T.R.N.C

NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

ALLELIC FREQUENCIES OF GENES INVOLVED IN HORMONAL REGULATION OF POLYCYSTIC OVARY SYNDROME

By

SANA MUNEEM MADHHER

Master of Science in Medical Biology and Genetics

Supervisor

Assoc. Prof. Pınar Tulay

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APPROVAL

Thesis submitted to the Institute of Graduate Studies of Near East University in partial fulfillment of the requirement for the degree of Master of Science in Medical Biology and Genetics.

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DECLARATION

I declare that, this thesis entitled as “Allelic frequencies of genes involved in hormonal regulation of polycystic ovary 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 “Allelic frequencies of genes involved in hormonal regulation of polycystic ovary syndrome” was written in accordance with the NEU Postgraduate thesis proposal and thesis writing directive.

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DEDICATION

I am dedicating this research work to the spirit of my first teacher, my soul, my ideals, and the ideal of fatherhood who cultivated love for knowledge and principles of life inside me, my father (God bless him).

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ABSTRACT

ALLELIC FREQUENCIES OF GENES INVOLVED IN HORMONAL REGULATION OF POLYCYSTIC OVARY SYNDROME

SANA MUNEEM MADHHER

Department of Medical Genetics

Thesis supervisor: Assoc. Prof. Pınar Tulay

Aim: The aim of this thesis was to investigate the allelic frequencies of *FSHR* gene for p.Ala307Thr (c.919G>A; rs6165) and p.Ser680Asn (c.2039C>T; rs6166) that are involved in the regulation of oogenesis.

Background: Polycystic ovary syndrome (PCOS) is a common complicated endocrine condition. PCOS causes a number of health problems. Hyperandrogenism, hirsutism, anovulation, infertility, insulin resistance, impaired glucose tolerance, gestational diabetes (GDM) and type 2 diabetes (T2DM), dyslipidemia, obstructive sleep apnea, and psychological disorders (increased anxiety, depression and poor quality of life) have been reported in females with PCOS. Therefore, PCOS is increasingly recognized that it is not only a reproductive problem but also a metabolic disease with significant health risks. The appearance of symptoms can vary by age. Reproductive and psychological symptoms predominate in young women. The prevalence of metabolic features increases with age and also occurs in young women, especially when they are overweight. The etiology of PCOS is complicated. It has been suggested that both environmental factors as well as genetic factors are involved in the development of PCOS. The hormonal regulation has proven to be important in the normal development of oocytes and any anomalies of the hormonal levels may lead to the development of PCOS.

Material and Methods: A total of 120 blood samples were collected from females with polycystic ovary syndrome and females without polycystic ovary syndrome as control group from the NEU Hospital. The clinical background and body mass index (BMI) were reported. DNA extraction was performed from whole blood of each sample. The analysis of allelic

frequencies were investigated by real-time PCR and SNPs in the *FSHR* gene in two regions associated with PCOS, p.Ala307Thr (c.919G>A; rs6165) and p.Ser680Asn (c.2039C>T; rs6166), were evaluated. The statistically program SPSS was used to conduct Fischer's exact test.

Results: The data was analyzed by SPSS program (version 25) by using Fischer's exact test. There were no statistically significant differences shown when PCOS patients compared with control group depending on the allelic frequency of *FSHR* p.Ala307Thr (c.919G>A; rs6165). The p-value was recorded as 0.999 and this is higher than alpha value 0.05. The p -value for *FSHR* p.Ala307Thr (c.919G>A; rs6165) was 0.498 between the patient and the control groups that is higher than alpha value 0.05. Majority of the patients were heterozygous for *FSHR* Ala307Thr while only two patients are found to be homozygous. On other hand, both the patients in the patient and the control group was found to be heterozygotes for *FSHR* p.Ser680Asn (c.2039C>T; rs6166).

Conclusions: In this study, the allelic frequency and genetic condition of *FSHR* gene p.Ala307Thr (c.919G>A; rs6165) and p.Ser680Asn (c.2039C>T; 6166) have shown no statistical significant difference when both groups were compared (PCOS and control groups, respectively). This would mean that the allelic frequency of *FSHR* gene including both p.Ala307Thr (c.919G>A; rs6165) and p.Ser680Asn (c.2039C>T; rs6166) was not associated with the pathogenicity of PCOS and cannot be considered as one of the factors in the oogenesis regulation.

Keywords: *FSHR*, PCOS, Ala307Thr, Ser680Asn, polymorphism.

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LIST OF ABBREVIATION

DNA	: Deoxyribonucleic acid
PCOS	: Polycystic Ovary Syndrome
FSHR	: Follicle Stimulating Hormone Receptor
T2DM	: Type 2 diabetes Mellitus
GDM	: Gestational diabetes Mellitus
BMI	: Body mass index
SNPs	: Single_nucleotide polymorphisms
GnRH	: Gonadotropin-releasing hormone
IR	: Insulin Resistance
IRS	: Insulin Receptor Substrate
CVS	: Cardiovascular Disease
CRP	: C-Reactive Protein
HDL	: High-Density Lipoprotein
EDTA	: Ethylenediaminetetra-acetic Acid
cDNA	: Complementary Deoxyribonucleic Acid
IVF	: *In vitro* Fertilization
POF	: Premature Ovarian Failure
FSH	: Follicle Stimulating Hormone
LH	: Luteinizing hormone
LHCGR	: luteinizing hormone/chorionic gonadotropin receptor
PPAR-y	: Peroxisome proliferator-activated receptor gamma
FTO	: Fat mass and obesity associated
VDR	: Vitamin D receptor
ER-α	: Estrogen receptor alpha
5-HTTLPR	: Serotonin-transporter-linked polymorphic region

PCOM	: Polycystic Ovarian Morphology
PCR	: Polymerase Chain Reaction
ND	: Nano Drop
HRM	: High resolution melting method
HPO	: Hypothalamic pituitary ovarian
Con.	: Concentration

CHAPTER ONE

1.1 Introduction:

Polycystic ovary syndrome (PCOS) is an endocrine condition. This can be seen in the various females. Anovulation, menstrual cycle disruption, increased androgen hormones, insulin resistance, abdominal fat gain and infertility are among the symptoms (Norman et al., 2007). PCOS is complex condition that is caused by both genetic factors (CM, 2011, Diamanti-Kandarakis et al., 2006) and environmental factors (Qu et al., 2012, Wang et al., 2014). PCOS affects approximately 7% of females in their reproductive years (Goodarzi et al., 2011). The polymorphism of genes may impact hormonal regulation and play a role in the normal development of oocytes and Polycystic ovary syndrome can develop as a result of hormonal imbalances (Goodarzi et al., 2011).

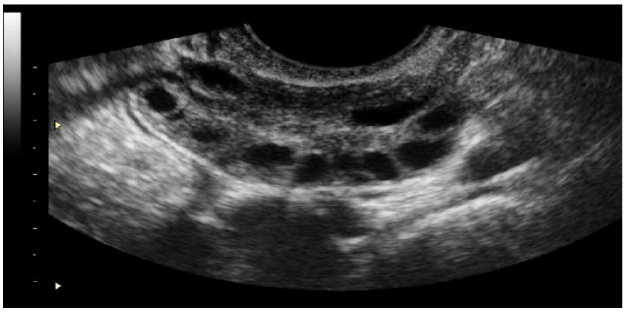
1.2 Clinical significance of polycystic ovary syndrome:

Females with polycystic ovary syndrome frequently experience irregular menstrual cycles, a high level of androgen and the inability to procreate. Menstrual cycle disarray is characterized by abnormally light menstrual bleeding , menstrual cycle suppression, and irregular menstrual bleeding lasting a long period (Farquhar, 2007). Around 30% of women suffering from polycystic ovary syndrome don't have any issues with menstruation (Balen et al., 1995). Nearly 85% to 90% of women with polycystic ovary syndrome have from abnormally light menstrual cycles, while 30% to 40% of women with polycystic ovary syndrome have suppressed menstruation (Hart et al., 2004).

Approximately 80% of women with polycystic ovary syndrome produce excessive amounts of androgen (Azziz et al., 2004). Women with polycystic ovary syndrome have high levels of androgen production and hirsutism in about 70% of cases (Fauser et al., 2012). The Feriman-Gallwey score system is used to calculate hirsutism (Ferriman et al., 1961). More than 90% of women have hirsutism with regular monthly cycle but have polycystic ovaries (Adams et al., 1986), while 50% of females suffering from polycystic ovary have excess unwanted body hair (Souter et al., 2004). Acne is another sign of excessive androgen production, although it is not common in polycystic ovary syndrome. However, 15%-30% of women with polycystic ovary syndrome still develop

acne (Azziz et al., 2004, Wijeyaratne et al., 2002, Eden, 1991)). Depending on the expression levels of 5α-reductase in the fatty gland and the hair follicle, the distribution of hirsutism and acne changes (Lowenstein, 2006). Additionally, weight gain is associated with polycystic ovary syndrome (Isikoglu M et al., 2007). Women with polycystic ovary syndrome are advised to follow a healthy diet to reduce weight gain, overall fat mass, testosterone and insulin levels (Teede et al., 2011).

Figure 1.1: The ultrasonography showing the PCOS (Nardo and Wadhwa, 2007).



1.3 Polycystic ovary syndrome associated abnormalities and diseases:

PCOS has been associated with various diseases. PCOS is linked to infertility, as previously stated. Furthermore, PCOS plays a role in the development of cardiovascular diseases and psychological disorders including, nervousness and stress. Additionally, it has been associated with cancers such as breast cancer and endometrial cancer. infertility and abortion affect nearly 20 percent of women (Diamanti-Kandarakis et al., 1998).

