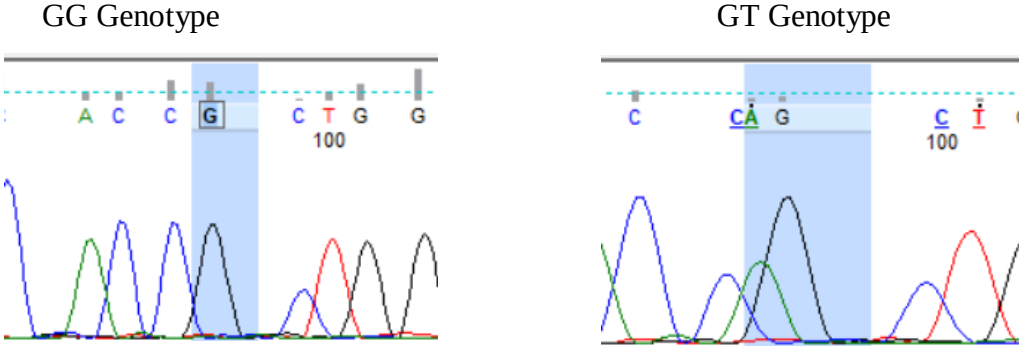
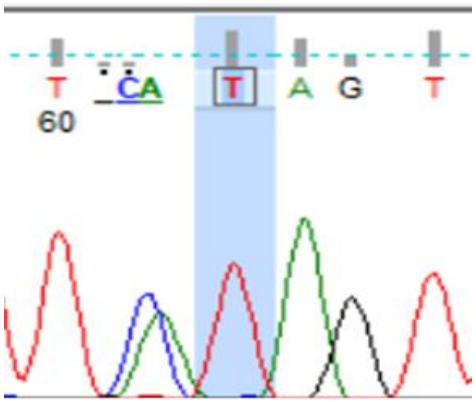


Figure 3.9 Sanger Sequencing Results For The Variant In The IRS1 Gene In F3. GG Genotype Is The Wildtype. Double Peaks Indicated A Heterozygous Genotype.

TT Genotype



CT Genotype

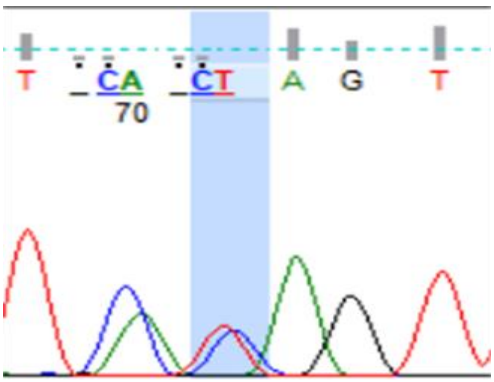
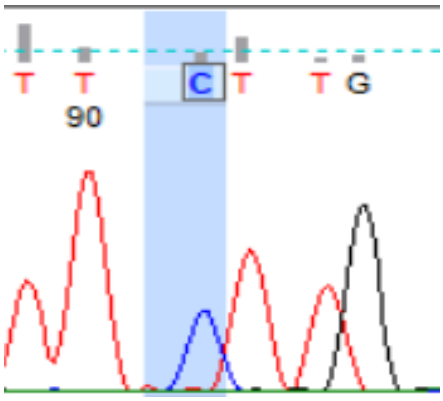


Figure 3.10 Sanger Sequencing Results For The Variant In STOX1 For F3. TT Genotype Is The Wildtypes. Double Peaks Indicated A Heterozygous Genotype.

GG Genotype



GA Genotype

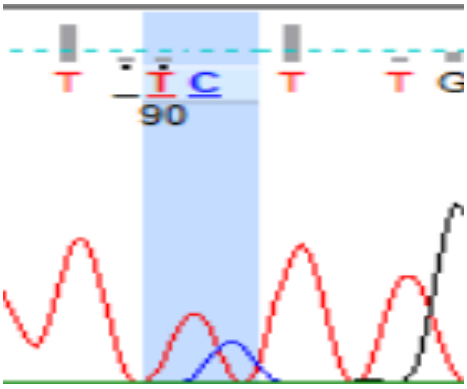


Figure 3.11 Sanger Sequencing Results For MC4R In F3. GG Is The Wildtype Genotype. Double Peaks Indicated A Heterozygous Genotype.

Family 4

Four variants were detected in family 4. IRS1 (c.3662G>C), IRS2 (c.195delG), FBN1 (c.3172G>A), and PON1 (c.380T>G). The FBN1 and PON1 variants identified are expressed in multiple affected individuals in a heterozygous manner. Both variants in FBN1 and PON1 segregate effectively within family members (Figures 17, 20, 21), supporting their significance in the disease. The IRS1 variant is expressed only in the proband, while the IRS2 variant seems to be a de novo mutation (Figures 17, 18, 19), where the deletion for the G allele occurred only in the proband and did not segregate from the paternal and maternal line. The segregation pattern of IRS1 and IRS2 indicated they could be insignificant and not associated with PCOS (Figure 17).

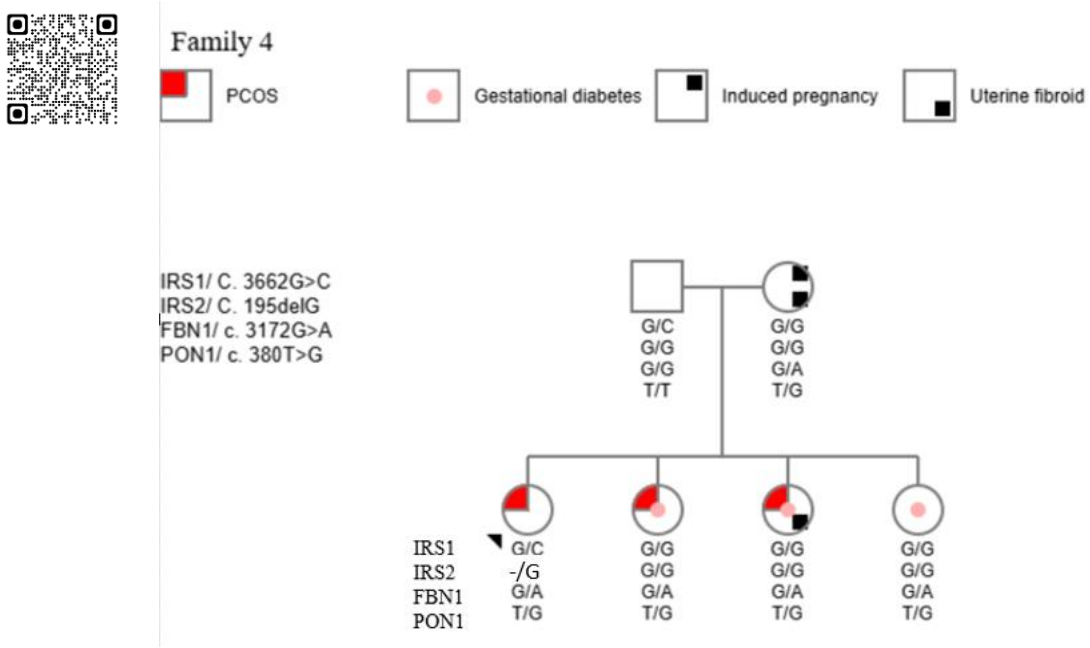


Figure 3.12 The Pedigree Of Family 4. The Pedigree Shows The Genotyping Results Of The IRS1, IRS2, FBN1 Genes For Each Member Of Family 4. The Colored Symbols Indicate The Phenotypes Within The Family Members, As Shown In The Figure, And The Black Arrow Indicates The Proband.

https://pedigree.progenygenetics.com/pedigree.png?X-XSRF-Cookie=1832304021&id=1717151033707

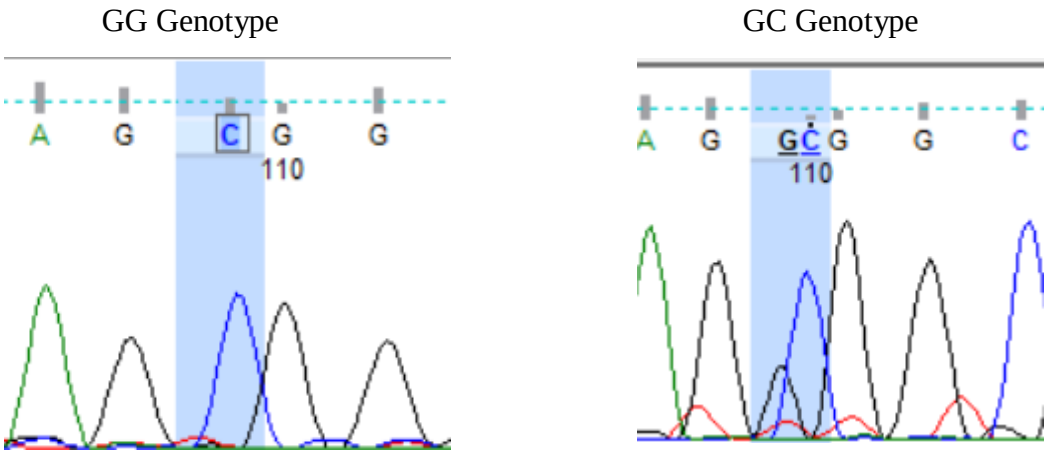


Figure 3.13 Sanger Sequencing Results Of IRS1 Variant. GG Is The Wildtype Genotype. Double Peaks Indicated A Heterozygous Genotype.

