T.R.N.C

NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

THE INVESTIGATION OF ALLELE FREQUENCIES OF POLYMORPHIC VARIANTS IN GENES THAT ARE RELATED TO POLYCYSTIC OVARIAN SYNDROME

By

SIHAD SALIM HAKEEM ALYOUSIF

Master of Science in Medical Biology and Genetics

Supervisor:

Assoc. Prof. Pinar Tulay

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APPROVAL

This thesis was submitted to the Institute of Graduate Studies at Near East University in partial fulfillment of the requirement for the dissertation of Master of Science in Medical Biology and Genetics.

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DECLARATION

I declare that, this thesis entitled as 'The Investigation of Allele Frequencies of Polymorphic Variants in Genes that are related to Polycystic Ovarian Syndrome' conducted by me under supervision of Assoc. Prof. Pinar Tulay, with respect to ethical guidelines.

I also declare that information obtained from published work of others had been cited in text and listed in the reference list.

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COMPLIANCE AND APPROVAL

Her master thesis ‘The Investigation of Allele Frequencies of Polymorphic Variants in Genes that are related to Polycystic Ovarian Syndrome’ was written in accordance with the NEU Postgraduate thesis proposal and thesis writing directive.

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DEDICATION

To the greatest prophet Mohammed (Pace be upon him), to the department of Medical Biology and Genetic to Assoc. Prof. Pinar Tulay to my parents the greatest example of love faithfulness, and patience to my life partner Elaf, to my brothers and sisters and best cousin Ciya Palo to my relatives uncle and aunts with love and respect and love to all friend. Who gave me a hand to complete this project and finally to all those who share even a word thanks to all of you.

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ABSTRACT

AIM: The main aim of this study was to investigate the allele frequencies of polymorphisms in genes *CYP11A1* rs4886595 and *CYP11A1* rs4887139 that are responsible for the steroidogenesis mechanism in PCOS patients and control females. We hypothesize that the allelic frequencies of SNPs would be different in two groups.

Background: Polycystic ovary syndrome (PCOS) is a multifactorial disease and is associated with metabolic and reproductive disorders. PCOS is marked by gonadotropin deficiencies and elevated androgen levels, insulin resistance, chronic anovulation and irregular menstrual cycle. Reproductive anomalies are habitual feature of PCOS. Furthermore, there is an elevated risk of developing metabolic disorder, cardiovascular diseases (CVD) and type II diabetes. The pathophysiology underlying PCOS is not fully deduced. However, increased insulin resistance and androgen levels are thought to play a role in the formation of metabolic diseases, reproduction to an affected woman. One of the main reasons of anovulation is thought to be the increase in androgen concentration. Also, hyperandrogenism can cause aberration in follicular development. An increase in adrenal androgen in 20-30% of PCOS patients may develop owing to errors in steroid biosynthesis enzymes and cortisol metabolism. Hyperandrogenism increases the risk of reproductive problems, especially cardiometabolic prophylaxis and cardiovascular diseases. Speculations exist that genetic factors presumably play crucial roles in the mechanism of the disease, since PCOS occurs in more than one member of the same family. Biomedical data from previous investigations suggest that alterations such as (single nucleotide polymorphisms) in the genes implicated in the steroidogenesis mechanism could be involved in the formation of the disease. This study aimed to investigate the allele frequencies of polymorphisms in genes, such as *CYP11A1* rs4886595 and *CYP11A1* rs4887139, involved in the steriodogenesis mechanism in PCOS patients.

Method: Patients at Near East University Hospital provided the samples needed for this research. Each patient were asked to sign an informed consent form. The patients' clinical information was obtained. Samples were divided into two groups for analysis. Non-obese women and normal ovulation, made up the control group, and PCOS patients made up the patient group. DNA was isolated from blood samples collected in an EDTA tube gathered from women in the control and patient groups. Real-time PCR

was used to for the analysis of SNPs in various genes linked to PCOS. The studies were carried out using samples obtained from 120 women, 55 of whom were non-obese and had normal ovulation, and 65 of whom were patients with PCOS. The allelic frequencies of SNPs in genes linked to PCOS were calculated using real-time-PCR outcomes.

Result: The findings showed that the patients and control group have no significant difference in Tm in *CYP11A1* rs4887139 G>A (p = 0.203), while the mean±SD of Tm in patients group was notably higher than the mean±SD of Tm in control group in *CYP11A1* rs4886595 C>A (86.45±1.58) (85.75±1.93) (p=0.01), respectively.

Conclusion: In this study, the differences in the heterozygosity status of the alleles at the rs4886595 C>A within *CYP11A1* involved in PCOS recorded significant difference in patients and control group, while the heterozygosity of rs4887139 G>A within *CYP11A1* was shown to be insignificant in both groups. Therefore, future research ought to focus on elucidating the susceptible causes of PCOS with wide range of SNPs and more sample size. More genome-wide association studies in PCOS patients of different origin will be important to recognize prospect genes as well as proteins that are implied in PCOS risk.

Keyword: Polycystic ovary syndrome, polymorphism, cytochrome P-450-11A1, Single nucleotide polymorphisms, Northern Cyprus.

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ACTH	Adrenocorticotrophic Hormone
ADT	Adipose Tissue
AES	Androgen Excess Society
AMH	Anti-mullerian Hormone
ASRM	American Society for Reproduction and Medicine
cAMP	Cyclic Adenosine Monophosphate
Ct	Cyclic of threshold
DNA	Deoxyribonucleic Acid
ESHRE	European Society of Human Reproduction and Embryology
FSH	Follicular Stimulating Hormone
FSHR:	Follicular Stimulating Hormone Receptor
GnRH	Gonadotropin Releasing Hormone
GnSAF	Gonadotropin Surge-attenuating Factor
HDL	High-density Lipoprotein
HRM	High Resolution Melting Method
IR	Insulin Resistance
IRS	Insulin Resistant Substrate
LDL	Low-density Lipoprotein
LH	Luteinizing Hormone
NIH	National Institute of Human

PCOS	Polycystic Ovary Syndrome
PCOM	Polycystic Ovarian Morphology
PCR	Polymerase Chain Reaction
SHBG	Sex Hormone Binding Globulin
SNP	Single Nucleotide Polymorphism
StRA	Steroidogenic acute Regulatory Protein
Tm	Melting Temperature

CHAPTER ONE

1. INTRODUCTION

Polycystic ovary syndrome (PCOS) is a complicated and frequent issue that affects 5 to 20 % of women of reproductive age. PCOS has been diagnosed in around 105 million women aged between approximately 15 to 49 all over the world (Azizz et al., 2016). PCOS is a complex disorder that can originate through alternations in genetic, hormonal and environmental factor. The most common outcomes of PCOS are female anovulation, marked by hyper-androgen and insulin resistance and it is among the most main sources of irregularities in menstruation, amenorrhea, and oligo-menorrhea which is the most common reason for female infertility (De Leo et al., 2016).

PCOS is the most prevalent endocrine condition amongst reproductive-age women, with symptoms presenting in prepubescent age in some instances and postmenopausal women in many situations (Thomson et al., 2011). Hormones play dominant role in the ovary's function and the menstrual cycle's management, which preserves fertility, if there is a chronic hormone level disruption in females, it can disrupt the activity of the ovary, contributing to the development of a cyst within the ovary, whereas androgen, a male hormone, has risen in females affected by PCOS beyond its normal range (Ranjith et al., 2015). The disease progresses from a reproductive condition to a more metabolic disorder with advancing maternal age. PCOS is more than a condition of reproduction, a wide range of metabolic disorder is associated with it. There are greater rates of cardiovascular disease, endometrial cancer, type II diabetes mellitus, dyslipidemia and glucose tolerance in women with the disease. Adolescence is the typical age of onset for PCOS, although most signs may not present till a woman is in her twenties or thirties, even if features may begin at menarche, the usual period of diagnosis is around the third or fourth decade of a woman's life (Dunaif, 1997). Two out of the following three PCOS characteristics determines if a woman has PCOS; the presence of a biochemical and/or clinical androgen surplus, polycystic ovarian morphology on ultrasound PCOM and anovulation or oligo-ovulation (Escobar et al., 2018). Genetic conditions account for approximately 79% of PCOS etiology, while the environment and lifestyle account for 21% (Franks & Berga, 2012). The genetic involvement of PCOS has gained importance and it is being extensively researched (Franks & Berga, 2012).

1.1. History

Irving Stein and Michael Leventhal first identified PCOS in 1935, disclosing seven patients with infertility, amenorrhea and swollen multi-cystic ovaries (Stein & Leventhal, 1935). The same year, testosterone was discovered by Laquer and Butenandt, and with the creation of the first androgen assay and a deeper perception of its biochemical and pathological origins, the field of biochemistry was upset (David et al 1935). Subsequently, androgens were found to be orchestrated by both the adrenal gland and ovaries, and notably, hyper-androgenism was responsible for excess androgens in patients (Finkelstein et al., 1961).

Until 1990, the PCOS definition remained somewhat confusing; the National Institute of Health (NIH) in the United States of America developed the first diagnostic criteria in April 1990. Hyper-androgenism and oligomenorrhea/amenorrhea is included. PCOS was described by those first diagnostic criteria chronic oligo-anovulation is consistent with the presence of hyper-androgenism in clinical and/or biological terminology, these early guidelines excluded morphological descriptions of the ovaries (Zawadski & Dunaif, 1991). In the 1970s and 1980s the evolution of ultrasound technology notable improvements has arisen in the diagnosis of PCOS. Enhancing hormonal monitoring and ultrasound for techniques have been important to change the diagnostic criterion for PCOS (Delcour et al., 2019). In 2003 the Rotterdam criteria included the finding of polycystic ovarian morphology (PCOM) on ultrasound as a criterion applied of previous criteria was necessary to support the diagnosis. In 2006, the Androgen Excess Society (AES) proposed a modification, in which hyperandrogenism would be a necessary condition for diagnosis accompanied by PCOM or oligomenorrhea, or both of them (Dennett & Simon, 2015).

The European Society For Human Reproduction and Embryology and American Society for Reproductive Medicine established the distinctive criteria for identifiying PCOS; two of the three characteristics needed for diagnosis that are; (i) clinical and/or biochemical hyperandrogenism, (ii) anovulation or oligoovulation, and (iii) ultrasound polycystic ovarian morphology (PCOM)(Mohammad & Seghinsara, 2017).