1.3.1 Infertility and PCOS:

Infertility affects nearly 40% of women with polycystic ovary syndrome (Teede et al., 2010). Around 90-95% of women with polycystic ovary syndrome do not have a mature egg and consequently do not ovulate. Despite having a normal number of primordial follicles, women are arrested at the pre-antral stage. While, the normal follicles remain at lower diameter of 4-8μm, resulting in anovulation (Brassard et al., 2008). PCOS impacts 42% to 73% of women who have abortions (Glueck et al., 2001, Jakubowicz et al., 2002). The polymorphisms of *FSHR* gene, such as p.Thr307Ala, have been considered to play a role in the development of polycystic ovary syndrome (Jakubowski, 2005, Layman et al., 1998).

1.3.2 Cardiovascular risk (CVD) and PCOS:

Polycystic ovary syndrome promotes a two-fold increase in the risk for cardiovascular disease in women. The risks include, high blood pressure, an abnormal amount of lipid in the blood, a high blood sugar level, and high body mass index. Furthermore, PCOS has also been associated with less prevalent risks, such as C-reactive protein (CRP), homocysteine, and tumor necrosis factor-alpha (TNF-alpha) (Toulis et al., 2011). Females of various ages with polycystic ovary syndrome have a major stranger sign for cardiovascular disease and these high risks can exist without an abnormal amount of lipid in the blood but with increased levels of lipid (Fauser et al., 2012). The polymorphism of *PPAR-y* gene affects lipid and high blood sugar levels (Falcão-Pires et al., 2009, Auwerx, 1999).

1.3.3 Metabolic risk and PCOS:

Obesity is an increasingly common symptom in women with PCOS. Women with polycystic ovary syndrome have high body mass index and abdominal fat (Bates et al., 2013), there is a strong link between vascular fat accumulation with increased insulin resistance (IR) (Carmina et al., 2007, Cascella et al., 2008). The accumulation of fat in the vascular system is related to high fasting insulin level and smaller insulin space than a detour (Carmina et al., 2007, Cascella et al., 2008). At the same time the accumulation of fat in the body related with a dyslipidemic profile, which includes elevated triglyceride levels in the blood and lower high-density lipoprotein levels (HDL) (Lord et al., 2006, Pasquali et al., 1994). Despite the fact that polycystic ovary syndrome is considered as one of the causes of type 2 diabetes mellitus (T2DM), women with polycystic ovary syndrome have a higher risk of developing T2DM (Alberti et al., 2007). The polymorphisms of *IR* and *IRS* play a role in the development of type 2 diabetes mellitus and polycystic ovary syndrome (Shi et al., 2016). The *FTO* gene polymorphisms may be involved in obesity-related polycystic ovary syndrome (Demirci et al., 2010, Shi et al., 2007). At the same time, a variation in the vitamin D receptor (*VDR*) gene, which is responsible for vitamin D levels and is correlated to polycystic ovary syndrome, may have a role in the development of PCOS (Ogunkolade et al., 2002, Mahmoudi and sterility, 2009).

1.3.4 Endometrial cancer and endometriosis risk and PCOS:

According to a meta-analysis, women with PCOS had a higher risk of developing endometrial cancer (Barry et al., 2014). While ovarian and breast cancers are less common, they are nonetheless possible (Barry et al., 2014). The polymorphism of the (*ER*)-α gene plays a significant role in endometrial cancer (Kang et al., 2005).

1.3.5 Psychiatric disorders and PCOS:

Psychological problems impact women with PCOS, lowering their quality of life (Moran et al., 2012). According to recent meta-analysis, it has been estimated that 14% to 67% of females with polycystic ovary syndrome had 4-fold increase risk of developing depression (Dokras et al., 2011). Women who have polycystic ovary syndrome experience anxiety (Dokras et al., 2011). The polymorphism in the *serotonin transporter* gene (*5-HTTLPR*) plays a role in the psychiatric disorders and polycystic ovary syndrome (Serretti et al., 2007).

1.4 Pathophysiology of PCOS:

Patients with PCOS have phenotypic manifestations linked to ovarian hormone production abnormalities and elevated androgen levels, as well as clinical features related to increased insulin resistance and hyperinsulinemia. insulin resistance and hormonal abnormalities play a significant role in the pathophysiology of PCOS in approximately 50-70% of cases (Skrgatic et al., 2013, Diamanti-Kandarakis et al., 2008).Hyperinsulinemia, hyperandrogenemia and ovarian hormone imbalances (with subsequent anovulation) have all been correlated to PCOS. Insulin interacts with gonadotropins and stimulates and enhances steroidogenesis by directly binding to its receptor in the ovary (Makker et al., 2012, Li et al., 2017).

1.5 Secretion of gonadotropins and their effects:

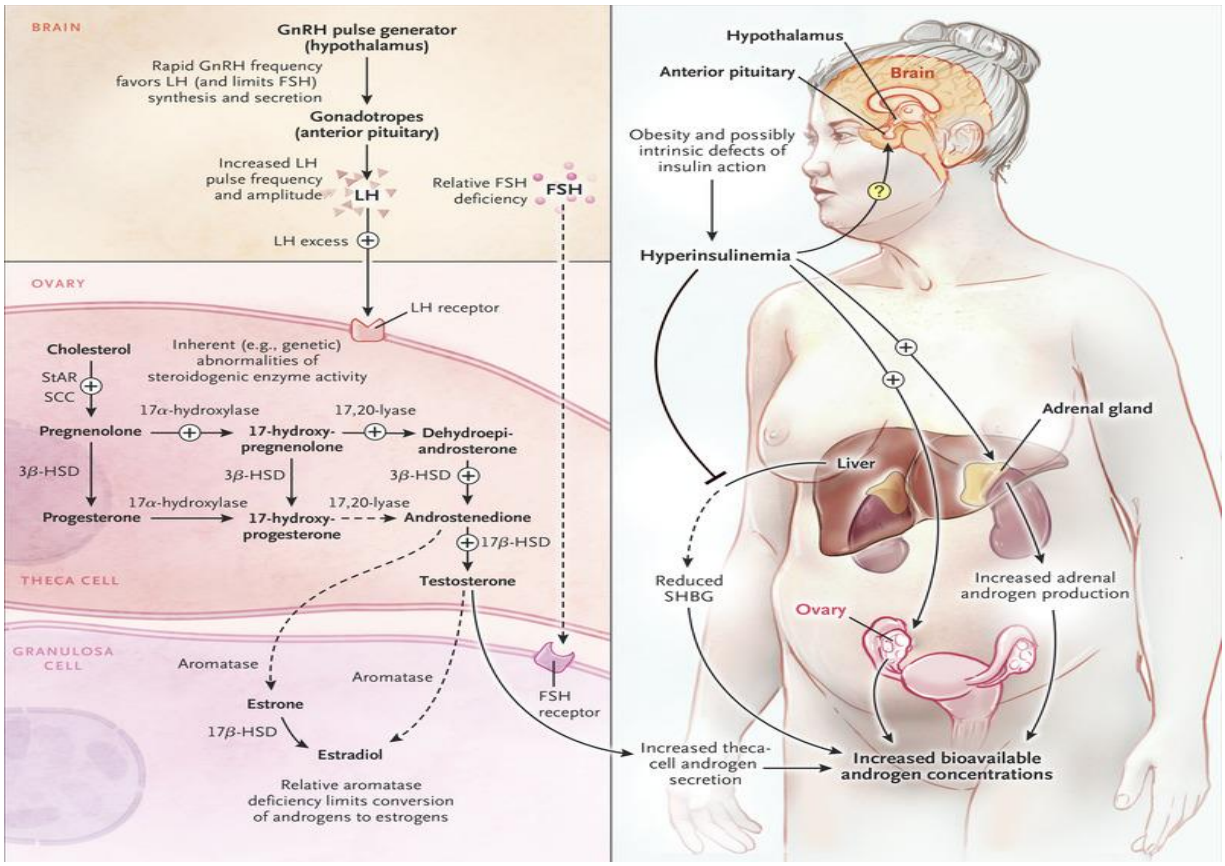
From an etiological point of view, PCOS could be caused by a dysfunction in neuronal pathways that control the hypothalamic-pituitary-ovarian (HPO) axis in the brain (Moore et al., 2016). In vast majority of mammals, the function of ovaries are controlled by GnRH neurons, which are found adjacent to optic in hypothalamus, (Moore et al., 2016) and produce GnRH neuropeptide into median eminence and portal vein,

stimulating adenohypophysis gland to secrete gonadotropins, hormones that control ovarian folliculogenesis and steroidogenesis (Carmel et al., 1976). Binding of follicular stimulation hormone (FSH) to its receptors on granulosa cells mediates the development of follicles in the ovary. Long-term FSH deficiency can cause impaired follicular maturation and ovulation failure resulting in subfertility. These immature follicles will appear as small cysts on ultrasound examination (Bafrouei et al., 2018, MELISSA et al., 2000).

Another gonadotropin, luteinizing hormone (LH), mediates follicular growth, stimulates steroidogenesis and develops corpus luteum (Shoham et al., 1993, Dufau, 1998). LH induces ovulation by binding to LH receptors and the luteinizing hormone/chorionic gonadotropin receptor (*LHCGR*) which is also the target receptor of HCG (Almawi et al., 2015). Improper production and release of gonadotropins (FSH and LH) is a major cause of PCOS (Sheykhha et al., 2007).

The main neuroendocrine change involved in the pathogenesis of PCOS is an elevation of GnRH levels, which is manifested as increased LH production. LH production is the most important factor in the etiology of PCOS (Sheykhha et al., 2007). This role can be explained by different mechanisms or hypotheses. The first mechanism is an elevation of circulating insulin that mediates overactivation of either GnRH nerve cells or the response of the pituitary gland to GnRH. The second mechanism is a decrease in serum progesterone, which causes anovulation, and this in turn cancels the effect of negative feedback of progesterone on GnRH release. The third mechanism is a rise in androgen levels that reduces negative feedback of steroid hormones (Moore et al., 2016). It has been found that, in contrast to *GnRH* and its *receptor* (*GnRHR*) genes, *FSH* and *LH receptor* (*FSHR/LHCGR*) genes have been correlated with an increased risk of development, regardless of ethnic differences (Almawi et al., 2015).