GG Genotype

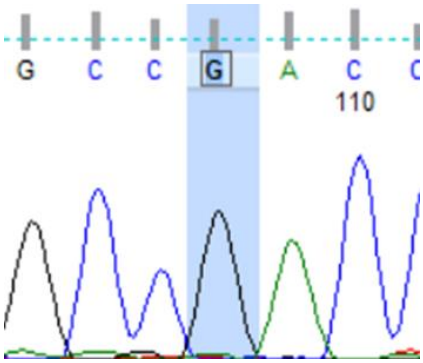
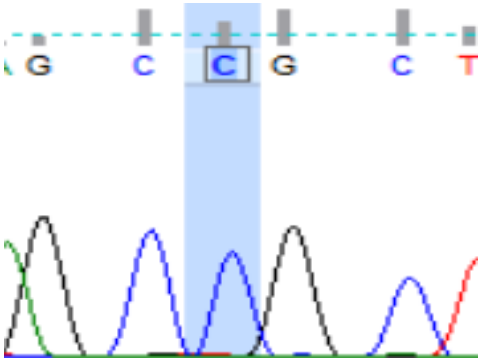


Figure 3.14 Sanger Sequencing Results Of IRS2 Variant. GG Is The Wildtype Genotype. Double Peaks Indicated A Heterozygous Genotype.

GG Genotype



GA Genotype

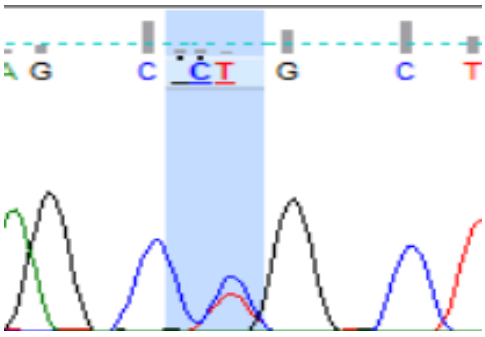
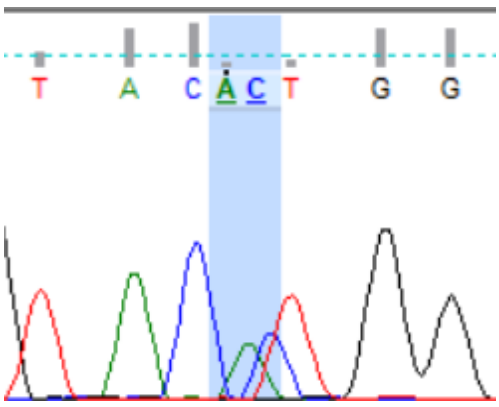


Figure 3.15 Sanger Sequencing Results Of The FBN1 Variant. GG Is The Wildtype Genotype. Double Peaks Indicated A Heterozygous Genotype.

TT Genotype



TG Genotype

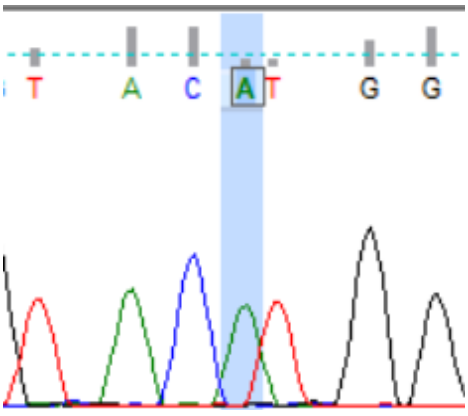
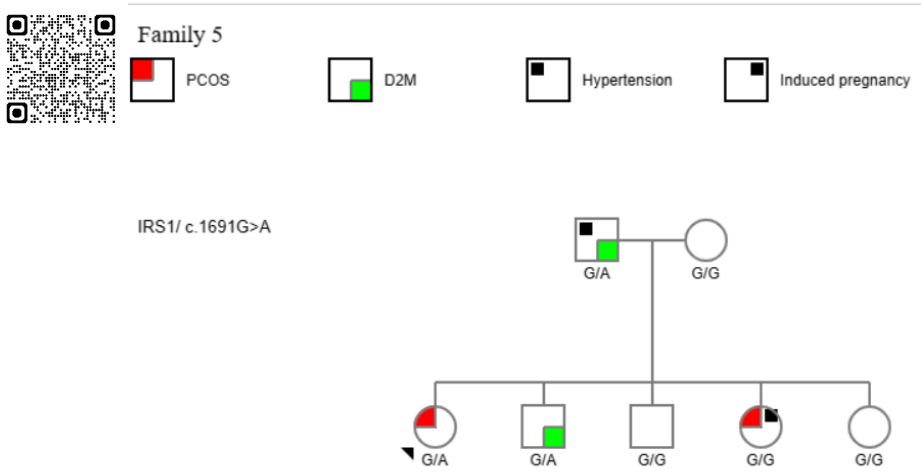


Figure 3.16. Sanger Sequencing Results Of The PON1 Variant. TT Is The Wildtype Genotype. Double Peaks Indicated A Heterozygous Genotype.

Family 5

One variant, IRS1 (c.1691G>A), was identified in the last family. The variant failed to segregate well within the family. The variant allele is inherited from the father affected

with T2DM. The proband and one brother who is affected with T2DM are heterozygous for the identified variant, while the rest of the siblings, even the affected sister, have wild-type genotypes, indicating that the variant seems insignificant to the disease (Figures 22, 23).



https://pedigree.progenygenetics.com/pedigree.png?X-XSRF-Cookie=1832304021&id=1717151881418

Figure 3.17. Pedigree of Family 5. The Pedigree Shows The Genotyping Results Of The IRS1 Gene For Each Family Member. The Colored Symbols Indicate The Phenotypes Within The Family Members, As Shown In The Figure, And The Black Arrow Indicates The Proband.

GG Genotype

GA Genotype

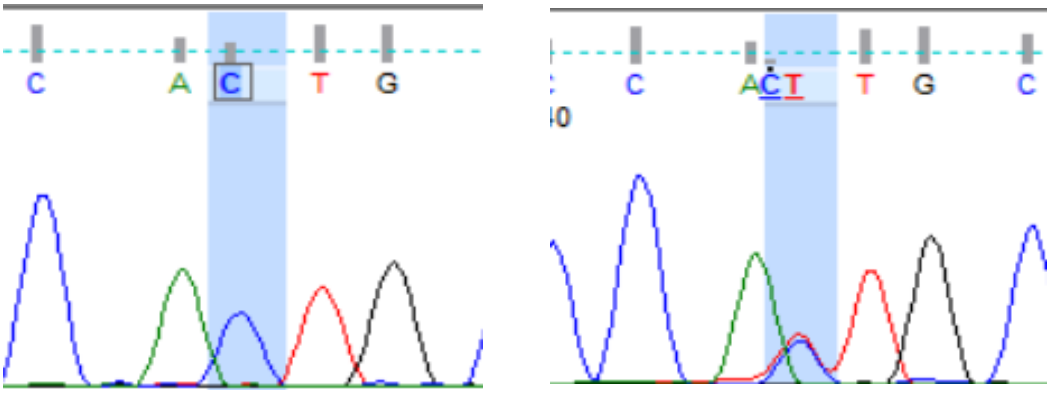


Figure 3.18 Sanger Sequencing Results For IRS1 In F5. GG Type Is The Wildtype Genotype. Double Peaks Indicated A Heterozygous Genotype.

3.5 In-silico Analysis of the Confirmed Variants

All variants, except CYP21A in family one and the variants of MCM6 and LDLR in the third family, were confirmed by Sanger sequencing and analyzed in silico using different bioinformatic tools to predict their conservation and pathogenicity.

3.6 Alignments of the Genes Using COBALT Alignment Tool

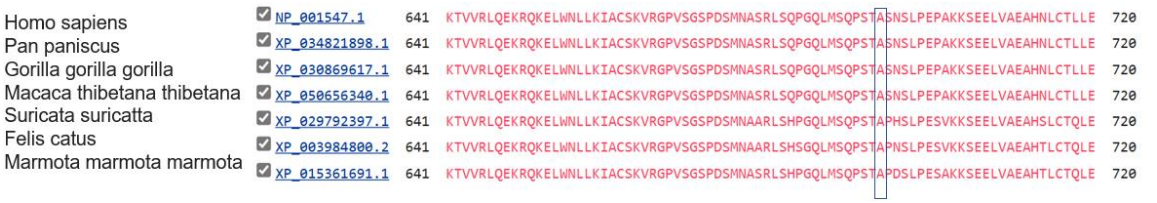
To understand the importance of the change sites, we did a conservation analysis using the COBALT alignment tool, which aligns the genes with different species. The results are summarized in the table (Table 4). The analysis shows that all analyzed sites are conserved because they lie in highly conserved regions (Figure 24), suggesting that the affected sites might be important for their protein functionality.