Table 1: Diagnostic criteria about polycystic ovary syndrome (Crespo et al.,2018)

NIH criteria in1990	Rotterdam standard in 2003	AES specification in 2006
• Clinical with or without biochemical signs of hyperandrogenis (Both criteria needed) • Chronic anovulation	• Oligo with or without anovulation • Hyperandrogenism and/or hyperandrogenemia • PCOM (Two of three criteria needed)	• Clinical and/or biochemical symptoms of hyperandrogenism • Ovarian dysfunction (Oligo-anovulation and/or polycystic ovarian morphology) (Both criteria needed)

1.2. Clinical features

PCOS is a hormonal disorder that can lead different illnesses and it is also a common cause of female infertility. There are different signs and symptoms, the three frequent characteristic correlated to PCOS that involves abnormalities in ovulation, increasing androgen levels, cystic ovaries when examined by ultrasound (Ndefo et al., 2013). Manifestations of PCOS are frequently recognized in puberty, the physiological effects of the disorder affect a woman throughout her life, with early onset manifestation of both hyperandrogenic and metabolic aberration, and reproductive dysfunction becoming more evident at adolescence age, as well as increasing cardiovascular and metabolic risk in mid and adulthood (Merkin et al., 2016). Two forms of the menstrual cycle are characterized by oligomenorrhea which is defined as a woman with menstrual cycle period of more than 35 days or 4 to 9 menstrual cycles per year. Second type, amenorrhea characterized as the absence of menstruation for more than 90 days. Oligo/amenorrhea is an oligo/anovulation indicator that is linked to infertility (Schmidt, 2011). Ovulation problems and excessive androgen levels affects majority of women with the syndrome. In comparison, high androgen concentrations are closely associated with hirsutism, acne, and alopecia, and there have been reports of polycystic ovaries on pelvic ultrasonography in about 70% of patients with the disease (Ndefo et al., 2013). A wide range of metabolic disorder are associated with PCOS, there are higher incidences of cardiovascular disease, dyslipidemia, and diabetes

mellitus type 2, glucose tolerance, endometrial cancer, high blood pressure, sleep apnea, and psychological characteristics such as anxiety, low standard of life, and mood disorder, in women with PCOS are also observed (Lynch, 2013). Patients with PCOS have multiple cysts in the ovaries that are 8 mm in diameter. The ovary has typically more than 12 cysts. About 70% of women are infertile as a result of this condition. Acne and hirsutism are caused by an increase in androgen levels, as previously discussed. Insulin resistance exists, resulting in type 2 diabetes and obesity. This condition lead to irregular menstrual cycle that lead to infertility (Ajmal et al., 2019). There are environmental-factors that lead to PCOS. While there is no data from human research on probable prenatal environmental causes, handful evidence indicates that postnatal sensitivity to environmental risk factors is correlated with PCOS. Also, harmful lifestyle including obesity and lack of proper exercise have been proposed as possible risk factors linked with PCOS-related cardio-metabolic disorders. Obesity was shown to be linked with exacerbated PCOS-related metabolic and ovulatory dysfunction, you have to lose weight in order to regain ovulation and raise hyper-androgenism levels. Furthermore, racial/ethnic heterogeneity in phenotypes often means that cultural variables as well as lifestyles play major roles in these PCOS metabolic implications (Merkin et al., 2016). It has been stated that testosterone levels are rising and LH levels are increasing, but there has been a decline in FSH levels. The hyperandrogenism may primarily be due to thecal cell hyperplasia that contributes to severe steroidogenesis of the ovary. Data shows that hyperandrogenism appears to be a major factor in follicular arrest-induced anovulation. High level of androgens has been linked to decreased oocytes competency for growth and rates of maturation. Moreover, in women with PCOS, testosterone was linked to low fertilization rate, embryo development, and spontaneous abortion. Additionally, the increase rate of adrenal androgen was also reported in 20-30% of PCOS women, which is most likely as a result of abnormal function of steroid pathway manufacturing enzymes or cortisol metabolism (Dadachanji et al., 2018).

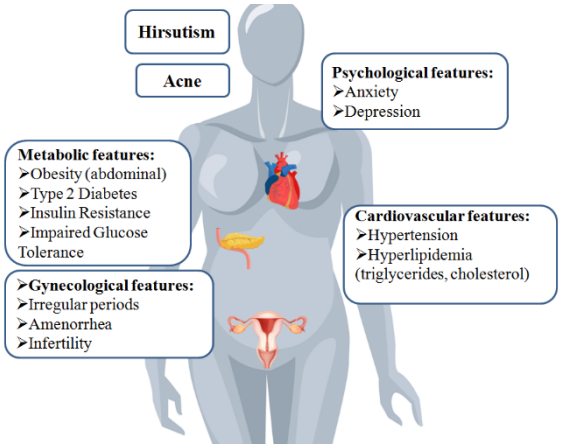


Figure 1: clinical features of PCOS (Al-Omar, Z., et al., 2020).

1.3. Etiology

PCOS is a multigenic disease in which the heterogeneous, physiological, and biochemical phenotype is associated with medley of both genetic and environmental influences. While the main cause of PCOS remains unclear, but a multifactorial disorder with a hereditary component has been identified. Approximately 40% of female first degree-relations of females with the disorder develop PCOS themselves, when compared to women in general population (with an incidence of 4-6%) (Goodarzi et al., 2011). Many women with PCOS, even though they have female PCOS families, never been diagnosed. As with type 2 diabetes, several genes are expected to make a minor contribution to PCOS etiology, and latest analyses of the genome-wide interaction have discovered candidate genes (Sirmans& Pate, 2014). Epigenetic and environmental influences, such as an unhealthy diet or low to absence of physical exercise, probably complicate any underlying genetic predisposition (Dennett & Simon, 2015). Latest studies support PCOS as a complicated endocrine disease arising from the association of vulnerable and defensive genomic variants under the control of environmental factors in multiple genes. Likewise, prospect genes associated with the synthesis of steroid hormones, the function of gonadal hormones and gonadotropins, alongside obesity, energy balance, insulin secretion and action have also been implicated. There may be multiple interlinking factors impacting the PCOS expression. PCOS is unlikely to have a single etiology (Branavan et al., 2018). The etiology of PCOS has been associated with both environmental and genetic factors. PCOS typically has the clinical manifestation including, oligo-anovulation, biochemical hyperandrogenism and cyst of in or both ovaries (Aversa et al., 2020).

Insulin resistance and its higher level create problems in the ovaries, which raises androgen level, contributing to anovulation, and the risk of PCOS is risen by an unhealthy lifestyle (such as diet) and other infectious mediators (Goodarzi et al., 2011). PCOS affects the levels of the following gonadotrophin-releasing hormone (GnRH); (i) luteinizing hormone (LH), (ii) prolactin, and (iii) follicular stimulating hormone (FSH). Polymorphism or any alteration of nucleotides triggers a transcriptional malfunction of a gene contributing to PCOS (Marx et al., 2003). The gene mutation disrupts the biochemical pathway and contributes to ovarian dysfunction. Polymorphisms, such as FSHR polymorphism, StAR polymorphisms, IRS and IR polymorphism, and GnRH polymorphisms are considered to be associated with PCOS. The development and severity of PCOS increases with the rise of both insulin and androgen levels. Hyperinsulinemia affects ovarian theca cells and raises androgen levels; as a result, SHBG hepatic biosynthesis is reduced. Increased androgen levels, on the other hand, stimulate the production of free fatty acids by visceral adipose tissue (ADT), which contributes to insulin resistance (Ajmal et al., 2019).

1.4. Pathophysiology

Several theories arose to describe the pathophysiology of PCOS. Initially, excess androgen intrauterine had been thought to be a principal perpetrator in the rise of the disorder (Ajmal et al., 2019). PCOS is a complicated disease in which there have been reports of associations between multiple factors such as gene-environment interactions as well as (gene to gene) interactions influencing PCOS development, physiology of this abnormality until now is not fully understood. The main causes of PCOS is abnormality of ovarian cells (theca cell) and the clinical and biochemical manifestations of the condition result in abnormal androgen synthesis. One of the primary ratios of PCOS is a high LH to FSH ratio, according to *Stein* and *Leventhal* hypothesis. Additionally, increased levels of these GnRH have also been hypothesized as the actual cause of PCOS and theca cells are stimulated to synthesis androgen, lower FSH concentration (and as such a defect in early follicular phases and the late luteal is observed). In addition, low levels of FSH in the pre-antral stage of follicular development are involved in the arrest. In essence, this causes disruptions in the negative feedback, contributing to the continuing imbalance in LH and FSH secretion. Insulin resistance, which contributes to hyperinsulinemia, has a dynamic interaction with PCOS, since hyperinsulinemia may be one of the causes of PCOS or one of its

outcomes. Increased levels of androgen induce increased tissue insulin resistance that leads to hyperinsulinemia. The stimulation of LH, which enhances the output of androgen in theca cells of the ovaries, involves hyperinsulinemia. In addition, hyperinsulinemia induces a decline in liver SHBG production that often contributes to hyperandrogenism. Insulin tolerance in the fat tissue and skeletal muscles from the pancreatic beta-cell dysfunction, a post-receptor defect (irregular phosphorylation of tyrosine kinase) alongside obesity makes it difficult to discern the exact causes PCOS and what has an impact on its pathogenesis (Bednarska & Siejka, 2017).