Figure 1.2: The diagram showing the GnRH cycle (McCartney and Marshall, 2016).



1.6 SNP mechanism:

A change in DNA sequence by single nucleotide (A, T, C, or G) is referred to as single nucleotide polymorphism (SNP). Around two out of three SNPs, Cytosine (C) is replaced by thymine (T). An SNP is found every 100-300 bases within the human genome. SNPs change less frequently from generation to generation in terms of evolution. SNPs constitutes about 90% of all human genetic variants and can be found in both coding and non-coding regions along the 3,200,000,000 base human genome with no obvious effect on cell function, meanwhile, some SNPs may have adverse effect on individuals or change their response to certain drugs. Scientists can use the SNP map to identify genes related to certain pathological conditions such as cancer, vascular disease, mental illness and diabetes.

There are many pathways involved in pathogenesis of PCOS, such as steroid synthesis (Carey et al., 1994, Gharani et al., 1997), gonadotropin action (Franks, 1995), signaling

pathways of insulin (Ciaraldi et al., 1992, Dunaif et al., 1995) and body weight regulation (Kiddy et al., 1992).

Although many SNPs within these genes involved in these pathways were investigated, (Carey et al., 1994, Gharani et al., 1997, O'Rahilly et al., 1991, Krook et al., 1996), only a few of them showed a correlation with the etiology of PCOS, while others did not (Carey et al., 1993). These inconclusive findings are not uncommon in diseases with complex genetic background like PCOS and this could be attributed to many factors including genetic heterogeneity, multiple underlying causes and environmental impact, some studies conducted to find the mode of inheritance in PCOS suggested a dominant inheritance of single gene and high penetrance (Govind et al., 1999), although others do not support this finding (Jahanfar et al., 1995). Previously published study on a group of 37 genes from 150 families showed that follistatin gene has the strongest evidence of linkage with PCOS (Urbanek et al., 1999). Another large-scale study on linkage between *FOLLISTATIN* gene and PCOS came to the same conclusion. This could have a role in functional defect of FSH-granulosa cell axis in PCOS. Many other genes involved in production of hormones like androgen, insulin and gonadotropin have been investigated and studied to find a possible linkage with PCOS (Urbanek et al., 1999).

1.7 The *FSHR* gene:

The *FSHR* gene is composed of nine introns and ten exons and located in chromosome 2-p21-p16 of human genome. Exons one through nine encode for extracellular domain of *FSHR*, while exon ten encodes for C-terminal end of all three domains of the receptor including extracellular, transmembrane and intracellular parts of the receptor. Exon ten is essential for transduction of signals but not for ligand binding. About 1800 SNPs in the *FSHR* gene have been identified and made available to researchers in National Center for Biotechnology information (NCBI) SNPs database (http://www.ncbi.nim.nih.gov). SNPs can be located anywhere in the gene. In case of *FSHR* gene, only eight SNPs were identified in coding regions (exons), with seven of them situated in exon ten at codon positions 307, 329, 449, 524, 567, 665, and 680. Six of these SNPs cause amino acid substitution so they are non-synonymous. Among these, p.Ala307Thr (c.919G>A; rs6165) and P.Ser680Asn (c.2039C>T; rs6166) are two most known and well characterized polymorphisms identified in exon ten of the

FSHR gene, both of which are linked and occurred during recombination (Tempfer et al., 2009).

There are different studies conducted to find if there is a link between polymorphisms identified in the *FSHR* gene and ovarian function. The amino acid serine polymorphism at codon 680 on both alleles results in elevation in endogenous serum FSH levels with subsequent prolonged follicular phase, which means that this variant is less sensitive to FSH. Accordingly, those who have this variant with serine on both alleles will need more FSH hormone to stimulate their ovaries during *in vitro* fertilization (IVF) cycles. In addition, Asn680Ser polymorphism was not linked to premature failure of ovary. Various studies have found different allelic variations in women with polycystic ovarian morphology (PCOM) (Tempfer et al., 2009, Valkenburg O et al., 2015).

1.8 The fertility treatment:

In mammals, the active oocyte is segregated from primordial follicles prior to delivery, it occasionally lingers in the ovary to become cystic (McLaughlin and McIver, 2009). Due to limited number of follicles that are ready for fertilization, obtaining functioning primordial follicles is difficult (McGee and Hsueh, 2000). The menopause begins around the age of 50 in females (Macklon and Fauser, 1999). Early menopause, which is defined as the cessation of the monthly cycle, is caused by premature ovarian failure (POF), which is induced by inactivation ovary and results in the absence of the premature follicle. This phenomenon happens in women around the age of 40, the rate of this occurrence is 1% of females, which is a heterogeneous defect (Okeke et al., 2013). POF can be a result of variety of conditions including genetic diseases, autoimmune defects and toxic exposure (Goswami and Conway, 2007). In cancer cases, the rate of the POF is increasing, due the chemotherapy and radiotherapy that have a negative impact on the ovarian function especially in young women (Linkeviciute et al., 2014). In POF cases, IVF may be an option for reproduction (Goswami and Conway, 2007). Many of the primordial follicles are not suitable for IVF, due to abnormal functioning of FSH receptor (McGee and Hsueh, 2000, Buratini et al., 2010).

1.9 Objectives of the study:

The main goal of this study was to investigate allelic frequencies of *FSHR* gene p.Ala307Thr (c.919G>A; rs6165) and p.Ser680Asn (c.2039C>T; rs6166) which are responsible for production of hormones that are involved in the regulation of oogenesis. Since hormonal regulation is very important in the development of PCOS, we hypothesized that specific alleles within *FSHR* gene that are involved in the hormonal regulation could cause PCOS.

1.10 Significance of the study:

Allelic frequencies of two *FSHR* genes involved in hormonal regulation, p.Ala307Thr (c.919G>A; rs6165) and p.Ser680Asn (c.2039C>T; rs6166), were investigated in this study. The results will have a great impact on both clinical and basic research. We would have a prediction strategy for PCOS patients' reproductive possibilities based on the data.

1.11 Thesis Structure:

The first chapter provides background information about the study. Basic information about allelic frequencies of the *FSHR* gene, which is involved in hormonal regulation of polycystic ovary syndrome, is available at this section. The second chapter discusses the methodology used for data collection and the statistical methods utilized to analyze data for the study. The basic information about the research findings will be provided in the third chapter. The fourth chapter delves deeper into the findings, comparing them to a literature review and making various recommendations for future research. Also, this chapter presents research's conclusions and a summary.

CHAPTER TWO

Materials and methods

2.1 The sample collection:

The samples used for this project were obtained from patients of NEU Hospital. The clinical information of each patient as well as their Body Mass Index (BMI) was provided. In this study, 65 females with polycystic ovary syndrome and 55 females without polycystic ovary syndrome were divided into two groups. The experiments were performed in DESAM laboratory in Near East University Hospital, Nicosia, North Cyprus, using the ethylenediaminetetra-acetic acid (EDTA) tube for blood sample collection

2.2 DNA extraction for Real Time Analysis:

DNA isolation and purification were conducted using the Invitrogen by thermo fisher scientific kit (pure link genomic DNA mini kit, USA). There was no need for mechanical-homogenization because the samples were lysed enzymatically. For this analysis, 200ml of peripheral blood sample was drawn from the study group and placed in EDTA tubes with no leukocytes separation. The (EDTA) tube acts as anti-coagulant in blood samples, minimizing the risk of hemolysis. ≤200µL fresh blood sample was added into a sterile microcentrifuge tube, followed by 20µL Proteinase K add 20µL RNase, By vortexing for a few seconds, the contents were thoroughly mixed, and incubated at room temperature for two minutes. Then, 200µL Purelink Genomic Lysis/Binding Buffer were added and mixed well by vortexing to obtain a homogenous solution, To promote protein digestion, samples were incubated at 55^{o}C for ten minutes. After the incubation period, 200µL of 96-100% ethanol (v/v) was added to the lysate. The solution was vortexed thoroughly to ensure that it was homogeneous. PureLink spin column was placed in a Collection tube, lysate (~ 640µL) with Lysis /Binding Buffer and ethanol was loaded and the column was centrifuged at 10,000 x g for one minute at room temperature, collection tube was discarded and spin column was placed into a new collection tube. The column was washed with 500µL Wash Buffer before being centrifuged at 10,000 x g for one minute at room temperature. The column was then placed in a new collection tube after the collection tube had been discarded. The column was rinsed with 500µL of Wash Buffer two that had been prepared with ethanol, centrifuged at maximum speed for three minutes at room temperature before being discarded. The spin column was placed in a sterile 1.5mL microcentrifuge tube. 200µL of PureLink Genomic Elution Buffer was used to elute DNA, which was then incubated the column at room temperature for one minute. Finally, the eluted DNA was obtained by centrifuging the column at high speed for one minute at room temperature.

2.3 DNA concentration:

The concentration of DNA was determined by the Nano Drop ND-200 (thermo scientific, Pittsburg, USA) for measuring the quantity of the DNA obtained. The wavelength for measuring the absorbance was between (260-280 nm) ratios.

2.4 Real- Time-Polymerase Chain Reaction (RT-PCR):

Real-time PCR was used to genotype the SNPs. A fluorescent reporter dye is used in real-time PCR that is linked to a probe or is a fluorescent molecule that is able to bind to double stranded DNA (eg. SYBR Green). The fluorescent signal corresponds to the amplified fragment concentration. When the run is completed, the results are analyzed using dedicated software associated with the kit used.