3.6.1 Family 1

A) LDLR (Arg 595 locus):

3.6.2 Family 2

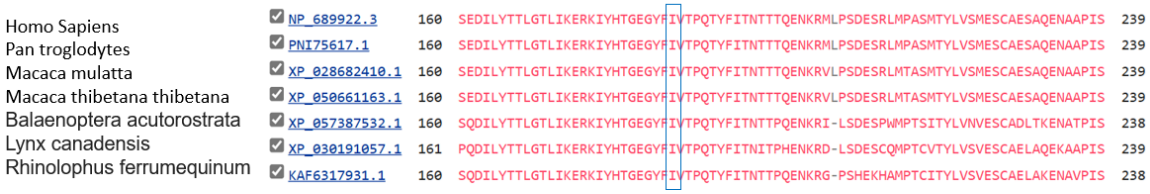
B) IKBKB (Ala 694 locus):



3.6.3 Family 3

C) IRS1 (Gly 669 locus):

D) STOX1 (Ile 186 locus):



E) MC4R (Glu 308 locus):

3.6.4 Family 4

F) IRS1 (Arg 1221 locus):

G) IRS2 (Ser 66 locus):

H) FBN1 (Gly 1058 locus):

I) PON1 (Met 127 locus):

3.6.5 Family 5

J) IRS1 (Ser 564 locus):

Figure 3.19 Conservation analysis of the studied variants according to the COBALT alignment tool. A) LDLR (Arg 595 locus) alignment in family one, where Arginine is highly conserved among Homo sapiens and other different species (F1). B) IKBKB (Ala 694 locus) in the second family (F2), in which Alanine is highly conserved among different species, including Homo sapiens. C) IRS1 (Gly 669 locus) in family three (F3), where the Glycine amino acid is highly conserved among different species. D) STOX1 (Ile 186 locus) in (F3), where the Isoleucine amino acid is highly conserved among different species. E) MC4R (Glu 308 locus) in (F3), Glutamic acid appears highly conserved. F) IRS1 (Arg 1221 locus) in the fourth family (F4), where Arginine is highly conserved in this locus among different species. G) IRS2 (Ser 66 locus) in (F4), in which Serine amino acid is highly conserved among different species. H) FBN1 (Gly 1058 locus) in (F4), Glycine amino acid is highly conserved. I) PON1 (Met 127 locus) in (F4), where Methionine amino acid is highly conserved among Homo sapiens and many different species. J) IRS1 (Ser 564 locus) in the last family (F5), where Serine amino acid is highly conserved in many different species.

4.7 Pathogenicity Prediction Tools

In order to access the potential pathogenicity of the identified variants, we used different pathogenicity tools. The results, summarized in table 14, show that some of the identified variants like LDLR c.1783C>T, STOX c/557T>C, MC4R c.922G>A, IRS2 c.195delG, PON1 c.380T>G, and FBN1 c.3172G>A, are probably protein function damaging variants and thus could be pathogenic (Table 13).

T

able 3.7 In-silico Analysis of the Variants Using Different Prediction Tools of Conservation Analysis and Pathogenicity Assessment.

Family No.	Variant	Conservation	Pathogenicity prediction tools						
		COBALT	SIFT	Mutation Taster	PolyPhen v2	Clinvar	Provean	GVGD	Fathmm
Family 1	**LDLR** (NM_000527.5) c.1783C>T	Conserved	Not tolerated	Disease-causing	Probably damaging	Pathogenic	Deleterious	Pathogenic	Damaging
Family 2	IKBKB (NM_001556.3) c.2081C>T	Conserved	Tolerated	Probably harmless	Benign	Likely benign	Neutral	pathogenic	Tolerated
Family 3	**IRS1** (NM_005544.3) c.2005G>T	Conserved	Not tolerated	Disease-causing	Benign	Uncertain significance	Neutral	Pathogenic	Tolerated
	STOX1 (NM_152709.5) c.557T>C	Conserved	Not tolerated	Disease-causing	Probably damaging	No information	Deleterious	Pathogenic	Damaging
	MC4R (NM_005912.3) c.922G>A	Conserved	Not tolerated	Disease-causing	Probably damaging	No information	Deleterious	Pathogenic	Tolerated
Family 4	**IRS1 (**NM_005544.3) c.3662G>C	Conserved	Not tolerated	Probably harmless	Benign	No information	Neutral	Pathogenic	Tolerated
	IRS2 (NM_003749.3) c.195delG	Conserved	Damaging	Disease-causing	No information	No information	Deleterious	Likely pathogenic	Tolerated
	FBN1 (NM_000138.5) c.3172G>A	Conserved	Tolerated	Disease-causing	Probably damaging	Conflicting classification of pathogenicity	Deleterious	Pathogenic	Damaging
	PON1 (NM_000446.7) c.380T>G	Conserved	Not tolerated	Disease-causing	Benign	Likely benign	Deleterious	Pathogenic	Tolerated
Family 5	**IRS1** (NM_005544.3) c.1691G>A	Conserved	Not tolerated	Probably harmless	Possibly damaging	No information	Neutral	Likely pathogenic	Tolerated

4.8 Study Population Characteristics

The percentage of PCOS frequency in different regions of Palestine was investigated among PCOS women visiting IVF centers and undergoing assisted reproductive techniques (ART). Approximately two hundred patients, including the study subjects, were selected randomly from different IVF centers in different countries in Palestine and classified according to diagnosis and age. Data was collected from the medical reports of

these patients, including affected and unaffected individuals. All PCOS patients were diagnosed according to Rotterdam Criteria. (Table 14).

Table 3.8 The Frequency of PCOS Among Palestinian Women. Data was Collected From IVF Clinics in West Bank Cities and the Gaza Strip.

Region	Ramallah	Hebron	Nablus	Toul Karem	Jenin	Gaza Strip	Bethlehem	Jerusalem
No. of PCOS cases	168	51	122	66	143	96	62	66
No. of non-PCOS	865	349	566	134	16	104	95	308
Total number	1063	400	688	200	159	200	157	374
Ages range (years)	24-41	20-39	22-39	20-39	21-41	19-43	25-43	22-42
Percentage	23%	15%	22%	33%	10%	48%	39%	21%

The prevalence of PCOS in Palestine was compared with different regions of North Africa and the Middle East (MENA) using the Institute for Health Metrics and Evaluation (IHME) tool (https://vizhub.healthdata.org/gbd-compare/). The prevalence and years lived with disability (YLDs) were retrieved from 1990 to 2021, compared with different regions, and presented with rates per 10,000 population, age, and years; the collected data from the Global Burden of Disease 2019 study (GBD) are interpreted in figures 25, 26, 27. The subnational estimation is for the annual percentage changes among females of all ages, diagnosed with PCOS from 1990 to 2021, and the prevalent cases are per 100,000 population in different states (Figure 25). The prevalent cases of PCOS per 10,000 population in Palestine were estimated according to years and ages, where PCOS is increasing by the years, and most cases are increasing during their reproductive ages (Figure 26). Finally, the percentage of the total Years Lived with Disability (YLDs) for PCOS patients was estimated according to the MENA region; 18 countries were chosen for this comparison (Figure 3.20).