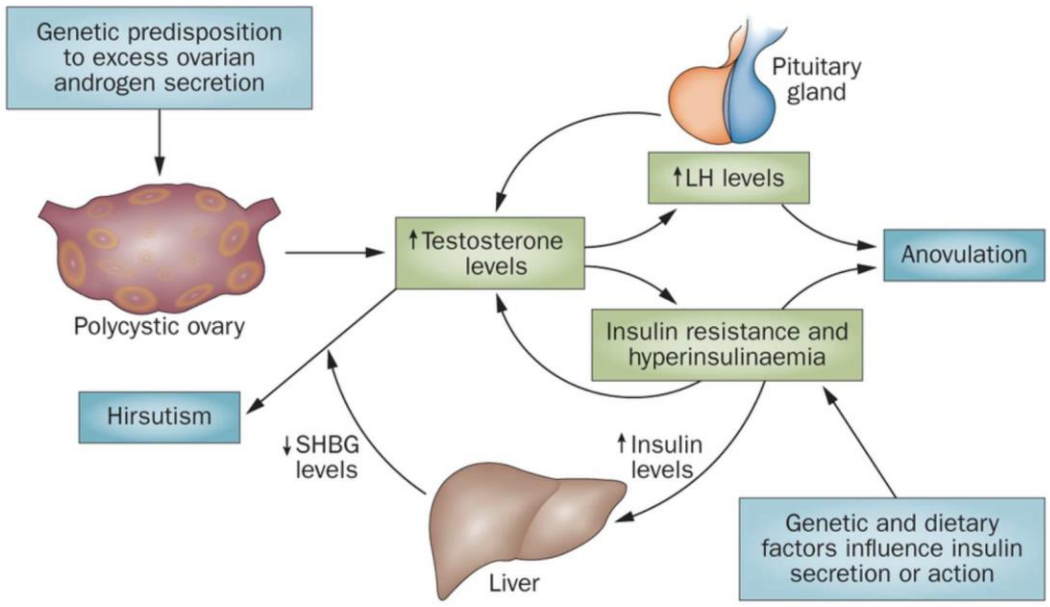


Figure 2: pathophysiology of PCOS (Bahadori et al., 2016)

1.5. Hypothalamus pituitary ovarian axis

Women's menstrual periods are marked by a regular pattern of hormonal changes mediated by feedback-mechanisms. The fundamental arbiters of ovarian impacts on the hypothalamic–pituitary framework are ovarian hormones, which are nonsteroidal or steroidal in nature. Ovarian steroids have been identified as the primary mediators of feedback processes during the menstrual cycle. However, new research has explained the more complex functions of these compounds in the pituitary secretion of the two gonadotrophins. With regards to nonsteroidal compounds produced by the ovaries, their involvement in the context of feedback mechanisms provides a novel approach to studying the physiology of pituitary gonadotrophin secretion. The

inhibitors A and B, as well as a factor called gonadotropin surge-attenuating factor (GnSAF), are among these compounds. In the follicular phases, progesterone and estradiol are the two major steroids released by the ovaries, whereas in the luteal phase, progesterone is produced. The function of each is not restricted to those phases of the menstrual cycle, however, and they are essential regulators of gonadotropin secretion during the entire cycle (Messinis et al., 2014). Menstrual cycle (28 days) divided into follicular phase, luteal phase and ovulation occurring between them. During follicular phase, the hypothalamus initiates the secretion of GnRH (Brents, 2016). This in turn signals the anterior pituitary to release (FSH) and (LH) into the ovaries. Within the ovarian follicle, two cell types are involved for hormone generation, granulosa cells have FSH receptor allowing FSH attachment, and theca cells have LH receptor allowing LH attachment. By activating the enzyme, cholesterol desmolase, LH triggers the theca cells to produce progesterone and androstenedione. The hormone diffuses to adjacent granulosa cells when androstenedione is secreted. FSH activates the aromatase enzyme, which converts androstenedione into testosterone and ultimately 17-beta-estradiol in the granulosa cells. This increase in FSH levels induces follicular development, which in turn stimulates the growth of a selected number of follicles. Towards the end of the follicular-phase, the selected follicles grow to a size of roughly 20-25 mm. The granulosa cells of the growing follicle secrete a number of peptides that may perform an autocrine/paracrine role in suppressing the development of neighboring follicles. The granulosa cell substance (anti-Müllerian hormone, AMH) has also been reported to play crucial role in the selection of dominant follicle during the follicular development (Crespo et al., 2018). At this time midcycle (ovulation occurs on day 14) is started. When estradiol levels are hypercritical, the expected negative feedback impact on LH and FSH change to a positive feedback effect, leading to a massive surge LH concentration and a decrease in the quantity of FSH (Filicori et al., 1986). About 36 hours upon LH surge, a mature oocyte is released or discharged by the ovaries and travels through the fallopian tube into the uterus (Adams et al., 1994). At luteal phase (from 14 to 28 day of the cycle) the remaining follicular tissue after oocyte expulsion (called corpus luteum) produces progesterone, which inhibits the synthesis of FSH and LH, and initiates the development of secretory endometrium (Stocco et al., 2007). In the absence of sperm cells, the deteriorating LH levels lead to a decrease in estradiol levels and progesterone (Taylor et al., 1995). The oocyte implants into the endometrium

in the presence of fertilization and releases chorionic gonadotropin that protects the corpus luteum (Filicori et al., 1986).

In the case of PCOS, the pulse rate of the GnRH is increased to one every 50-60 minute, the quantity of GnRH increases, because of the enhanced response to LH by endogenous GnRH. This sequence of GnRH secretion contributes to raising ratio of LH and FSH. Aromatase cytochrome P450 and 17–hydroxysteroid dehydrogenase convert androstenedione to testetrone, estadiole, or estrone in granulosa cells. In general, to promote the androstenedione and testosterone production of thecal cells, LH binds to its receptor. The relative deficit of FSH-concentration causes the activity of the aromatase enzyme in granulosa-cells to decline, which results in testosterone not being aromatized into estrogen. Adrenal androgens have a negative influence on LH production in the hypothalamus, which successively leads to extend the blend of ovarian androgen, therefore, the adrenal androgen in the ovary can lead alteration in steroid synthesis, and the layer of theca cells become thicker than normal, cholesterol is transformed into androgens in theca cell layer by a sequence of intermediate stages, and the unregulated biosynthesis of androgen enzyme (P450c17) results in hyperandrogenism. Androgens are released from the ovarian and adrenal organs and transported into adipose tissue, in which they are transformed into estrogen. Excess serum estrogen has a continual feedback effect on the pituitary gland, which have a positive feedback effect on LH production and a negative-feedback effect on FSH secretion, contributing to an increase in the ratio of either LH or FSH. Low basal FSH levels promote follicle development, but not mature follicles, increased LH releasing, but no cyclical fluctuations, no LH peak, so LH peak is as a result of oligo-ovulation, which contributes to infertility (Picchio et al., 2020).

The absence of FSH in PCOS contributes to a decline in aromatase activity in granulosa cells, which inhibits testosterone from being converted into estrogen. Progesterone is a precursor to the creation of androgen and estrogen. Progesterone release increased in luteinized granulation cells after ovulation in response to LH stimulation, whereas estrogen secretion decreased, facilitating the inability to identify the dominant follicle and resulting in infertility (Picchio et al., 2020).

1.6. The steroidogenic pathway in the ovary

In the human body, steroid hormones play a variety of functions, including inducing male and female secondary sexual traits (androgens and oestrogens, respectively), as well as endometrial receptivity (progesterone). The utilization of cholesterol, which is the starting point of the steroidogenic cascade, is essential to the development of androgen, estrogen, and progesterone. The synthesis of cholesterol *de novo* from acetate or from high or low-density lipoproteins (HDL)/ (LDL) is one of the first steps in steroid synthesis. In human, cholesterol reaches the mitochondria of the thecal cell, the site of union of numerous steroidogenic catalyst synthases, supported by the action of the steroidogenic-acute-regulatory protein (StAR). In the biosynthesis of steroid hormones in the ovary's thecal cell, multiple enzymes work in a sequential order (Miller, 1988).

The *CYP11A1* gene encodes for a cleavage enzyme known as (P450scc), which is a crucial element of the steroidogenesis pathway. P450scc uses three chemical reactions to convert cholesterol to pregnenolone; C20, 22 bond cleavages and 20, 22 hydroxylation. The cholesterol side-chain-cleavage process is considered as the first rate-limiting step in steroidogenesis. Pregnenolone is transformed into testosterone by a chain of chemical reactions catalyzed by the enzymes P450C17, 3ß hydroxysteroid-dehydrogenase (3ßHSD) and 17ß hydroxysteroid-dehydrogenase (171HSD). P450scc is also found in theca cells of small-antral-follicles, while levels in granulosa cells of these follicles are low but grow during FSH-induced preovulatory follicle growth. After luteinization, when progesterone development is still high, P450scc levels are at their peak. Androstenedione and testosterone are converted to estrogen and estradiol in the ovarian granulosa cells after passing the basal lamina from the theca cell into the granulosa cell. This is due to the enzyme aromatase, which is encoded by *CYP19* (Miller, 1988).