Reaction mixture was prepared by adding 5µl maser mix, 0.8 µl Forward and Reverse Primer (final concentration of 25µM), 1.4 µl water and 2µ of DNA into a microtube. The mixture was vortexed briefly and spun for 5 seconds before being placed in RT-PCR machine. The RT-PCR was started by selecting the program whose temperatures and durations were determined. Norgen's Transcript-First Strand cDNA synthesis kit (Norgen, Canada) was used for reverse transcription from RNA to synthesize cDNA following manufacturer's protocol with no modification. All the steps of PCR preparation performed in laminar flow hood to avoid contamination. The Real-time PCR was used to analyze the allelic frequency of two SNPs within the *FSHR* gene followed by high resolution melting method (HRM). The primers were designed to flank the exon-exon boundaries to avoid amplification of any DNA that may be contaminating the samples. The primer sequences are listed in table 2.1 a and the PCR steps are listed in table 2.1 b.

2.1. a Details of primer sequences:

Primer name	Sequence 5'-3'
FHSR_rs6165wt_F	CAG AGA GAA TCT CTG AAC CCT AGT
FSHR_rs6165wt_R	ATCAGTGCTGTCGCTGTCAC
FHSR_rs6165mt_F	CAG AGA GGG TCT CTG AGC CCT AGC
FHSR_rs6165mt_R	GGC AAG AAG TTG ATT ATA TGA CTC AG
FHSR_rs6166wt_F	AGG GAC AAG TAT GTA AGT AGA ACC AT
FHSR_rs6166mt_F	AGG GAC AAG TAT GTG AGT GGA ACC AC
FHSR_rs6166_R	CTC TTC AGC TCC CAG AGT CAC CA

2.1. b Table showing the PCR steps:

PCR steps	Temperature C°/ Time sec	Cycles	HRM
Denaturing	94 C° for 30sec	1	95 for 1 hour 40 for 1 hour
Annealing	52-58C° for 30sec	40	65 for 1 seconds 97 for 1 seconds
Elongation	72C° for 30sec		

2.5 Statistical analysis:

Data were analyzed by using the statistical package for Social Sciences (SPSS, version 25). Fisher's exact test was used to compare the genetic condition and allelic frequency in two groups (PCOS and control groups, respectively). A p-value of ≤ 0.05 is considered as statistically significant.

CHAPTER THREE

3.1 RESULTS:

The main aim of this study was to investigate the allelic frequencies of genes involved in the hormonal regulation of polycystic ovary syndrome. A total number of 120 blood samples, 65 from women diagnosed with PCOS and 55 from healthy women, were collected. The table 3.1 shows the details of each patient with PCOS and the table 3.2 involves the details of control group patients, respectively. The PCOS patients were diagnozed by measuring the hormonal levels and vaginal ultrasonography. In this study, the average age was 20 and the average body mass index was 17 for both groups. Table 3.1 shows the details for PCOS patients and table 3.2 for control patient, respectively.

The amplification of the *FSHR* gene for two alleles at the p.Ala307Thr (c.919G>A; rs6165) and p.Ser680Asn (c.2039C>T; rs6166) were carried out. The principle of real time PCR is the detection of fluorescent signals when SYBR Green binds to dsDNA following each amplification. Sample signals are then assessed based on the threshold. In this study, high resolution melting analysis was also performed, in such after PCR amplification the amplicon produced melted gradually. Each melting analysis was performed when the DNA was 50% double-stranded and 50% single-stranded**.**

The results of this study were shown in the table table 3.1 for PCOS patients and table 3.2 for control patients, respectively. SPSS program was used to determine the statistical significant differences of allelic frequency for FSHR gene *FSHR* gene p.Ala307Thr (c.919G>A; rs6165) and p.Ser680Asn (c.2039C>T; rs6166), respectively. A p-value of ≤ 0.05 is considered as statistically significant.

Table 3.1 Describe the details for PCOS group:

P.code	Date of birth	Oligomenore	PCOS	Hyperandrogenism	Heigh	Weight	BMI	FSH	LH	T.Testesterone	S.Testtestersone	Amp.allel FSH Rrs6165 mt	Amp.allel FSH Rre6165 wt	Genetic condition	Amp.allel FSH Rre6166 mt	Amp.allel FSH Rrs6166 wt	Genitic condition
1	27.0,11996	YES	YES	YES	158	46	18.4	5.64	7.20	1.70	1.55	19.34	21.81	Heterozygous	14.56	16.7	Heterozygous
2	20.11.1987	NO	YES	YES	169	55	19.3	8.08	21.58	1.44	1.19	18.88	21.87	Heterozygous	13.88	16.8	Heterozygous
3	05.12.1995	NO	YES	YES	168	60	21.3	4.24	6.02	39.85(Y)	2.87 (Y)	23.66	24.95	Heterozygous	14.32	22.07	Heterozygous
4	23.09.1996	YES	YES	YES	164	57.5	9.7	2.55	1.15	1.73	5.3	23.38	23.01	Heterozygous	13.73	19.32	Heterozygous
5	22.11.1992	YES	YES	YES	172	64	9.8	3.46	3.8	49.20(Y)	1.27	19.04	20.84	Heterozygous	16.59	16.85	Heterozygous
6	10.12.1997	YES	YES	YES	165	63	9.3	5.46	4.76			19.87	23.31	Heterozygous	17.38	16.11	Heterozygous
7	15.05.1992	YES	YES	NO	171	58	9.0	3.49	1.54			19.75	21.55	Heterozygous	15.91	17.45	Heterozygous
8	07.04.1994	NO	YES	YES	168	68	10.9	4.83	3.89	1.32	1.73	18.55	22.1	Heterozygous	14.68	21.19	Heterozygous
9	27.03.2000	YES	YES	YES	178	82	25.88	3.37	1.83	1.04	1.75	19.02	20.88	Heterozygous	13.56	16.1	Heterozygous
10	07.01.1988	NO	YES	YES	164	65	24.2	4.29	4.9	0.3		23.86		Homozygous	14.33	19.84	Heterozygous
11	12.09.1995	YES	YES	YES	170	65	22.5	5.22	4.77	1.55	1.75						
12	06.10.1997	NO	YES	YES	158	62	24.8										
13	05.04.2001	YES	YES	YES	162	57	25.5	5.10	2.93	0.89	0.69	19.51	22.25	Heterozygous	14.11	16.52	Heterozygous
14	10.04.1997	YES	YES	NO	171	93	31.8					17.84	22.03	Heterozygous	15.47	16.31	Heterozygous
15	17.02.1988	YES	YES	YES	176	74	23.9	4.09	5.39	1.12	1.59	18.34	22.27	Heterozygous	14.15	16.49	Heterozygous
16	29.11.1986	YES	YES	NO	160	76	29.69	4.59	4.43			19.08	23	Heterozygous	14.18	16.63	Heterozygous
17	29.02.2000	YES	YES	YES	153	42	17.9	9.48	6.39	2.26(Y)	2.74	19.65	21.79	Heterozygous	15.01	15.64	Heterozygous
18	29.03.1991	YES	YES	NO	150	73	32.4	5.41	4.11	2.10(Y)	2.32	18.87	23.68	Heterozygous	15.06	17.63	Heterozygous
19	08.11.1995	NO	YES	YES	163	83	31.2	3.91	2.18	1.39	1.49						
20	25.11.1997	YES	YES	YES	167	86	30.84	4.66	8.18	2.48	2.29	19.88	21.96	Heterozygous	14.73	16.97	Heterozygous
21	11.11.1996	NO	YES	YES	172	64	21.8	4.22	6.84	2.89(Y)	2.49	19.94	21.23	Heterozygous	13.65	16.7	Heterozygous
22	25.04.1992	YES	YES	YES	171	133.25	45.5	1.25	0.41	0.79	0.83	21.87	24.96	Heterozygous	14.2	19.19	Heterozygous
23	02.01.1989	YES	YES	YES	167	82.2	29.4	4.85	4.17	0.95	1.36	18.64	21.28	Heterozygous	14.5	16.51	Heterozygous
24	22.02.1996	YES	YES	YES	158	53	21.2	4.30	2.55	1.56	1.43	18.65	22.48	Heterozygous	12.72	16.39	Heterozygous
25	1.3.1996	NO	YES	YES	150	48	21.3	4.53	2.93	1.79	2.23	18.19	21.37	Heterozygous	14.56	16.82	Heterozygous
26	25.02.1987	YES	YES	NO	172	106	35.8	5.22	6.44			19.04	21.36	Heterozygous	14.33	16.54	Heterozygous
27	20.04.2004	YES	YES	YES	161	39.2	15	6.96	10.24	0.77	1.42	19.41	21.55	Heterozygous	15.01	16.53	Heterozygous
28	12.02.1997	YES	YES	NO	160	69.2	27.0					18.1	21.69	Heterozygous	14.07	16.96	Heterozygous
29	22.10.1994	YES	YES	YES	163	56	21.08	4.07	8.63	1.76	1.96	21.97	21.3	Heterozygous	16.91	19.43	Heterozygous
30	17.06.1995	NO	YES	NO	170	61	21.11	4.66	3.27		1.91	18.61	22.47	Heterozygous	15.43	16.96	Heterozygous
31	26.06.1995	YES	YES	YES	173	69	23.5	4.09	3.95	1.16	1.27						
32	30.06.1984	YES	YES	NO	172	92	31.1	4.7	4.02	1.14	1	18.98	21.79	Heterozygous	13.73	16.1	Heterozygous
33	28.08.1997	YES	YES	YES				3.01	9.99	3.27	2.75	18.3	21.95	Heterozygous	14.09	20.94	Heterozygous
34	18.01.1997	YES	YES	YES	159	79	31.25					23.1	25.46	Heterozygous	14.2	19.85	Heterozygous
35	28.09.1997	YES	YES	YES	162	69	28.29	5.3	8.1	2.09	2.34	24.64	24.7	Heterozygous	14.07	19.63	Heterozygous
36	24.09.1997	YES	YES	YES	172	50	18.9	6.02	5.39	1.43	1.09	24.48	25.91	Heterozygous	14.17	20.07	Heterozygous