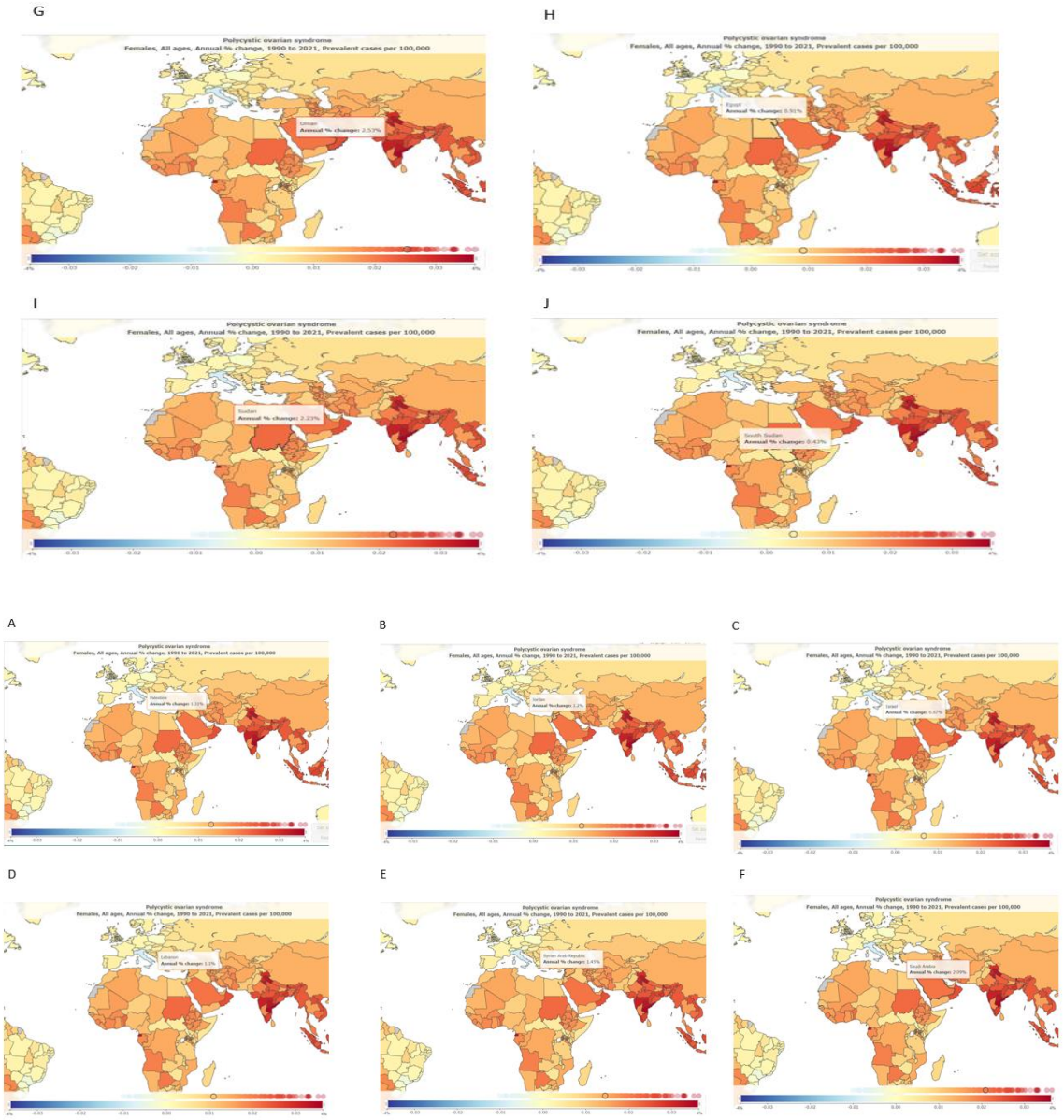


Figure 3.20 GBD Comparison: A Chart Visualizes County Or Subnational Estimates On A Map. The Estimation Is For The Annual Percentage Change Among Females Of All Ages, Diagnosed With PCOS From 1990 To 2021. The Prevalent Cases Per 100,000 Population. The Red Color Is Recorded As The Highest Percentage; The Blue Color Is Recorded As The Lowest Percentage, While The Orange Color Is In The Middle. A) Annual % Change In Palestine Is 1.32%. B) Annual % Change In Jordan Is 1.2%. C) Annual % Change Is 0.67% Among The Israeli Population. D) The Annual % Change In Lebanon Is 1.1%. E) Annual % Change In Syria Is 1.45%. F) Annual % Change In Saudi Arabia Is 2.09%. G) Annual % Change In Oman Is 2.53%. H) Annual % Change In Egypt Is 0.91%. I) Annual % Change In Sudan Is 2.23%. J) Annual % Change In South Sudan Is 0.43%. Https://Vizhub.Healthdata.Org/Gbd-Compare/

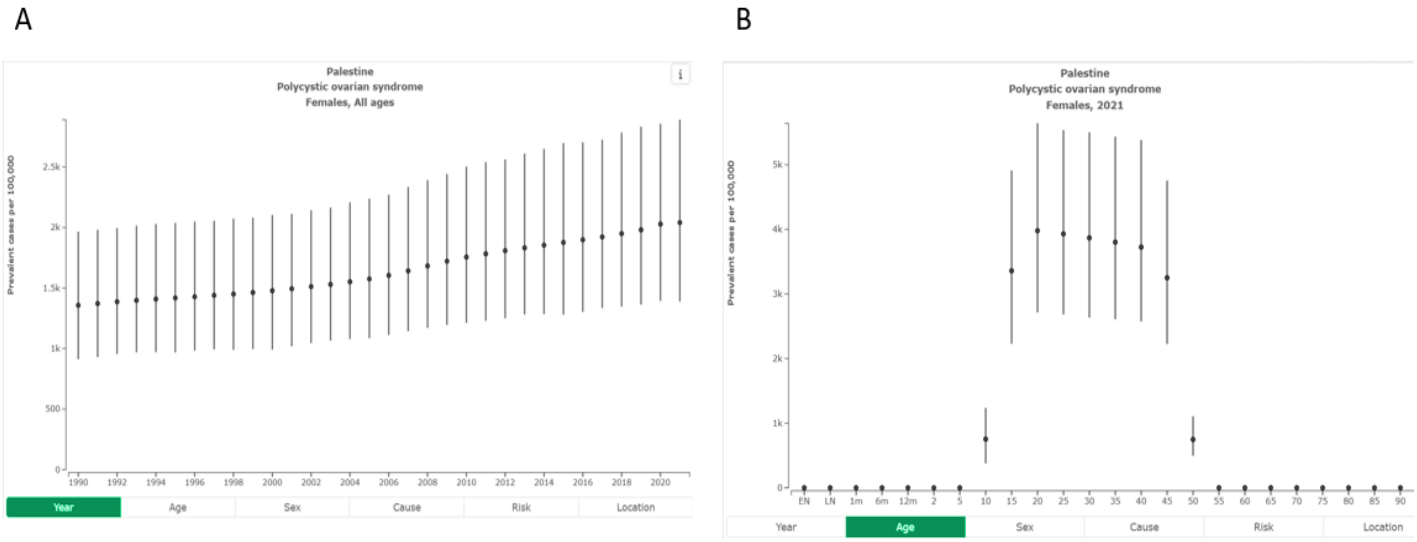


Figure 3.21 The plot chart displays mean estimates (points) and 95% uncertainty intervals (lines) by years, sex, risk, cause, or location. A) shows the prevalence of PCOS per 100,000 of the population in Palestine for all ages according to the years from 1990 to 2021. B) shows the prevalence of PCOS among Palestinian females in the year 2021 according to their ages.
https://vizhub.healthdata.org/gbd-compare/

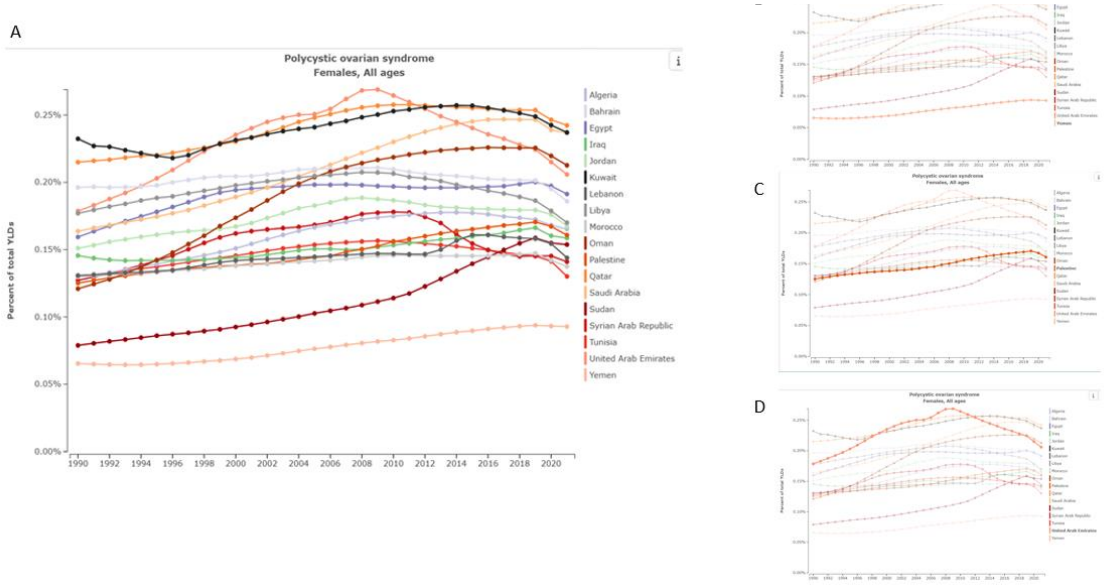


Figure 3.22 The Line Chart Displays Estimates for Causes and Risks by Years for Selected Ages and Locations. This Chart Shows the Percentage of The Total Years Lived with Disability (Ylds) For Females with PCOS Of All Ages Over the Years (1990-2021) According to MENA Region. A) Shows the Ylds for 18 Countries Were Chosen Randomly for Comparison. B) Shows the Ylds In Yemen, Which Recorded the Lowest Percentage. C) Shows the Ylds In Palestine. D) Shows the Ylds In the United Arab Emirates, Which Recorded the Highest Percentage. Https://Vizhub.Healthdata.Org/Gbd-Compare/.

Chapter Five: Discussion

Polycystic ovary syndrome (PCOS) is a chronic heterogeneous disorder that leads to reproductive and metabolic disorders in women during the reproductive age. It affects 8% to 20% of women worldwide during their reproductive age. Due to its ambiguous etiology, it is considered a multigenic and multifactorial disease. It is the most common but the least understood endocrinopathy. The significant factors contributing to PCOS pathogenesis are insulin resistance, oxidative stress, and obesity. (Dadachanji et al., 2015). PCOS phenotypes are expressed when the female adrenal glands produce excess male hormones, leading to hyperandrogenism. However, the molecular mechanisms underlying PCOS development are unclear (M. R. Islam et al., 2020). The most common feature of PCOS is polycystic ovary morphology (PCOM), oligomenorrhea, and hyperandrogenism. PCOS patients usually suffer from risk factors with cardiovascular disease, including IR, unfavorable lipid profile, obesity, endothelial dysfunction, impaired fibrinolysis, and increasing coagulation cascade (Dursun et al., 2006; Gu et al., 2022).