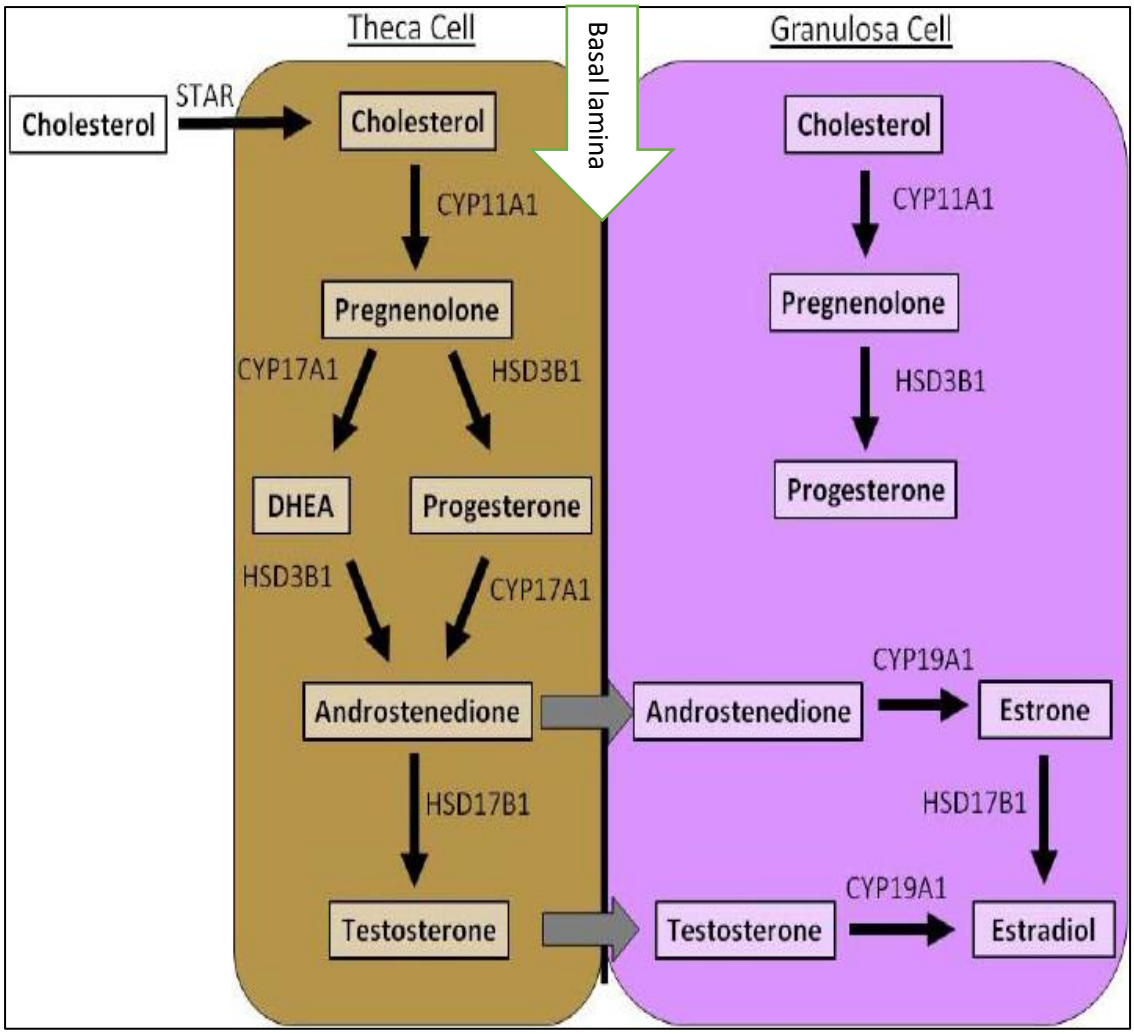


Figure 3: Androgen synthesis pathway that occurs in the ovary (Patel et al., 2015)

1.7. The cytochrome p-450 11a1 (*CYP11A1*) gene

The *CYP11A1* (cytochrome P-450 11A1) gene belongs to the Cytochrome P450, family 11, the gene is cytogenetically located on chromosome 15q23-q24. *CYP11A1* regulate the synthesis of pregnenolone from cholesterol in the inner membrane of the human mitochondria, and it is one of the most promising candidate genes for PCOS enzyme codes, which cultivate the first and most important step in the production of steroid hormones (Ajmal et al., 2019).

CYP11A1 (cytochrome P-450 11A1) composed of nine exons separated by eight introns and spanning a total of 29,864 base pair. *CYP11A1* genomic structure was first described in 1986, and it was subsequently mapped to chromosome 15q23-q2 with greater precision (Sparkes et al., 1991); (Mount, 1982). The gonads, placenta and

adrenal cortex are among the steroidogenic tissues that express *CYP11A1* (Shan et al., 2016). In the adrenal cortex, *CYP11A1* is expressed in all zones (zona glomerulosa, zona fasciculate and zona reticularis). Both the granulosa and theca cells of the ovary express *CYP11A1*. In males, only the Leydig cells in the testis express *CYP11A1* (Payne & Hales, 2004). This gene codes for an enzyme P450scc, which belongs to the cytochrome-P450-superfamily of enzymes (Ajmal et al., 2019). While P450 is still active, *CYP11A1* genetic variants can cause changes in its expression and activities, which can lead to various hormonal related diseases. Alteration in the *CYP11A1* gene have been reported as important markers in a variety of hormone-dependent diseases, including breast cancer, endomaterial cancer, prostate cancer and PCOS (Shan et al., 2016).

1.7.1 Function

As mentioned above, in the mitochondria, *CYP11A* catalyzes the first rate-limiting step in the synthesis of androgens. StAR is a protein that aids in the transport of cholesterol into the mitochondria. To generate pregnenolone, *CYP11A* mediates the 20- and 22-hydroxylations of cholesterol as well as the cleavage of the C20-C22 bond on the inner mitochondrial membrane. Many tissue and species-specific variations have been found in transcriptional regulation (Moore et al., 1992); (Rodriguez et al., 1997); (Ben Zimra et al., 2002); (Lui and Simpson, 1997).

1.7.2 Gene expression

In the ovary, gonadotrophins control *CYP11A1* expression *via* cAMP (Cyclic adenosine monophosphate), and in the adrenal cortical cells, ACTH (adrenocorticotrophic hormone) regulates *CYP11A1* expression *via* cAMP. The stimulatory hormone attaches to the cell surface receptor, stimulates a G-protein, and raises cellular cAMP as a result. cAMP controls *CYP11A1* gene transcription directly by binding to multiple cAMP responsive elements (CRE, TGATGTCA) (Chung et al., 1989). PCOS susceptibility variants have been identified in seven single nucleotide polymorphism (SNPs) in the *CYP11A1* gene. PCOS vulnerability has been linked to the homozygote CC genotype of rs4886595 g.200193 C>A and the homozygote GG genotype of rs4887139 g.198873G>A (Shan et al., 2016).

1.7.3 *CYP11A1* and PCOS

Based on the action of this enzyme in the cell, *CYP11A1* remains a strong biological candidate gene. As disscused above, In the ovary's thecal cell, as well as the adrenal cortex and the placenta, *CYP11A1* transforms cholesterol to pregnenolone. Furthermore, studies have shown that PCOS patients' thecal cells produce more androgen, as well as having higher *CYP11A1* mRNA expression (Gilling-Smith el al., 1997); (Jakimiuk et al., 2001); (Daneshmand et al., 2002). Several *CYP11A1* SNPs have been implicated in the aetiopathogenesis of PCOS and have been linked to increases in testosterone levels. Many studies have focused on polymorphisms in the *CYP11A1* gene, although the majority have focused on the pentanucleotide [TTTTA]n repeat (*D15S520*), which has six typical polymorphisms of four, six, seven, eight, nine, or ten repeats and is located at 528-(bp upstream) of the translational- initiation-site. The four-, six- and eight-repeat alleles are the most common variants in the Chinese population, and they are linked to breast cancer and PCOS (Shan et al., 2016).

1.8. Study aim and hypothesis

The main aim of this study was to investigate the allele frequencies of polymorphisms in genes *CYP11A1* rs4886595 and *CYP11A1* rs4887139 that are responsible for the steroidogenesis mechanism in PCOS patients and control females. We hypothesize that the allelic frequencies of SNPs would be different in two groups.

1.9. Intended outcome of thesis and significance

PCOS is a multifactorial disease and its molecular regulation is not very well understood. Due of the clusters identified in families, speculations still exist on the involvement of genetic factors in the etiology of the syndrome. Up till date, handful published data have emphasized the importance of single nucleotide polymorphisms in influencing PCOS susceptibility. Therefore, this study intended to elucidate more on the mechanism of PCOS. Furthermore, evaluation of susceptibility to PCOS is of great importance in early diagnosis and treatment.

CHAPTER TWO

2. MATERIAL AND METHOD

2.1. Sample collection

Patients at Near East University Hospital provided the samples needed for this research. Each patient was asked to sign an informed consent form. The patients' clinical information was obtained. Samples were divided into two groups for analysis. Non-obese women and normal ovulation were made up of the control group, and non-obese PCOS patients were made up of the patient group. DNA was isolated in an EDTA tube from blood samples gathered from women in the control and patient groups, and samples were studied in the DESAM laboratory. DNA was extracted from the entire blood. Real-time-PCR was used to analyze the heterozygosity of SNPs in various genes linked to PCOS. The study group included a total 120 women, 55 of whom were non-obese and had normal ovulation, and 65 of whom were patients with PCOS. DNA was obtained from all whole blood. The allelic frequencies of single nucleotide polymorphisms in genes linked to PCOS were evaluated using real-time PCR.

2.2. Material

In this study, the following equipment and instruments were used; Thermo Fisher Scientific kit (pure link genomic DNA mini kit), RNase (supplied with kit), protein K (supplied with kit), genomic lysis/binding buffer (supplied with kit), ethanol (included in the kit), Wash buffers 1 and 2 (included in the kit), pure link genomic elution buffer and spin column (both also included in the kit), forward and reverse primers, light cycler SYBR Green 480 H2O (USA 001), light cycler SYBR Green 480 high resolution melting master mix 2x concentration (included in the kit), vortex, incubator, water bath or heat block, Nano-Drop 2000/2000c spectrophotometers, pipette and pipette tips, laminar flow hood, and thermal cycler PCR (all in DESAM laboratory), statistical packages for the social sciences (SPSS).

2.3. Methodology

2.3.1 DNA isolation from whole blood samples

Using the Invitrogen pure link genomic DNA mini kit (USA), DNA was extracted from each sample. Room temperature (15-20°C) was used to thaw frozen

blood samples. 200 µl of thawed blood was pitted into a 1. 5ml micro-centrifuge tube. After that added 20 µl proteinase K (w/v) (Proteinase K is a proteinase that is used to break down proteins in cell lysates). 20 µl of RNase (to remove RNA from genomic DNA) was added to a tube containing a solution. Then adding 200 µl of pure link genomic lysis/binding buffer, all samples were (vortexed) for 10 seconds to create a homogenous solution, and then incubated at 55^{o}C for 10 minutes to facilitate protein digestion. After incubation, 200 µl of 96-100 % ethanol (v/v) was added to the tube and vortexed for 5 seconds to generate a homogenous solution. After transferring 640 µl of the samples into a fresh pure link spin column and centrifuging at 10,000xg for 1 minute, after which both the flow-through and filtrate/collection tubes were disposed. The spin-column was placed in a new collection tube and 500 µl of wash buffer 1 was added to the tube. The collection tube was centrifuged at 10,000xg at room temperature for one minute. Then 500 µl of wash buffer 2 was added at room temperature and centrifuged at high speed for three minutes. Finally, the spin column was relocated to a new 1.5-mL micro-centrifuge tube, 200 µl of Pure Link Genomic Elution Buffer was added to the solution, and the column was incubated at room temperature for one minute before being centrifuged at maximum speed for one-minute at room temperature to extract the eluted DNA.