37	22.04.1998	YES	YES	YES	164	75	27.89	3.87	2.81	2.41	2.18	19.15	21.73	Heterozygous	14.27	21.52	Heterozygous
38	04.06.1997	YES	YES	NO	159	52	20.57					22.79	24.9	Heterozygous	12.72	19.4	Heterozygous
39	18.07.1994	YES	YES	NO				3.52	0.8	1.9		23.91	24.03	Heterozygous	15.01	19.7	Heterozygous
40	02.12.1998	YES	YES	YES	176	89	28.73	5.38	2.53	1.13	1.42	25.44	22.37	Heterozygous	14.84	19.18	Heterozygous
41	09.05.1997	YES	YES	YES	170	68	23.53										
42	16.10.1998	YES	YES	YES	175	74	24.16	5.27	5.74	1.72	2.17	18.63	21.08	Heterozygous	15.22	16.31	Heterozygous
43	12.11.1998	YES	YES	YES								19.05	21.23	Heterozygous	15.27	16.55	Heterozygous
44	17.04.1992	YES	YES	YES	160	76	29.69	3.97	6.63	2.31		18.47	11.91	Heterozygous	16.59	13	Heterozygous
45	02.01.1995	YES	YES	NO	162	52	19.81	2.99	1.72	0.81							
46	08.08.1998	YES	YES	YES	170	60	20.76	0.05	2.66	0.87		18.63	20.83	Heterozygous	15.5	21.35	Heterozygous
47	03.02.1999	YES	YES		168	77	27.3	0.52	0.81	1.37		19	21.64	Heterozygous	15.27	16.62	Heterozygous
48	29.11.1995	YES	YES	YES	165	60	22	3.82	6.43	1.6		18.06	21.04	Heterozygous	13.88	15.62	Heterozygous
49	21.07.1997	YES	YES	YES	164	72	26.8	3.94	2.54	1.73		19.61	21.21	Heterozygous	15.53	21.13	Heterozygous
50	11.03.1997	YES			165	69.4	25.5					21.88	20.77	Heterozygous	16.28	20.8	Heterozygous
51	06.01.1998	YES	YES	YES	167	60	21.5	4.18	11.31	1.34		18.7	22.54	Heterozygous	15.63	21.08	Heterozygous
52	04.02.2001	YES	YES	YES	170	65	22.5					15.34	33.96	Heterozygous	14.93	25.29	Heterozygous
53	27.11.1997	YES	YES	YES	167	81	29	3.06	2.17	1.37		18.74	21.48	Heterozygous	14.42	16.11	Heterozygous
54	12.03.1997	YES	YES	YES	173	59						18.95	21.81	Heterozygous	21.64	17.77	Heterozygous
55	14.09.2000	YES	YES	YES	158	70	28	2.91				18.06	21.28	Heterozygous	14.7	15.72	Heterozygous
56	10.01.2001	YES	YES	YES	162	73	27.8	4.25	6.15	2.07		18.49	21.95	Heterozygous	14.14	15.82	Heterozygous
57	30.05.1991	NO	YES	NO	178	80	25.22					18.85	21.99	Heterozygous	20.3	16.87	Heterozygous
58	15.07.1997	YES	YES	YES	173	59							16.32	Homozygous	21.72	22.38	Heterozygous
59	03.08.1997	NO	YES	YES	163	72						18.17	22.06	Heterozygous	22	16.75	Heterozygous
60	17.01.1989	YES	YES	YES	167	52						18.07	21.84	Heterozygous	13.64	31.63	Heterozygous
61	31.07.1998	YES	YES	YES	158	53						22.44	21.85	Heterozygous	14.29	17	Heterozygous
62	11.01 1989	YES	YES	NO	161	53						22.46	22.34	Heterozygous	16.45	17.44	Heterozygous
63	7.01.1981											19.51	21.6	Heterozygous	16.59	16.77	Heterozygous
64												18.81	22.71	Heterozygous	15.22	16.46	Heterozygous
65												18.71	21.7	Heterozygous	14.39	16.67	Heterozygous

Table 3.2 Describe the details for control group:

P.code	Date of birth	Oligomenore	Control	Hyperandrogenism	Height	Weight	BMI	FSH	LH	T.Testesterone	S.Testesteron	Amp.allelFSHRre6165mt	Amp.allelFSHRrs6165wt	Genetic condition	Amp.allelFSHR6166mt	Amp.allelFSHRrs6166wt	Genetic condition
1	15.06.1995	NO	NO	NO								19.47	20.84	Heterozygous	15.41	15.96	Heterozygous
2	24.10.1996	NO	NO	NO								19.27	22.32	Heterozygous	14.68	16.11	Heterozygous
3	17.06.1996	NO	NO	NO								18.2	21.41	Heterozygous	14.1	20.98	Heterozygous
4		NO	NO	NO								18.44	21.49	Heterozygous	14.25	16.54	Heterozygous
5	09.12.1994	NO	NO	NO								18.97	21.18	Heterozygous	15.14	17.2	Heterozygous
6	03.01.1996	NO	NO	NO													
7	30.05.1996	NO	NO	NO	165	65	25.4					21.32	20.83	Heterozygous	14.85	15.95	Heterozygous
8	28.02.1995	YES	NO	YES	168	96		4.34	2.96	1.92	2.36	18.44	21.36	Heterozygous	14.5	15.9	Heterozygous
9	21.07.1995	YES	NO	YES	172	67	22.6	4.21	3.18	1.68	2.10	18.49	22.19	Heterozygous	14.2	16.55	Heterozygous
10	02.03.1989	YES	NO	YES	158	68	27.2	5.11	6.19	1.49	2.13	18.28	20.96	Heterozygous	14.17	15.95	Heterozygous
11	24.07.2006	YES	NO	YES				4.14	9.17	2.04	1.57	29.96	21.25	Heterozygous	14.32	16.97	Heterozygous
12	12.07.1993	NO	NO	NO	168	52	18.4					18.95	22.54	Heterozygous	21.37	17.46	Heterozygous
13	21.01.1995	NO	NO	NO	160	65	25.24					18.34	21.67	Heterozygous	21.86	16.85	Heterozygous
14	15.01.1997	NO	NO	NO	166	72	26.1					17.9	21.56	Heterozygous	15.07	21.28	Heterozygous
15	15.11.1997	NO	NO	NO	163	56	21.1					18.16	22.3	Heterozygous	22.54	22.34	Heterozygous
16	01.01.1997	YES	YES	NO	168	90						18.15	21.32	Heterozygous	14.29	15.97	Heterozygous
17	23.05.1998	NO	NO	NO	156	68						19.3	20.98	Heterozygous	15.38	16.78	Heterozygous
18	10.07.1998	NO	NO	NO	168	59						19.2	22.13	Heterozygous	14.77	16.55	Heterozygous
19	07.10.1995	NO	NO	NO	160	56						18.85	22.85	Heterozygous	15.98	16.44	Heterozygous
20	03.05.1995	NO	NO	NO	156	39						18.41	20.87	Heterozygous	15.25	17.17	Heterozygous
21	12.06.1996	NO	NO	NO	160	59						22.18	11.19	Heterozygous	14.69	17	Heterozygous
22	21.06.1997	NO	NO	NO	170	52.5						18.8	20.72	Heterozygous	14.62	16.58	Heterozygous
23	20.03.1995	NO	NO	NO	172	60						17.78	20.12	Heterozygous	14.76	15.63	Heterozygous
24	20.02.1995	NO	NO	NO	162	73						18.93	22.35	Heterozygous	15.96	16.63	Heterozygous
25	30.09.1999	NO	NO	NO	158	56						18.98	21.78	Heterozygous	14.78	16.34	Heterozygous
26	08.02.1996	NO	NO	NO	174	60						17.99	20.12	Heterozygous	13.94	15.29	Heterozygous
27	27.09.1991	NO	NO	NO	156	62						22.67	21.15	Heterozygous	15.16	16.81	Heterozygous
28	04.04.1996	NO	NO	NO	159	50						21.56	21.79	Heterozygous	15	16.93	Heterozygous
29	11.09.1996	NO	NO	NO	165	72						17.83	21.64	Heterozygous	14.24	16.32	Heterozygous
30	22.11.1995	NO	NO	NO	170	62						21.79	21.87	Heterozygous	14.68	33.28	Heterozygous
31	20.03.1996	NO	NO	NO	174	64						22.05	21.76	Heterozygous	15.12	17.27	Heterozygous
32	20.01.1999	NO	NO	NO	167	56						18.89	22.38	Heterozygous	15.4	18.52	Heterozygous
33	16.05.1996	NO	NO	NO	161	72						18.75	22.39	Heterozygous	15.39	17.06	Heterozygous
34	28.08.1998	NO	NO	NO	160	58						19.55	31.31	Heterozygous	21.42	21.76	Heterozygous
35	06.07.1989	NO	NO	NO	160	61						18.7	22.75	Heterozygous	21.66	17.06	Heterozygous
36	20.01.1991	NO	NO	NO	164	62						18.42	22.6	Heterozygous	21.18	16.5	Heterozygous
37	30.09.1991	NO	NO	NO	177	72						18.69	22.1	Heterozygous	21.46	16.49	Heterozygous
38	06.02.1	NO	NO	NO	164	68						18.53	22.14	Heterozyg	21.93	16.53	Heteroz