The morphological characteristics of PCOS are the accumulation of 2-8 mm diameter follicles. This means the large antral follicles have been blocked during development, and the dominant follicle selection has failed. The follicle atresia is related to endocrine abnormalities, like excessive latinizing hormone (LH), androgen, and insulin secretions. This abnormality leads to secondary inhibition of the follicular stimulating hormone (FSH), which, in turn, inhibits follicles' maturation (Khan et al., 2018).

The complexity of the disease resides in its multifactorial and heterogeneous nature, where several factors, including hormonal, environmental, and genetic factors, contribute to its development, in addition to various clinical manifestations that show high heterogeneity and familial aggregation phenomenon of the disease. (Zhong et al., 2021). Although the etiological causation behind PCOS is unclear and not well elucidated, it is believed that PCOS is heritable, where genetic factors play an essential role in its etiology. Several studies support this idea and documented ethnic predisposition, concordant twin studies, familial aggregations, and association with other Mendelian disorders. (Nicolaides et al., 2020).

The prevalence of PCOS is constantly increasing among adolescents and adult women in Palestine, and there is a large gap in precise diagnosis and individualized therapies due to a lack of knowledge and awareness (Abu-Taha et al., 2020). This highlights the

importance of investigating the genetic and epigenetic factors that could lead to PCOS development. In recent years, researchers and clinicians have been interested in looking for the relevant genes that contribute to the pathogenesis of PCOS.

The presented study aimed to screen for genetic factors associated with PCOS pathogenesis among a cohort of women in the Palestinian population. Furthermore, the study aimed to measure the percentage of PCOS frequency among the affected women who visit fertility clinics to pursue conception and solve infertility questions.

In the first part of our research, we studied five PCOS women diagnosed according to the Rotterdam Criteria. The hormonal, anthropometric, and clinical parameters were conducted. The laboratory results of the biochemical parameters, including LH, FSH, FT, and LH/FSH ratio, provided evidence of the disease spectrum. As anticipated in most PCOS patients, LH levels were significantly increased, while FSH levels were in the normal range. LH/FSH ratio also increases dramatically in most cases, while it is usually 1:1 in normal individuals. The elevated levels of LH and the high LH/FSH ratio are hallmarks of PCOS patients. Earlier studies reported the increase in follicle numbers and ovarian volume is possibly correlated with high LH levels, which was found in most PCOS cases in the study, indicating LH hypersecretion had a potential effect on ovulation, pregnancy complications, fertility, and subfertility problems in affected women. (Deswal et al., 2019).

Evidently, free testosterone levels showed normal ranges in four of our patients except in patient number five, who was the only adolescent patient in this study who showed the highest BMI (BMI is 35), which is a common feature among PCOS women. Also, she has the highest levels of testosterone (882 pg/ml) among the study subjects. Significantly, more adolescents among PCOS patients show markers of more PCOS features and poor metabolic health (Jabeen et al., 2022; Lidaka et al., 2021).

Although the levels of testosterone are standard in four of our patients, they show hyperandrogenism as they share most PCOS symptoms. Hyperandrogenism is the hallmark feature of PCOS that occurs due to excessive production of androgens. However, some PCOS women have normal testosterone levels, albeit they are exhibiting clinical hyperandrogenism symptoms. This could be due to low levels of Progesterone hormone that are exhibited in most PCOS cases due to chronic anovulation. The lack of Progesterone increases LH levels, stimulating the ovaries to produce more androgens. (Abbott et al., 2019; Azziz et al., 2009).

After following the medical histories of the participants and their families, it has been found that the probands had a familial background for PCOS.

To the best of our knowledge, genetic variants that may contribute to the etiology of PCOS have never been reported among the Palestinian population. This is the first study to highlight the significant association of genetic polymorphisms with increased risks of PCOS development in Palestine.

Family1

Two variants were identified in the first proband of family number one (F1). The first one is the CYP21A2 c.*2405_*2406delGTinsTC variant. This indel is located in exon 36 of the 44 exons of chromosome number six. The nucleotides GT were deleted, and new TC nucleotides were inserted, causing a missense mutation at positions 2405 and 2406 within the 3'UTR, the stop codon of the CYP21A2 gene. This variant was reported in ClinVar and classified as uncertain significance. After a deeper look into this variant, it seemed to be related to the Tenascin-X (TNXB) gene but not the CYP21A2 gene. In the gene bank, TNXB and TNXA lie on the opposite strand of DNA from the C4 and CYP21 genes. The last exon of TNXA and TNXB lies within the 3' UTR region of exon 10 in CYP21A1P and CYP21A2. TNXB gene was not reported before to be relevant to PCOS disorder. Thus, this patient was probably misdiagnosed. Further diagnosis and genetic analyses were recommended to follow up with the patient. PCOS could be misdiagnosed with other diseases like congenital adrenal hyperplasia (CAH) because their symptoms are similar. CAH is related to a 21-hydroxylase deficiency, which results in symptoms that are similar to PCOS but with more severe outcomes. This deficiency is associated with Ehlers-Danlos syndrome in patients with CAH. (Merke et al., 2013). In their study, these authors reported that the CYP21A2 gene is flanked with the gene encoding TNXB. In fact, CYP21A2 and a highly homologous pseudogene, CYP21A1P, are mapped to the short arm of chromosome 6, where the TNXB gene is located. The same study was conducted on 192 CAH patients and found 13 patients with deleterious TNXB mutation, and 13 patients were either homozygous or heterozygous deletion of the CYP21A2 gene. The remaining deletions resulted in chimeric CYP21A2 and CYP21A1P genes, which do not affect the TNXB gene. After sequencing, it was found that a premature stop codon variant was predicted, resulting in haplotype deficiency in one patient with a homozygous CYP21A2 variant. Hence, the premature stop codon mutation in TNXB indicates that CYP21A2 mutations lead to increased TNXB mutation risks due to genetic recombination. (Merke et al., 2013). A study conducted by Maffziolo, Giovana DN, et al.

demonstrated that steroid level analysis, in addition to testing the level of some electrolytes, can differentiate between CAH and PCOS. CAH leads to electrolyte imbalance, where Potassium levels become high while Sodium levels become low. CAH is also characterized by high levels of cortisol, aldosterone, and androgens. This study concluded that basal 17-OHP levels >5.4ng/mL can diagnose PCOS and CAH differently. At the same time, the 17-OHP/Cortisol ratio is higher in PCOS patients than in CAH patients. However, the basal 17-OHP/Cortisol ratio was not superior to basal levels of 17-OHP (Maffazioli et al., n.d.). Because the variant identified in family one could be related to non-classical CAH and not to PCOS, this variant seems unrelated to PCOS, and the patient in family number one was asked for further blood tests, such as cortisol and electrolytes, to be appropriately diagnosed.

Interestingly, a previous study reported that a heterozygous variant in CYP21A2 plays a crucial role in the pathogenesis of PCOS, with 14 variants detected in CYP21A2 in progressing PCOS (Ajmal et al., 2019).

The second variant was the rs373371572 polymorphism found in exon 12 of the LDLR gene (c.1783C>T). This variant leads to a missense effect, causing an Arginine substitution with Tryptophan (Arg595Trp). The identified variant was located within a highly conserved region. This variant was predicted to be disease-causing. It is worth mentioning that this polymorphism has never been identified before to be associated with PCOS, especially among the Palestinian population.

Lipid metabolic disorders are the most frequent clinical complication associated with PCOS. Several studies have demonstrated that LDLR polymorphisms may be associated with the development of PCOS. In these studies, it was shown that LDLR variations are associated with hyperandrogenism, obesity, insulin resistance, and glucose intolerance, which are hallmarks of PCOS and play essential roles in developing metabolic disorders and CVDs (Pruett et al., 2023). In a previous study, Mondal *et al.* demonstrated that dyslipidemia is the standard intermediate in translating the metabolic effects of these factors, and they reported that hyperandrogenism and hyperhomocysteinemia (Hcy) together might disrupt lipid homeostasis in PCOS, which involves a complex interaction between many factors of which Serine protease (PCSK9) plays an essential role. The liver produces PCSK9 normally, which has an inhibitory effect on the expression of the receptors of LDL (LDLR), which is essential for LDL metabolism. These results showed that Hcy and androgens, like testosterone and Dihydrotestosterone **(**5α-DHT), potentially

affect the PCSK9/LDLR pathway, causing PCSK9 overexpression and reduced LDLR levels. (Mondal et al., 2018).

Another link that connects LDLR to PCOS is that up to 70% of PCOS patients have dyslipidemia, which is implicated in anovulation among these patients. It was found that women with anovulatory PCOS have higher concentrations of TGs, LDL-C, and lower concentrations of HDL-C than women with ovulatory PCOS (Liu et al., 2019).

There is a close relationship between lipid metabolism and ovarian function, where cholesterol is crucial for endocrine, ovarian, and follicular development and maturation. Recently, it was reported that plasma lipoproteins are the major source for steroidogenesis in ovaries. LDL-C can be absorbed by granulosa cells (GCs) and theca-interstitial cells (TICs), and can pass into follicular fluid (FF) through LDLR. Any lipid metabolism disruption can directly or indirectly impact follicular growth and function by changing follicular fluid environment (M. Wang et al., 2019).