2.3.2 DNA concentration

Nano drop (Nanodrop ND200, Thermo Scientific, Pittsburgh, USA was used to measure the concentration of DNA at 260 nm (OD260). The 260/280 ratio was used to determine the purity and quality.

2.3.3 PCR amplification

Allelic frequencies at specific SNP locations in the genes *CYP11A1* rs4886595 and *CYP11A1* rs4887139, both of which are associated with PCOS, were determined using real-time PCR. These primers were designed by Associate Professor Pinar Tulay of Near East University (Intron, Turkey). The reaction mixture contained 5 µl of master mix, 0.8 µl of both forward and reverse primers (0.25 µM final concentration), and 1.4 µl of H2O, respectively. Each reaction received 2 µl of isolated DNA. All of the PCRs were performed in a laminar flow hood to avoid contamination. The high resolution-melting-method (HRM) analysis was used to investigate the allelic frequencies of the two SNPs within *CYP11A1*, and thermal cycler software was used to calculate the cycle

of threshold (Ct) and melting temperature (Tm) values. The PCR conditions for amplification are presented in table 2.

Table 2: The conditions for PCR analysis

PCR Step	Temperature C^0 / time (second)	Cycles
Initial Denaturing	95 C^0 /10 Minutes	1
Denaturation	95 C^0 /10 Seconds	40 Cycles
Annealing	56 C^0 /30 Seconds	
Elongation	72 C^0 / 30 Seconds	
HRM	95 C^0 For 1 Minute 40 C^0 For 1 Minute 65 C^0 For 1 Second 97 C^0 For 1 Second	

Table 3: Table summarizing the primer sequences

Genes	Primer sequences	Primer sequences
CYP11A1rs4886595	F:AAGTCCCACACGAGTCCAAC	R:TGTGGCCTGTATGTTGCCTA
CYP11A1rs4887139	F:GGCATGGAAGAACTTGTGGT	R:AACTGAATCGCTGGCCTATG

2.3.4. Statistical analysis

Statistical packages for the social sciences (SPSS version 10, Chicago, USA) were used in this study. Descriptive statistics and the Mann Whitney Test were applied. Samples with p-values ≤ 0.05 were regarded as significant. To analyze the data, a standadized descriptive statistics was conducted on the sample to find the following variables; (i) frequency, (ii) percentage for categorical variables, (iii) the weighted mean, (iv) the median, (v) the standard deviation (SD), and (vi) the minimum and maximum for continuous variables were calculated.

The both Kolmogorov-Smirnov test of normality and the Mann–Whitney U test were implimented where applicable because the data did not support parametric assumptions. ($\alpha = 0.05$) was chosen as the degree of significance.

CHAPTER THREE

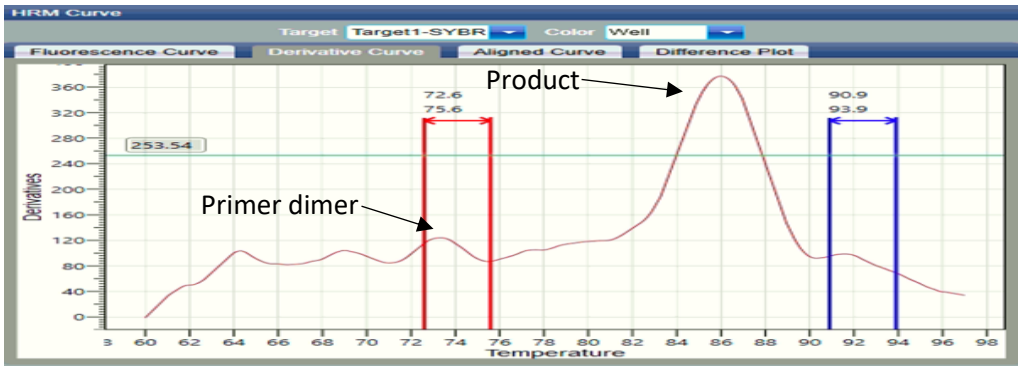
3. RESULTS

A total of 120 participants were recruited for the study. The participants were divided into two groups; patients (n=65) and controls (n=55). The whole blood sample was obtained from the participants to investigate the allelic frequencies for the polymorphic variant genes that are associated with PCOS. Both hormonal level and vaginal ultrasonography were used to assess the PCOS patients and control groups, respectively. The demographic details of each participant are represented in tables 4 and 5. The average age was 20 and the average body mass index for all the patients and the control group was 17, respectively.

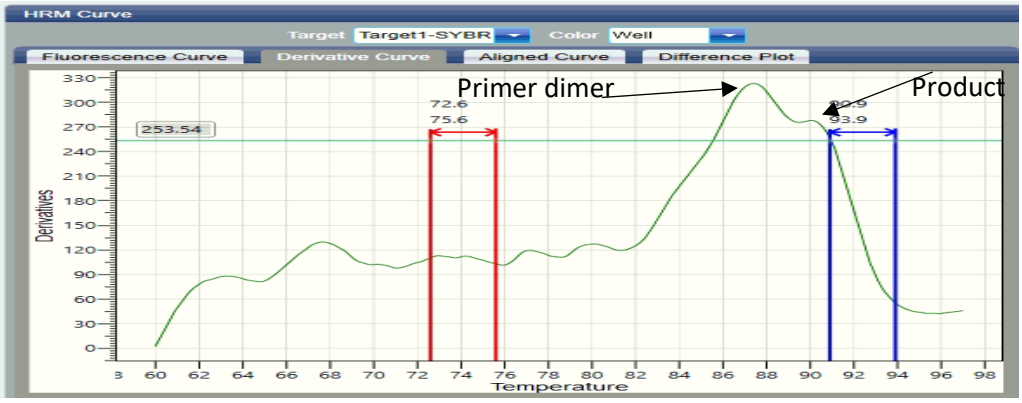
The heterozygosity status of the SNPs was investigated using real time PCR analysis by evaluating the cycle of threshold (Ct) for each amplification. The whole number of cycles required for the fluorescent signal to cross the threshold is shown by the Ct values. Melting temperature (Tm) values were also recorded for each amplification. When the DNA is 50 percent double-stranded and 50 percent single-stranded, Tm shows the melt curve. In high resolution melting (HRM) analysis, following PCR amplification, the amplicons produced is melted gradually. This allows fluorescence to be emitted, which is detected by the software of real-time PCR equipment. Due to the variances in Tm values, these melt curves have various shapes.

The results of the real-time PCR-HRM analysis were presented graphically and in tabular formats (figure 4). The primer-dimer was found in a number of cases, each with a distinct melting temperature than the product's melting temperature (figure 4 A & 4 B). PCR-HRM image showing the heterozygosity of the PCR product (figure 4 C).

A:



B:



C:

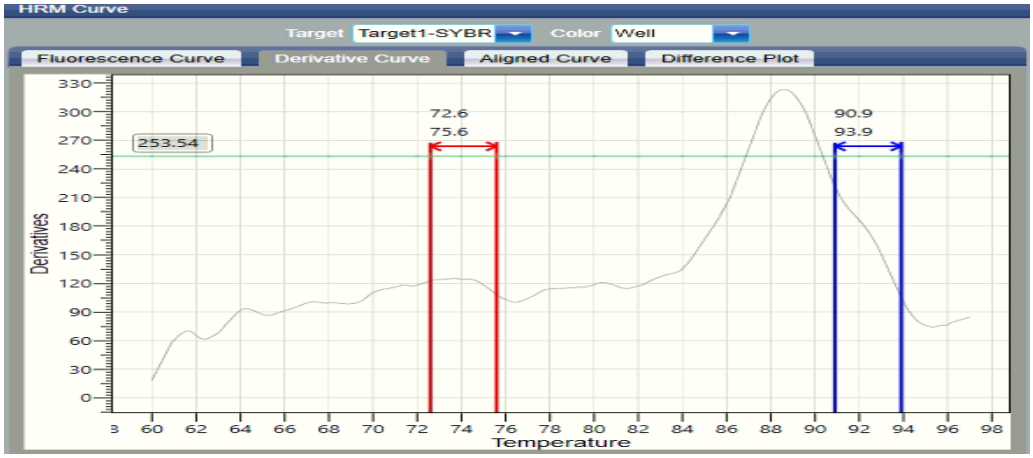


Figure 4: PCR-HRM image showing melt curve analysis of the PCR product

Table 4: Information of each patient

Patients code	Date of birth	Oligomenore	Hyprandrogenism	Height	Weight	BMI	FSH	LH	T.Testesterone	S.Testesterone
1	27.0,11996	YES	YES	158	46	18.4	5.64	7.2	1.7	1.55
2	20.11.1987	NO	YES	169	55	19.3	8.08	21.58	1.44	1.19
3	05.12.1995	NO	YES	168	60	21.3	4.24	6.02	39.85(Y)	2.87(Y)
4	23.09.1996	YES	YES	164	57.5	9.7	2.55	1.15	1.73	5.3
5	22.11.1992	YES	YES	172	64	9.8	3.46	3.8	49.20(Y)	1.27
6	10.12.1997	YES	YES	165	63	9.3	5.46	4.76		
7	15.05.1992	YES	NO	171	58	9	3.49	1.54		
8	07.04.1994	NO	YES	168	68	10.9	4.83	3.89	1.32	1.73
9	27.03.2000	YES	YES	178	82	25.88	3.37	1.83	1.04	1.75
10	07.01.1988	NO	YES	164	65	24.2	4.29	4.9	0.3	
11	12.09.1995	YES	YES	170	65	22.5	5.22	4.77	1.55	1.75
12	06.10.1997	NO	YES	158	62	24.8				
13	05.04.2001	YES	YES	162	57	25.5	5.1	2.93	0.89	0.69
14	10.04.1997	YES	NO	171	93	31.8				
15	17.02.1988	YES	YES	176	74	23.9	4.09	5.39	1.12	1.59
16	29.11.1986	YES	NO	160	76	29.69	4.59	4.43		
17	29.02.2000	YES	YES	153	42	17.9	9.48	6.39	2.26(Y)	2.74
18	29.03.1991	YES	NO	150	73	32.4	5.41	4.11	2.10(Y)	2.32
19	08.11.1995	NO	YES	163	83	31.2	3.91	2.18	1.39	
20	25.11.1997	YES	YES	167	86	30.84	4.66	8.18	2.48	2.29
21	11.11.1996	NO	YES	172	64	21.8	4.22	6.84	2.89(Y)	2.49
22	25.04.1992	YES	YES	171	133.25	45.5	1.25	0.41	0.79	0.83
23	02.01.1989	YES	YES	167	82.2	29.4	4.85	4.17	0.95	1.36
24	22.02.1996	YES	YES	158	53	21.2	4.3	2.55	1.56	1.43
25	1.3.1996	NO	YES	150	48	21.3	4.53	2.93	1.79	2.23
26	25.02.1987	YES	NO	172	106	35.8	5.22	6.44		
27	20.04.2004	YES	YES	161	39.2	15	6.96	10.24	0.77	1.42
28	12.02.1997	YES	NO	160	69.2	27				
29	22.10.1994	YES	YES	163	56	21.08	4.07	8.63	1.76	1.96
30	17.06.1995	NO	NO	170	61	21.11	4.66	3.27		1.91
31	26.06.1995	YES	YES	173	69	23.5	4.09	3.95	1.16	1.27
32	30.06.1984	YES	NO	172	92	31.1	4.7	4.02	1.14	1
33	28.08.1997	YES	YES				3.01	9.99	3.27	2.75
34	18.01.1997	YES	YES	159	79	31.25				
35										
36	28.09.1997									
37	24.09.1997									
38	22.04.1998	YES	YES	162	69	28.29	5.3	8.1	2.09	2.34
39	04.06.1997	YES	YES	172	50	18.9	6.02	5.39	1.43	1.09
40	18.07.1994	YES	YES	164	75	27.89	3.87	2.81	2.41	2.18
41	02.12.1998	YES	NO	159	52	20.57				
42	09.05.1997	YES	NO				3.52	0.8	1.9	
43	16.10.1998	YES	YES	176	89	28.73	5.38	2.53	1.13	1.42
44	12.11.1998	YES	YES	170	68	23.53				
45	17.04.1992	YES	YES	175	74	24.16	5.27	5.74	1.72	2.17
46	02.01.1995	YES	YES							
47	08.08.1998	YES	YES	160	76	29.69	3.97	6.63	2.31	
48	03.02.1999	YES	NO	162	52	19.81	2.99	1.72	0.81	
49	29.11.1995	YES	YES	170	60	20.76	0.05	2.66	0.87	
50	21.07.1997	YES		168	77	27.3	0.52	0.81	1.37	
51	11.03.1997	YES	YES	165	60	22	3.82	6.43	1.6	
52	06.01.1998	YES	YES	164	72	26.8	3.94	2.54	1.73	
53	04.02.2001	YES		165	69.4	25.5				
54	27.11.1997	YES	YES	167	60	21.5	4.18	11.31	1.34	
55	12.03.1997	YES	YES	170	65	22.5				
56	14.09.2000	YES	YES	167	81	29	3.06	2.17	1.37	
57	10.01.2001	YES	YES	173	59					
58	30.05.1991	YES	YES	158	70	28	2.91			
59	15.07.1997	YES	YES	162	73	27.8	4.25	6.15	2.07	
60	03.08.1997	NO	NO	178	80	25.22				
61	17.01.1989	YES	YES	173	59					
62	31.07.1998	NO	YES	163	72					
63	11.01 1989	YES	YES	167	52					
64		YES	YES	158	53					
65		YES	NO	161	53					

Table 5: Information of the control participants

Patients code	Date Of Birth	Oligomenore	Hyperandrogenism	Height	Weight	BMI
1	15.06.1995	NO	NO			
2	24.10.1996	NO	NO			
3	17.06.1996	NO	NO			
4		NO	NO			
5	09.12.1994	NO	NO			
6	03.01.1996	NO	NO			
7	30.05.1996	NO	NO	165	65	25.4
8	28.02.1995	YES	YES	168	96	
9	21.07.1995	YES	YES	172	67	22.6
10	02.03.1989	YES	YES	158	68	27.2
11	24.07.2006	YES	YES			
12	12.07.1993	NO	NO	168	52	18.4
13	21.01.1995	NO	NO	160	65	25.24
14	15.01.1997	NO	NO	166	72	26.1
15	15.11.1997	NO	NO	163	56	21.1
16	01.01.1997	YES	NO	168	90	31.89
17	23.05.1998	NO	NO	156	68	27.94
18	10.07.1998	NO	NO	168	59	20.9
19	07.10.1995	NO	NO	160	56	21.88
20	03.05.1995	NO	NO	156	39	16.03
21	12.06.1996	NO	NO	160	59	23.05
22	21.06.1997	NO	NO	170	52.5	18.17
23	20.03.1995	NO	NO	172	60	20.28
24	20.02.1995	NO	NO	162	73	27.82
25	30.09.1999	NO	NO	158	56	22.43
26	08.02.1996	NO	NO	174	60	19.82
27	27.09.1991	NO	NO	156	62	25.48
28	04.04.1996	NO	NO	159	50	19.78
29	11.09.1996	NO	NO	165	72	26.45
30	22.11.1995	NO	NO	170	62	21.45
31	20.03.1996	NO	NO	174	64	21.14
32	20.01.1999	NO	NO	167	56	20.08
33	16.05.1996	NO	NO	161	72	27.78
34	28.08.1998	NO	NO	160	58	22.66
35	06.07.1989	NO	NO	160	61	23.83
36	20.01.1991	NO	NO	164	62	23.05
37	30.09.1991	NO	NO	177	72	22.98
38	06.02.1991	NO	NO	164	68	25.28
39	22.09.1990	NO	NO	155	50	20.81
40	21.04.1996	NO	NO	163	56	21.08
41	03.12.1995	NO	NO	157	58	23.53
42	09.07.1997	NO	NO	164	62	23.05
43	23.01.1994	NO	NO	162	62.5	23.81
44	28.02.1999	NO	NO	169	63	22.06
45	29.11.1993	NO	NO	159	67	26.5
46	24.03.1997	NO	NO	160	62	24.22
47	0.8.10.1998	NO	NO	166	58	21.05
48	02.11.1998	NO	NO	148	54	24.65
49	02.09.1995	NO	NO	150	46	20.44
50	11.01.1991	NO	NO	170	68	23.53
51	12.11.1998	NO	NO	155	58	24.14
52	24.06.2000	NO	NO	178	48	15.15
53	03.01.1999	NO	NO	160	59.5	23.24
54	10.11.1991	NO	NO	154	54	22.77
55	15.01.1997	NO	NO	151	46	20.17

Referred to the PCOS group for the *CYP11A1* rs4887139 polymorphism, a higher percentage (89.2%) of patients was found to be homozygous, while the heterozygotes recorded to be in low percentage of the patients with about 3.0 % (table 6).

Table 6: The percentages of *CYP11A1* rs4887139 heterozygosity in PCOS.

	Number of patients	Percentage
Homozygous	58	89.2
Heterozygous	2	3.0
Total	60	92.3
No result	5	7.7
Total	65	100.0

Figure 5 below illustrated the heterozygosity of the allele at the *CYP11A1* rs4887139 in the patient group. As shown, the higher number of allelic frequencies were found as homozygous (table 6).

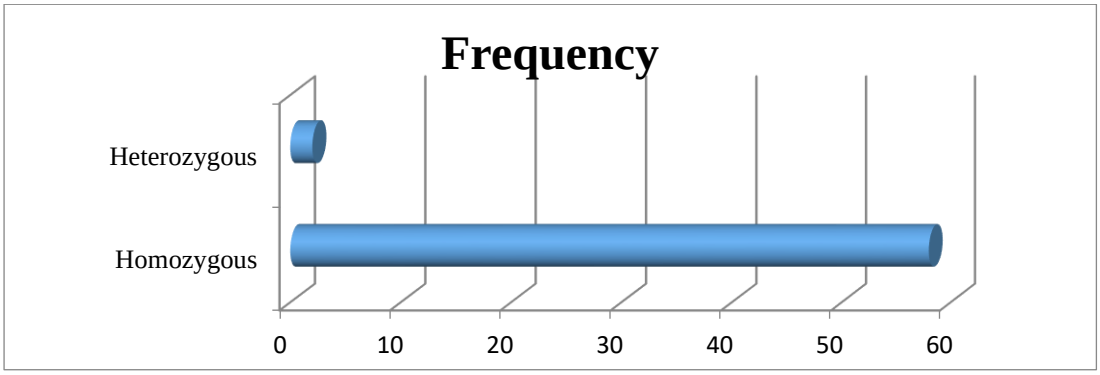


Figure 5: The frequency of heterozygosity and homozygosity of CYP11A1 rs4887139 in patient group.

The homozygosity of *CYP11A1* rs4486595 was recorded high, of about 69.2 %, while the heterozygosity was recorded lower with about 21.5% (table 7).

Table 7: The percentages of heterozygosity of *CYP11A1* rs4886595 in patients.

	Number of patients	Percentage
Homozygous	45	69.2
Heterozygous	14	21.5
Total	59	90.7
No amplification	6	9.3
Total	65	100.0

Figure 6 below illustrated the heterozygosity of alleles at the SNP site *CYP11A1* rs4886595 in patient group. The rate of homozygosity is higher in this group (Table 7).

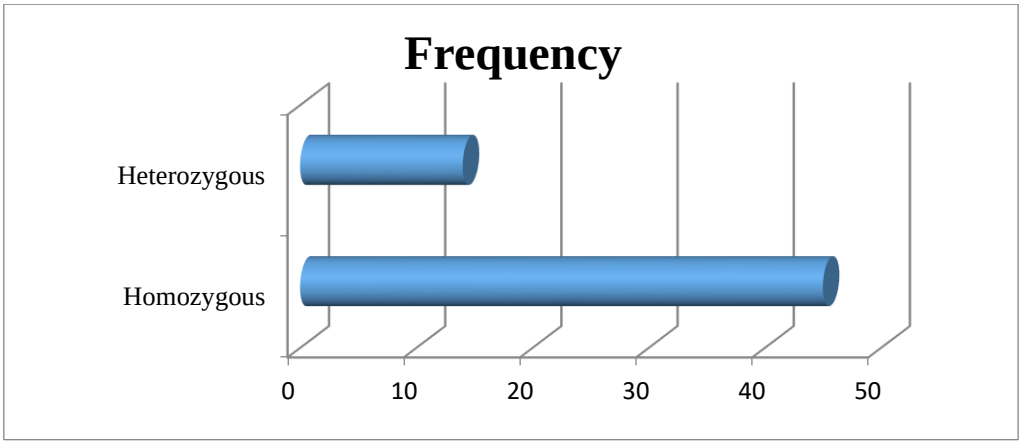


Figure 6: The frequency of heterozygous and homozygous of CYP11A1 rs4886595 in patient group.