	991													ous			ygous
39	22.09.1990	NO	NO	NO	155	50						18.13	21.59	Heterozygous	21.25	15.97	Heterozygous
40	21.04.1996	NO	NO	NO	163	56						18.13	33.27	Heterozygous	21.32	16.29	Heterozygous
41	03.12.1995	NO	NO	NO	157	58						18.66	22.54	Heterozygous	20.96	16.61	Heterozygous
42	09.07.1997	NO	NO	NO	164	62						18.26	21.44	Heterozygous	20.25	16.34	Heterozygous
43	23.01.1994	NO	NO	NO	162	62.5						18.24	21.89	Heterozygous	26.23	16.13	Heterozygous
44	28.02.1999	NO	NO	NO	169	63						18.93	22.18	Heterozygous	13.94	16.48	Heterozygous
45	29.11.1993	NO	NO	NO	159	67						18.86	22.67	Heterozygous	16.05	17	Heterozygous
46	24.03.1997	NO	NO	NO	160	62						20.1	21.79	Heterozygous	14.71	17.05	Heterozygous
47	0.8.10.1998	NO	NO	NO	166	58						18.18	22.05	Heterozygous	16.17	16.8	Heterozygous
48	02.11.1998	NO	NO	NO	148	54						18.49	22.46	Heterozygous	15.4	17.35	Heterozygous
49	02.09.1995	NO	NO	NO	150	46						18.42	22.44	Heterozygous	15.63	16.26	Heterozygous
50	11.01.1991	NO	NO	NO	170	68						18.72	22.39	Heterozygous	20.58	16.28	Heterozygous
51	12.11.1998	NO	NO	NO	155	58						18.86	22.27	Heterozygous	20.71	21.87	Heterozygous
52	24.06.2000	NO	NO	NO	178	48						17.95	21.88	Heterozygous	21.32	21.79	Heterozygous
53	03.01.1999	NO	NO	NO	160	59.5							18.32		34.69	21.84	Heterozygous
54	10.11.1991	NO	NO	NO	154	54							16.85		21.6	21.85	Heterozygous
55	15.01.1997	NO	NO	NO	151	46							16.69		34.01	22.39	Heterozygous

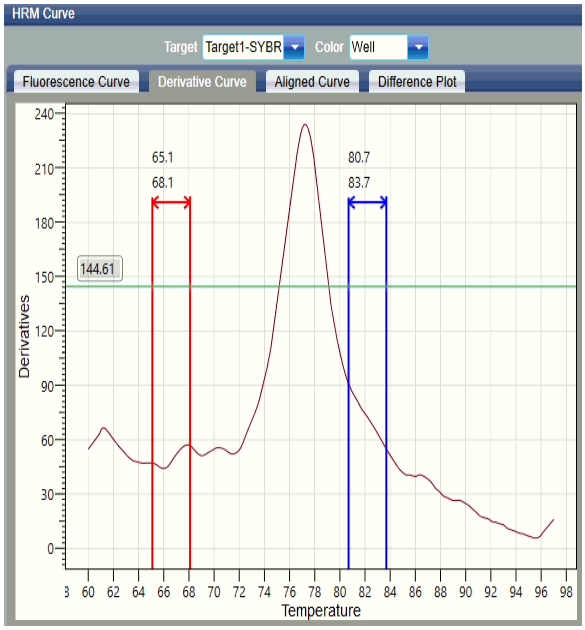


Figure 3.1a

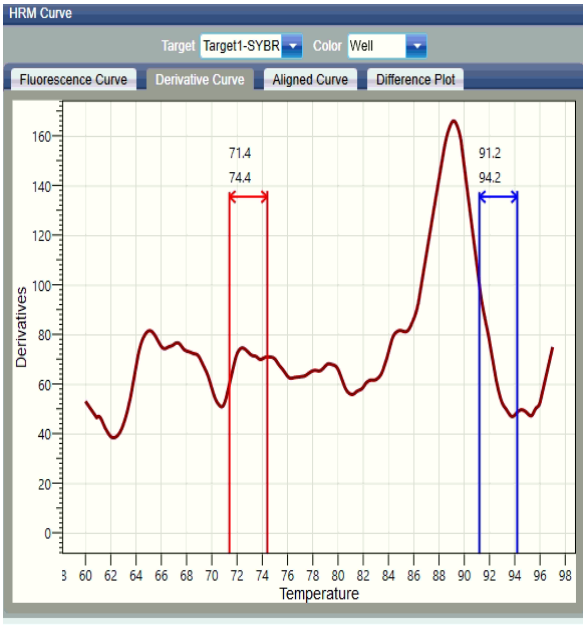


Figure 3.1b

Figure 3.1a Real-time PCR image showing the amplification of mutant type allele p.Ala307Thr (c.919G>A; rs6165).

Figure 3.1b Real-time PCR image showing the amplification of wild type allele p.Ala307Thr.

3.2 Fischer's exact test for *FSHR* p.Ala307Thr gene in two groups (PCOS & Control):

Table 3.3 summarizes the percentage of alleles detected at the *FSHR* Ala307Thr (c.919G>A; rs6165) gene in two groups (PCOS & Control) with 96.6% and 3.4% for heterozygous and homozygous in the PCOS group, respectively (Figure 3.2). The heterozygosity was 100% in the control group. The p-value of was 0.498. Six samples in PCOS group and four samples in the control group have been excluded from the study since the PCR amplification was not detected. Thus, no statistical significance was observed between both groups.

Table 3.3: Summary table for the percentage of genetic condition *FSHR* Ala307Thr (c.919G>A; rs6165) gene in two groups (PCOS & Control):

	PCOS		**Control**		**Total**		**P value**
	Number of patients	**%**	**Number of patients**	**%**	**Number of patients**	**%**	0.498
Heterozygous	**57**	**96.6**	**51**	**100.0**	**108**	**98.2**	
Homozygous	**2**	**3.4**	**0**	**0.0**	**2**	**1.8**	
Total	**59**	**100.0**	**51**	**100.0**	**110**	**100.0**	

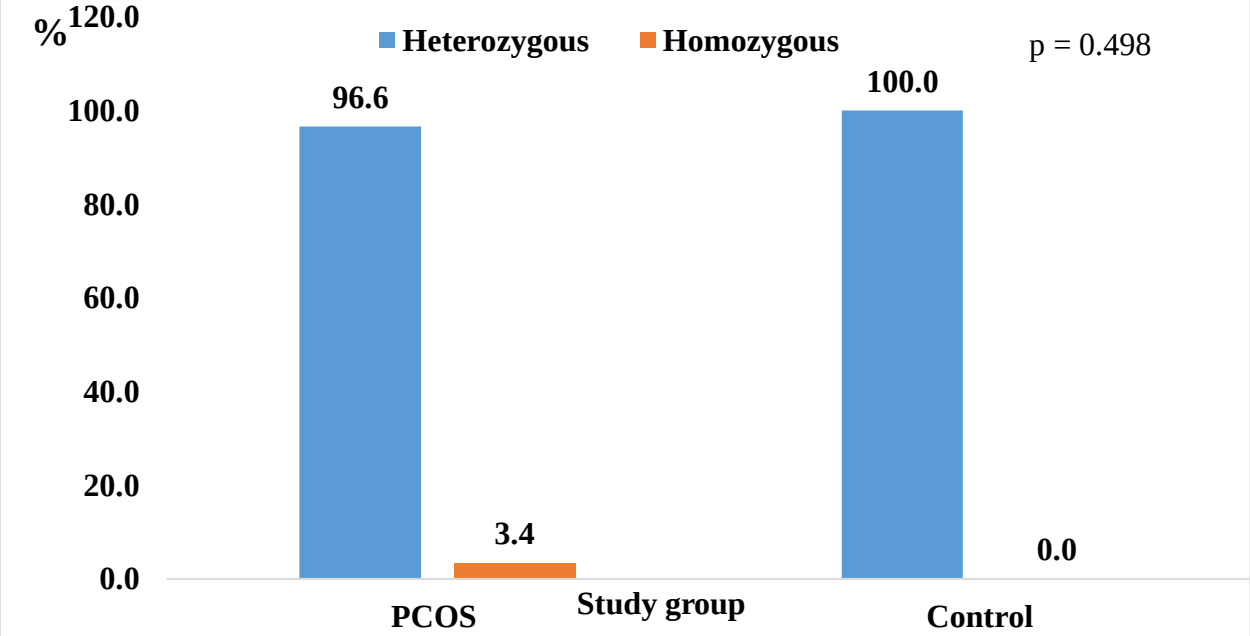


Figure 3.2 The percentage for alleles detected at the *FSHR Ala307Thr* in two groups (PCOS & Control)

Table 3.4 summarizes the percentage alleles detected at the *FSHR* Ala307Thr (c.919G>A; rs6165) gene in two groups (PCOS & Control) with 96.6% and 100% for GA in both PCOS and control groups, respectively (Figure 3.3). Furthermore, 1.7% of homozygous GG and homozygoes AA was detected in the PCOS group, respectively. The p-value was 0.999. Thus, there no statistically significant difference was observed for the alleles detected at the *FSHR* gene p.Ala307Thr (c.919G>A; rs6165).

Table 3.4: Summary table for the percentage of SNP of *FSHR* Ala307Thr (c.919G>A; rs6165) gene in two groups (PCOS & Control):

Genotypes	PCOS		Control		Total		P value
	Number of patients	%	Number of patients	%	Number of patients	%	
GA	57	96.6	51	100.0	108	98.2	>0.999
GG	1	1.7	0	0.0	1	0.9	
AA	1	1.7	0	0.0	1	0.9	
Total	59	100.0	51	100.0	110	100.0	