It was revealed recently that dyslipidemia, which is common among PCOS patients, leads to increased production of androgens which are then converted into Estradiol (E2), which secreted by follicles. E2 influences on hypothalamus and pituitary gland, stimulating LH release. High LH levels lead to abnormal development to follicles. As a result, immature follicles do not mature properly and may either turn cystic or degenerate, resulting on ovarian polycystic changes.

Family2

Three variants have been identified in the patient from second family (F2). The first variant was MCM6 gene (c.1917+323T>C). The potential implications of this variant are significant. Minichromosome maintenance complex (MCM) is essential in regulating DNA replication, and dysregulation of this complex can induce multiple human cancers and ovarian cancer (OC) (Y. Li et al., 2021). However, this variant (rs41456145) in the MCM6 gene was not reported as relevant to PCOS. Since this SNP resides in intronic region, it may affect mRNA stability, gene expression, and the formation of an alternative protein isoform. However, because it is predicted that a real pathogenic variant in this gene could result in severe outcomes, this intronic variant was filtered out and excluded from in-silico pathogenicity predictions.

The second identified variant was IKBKB (c.2081C>T), a nonsynonymous codon variant replacing Alanine with Valine. The Inhibitor of Nuclear Factor Kappa B Kinase Subunit Beta gene encodes a protein kinase that regulates the signaling pathway of nuclear factor

Kappa B (NF-κB). This pathway regulates many cellular processes, including immune responses, inflammation, and cell survival. A previous study using knockout and transgenic mice models to study the IKK-IKB-NF-KB signaling system found that IKB-NF-KB plays an essential role in embryogenesis development and inflammatory and stress responses (P, n.d., et al., 2014).

A previous study in the Chinese Han population, based on a genome-wide association study (GWAS), reported that rs12676482 SNP in the IKBKB gene is associated with systemic lupus erythematosus (SLE). It has been reported that autoimmune disorders like SLE are related to the etiology of PCOS. Ovarian failure is common in patients with SLE as well as in PCOS (Y. Li et al., 2015)However, there is no direct evidence of a link between the IKBKB gene and PCOS development. Due to ethnicity and variability, it is important to test whether the identified SNP in IKBKB is genuinely associated with SLE and PCOS among different populations.

IKBKB c.2081C>T, identified in this family (F2), is a nonsynonymous codon variant that changes Alanine amino acid into Valine. Although this variant falls in a highly conserved region, according to in-silico tools, the pathogenicity of IKBKB is benign and probably harmless. Thus, it might not be relevant to PCOS pathogenicity. This variant also failed to segregate appropriately within the family.

The third identified variant was LDLR c.-18C>T. It is located in the 5'-UTR 18 nucleotides upstream of the first codon of the gene. This variant does not change any amino acids and is unlikely to have a pathogenic effect; hence, it was filtered out from in-silico analysis.

Family3

Three variants were detected in the third family (F3). The first one is IRS1 c.2005G>T. This variant is highly conserved among different species and was predicted to be pathogenic and damaging; however, it is regarded with unknown significance. Several studies reported different polymorphisms related to IRS1 and their relation to insulin signaling problems, which is one of PCOS-associated complications.

The second variant was in STOX1 (Storkhead-Box1 Protein) gene (c.557T>C). It is located in exon 3 of the gene. It is located in a highly conserved region and predicted to be pathogenic and damaging. However, based on published literature, this variant is classified as a variant of uncertain significance. This gene is known to be associated with preeclampsia. A recent genetic linkage study on 67 patients reported that the most

frequent variant of the STOX1 gene was (rs1341667). This variant results in replacement of Tyrosine amino acid with Histidine in the DNA binding domain of the protein. Moreover, this study revealed that pathogenic STOX1 gene variations are associated with early-onset preeclampsia and premature births (Gurbuz et al., 2021). Thus, in the current study, the identified variant in the third proband could explain the proband's recurrent failure of IVF attempts; however, regarding its role in PCOS pathogenesis, this variant is not related to PCOS since it failed to segregate properly in affected family members.

The third identified mutation was Melanocortin 4 receptor (MC4R) (c.922G>A) variant. This gene is composed of only one exon and is associated with increasing BMI, which is one of the complications of PCOS. It is essential in regulating the central melanocortin neuronal pathway and may contribute to autosomal dominant obesity (Batarfi et al., 2019). The role of MC4R variants in obesity was demonstrated in different studies. More than 150 variants within the MC4R gene have been reported to be relevant to metabolic disorders, and they are considered the most frequent genetic cause of obesity in different ethnic origins. These variants in the MC4R gene have been found in 2%-6% of severe cases of obesity (Batarfi et al., 2019; Bradnová et al., 2015; Hammad et al., 2020). Therefore, we suggest that the identified variant in MC4R gene is correlated with obesity since it is consistent with the measured anthropometric parameters for the patient affected by this variant, who has a very high IBM (IBM is 30), one of the PCOS comorbidities. This variant is highly conserved, predicted as pathogenic, and failed to be segregated within the family.

A study by Batarfi et al. demonstrated a significant association of variant rs17782313 (C/T) with obesity and the association of variant rs12970134 (A/G) with obesity and insulin resistance among Saudi Arabis population (Batarfi et al., 2019). Depending on the fact that PCOS is a polygenic disease, a previous study enrolled on PCOS patients to study association of obesity-related traits and MC4R rs17781313 in addition to variant FTO rs9939609. It has been found that there is a combined pathogenic effect of FTO and MC4R in developing PCOS. Although MC4R rs17782313 was not found to be related to increased PCOS susceptibility, they have found that patients who were affected with both FTO and MC4R risk alleles are suffering from PCOS more than those patients who were affected with one risk allele (Yuan et al., 2015). These findings lead us to think that although rs375095163 polymorphism of MC4R and rs557319201 of IRS1 identified in our patient cannot, each alone, explain the development of PCOS, they could work synergistically and maybe lead to PCOS development.

Family4

Four variants were identified in family four (F4). The coding sequence variant of IRS1 (c.3662G>C) is located in a highly conserved region and predicted to be pathogenic. The correlation between IRS1 and PCOS was discussed previously above.

The second detected variant was IRS2 c.195delG. The G allele deletion is damaging because it leads to a frameshift mutation that disrupts the reading frame from position 66, producing a truncated protein, most likely causing a loss of function mutation. The identified variant is located in a highly conserved position and was predicted to be deleterious. According to the SIFT tool, this variant causes nonsense-mediated decay (NMD), which is evidence that this mutation leads to a loss of function of the protein. The relationship between IRS2 and PCOS will be discussed later on.

The third variant was the Fibrillin-1 gene (FBN1 c.3172G>A) (rs144390654). Although the G/A substitution is a nonsynonymous variant, it is located within a highly conserved region and predicted to be pathogenic. The G/A allele of the rs144390653 polymorphism in the FBN1 gene segregated well in all family members. This is evidence that this variant may play a role in the pathogenesis of PCOS. However, no studies have reported the association between this variant and the etiology of PCOS. In a previous case-control study, Saud et al. examined the expression of FBN1 and LTBP1 transcripts among Iraqi women diagnosed with PCOS to investigate the potential role of these two genes in the pathogenesis of PCOS. (Saud et al., 2020). They reported a novel SNP in FBN1, likely to have a predominant effect on developing the disease in combination with the LTBP1 gene. The expression of both genes was higher in PCOS patients compared with the control subjects. FBN1 overexpression was statistically significance among PCOS subjects, and it could affect the hormonal regulation and reduce the function of transforming-growth factor (TGFβ) by preventing the release of TGFβ disrupting the signaling pathway. This may lead to an increase in susceptibility to metabolic disorders and CVDs in PCOS women. (Saud et al., 2020). In this sense, it is not clear whether this identified variant may lead to increased protein stability.

The fourth detected variant was a missense variant in the Paraoxonase-1 gene (PON1 c.380T>G). This variant is also located in a highly conserved region and is predicted to be deleterious and disease-causing.

The PON1 gene is expressed in the liver and encodes the antioxidant high-density lipoprotein-associated enzyme (Paraoxonase). Proinflammatory mediators and androgens reduce PON1 expression. PCOS patients usually have inflammation and

hyperandrogenism, which reduce PON1 expression and lead to an increase in oxidative stress (OS), one of the main features of PCOS. This indicates that PON1 might be related to the pathogenesis of PCOS (San Millán et al., 2004). Reduced serum PON1 activity could also contribute to insulin resistance since oxidative stress deteriorates insulin actions, which may explain why reduced serum PON1 can be found in T2DM, CVDs, and IR disorders, which are comorbid complications in PCOS patients (San Millán et al., 2004). Limited studies were conducted to investigate the association between PON1 polymorphisms and PCOS. In 2004, San Millan et al. genotyped three variants in PON1 among PCOS patients and reported that PCOS patients were homozygous with the PON1 gene (-108T) allele compared to the control subjects (San Millán et al., 2004). The findings in this study are in agreement with another study conducted by Durnus et al., who investigated the association between PON1 and the risk of atherosclerotic heart disease (AHD) among PCOS patients. They reported that PCOS patients have a homozygous genotype with the PON1 (-108 C>T) SNP and found significant signs of hyperandrogenism, like high hirsutism scores, high concentrations of androstenedione, free testosterone (FT), and total testosterone (TT) (Dursun et al., 2006)San Millan et al. revealed that variants in the PON1 gene decrease its expression, leading to increased OS, which might result in IR. Hence, the PON1 gene is probably associated with the development of PCOS and is involved in increasing IR, OS, and HA in PCOS patients. (San Millán et al., 2004). The PON1 (380T>G) identified in this study has not been reported before, and there is no evidence of its association with PCOS pathogenesis. Thus, more investigations are crucial to prove whether this variant could be relevant to PCOS, especially since the affected patient in the current study suffers from severe symptoms of PCOS since she experienced recurrent IVF failure trials without a single successful one.