Referred to the control group for the *CYP11A1* rs4887139 polymorphism, a higher percentage (90.9%) of patients was found to be homozygous, while the heterozygous recorded to be in low percentage of the patients with about 3.6 % (table 8).

Table 8: *CYP11A1* rs4887139 status for control group

	Number of control group	Percentage
Homozygous	50	90.9
Heterozygous	2	3.6
Total	52	94.6
No results	3	5.4
TOTAL	55	100.0

Figure 7 illustrated the heterozygosity of alleles at the SNP site *CYP11A1* rs4887139 in control group. As shown, the higher number of allelic frequencies were found as homozygous (table 8).

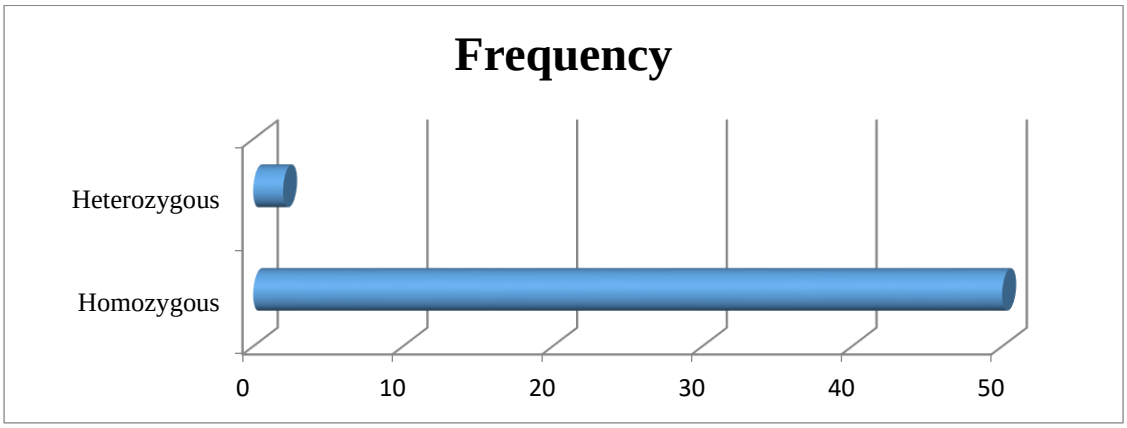


Figure 7: The frequency of heterozygous and homozygous of CYP11A1 rs4887139 in control group

Referred to the control group for the *CYP11A1* rs4886595 polymorphism, a higher percentage of 76.4% of patients was found to be homozygous, while the heterozygous recorded to be in low percentage of the patients with about 18.2% (table 9).

Table 9: *CYP11A1* rs4886595 homozygosity status for control group

	Number of control group	Percentage
Homozygous	42	76.4
Heterozygous	10	18.2
Total	52	94.6
System	3	5.4
Total	55	100.0

Figure 8 illustrated the heterozygosity of alleles at the SNP site *CYP11A1* rs4886595 in control group. As shown, the higher number of allelic frequencies were found as homozygous (table 9).

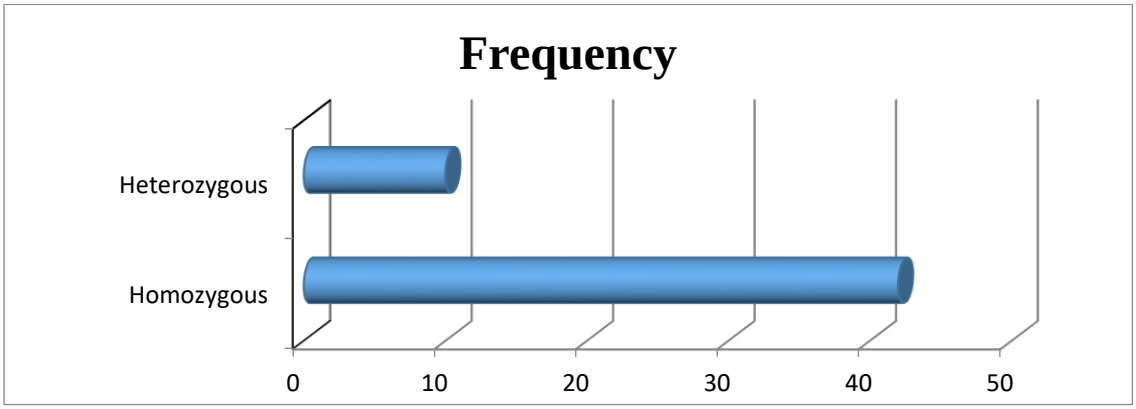


Figure 8: The frequency of heterozygous and homozygous of CYP11A1 rs4886595in control group

3.1. The heterozygosity status among patients in *CYP11A1* rs4887139 gene polymorphism

Mann Whitney U test was conducted on the results to investigate the difference between homozygosity and heterozygosity of the patients using the melting temperature (Tm) analysis. The *CYP11A1* rs4487139 Tm in the patients group showed that the heterozygosity was significantly higher than the homozygosity (mean ± SD; 90.70 ± 0.28; mean ± SD; 88.29 ± 0.95; $p<0.05$, respectively; Tables 10).

Table 10: Descriptive statistics for heterozygosity evaluation of *CYP11A1* rs4887139 using Tm values in patient group

	N	Mean	S.D	Median	Min	Max
Homozygous	58	88.29	0.95	88.8	86.0	89.30
Heterozygous	2	90.70	0.28	90.70	90.50	90.70

3.2. The heterozygosity status among control participants in *CYP11A1* rs4887139 gene polymorphism

Mann Whitney U test was implemented to investigate the difference between homozygosity and heterozygosity in the control group using the melting temperature (Tm) of *CYP11A1* rs4887139 gene polymorphism. The Tm of *CYP11A1* rs4487139 for the patients group showed the heterozygosity was significantly higher than mean homozygosity (91.50 ± 1.97; 88.15 ± 1.07; $p<0.05$, respectively Tables 11).

Table 11: Descriptive statistics for heterozygosity evaluation of *CYP11A1* rs4887139 using Tm values in control group

	N	Mean	S.D	Median	Min	Max
Homozygous	50	88.15	1.07	88.70	86.20	90.70
Heterozygous	2	91.50	1.97	91.50	90.10	92.90

3.3. The heterozygosity status among patients in *CYP11A1* rs4886595 gene polymorphism

Mann Whitney U test was implemented to investigate the difference between homozygosity and heterozygosity in the patients group using the melting temperature (Tm) of *CYP11A1* rs4886595 gene polymorphism. The Tm of *CYP11A1* rs4886595 for the patients group showed the heterozygosity was significantly higher than mean homozygosity (87.80 ± 1.39; 86.04 ± 1.41; $p<0.05$), respectively, Table 12).

Table 12: Descriptive statistics for heterozygosity evaluation of *CYP11A1* rs4886595 using Tm values in patient group

	N	Mean	S.D	Median	Min	Max
Homozygous	45	86.04	1.41	86.10	80.10	89.60
Heterozygous	14	87.80	1.39	87.70	84.60	90.30

3.4. The heterozygosity status among control participants in *CYP11A1* rs4886595 gene polymorphism

Mann Whitney U test was implemented to investigate the difference between homozygosity and heterozygosity in the control group using the melting temperature (Tm) of *CYP11A1* rs4886595 gene polymorphism. The Tm of *CYP11A1* rs4486595 for the patients group showed the heterozygosity was significantly higher than mean homozygosity (87.34 ± 2.49) (85.38 ± 1.59) ($p<0.05$), respectively (Table 13).

Table 13: Descriptive statistics for heterozygosity evaluation of *CYP11A1* rs4886595 using Tm values in control group

	N	Mean	S.D	Median	Min	Max
Homozygous	40	85.38	1.59	85.95	82.10	88.60
Heterozygous	3	87.34	2.49	87.60	82.70	90.60

3.5. The heterozygosity status among patients and control participants in both *CYP11A1* rs4886139 and *CYP11A1* rs4886595 gene polymorphism

The findings showed that the patients and control groups have no significant difference in Tm in *CYP11A1* rs4887139 ($p = 0.203$), while the mean±SD of Tm in patients group was remarkably higher than the mean±SD of Tm in the control group in *CYP11A1* rs4886595 (86.45±1.58; 85.75±1.93; $p=0.01$), respectively. Student's T-test was performed to confirm the Mann Whitney U test, in which both test showed similar

findings comparing the patient and control groups in Tm values for rs4887139 and rs4885595, respectively. The test showed significant difference in Tm rs4886595 among patient and control groups, while no significant difference was observed for the Tm rs4887139.

CHAPTER FOUR

4.1. Discussion

The ovary is considered a major organ in the female productive system and its interruption due to endocrine anomalies may lead to female infertility. Polycystic ovarian syndrome is a metabolic and hormonal disorder that devastates women throughout their reproductive years (Azziz R, et al., 2004). Nonetheless, regarding its heterogeneity and complex structure, PCOS remains unclear in commonly recognized clinical significance. Moreover, the existence of PCOM in these patients happens to be usual occurrences in most individuals with PCOS (Legro RS, et al., 2003). Approximately (95%) of females with this syndrome have decreased degree of follicle stimulating hormonal and polycystic ovaries at the initial follicular stage which prompt antral-follicle development and elevate LH expression (Lujan ME, et al., 2008). In such manner, PCOS is considered as an unpredictable androgen overabundance related by an assortment of levels of gonadotropic and metabolic dysregulation constrained by numerous genes collaboration and ecological angles (Azziz R, et al., 2009). However, the degree to which this feature is passed generation to generation, as well as the fundamental molecular factors that stress PCOS are uncertain. The pathogenic origin of anovulation, aberrant follicular development and metabolic problem alongside other heterogeneous rational abnormalities in PCOS patients requires specific investigations. It is claimed that the daughters of women with PCOS features may have an increased possibility of hyperandrogenism and other PCOS phenotypes (Goodarzi MO, et al., 2007).