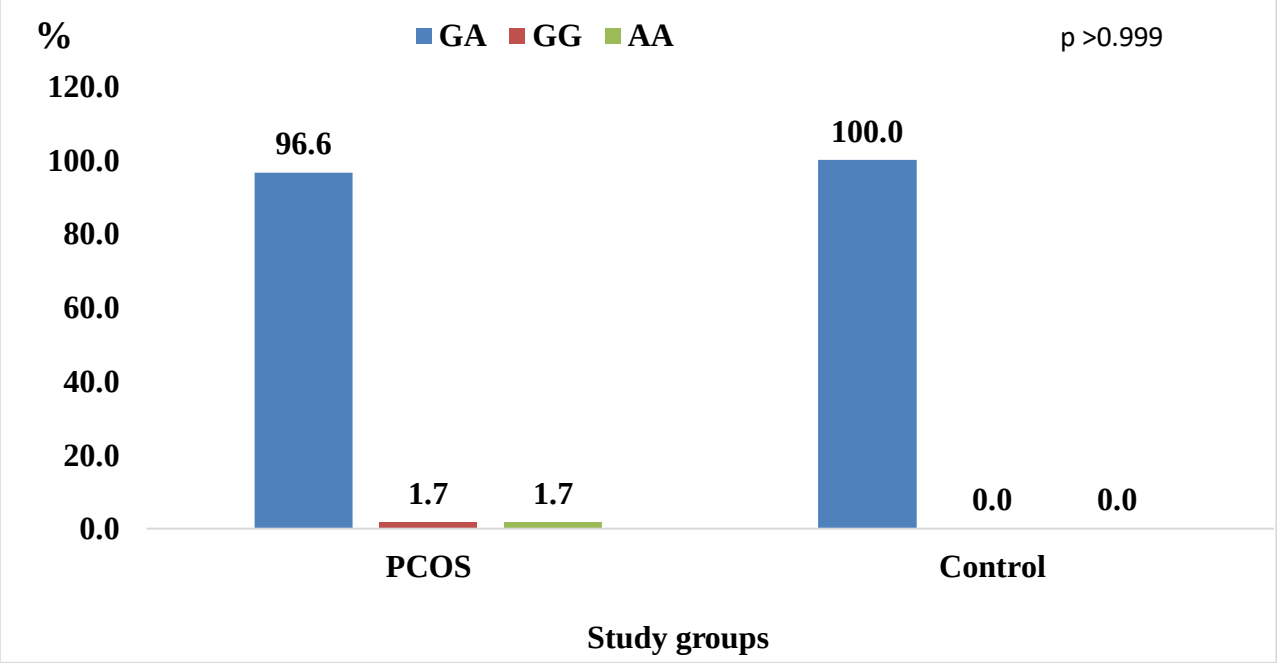


Figure 3.3 The percentage of alleles detected at the *FSHR Ala307Thr* in two groups (PCOS & Control)

3.3 Fischer's exact test for *FSHR* p.Ser680Asn in two groups (PCOS & Control):

Table 3.5 summarizes the percentage of genotypic status for *FSHR* Ser680Asn (c.2039C>T; rs6166) gene in two groups (PCOS & Control) with 100% for heterozygous status in both groups, that is why the p-value is not available. Six samples in PCOS group and one sample in control group have been excluded as amplification was obtained.

Table 3.5: Summary table for the percentage of alleles detected at the *FSHR* Ser680Asn (c.2039C>T; rs6166) in two groups (PCOS & Control):

	PCOS		Control		Total		P value
	Number of patients	%	Number of patients	%	Number of patients	%	NA
Heterozygous	**59**	**100.0**	**54**	**100.0**	**113**	**100.0**	
Homozygous	**0**	**0.0**	**0**	**0.0**	**0**	**0.0**	
Total	**59**	**100.0**	**54**	**100.0**	**113**	**100.0**	

Table 3.6 summarizes the percentage of alleles detected at the *FSHR* Ser680Asn (c.2039C>T; rs6166) gene in two groups (PCOS & Control) with 100% for heterozygous CT in both PCOS and control groups.

Table 3.6: Summary table for the percentage of SNP of *FSHR* Ser680Asn (c.2039C>T; rs6166) gene in two groups (PCOS & Control):

Genotypes	PCOS		Control		Total		P value
	Number of patients	%	Number of patients	%	Number of patients	%	NA
CT	**59**	**100.0**	**54**	**100.0**	**113**	**100.0**	

CHAPTER FOUR

4.1 DISCUSSION

Polycystic ovary syndrome (PCOS) is one of the most common disease affecting females during childbearing age that results from abnormal endocrine function. About 5-10% of women in the population complain of some features of PCOS (Franks, 1995). According to the Rotterdam criteria (Franks, 1995), a clinical diagnosis of PCOS requires that a patient present with two of the following symptoms; oligo-ovulation or anovulation, hyperandrogenism, clinical (including sign such as hirsutism) or biological (including a raised free androgen index or free testosterone). Both genetic and environmental factors are considered to play an important role in the etiology and pathogenesis of PCOS (Shen et al., 2011).

FSH mediates its function through follicle stimulation hormone receptor *FSHR* (Fauser and van Heusden, 1997) and it is responsible for development of follicles, regulation of steroid synthesis and maturation of oocytes (Gu et al., 2010). *FSHR* is a member of G-protein coupled receptor family. *FSHR* gene is located on chromosome 2p21 and composed of ten exons and nine introns (Gromoll et al., 1996). *FSHR* expression affects FSH secretion and its level in blood, and so any change in *FSHR* gene and protein may affect ovarian function (Gu et al., 2010). A number of SNPs have already been identified in the *FSHR* gene and no major mutations have been characterized as revealed by many studies (Simoni et al., 2002, Mayorga et al., 2000). Ala307Thr and Ser680Asn polymorphisms in exon ten are interesting examples of SNPs in the *FSHR* gene that result in alteration of amino acids and *FSHR* protein configuration. Many studies revealed that these *FSHR* SNPs at 307 and 680 positions could be clinically relevant because of their role in ovarian response to FSH, ovarian overstimulation, menstrual cycle alteration and pathogenesis of premature ovarian failure (POF) and PCOS (Lussiana et al., 2008).

In this study, the allelic frequency of *FSHR* Ala307Thr and *FSHR* Ser680Asn were analysed using SPSS program by Fischer's exact test. The results of this study have shown no statistically significant difference in the genotypes of *FSHR* p.Ala307Thr (c.919G>A; rs6165) in two groups (PCOS & Control). Furhtermore, the allelic frequency of *FSHR* Ala307Thr did not show any statistical significance between the PCOS and the control groups. The SNP is represented by C/T

100% in PCOS and C/T 100% in the control groups, respectively. Thus, no statistical analysis was available. This indicates that *FSHR* gene polymorphisms of p.Ala307Thr and p.Ser680Asn are not associated with pathogenicity of PCOS.

Previously published studies genotyped the *FSHR* Ala307Thr and *FSHR* Ser680Asn and analysed the association with PCOS (Kim et al., 2017). The genotype distribution of the PCOS group shown statistically significant difference from control group. The allelic frequency for genotype of *FSHR* Ala307Thr was shown to be Thr/Thr 38.5%, Thr/Ala 46.7% and Ala/Ala 14.9% in the PCOS group and Thr/Thr 46.6%, Thr/Ala 45.4%, and Ala/Ala 8.0% in the control groups with a p-value of 0.05, respectively. While the allelic frequency of *FSHR* Ser680Asn was Asn/Asn 39.5%, Asn/Ser 47.2%, and Ser/Ser 13.3% in the PCOS group and Asn/Asn 46.4%, Asn/Ser 45.4%, and 8.2 % in the control groups with a p-value of 0.035, respectively. Thus, contradictory to our results, this study showed a statistically significant correlation between *FSHR* Ala307Thr and *FSHR* Ser680Asn polymorphisms and PCOS patients (Kim et al., 2017). Additionally, results obtained from other studies supported the fact that there is an association between *FSHR* polymorphisms and PCOS. An association between Ser680Asn of FSHR and PCOS in was reported while such association was not found between Ala307Thr and PCOS in Korean females (Gu et al., 2010). Another study from Shanghai in china revealed a clear correlation between Ser680Asn and PCOS (Du et al., 2010). On other hand, another study reported that Ala307Thr is statistically correlated with PCOS in Italian females (Dolfin et al., 2011). Similarly, statistical difference in the allelic frequencies of both Ala307Thr and Ser680Asn between PCOS patients and controls were not reported in the Turkish population (Unsal et al., 2009). Studies performed on Han ethnic from Shanxi province in China (Fu et al., 2013) and Netherlands (Valkenburg et al., 2009) showed no association between Ser680Asn polymorphism and PCOS patients. This variability in results obtained from different studies may support the fact that correlation between PCOS and *FSHR* polymorphisms might be attributed to race and geography. In conclusion different results concerned with association between SNPs in the *FSHR* gene and PCOS have been obtained from different studies that performed on different ethnic and geographic groups.

4.2 Conclusion:

In summary, in this study the allelic frequency of *FSHR* gene including both p.Ala307Thr (c.919G>A; rs6165) and p.Ser680Asn (c.2039C>T; rs6166) have shown statistically no significant difference when both groups were compared (PCOS & Control). This means that allelic frequency of *FSHR* p.Ala307Thr (c.919G>A; rs6165) and p.Ser680Asn (c.2039C>T; rs6166) are not associated with the pathogenicity of PCOS and cannot be considered as a factor in oogenesis regulation. Therefore, the aim for future studies has to focus on other susceptible alleles of *FSHR* gene using higher sample size to find possible association with PCOS.