Family5

IRS1 (c.1691G>A) polymorphism was found in family five (F5). This missense variant resides within a highly conserved region and was predicted as likely pathogenic or possibly damaging. Individuals in this family with the A allele were diagnosed with T2DM. The proband is an adolescent with IR and has the highest BMI (35) amongst all the study subjects.

In our study, different polymorphisms in IRS genes were evident in three families (F3, F4, F5). Many polymorphisms of IRS1 and IRS2 have been entangled with insulin

homeostasis. Such polymorphisms are linked with increased susceptibility to T2DM and are correlated with PCOS pathogenesis (Jones & Goodarzi, 2016).

It was reported that 50% - 90% of PCOS have IR, and 50% are obese. (F. Wang et al., 2014). Other studies have demonstrated that IR appears in all phenotypes of PCOS, while insulin sensitivity varies depending on the phenotypes of the disease. For example, IR is the most common among the classical phenotypes (types A and B), which account for 80% of the cases, and detected in 65% of ovulating PCOS and in 28% of non-hyperandrogenic PCOS (Zhao et al., 2023).

Our results, showed the presence of different IRS polymorphisms in multiple family members in the families we studied which is consistent with the fact that IR gene alteration is the most prevalent feature among PCOS patients, attributed to impaired insulin transduction pathways as reported previously. (Jamshidi et al., 2021). The most common polymorphisms in the IRS genes associated with PCOS and T2DM susceptibility are IRS1 Gly972Arg (rs1801278) polymorphism and IRS2 Gly1057Asp (rs1805097) polymorphism. (Ruan et al., n.d.). Two polymorphisms in the INSR and IRS1 genes, which are key genes strongly relevant to PCOS's pathogenicity, including IRS1 (rs1801278) and INSR (rs1799817) polymorphisms, were detected among PCOS patients, with no significant difference in the study population due to the small sample size, the limited number of the studied polymorphisms, and the high heterogeneity of the disease (Adam et al., 2022). In a study conducted on Turkish cohort, significantly different frequencies of the IRS1 Gly972Arg polymorphism were found among Turkish PCOS patients. They noticed the variant carriers were obese and suffered from IR (Dilek et al., 2005). Another meta-analysis investigated the association of IRS1 and IRS2 polymorphisms with PCOS, revealed the A allele of Gly972Arg in IRS1 significantly increased the risk of PCOS compared with the G allele. In contrast, the IRS2 Gly1057Asp polymorphisms showed no significant association with PCOS (Ruan et al., n.d.).

Finally, in this project, PCOS frequency among Palestinian women who attend IVF centers. Studying the prevalence of the disease may provide specific geographic factors with racial and ethnic variations that may affect the clinical presentation of the disease. Previous epidemiological studies reported variations in the prevalence of PCOS in different populations in different geographic regions based on various PCOS definition criteria. For example, the prevalence of PCOS in one region was 5% to 10% according to NIH 1990 criteria, 10% to 15% according to the AE-PCOS 2006 criteria, and from 6% to 21% according to the ESHRE/ASRM 2003 criteria. These findings indicate that the

estimated prevalence of PCOS depends on different diagnostic criteria (Lizneva et al., 2016; Rao et al., 2020). In the current study, the prevalence of PCOS was estimated in different geographic regions in Palestine by choosing PCOS patients who have been diagnosed in IVF clinics according to the Rotterdam criteria. Interestingly, PCOS frequency differs from one area to another in Palestinian cities. For example, in Jenin, PCOS cases were 10%, showing a minor frequency in Palestine; Ramallah, Jerusalem, and Nablus showed convergent percentages of 23%, 21%, and 22%, respectively.

Variations in the reported frequency percentages across different countries and geographical areas are due to ethnic differences, environmental factors, psychological disturbances, and the use of different criteria for diagnosing the disease due to its complexity. PCOS phenotype assessments are complex and require extensive follow-up. The prevalence of PCOS in Palestine was then assessed using the Institute for Health Metrics and Evaluation (IHME) tool, and the results were compared with those of multiple MANA regions.

The annual percentage changes were conducted among females of all ages, and the prevalent cases were studied per 100,000 population. The percentages were consistent, including 1.32% in Palestine, 1.2% in Jordan, 1.2% in Lebanon, and 1.45% in Syria. In the Gulf region, the percentages were higher than in the Levant, including 2.09% in Saudi Arabia and 2.53% in Oman, while in Egypt, it was 0.91%, 2.23% in Sudan and 0.43% in South Sudan.

The prevalence of PCOS in Palestine has also been studied over the years. The presented results show that the prevalence is constantly increasing over the years. Also, when we studied PCOS in different age groups, we found that the prevalence of PCOS among females is elevated during reproductive years, starting from puberty until menopause. In addition to its prevalence, our study shows that PCOS increases death risk by 1.3%, which is very high compared to YLDs among other states. For example, while YLDs are approximately 1.3% in Palestine, the percentage was 0.06% in Yemen and >0.25% in the United Arab Emirates in 2006 and 2010.

The prevalence of PCOS is relatively high among young Palestinian women and parallels PCOS prevalence in Caucasian and Mediterranean populations. In this regard, further studies are needed in Palestine on a large scale, which will help improve the diagnosis, treatment, management, and awareness among PCOS patients and physicians. Since PCOS cannot be fully cured, it is regarded as irreversible disorder. However, with appropriate treatment and lifestyle modifications, symptoms can be managed effectively.

In other words, PCOS is manageable, allowing patients to lead healthy and symptom-free lives.

The results reported in the current study regarding the identified familial genetic risk factors are in conceptual agreement with former studies. All identified variants showed heterozygous genotypes, and none of the participants in this study carried a variation in a homozygous state. The inheritance pattern was difficult to explain because different genetic and environmental factors potentially affect the etiology of the disease. The present findings extend our understanding of the molecular events in PCOS and the genetic association with the pathogenesis of PCOS, providing advanced treatment protocols. That is because WES and family segregation analysis had significant advantages for targeted therapies, where identifying specific genetic causes of PCOS disorder can develop personalized treatment protocols, which may include gene therapy or pharmacogenomics, which is helpful in providing some drugs that can target specific genetic mutations. By addressing the underlying genetic causes of the disease, gene-target therapy can improve treatment outcomes rather than just managing symptoms.

While our study is the first of its own to shed light on PCOS genetics in Palestine, our study has some limitations, including the small sample size, misdiagnosis cases due to the disease's complexity, related to the presence of many comorbid diseases, and presence of many genes that could be involved in PCOS pathogenesis. Thus, further investigations into the genetic basis of PCOS using a larger sample size are recommended to elucidate a clear understanding of the molecular genetic basis of the disease and provide further analysis and follow-up of patients involved in the study. Genome-wide association Studies (GWAS) are also recommended to identify the associated genes by detecting the SNPs in a large group of people.

Conclusion

In conclusion, the present analytical study aimed to screen for potential genetic factors associated with the pathogenesis of PCOS among selected Palestinian women. WES was conducted on five probands, and several variants have been revealed. The most relevant genes defined in three families were IRS1 and IRS2, which were previously reported to be significantly associated with PCOS. The variants in the PON1 and FBN1 genes showed strong evidence of association with PCOS. Some resulting variants showed no significance to PCOS pathogenesis, like MCM6. However, further investigations are needed. CYP21A2 was excluded, too, due to misdiagnosis and technical error. The patient will be followed up, and further analysis will be conducted.

The main genetic contributions are suspected, but since limited genotyping studies exist, no evident genes can be identified as contributors to PCOS. The phenotypic heterogeneity among the study subjects limited the ability to explain the disease's etiology and pathogenicity. Overlapping symptoms increase the disease's complexity because the more distinct phenotypes within the affected groups, the more complex the genetic analysis and the more significant the gap in diagnosis and individualized treatment. Consequently, it leads to different conclusions.

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Appendices

Appendix (1) Consent Form

نموذج الموافقة على المشاركة في البحث العلمي

عنوان البحث العلمي:

البحث عن عوامل جينية محتملة تسبب متلازمة المبيض متعدد الأكياس لدى النساء الفلسطينيات.

اسم الباحث الرئيسي: رولا روحي أحمد فتوح

اسم المشرف على البحث: دكتور زيدون صلاح

ملخص البحث:

ان متلازمة تكيس المبيض عبارة عن مرض هرموني متعدد العوامل، ومنتشر بكثرة عند النساء في سن الانجاب. ان هذه المتلازمة في تزايد مستمر ضمن النساء الفلسطينيات مسببا العقم في حوالي 90% من الحالات، كما يسبب اضطرابات الايض والسكري، وارتفاع ضغط الدم، وامراض القلب. تكمن صعوبة هذا المرض في وجود عدة عوامل مسببة له، حيث يوجد عوامل بيئية، هرمونية، ووراثية. ان العوامل الجينية تعتبر من اهم العوامل المؤثرة، ولكن لا يوجد حتى الان اي دراسة حول العوامل الوراثية التي تسبب المرض في فلسطين. لذلك، تهدف هذه الدراسة الى متابعة مدى انتشار متلازمة تكيس المبيض ضمن مجموعة من النساء الفلسطينيات اللاتي يتلقين الرعاية الصحية في مراكز المساعدة على الحمل، والبحث عن العوامل الجينية المتوارثة في مجموعة من العائلات الفلسطينية.