Another suggestion of a genetic cause is to have a single change in nucleotide base of any of the following genes; *THYROID ADENOMA LINKED (THADA), INTERLEUKIN 6 (IL6), ADIPONECTIN GENES* and *(DENN/MADD DOMAIN CONTAINING 1A (DENND1A))*. Moreover, a modified expression was observed in *in vitro* investigations of both the *CYP11A* and *CYP17* genes in theca-cells of PCOS patients (Salilew-Wondim, D., et al., 2015). New episode in the genetic field alluded to broad reconstructing of each part of the definite natural systems of the turn of events and movement of PCOS (Gupta AR, et al., 2007).

The chemical transformation of cholesterol to pregnenolone that is facilitated by the cytochrome enzyme (P450scc) is the most important step in the biosynthesis of

steroid hormones in the ovary. Genetic alterations in the regulatory region of *CYP11A1* and *CYP17A1* genes are associated with pathogenesis of PCOS. Common polymorphisms of *CYP17A1* and *CYP11A1* genes are hypothesized to predict the individual's susceptibility to PCOS (Mendoza N, 2011).

In our study, two SNPs of the *CYP11A1* (rs4887139 and rs4886595 variant) were chosen to examine the possible association with the disorder. *CYP11A1* rs4887139 a single nucleotide variation located on 15:74369604 (GRCh38) 15:74661945 (GRCh37), position: chr15:74661945-74661945, band: 15q24.1, genomic size: 1, global allelic frequency of this variation is 0.8902. *CYP11A1* rs4886595 is a single nucleotide variation located on 15:74370924 (GRCh38) 15:74663265 (GRCh37)**,** position: chr15: 74663265-74663265**,** band: 15q24.1, genomic size: 1**,** global allelic frequency of this variant 0.8291. Both variants were not pathogenic since neither had been reported in ClinVar. These variants are located in the non-coding regions of the genes.

In order to analyze the $[TTTA]_n$ repeat polymorphism of the *CYP11A1*, 100 PCOS cases and 100 healthy were analyzed, and there was a correlation between $[TTTA]_n$ repeats and the risk of PCOS (Gao GH et al., 2010).

CYP11A1 is extended around 20K and is localized at 15q24. P450scc is a crucial rate-limiting enzyme and it is encoded by this gene, which has nine exons and eight introns. P450scc-enzyme catalyzes the initial step in the production of androgens and estrogens, converting cholesterin to pregnenolone, an androgen precursor (Zhang, C. W., et al., 2012). Theca cells from polycystic ovaries generate more progesterone, 17-hydroxyprogesterone, and androstenedione than normal theca cells, according to i*n vitro* studies. Several *CYP11A1* SNPs have been implicated in the aetiopathogenesis of PCOS, with the potential to modify testosterone levels. The hyperandrogenic condition in PCOS has been linked to microsatellite allelic variation $[TTTA]_n$ of this gene, a region comprising several cAMP-regulated components that control its expression (Reddy et al., 2014).

Several studies analyzed the association of PCOS and *CYP11A1* gene polymorphisms, focusing on the $[TTTTA]_n$ pentanucleotide repeat (*D15S520*) situated 528-bp-upstream of the translational initiation site, resulting in six variants with ranging from (four, six, seven, eight, nine, or ten repeats). The primary variations found in the

Chinese population are the four-, six-, and eight-repeat alleles, which are linked to breast cancer and polycystic ovary syndrome risks (Gao GH, et al.,2010), (Kim JW., et al., 2005). Furthermore, In Caucasians, there were also substantial relationships between the $[TTTA]_n$-repeats and PCOS risk (Shen, W., et al., 2014). In fact, a previous study suggested that the $[TTTTA]_6$ variation is involved in the etiology of PCOS in Chinese women (Zhang 2012).

In this study, melting temperature (Tm) analysis was used to determine the homozygosity and heterozygosity status of SNPs at *CYP11A1* rs4887595 site. The (Mann Whitney U) test was used to investigate at the statistical differences. The results of this investigation showed that there was a substantial difference in *CYP11A1* rs4886595 heterozygosity and homozygosity between the patients and the control group. Similar studies were performed previously, in such seven SNPs within *CYP11A1* (rs12917295, rs11632698, rs1484215, rs6495096, rs4887139, rs9806234, and rs4886595) in PCOS patients were genotyped. The results show that the genotype "GG" of rs4887139 was associated with high PCOS risk with OR = 1.79, 95 percent CI = 1.04-3.10, P = 0.035, and the genotype "CC" of rs4886595 with increased PCOS risk with OR = 4.29, 95 percent CI = 0.90-20.36, P = 0.04 (B. Shan et al., 2016). Similar results were also reported previously, in such genotypic distributions of the SNP *CYP11A1* rs4077582 in PCOS patients were substantially different from the controls in 106 Egyptian females between the ages of 18 and 45. Thus, they concluded that *CYP111A1* rs4077582 is linked to the pathophysiology of PCOS, implying that *CYP11A1* rs4077582 may alter the P450scc compound activity and as a result, androgen production (Abdel-Mageed, W. S., et al., 2016).

Another study performed in Chinese women found that the frequency of rs4077582, D15S1547, D15S1546, and rs11632698 in *CYP11A1* gene between two groups (patients and control) showed statistical significance (P = 0.010, 0.044, 0.018, and 0.026). There was a notably difference in allele frequencies between the two groups for *D16S520* and rs4077582, rs4887139, rs1843090, D15S1547 (P = 0.002, 0.048, 0.030, 0.001, respectively). On the other hand, another study showed insignificant difference in Tm among patient and control groups in rs4887139 (GH Gao et al., 2010). There have also been studies performed in Chinese women in $[TTTTA]_n$ polymorphism, but no definitive conclusions have been reached. Tan and Chen found no link between this polymorphism and PCOS (Abdel-Mageed, W. S., et al., 2016).

Another study performed in South India a total of 542 participants were included in the study, including 267 PCOS and 275 healthy controls. DNA was obtained from whole blood samples and the *CYP11A1* $[TTTA]_n$ polymorphism was genotyped using PCR-PAGE. In this demographic, the *CYP11A1* (TTTA)n repeat polymorphism seemed to be a possible molecular marker for PCOS risk. In the case of obesity, gene-gene and gene-environmental interactions may play a role in the development of this complex disease (Reddy et al., 2014).

CYP11A1 gene of the cholesterol side chain cleavage enzyme has been implicated and required for the production of sex hormones in the pathophysiology of PCOS. A case–control research was performed using the polymerase chain reaction restriction fragment length polymorphism technique on 314 PCOS patients and 314 controls to determine the relationship between the SNPs rs11632698 and rs4077582 in *CYP11A1* with PCOS. Following that, for validation, 100 DNA samples were re-genotyped using direct sequencing. In women with PCOS, the genotypic distribution of rs4077582 was different than in controls (P = 0.002). In rs11632698 (P = 0.912), no such distributional difference was recorded.

The results of this study were merged with data from a prior research in which two SNPs were also investigated in 290 PCOS patients and 344 controls (Zhang et al., 2012). The combined study (1262 individuals, 604 control women and 658) revealed a significantly greater difference in the genotypic distribution of rs4077582 between PCOS and controls (P<0.001). The T allele was more common in patients with PCOS (odds ratio = 1.314; 95 percent confidence interval [CI] = 1.122–1.540). In the control group, testosterone levels, LH levels, and the LH/FSH ratios were all significantly different among the three rs4077582 genotypes. As a result, the SNP rs4077582 in *CYP11A1* is significantly associated with PCOS susceptibility and may affect testosterone levels in various genotypes via LH modulation. In rs11632698, no correlation was found (Zhang et al., 2012).

4.2. Conclusion

In this study, the differences in the heterozygosity status of the alleles at the rs4886595 C>A within *CYP11A1* involved in PCOS recorded significant difference in patients and control group, while the heterozygosity of rs4887139 G>A within *CYP11A1* was shown to be insignificant in both groups. Therefore, future research

ought to focus on elucidating the susceptible causes of PCOS with wide range of SNPs and more sample size. More genome-wide association studies in PCOS patients of different origin will be important to recognize prospect genes as well as proteins that are implied in PCOS risk.

4.3. Limitation

The study had numerous possible limitations, one of which was sample size; the small sample size in both the case and control groups might impair the reproducibility of the findings. Although a few research studies aimed to investigate the heterozygosities at these two polymorphisms (rs4887139 G>A and rs4886595 C>A), further analysis is required to establish the correlation between these polymorphisms and PCOS. Another limitation is that just two SNPs were chosen, perhaps resulting in insufficient coverage of variants.

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Ethical Approval

EK-859-2019

YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi :28.03.2019
Toplantı No : 2019/67
Proje No : 784

Yakın Doğu Üniversitesi Tıp Fakültesi öğretim üyelerinden Doç. Dr. Pınar Tulay'ın sorumlu araştırmacısı olduğu, YDU/2019/67-784 proje numaralı ve **"İnfertilite, Spontan Düşük Ve Polikistik Over Sendromu İle İlişkili Polimorfizmlerin Araştırılması"** başlıklı proje önerisi kurulumuzca değerlendirilmiş olup, etik olarak uygun bulunmuştur.

1. Prof. Dr. Rüştü Onur (BAŞKAN)
2. Prof. Dr. Nerin Bahçeciler Önder (ÜYE) KATILMADI
3. Prof. Dr. Tamer Yılmaz (ÜYE)
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7. Prof. Dr. Kaan Erler (ÜYE) KATILMADI
8. Prof. Dr. Atalay Arkan (ÜYE) KATILMADI
9. Doç. Dr. Ümran Dal Yılmaz (ÜYE) KATILMADI
10. Doç. Dr. Nilüfer Galip Çelik (ÜYE)
11. Doç.Dr. Emil Mammadov (ÜYE)
12. Doç. Dr. Mehtap Tınazlı (ÜYE)