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Appendix

Table showing the genetic condition and SNP of FSHR gene p.Ala307Thr and p.Ser680Asn in PCOS group:

Patient code	CT of FSHR rs6165 mt	CT of FSHR rs6165 wt	Heterozygosity	Genotype	CT of FSHR rs6166 mt	CT of FSHRrs6166 wt	Heterozygosity	Genotype
1	19.34	21.81	Heterozygous	GA	14.56	16.7	Heterozygous	CT
2	18.88	21.87	Heterozygous	GA	13.88	16.8	Heterozygous	CT
3	23.66	24.95	Heterozygous	GA	14.32	22.07	Heterozygous	CT
4	23.38	23.01	Heterozygous	GA	13.73	19.32	Heterozygous	CT
5	19.04	20.84	Heterozygous	GA	16.59	16.85	Heterozygous	CT
6	19.87	23.31	Heterozygous	GA	17.38	16.11	Heterozygous	CT
7	19.75	21.55	Heterozygous	GA	15.91	17.45	Heterozygous	CT
8	18.55	22.1	Heterozygous	GA	14.68	21.19	Heterozygous	CT
9	19.02	20.88	Heterozygous	GA	13.56	16.1	Heterozygous	CT
10	23.86		Homozygous	AA	14.33	19.84	Heterozygous	CT
11								
12								
13	19.51	22.25	Heterozygous	GA	14.11	16.52	Heterozygous	CT
14	17.84	22.03	Heterozygous	GA	15.47	16.31	Heterozygous	CT
15	18.34	22.27	Heterozygous	GA	14.15	16.49	Heterozygous	CT
16	19.08	23	Heterozygous	GA	14.18	16.63	Heterozygous	CT
17	19.65	21.79	Heterozygous	GA	15.01	15.64	Heterozygous	CT
18	18.87	23.68	Heterozygous	GA	15.06	17.63	Heterozygous	CT
19								
20	19.88	21.96	Heterozygous	GA	14.73	16.97	Heterozygous	CT
21	19.94	21.23	Heterozygous	GA	13.65	16.7	Heterozygous	CT
22	21.87	24.96	Heterozygous	GA	14.2	19.19	Heterozygous	CT
23	18.64	21.28	Heterozygous	GA	14.5	16.51	Heterozygous	CT
24	18.65	22.48	Heterozygous	GA	12.72	16.39	Heterozygous	CT
25	18.19	21.37	Heterozygous	GA	14.56	16.82	Heterozygous	CT
26	19.04	21.36	Heterozygous	GA	14.33	16.54	Heterozygous	CT
27	19.41	21.55	Heterozygous	GA	15.01	16.53	Heterozygous	CT
28	18.1	21.69	Heterozygous	GA	14.07	16.96	Heterozygous	CT
29	21.97	21.3	Heterozygous	GA	16.91	19.43	Heterozygous	CT
30	18.61	22.47	Heterozygous	GA	15.43	16.96	Heterozygous	CT
31								
32	18.98	21.79	Heterozygous	GA	13.73	16.1	Heterozygous	CT
33	18.3	21.95	Heterozygous	GA	14.09	20.94	Heterozygous	CT
34	23.1	25.46	Heterozygous	GA	14.2	19.85	Heterozygous	CT
35	24.64	24.7	Heterozygous	GA	14.07	19.63	Heterozygous	CT
36	24.48	25.91	Heterozygous	GA	14.17	20.07	Heterozygous	CT
37	19.15	21.73	Heterozygous	GA	14.27	21.52	Heterozygous	CT
38	22.79	24.9	Heterozygous	GA	12.72	19.4	Heterozygous	CT

39	23.91	24.03	Heterozygous	GA	15.01	19.7	Heterozygous	CT
40	25.44	22.37	Heterozygous	GA	14.84	19.18	Heterozygous	CT
41								
42	18.63	21.08	Heterozygous	GA	15.22	16.31	Heterozygous	CT
43	19.05	21.23	Heterozygous	GA	15.27	16.55	Heterozygous	CT
44	18.47	11.91	Heterozygous	GA	16.59	13	Heterozygous	CT
45								
46	18.63	20.83	Heterozygous	GA	15.5	21.35	Heterozygous	CT
47	19	21.64	Heterozygous	GA	15.27	16.62	Heterozygous	CT
48	18.06	21.04	Heterozygous	GA	13.88	15.62	Heterozygous	CT
49	19.61	21.21	Heterozygous	GA	15.53	21.13	Heterozygous	CT
50	21.88	20.77	Heterozygous	GA	16.28	20.8	Heterozygous	CT
51	18.7	22.54	Heterozygous	GA	15.63	21.08	Heterozygous	CT
52	15.34	33.96	Heterozygous	GA	14.93	25.29	Heterozygous	CT
53	18.74	21.48	Heterozygous	GA	14.42	16.11	Heterozygous	CT
54	18.95	21.81	Heterozygous	GA	21.64	17.77	Heterozygous	CT
55	18.06	21.28	Heterozygous	GA	14.7	15.72	Heterozygous	CT
56	18.49	21.95	Heterozygous	GA	14.14	15.82	Heterozygous	CT
57	18.85	21.99	Heterozygous	GA	20.3	16.87	Heterozygous	CT
58		16.32	Homozygous	GG	21.72	22.38	Heterozygous	CT
59	18.17	22.06	Heterozygous	GA	22	16.75	Heterozygous	CT
60	18.07	21.84	Heterozygous	GA	13.64	31.63	Heterozygous	CT
61	22.44	21.85	Heterozygous	GA	14.29	17	Heterozygous	CT
62	22.46	22.34	Heterozygous	GA	16.45	17.44	Heterozygous	CT
63	19.51	21.6	Heterozygous	GA	16.59	16.77	Heterozygous	CT
64	18.81	22.71	Heterozygous	GA	15.22	16.46	Heterozygous	CT
65	18.71	21.7	Heterozygous	GA	14.39	16.67	Heterozygous	CT

Table showing the genetic condition and SNP of FSHR gene p.Ala307Thr and p.Ser680Asn in control group:

Patient code	CT of FSHR rs6165 mt	CT of FSHR rs6165 wt	Heterozygosity	Genotype	CT of FSHR rs6166 mt	CT of FSHR rs6166 wt	Heterozygosity	Genotype
1	19.47	20.84	Heterozygous	GA	15.41	15.96	Heterozygous	CT
2	19.27	22.32	Heterozygous	GA	14.68	16.11	Heterozygous	CT
3	18.2	21.41	Heterozygous	GA	14.1	20.98	Heterozygous	CT
4	18.44	21.49	Heterozygous	GA	14.25	16.54	Heterozygous	CT
5	18.97	21.18	Heterozygous	GA	15.14	17.2	Heterozygous	CT
6								
7	21.32	20.83	Heterozygous	GA	14.85	15.95	Heterozygous	CT
8	18.44	21.36	Heterozygous	GA	14.5	15.9	Heterozygous	CT
9	18.49	22.19	Heterozygous	GA	14.2	16.55	Heterozygous	CT
10	18.28	20.96	Heterozygous	GA	14.17	15.95	Heterozygous	CT
11	29.96	21.25	Heterozygous	GA	14.32	16.97	Heterozygous	CT
12	18.95	22.54	Heterozygous	GA	21.37	17.46	Heterozygous	CT
13	18.34	21.67	Heterozygous	GA	21.86	16.85	Heterozygous	CT
14	17.9	21.56	Heterozygous	GA	15.07	21.28	Heterozygous	CT
15	18.16	22.3	Heterozygous	GA	22.54	22.34	Heterozygous	CT
16	18.15	21.32	Heterozygous	GA	14.29	15.97	Heterozygous	CT
17	19.3	20.98	Heterozygous	GA	15.38	16.78	Heterozygous	CT
18	19.2	22.13	Heterozygous	GA	14.77	16.55	Heterozygous	CT
19	18.85	22.85	Heterozygous	GA	15.98	16.44	Heterozygous	CT
20	18.41	20.87	Heterozygous	GA	15.25	17.17	Heterozygous	CT
21	22.18	11.19	Heterozygous	GA	14.69	17	Heterozygous	CT
22	18.8	20.72	Heterozygous	GA	14.62	16.58	Heterozygous	CT
23	17.78	20.12	Heterozygous	GA	14.76	15.63	Heterozygous	CT
24	18.93	22.35	Heterozygous	GA	15.96	16.63	Heterozygous	CT
25	18.98	21.78	Heterozygous	GA	14.78	16.34	Heterozygous	CT
26	17.99	20.12	Heterozygous	GA	13.94	15.29	Heterozygous	CT
27	22.67	21.15	Heterozygous	GA	15.16	16.81	Heterozygous	CT
28	21.56	21.79	Heterozygous	GA	15	16.93	Heterozygous	CT
29	17.83	21.64	Heterozygous	GA	14.24	16.32	Heterozygous	CT
30	21.79	21.87	Heterozygous	GA	14.68	33.28	Heterozygous	CT
31	22.05	21.76	Heterozygous	GA	15.12	17.27	Heterozygous	CT
32	18.89	22.38	Heterozygous	GA	15.4	18.52	Heterozygous	CT
33	18.75	22.39	Heterozygous	GA	15.39	17.06	Heterozygous	CT
34	19.55	31.31	Heterozygous	GA	21.42	21.76	Heterozygous	CT
35	18.7	22.75	Heterozygous	GA	21.66	17.06	Heterozygous	CT
36	18.42	22.6	Heterozygous	GA	21.18	16.5	Heterozygous	CT
37	18.69	22.1	Heterozygous	GA	21.46	16.49	Heterozygous	CT
38	18.53	22.14	Heterozygous	GA	21.93	16.53	Heterozygous	CT
39	18.13	21.59	Heterozygous	GA	21.25	15.97	Heterozygous	CT

40	18.13	33.27	Heterozygous	GA	21.32	16.29	Heterozygous	CT
41	18.66	22.54	Heterozygous	GA	20.96	16.61	Heterozygous	CT
42	18.26	21.44	Heterozygous	GA	20.25	16.34	Heterozygous	CT
43	18.24	21.89	Heterozygous	GA	26.23	16.13	Heterozygous	CT
44	18.93	22.18	Heterozygous	GA	13.94	16.48	Heterozygous	CT
45	18.86	22.67	Heterozygous	GA	16.05	17	Heterozygous	CT
46	20.1	21.79	Heterozygous	GA	14.71	17.05	Heterozygous	CT
47	18.18	22.05	Heterozygous	GA	16.17	16.8	Heterozygous	CT
48	18.49	22.46	Heterozygous	GA	15.4	17.35	Heterozygous	CT
49	18.42	22.44	Heterozygous	GA	15.63	16.26	Heterozygous	CT
50	18.72	22.39	Heterozygous	GA	20.58	16.28	Heterozygous	CT
51	18.86	22.27	Heterozygous	GA	20.71	21.87	Heterozygous	CT
52	17.95	21.88	Heterozygous	GA	21.32	21.79	Heterozygous	CT
53		18.32			34.69	21.84	Heterozygous	CT
54		16.85			21.6	21.85	Heterozygous	CT
55		16.69			34.01	22.39	Heterozygous	CT