المطلوب للمشاركة في البحث:

تعبئة استمارة عن التاريخ الطبي للمشارك في البحث وللعائلة.

سحب عينات دم من المشارك لفحص بعض الهرمونات الضرورية في تشخيص المرض، و لفحص الحمض النووي.

الفائدة المرجوة من البحث:

البحث العلمي سيساعد في المجال الطبي في ايجاد السبل العلاجية الملائمة للمريضات اللاتي يعانين من متلازمة تكيس المبيض وبالتالي ايجاد حلول للعقم المنتشر في المجتمع الفلسطيني.

المخاطر المتوقعة من البحث:

لا يوجد اي مخاطر تذكر على المشاركين خلال هذا البحث العلمي.

الخصوصية:

حرصا على خصوصية المشاركين في البحث العلمي سيتم التعامل مع العينات بأرقام تحدد هوية المشارك بدون ذكر الأسماء.

حقوق المشاركين في البحث:

يحق للمشاركين التوقف عن المشاركة في هذا البحث لأي سبب شخصي.

يحق للمشاركين بمعرفة النتائج النهائية للبحث العلمي عن طريق التواصل المباشر مع الباحث.

في حال وجود أي استفسارات عن البحث يستطيع المشتركون التواصل المباشر مع الباحث عبر البريد الالكتروني:

Rula_rf@hotmail.com

في حال عدم الشعور بالراحة او الرضى عن كيفية اجراء الدراسة يمكن للمشتركين التواصل مع:

Ethical Review Committee

Deanship of Scientific Research

Arab American University-Palestine (AAUP)

Email: src@aaup.edu

الموافقة على المشاركة:

بعد قراءة كل المعلومات المذكورة أعلاه وفهم جميع البنود أنا الموقع أدناه أوافق على المشـــاركة في هذا البحث العلمي مع احتفاظي بجميع الحقوق المذكورة.

اسم المشارك:

التاريخ:

التوقيع:

Questionnaire

PCOS QUESTIONNAIRE FORM

Number: ----------

Date: ----------

Name: ----------

Age: ----------

Residence: ----------

Occupation: ----------

Weight: ----------

Height: ----------

BMI: ----------

Menarche: ----------

Menstrual cycle status:

- **Regular**
- **Irregular**
- **Period of the cycle:**

Year of Marriage: ----------

-Obstetric history: ☐ **G** + ☐ **P** + ☐ **A** + ☐ **E**

Pregnancy Status:

☐ **Spontaneous**

☐ **Induced**

Notes: ----------

Infertility factors:

- **Male Factor**
- **Female Factor:**

☐ **Tubal Factor** ☐ **Uterine Factor** ☐ **Central Factor** ☐ **Ovarian Factor**

IVF Trials: ----------

IVF Successful trials: ----------

Abdominal Fats:

- **Yes**
- **No**

PCOS Symptoms:

- **Polycystic ovaries**
- **Irregular/No period**
- **Hirsutism**
- **Weight gain**
- **Acne**
- **Sleep Apnea**

Diseases:

- **Diabetes mellitus**
- **Heart diseases**
- **Hypertension**
- **Cholesterol**
- **Triglycerides**
- **Thyroid diseases**
- **Hormonal diseases(if yes, please mention):** ----------

Other Diseases (if yes, please mention): ----------

Hormonal Profile:

Free Testosterone: ----------

LH: ----------

FSH: ----------

Medications: ----------

Family Status:

Number of sisters:

Ages of sisters:

Number of paternal aunts:

Number of maternal aunts:

Pregnancy status for mother: ☐ **Spontaneous** **Induced** ☐

Pregnancy status for sisters: ☐ **Spontaneous** **Induced** ☐

Pregnancy status for aunts: ☐ **Spontaneous** **Induced** ☐

PCOS (Please mention family members with PCOS): ----------

Family Disease:

Diabetes Mellitus	☐	**Paternal**	**Maternal**	☐
Heart diseases	☐	**Paternal**	**Maternal**	☐
Hypertension	☐	**Paternal**	**Maternal**	☐
Cholesterol	☐	**Paternal**	**Maternal**	☐
Triglycerides	☐	**Paternal**	**Maternal**	☐
Thyroid diseases	☐	**Paternal**	**Maternal**	☐
Hormonal disorders	☐	**Paternal**	**Maternal**	☐

Number of Brothers: ----------

Ages: ----------

Marital status: ----------

Fertility Status: ----------

Balding: ----------

Diseases: ----------

Signature:

متلازمة المبيض المتعدد الكيسات هي من أكثر اضطرابات الغُدد الصمَّاءِ شيوعاً والتي تصيب النساء خلال سن الإنجاب

رولا روحي أحمد فتوح

أسماء لجنة المناقشة

د. زيدون محمود حسن صلاح

أ. د. هشام درويش

أ. د. فواز عوض

ملخص

إن متلازمة المبيض المتعدد الكيسات هي من أكثر اضطرابات الغُدد الصمَّاءِ شيوعاً والتي تصيب النساء خلال سن الإنجاب. كان أن المتلازمة تعتبر أنها المسبِّب الرئيسي لفرط الأندروجينية والعقم الإباضي لحوالي تسعين بالمئة من الحالات. إن تكيّس المبيض يعتبر مرضاً معقداً، ويكمن تعقيد هذا المرض في أنه غير متجانس للغاية، ومتعدد الجينات والعوامل، كما ويرافقه عدّة أمراض مصاحبة، مثل اضطرابات التمثيل الغذائي، ومقاومة الإنسولين، وعدم تحمّل الجلوكوز، وداء السكري من النوع الثاني، وارتفاع ضغط الدم، واضطرابات القلب والأوعية الدموية. إن انتشار متلازمة المبيض متعدد الكيسات في تزايدٍ مستمر اعتماداً على العرق، ويُعتقد أن مسبباته متجذرة في العوامل البيئية والوراثية. ومع ذلك، يصعب تفسير نمط الوراثة بسبب الجينات المختلفة ذات الصلة. لقد سُجِّلت حالات متزايدة من متلازمة تكيس المبيض بين الإناث الفلسطينيات اللّاتي يعانين من نقصٍ حاد في المعلومات الوراثية. إذاً، تهدف هذه الرسالة إلى فحص المتغيرات الجينية المسبّبة للأمراض المحتملة المرتبطة بمتلازمة تكيس المبايض وقياس انتشار المرض بين النساء الفلسطينيات اللاتي يتردّدن على عيادات الأمراض النسائية للتلقيح الاصطناعي للحصول على تقنيات الإنجاب المساعدة. تم تطويع خمس نساء مصابات بمتلازمة تكيس المبيض وقد شُخِّصن وفقاً لمعايير روتردام للدراسة. تم إجراء تسلسل كامل للإكسوم على الحمض النووي من المُستَلفتين الخمسة للكشف عن المتغيرات ذات الصلة بمتلازمة تكيس المبايض العائلية، حيث تم تصنيف عينات الحمض النووي لأفراد أُسرهم وراثياً للمتغيرات المُكتشفة. ثم تم استخدام أدوات التوقع الحاسوبية لتقييم الآثار الضارة المحتملة للطفرات التي تمّ تحديدها على البروتينات. كما وتم تقييم انتشار المتلازمة بين مجموعة من المرضى من عدّة مناطق مختلفة باتباع معايير التشخيص المعتمدة. كشف البيانات المقدّمة عن وجود ثلاث عشرة متغيراً غير متجانس الأمشاج في جينات مختلفة، وهيCYP21A2, LDLR, MCM6, IKBKB, STOX1, MC4R, IRS1, IRS2, PON1, FBN1. وأظهرت النتائج أربع متغيرات ذات أهمية غير مؤكدة وسبع متغيرات مسبّبة للأمراض. لقد أظهروا ارتباطًا محتملاً بين متلازمة تكيس المبيض وتعدّد الأشكال الجينية التالية LDLR Arg595Trp, FBM1 : Gly1058Ser, PON1 Met127Arg, STOX Ile186Thr, MC4R Glu308 Lys, IRS1 Ser564Asn, وGlu308Lys, وGly669Cys, IRS2 Arg1221Pro, و Ser66fs*27. مما يشير إلى أن الطفرات في هذه الجينات تساهم في التسبب لمتلازمة المبيض متعدد الكيسات. ومع ذلك، لاتزال الدراسات حول الجينات المرتبطة بالمتلازمة جدلية. هذه الدراسة الواعدة تعتبر منارة جديرة للبحث والتي تلقي الضوء على المبادئ التوجيهية للتشخيص والعلاج والإدارة المناسبة.

الكلمات المفتاحية: متلازمة المبيض متعدد الكيسات، مقاومة الانسولين، متغير غير متجانس الأمشاج، العقم الاباضي، فرط الأندروجينية.