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 (PCOS)

by

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Master Thesis

Medical Biology and Genetics

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Nicosia, North Cyprus 2021 January

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APPROVAL

Thesis submitted to the Institute of Graduate Studiesof Near East University in partial fulfillment of the requirement for the degree of Master of Science in Medical Biology and Genetics. This thesis is approved by the jury members in accordance with the NEU postgraduate education, training and examination regulations and has been accepted by the decision of the board of the Institute.

Thesis Committee;

Chair of the committee/Supervisor: Assoc. Prof. Pinar Tulay

                                            Near East University

                                            ……………….

Jury Members: Assoc. Prof. Mahmut Cerkez Ergoren

                                            Near East University

                                            ……….............

                                            Assist. Prof. Ozel Yuruker

                                            Girne University

                                            ……….........

Approved by: Prof. Kemal Hüsnü Can Ba er

                                            Director of Institute of Graduate Studies

                                            Near East University

                                            ....

DECLARATION

I declare that this thesis was conducted by me under supervision of Assoc. Prof. Pinar Tulay with respect to ethical guidelines. I also declare that information obtained from previously published studies have been cited in the text and listed in the reference list.

Abdulkadir Rabiu Adam

DEDICATION

I give thanks to Allah (S A W) for making it possible to complete my studies successful, i am dedicating this research work to my family the Arak family and my friends Sana, Mallam kabiru bala ,Nafiu for their love, support and encouragement throughout my education.

ACKNOWLEDGMENT

ABSTRACT

Background: Polycystic ovary syndrome (PCOS) is a hormonal disorder that normally affects woman of reproductive age. The prevalence of PCOS is estimated to be around 8 to 10% worldwide. Those woman associated with PCOS may observe irregular menstrual periods, excessive male hormone levels (androgens), short number of follicles. PCOS leads to formation excessive fat in the body, resistance to insulin and lastly it's associated with severe risk factors such as diabetes and cardiovascular disease. This study aimed to investigate the allelic frequencies of genes, *IRSrs18012781 and INSRrs1799817*,involved in PCOS. The allelic frequencies in the PCOS and control group was examined to determine if the frequency of a particular allele is evident in the PCOS patients.

Methods: The samples required for this study were obtained from patients of Near East University Hospital, Department of Gynecology and Obstetrics. Informed consent was taken from each patient. Clinical information of the patient was collected and body mass indexes were reported. The samples to be studied were divided into two groups. The control group was consisting of normal ovulation and non-obese women and the patient group includedthe PCOS patients. Blood samples were collected from 55 women in the control and 65 samples from the patient group, respectively. DNA from the whole blood was obtained. The genotype of SNPs in different genes associated with PCOS was determined using real time PCRin a total of 120 women participated. Results were presented as the heterozygous and homozygous state of the SNPs. Mann Whitney statistical analysis was performed using SPSS and the P-value of less than 0.5 was considered to be statistically significant.

Results: There were no significant differences recorded between patients and the control groups in neither of the SNPs investigated.The *INSR* Tm was also analyzed using the Mann Whitney test value and P-value (0.059) higher than alpha value (0.05). The result revealed that *INSRrs1799817* Tm values for homozygous is not equal to *INSRrs1799817* Tm values for heterozygous, thus, there was significantly no difference between *INSR* Tm values among the patients and control group.

Conclusions: The result shows that there was no statistically significant difference between *IRS1rs1801278*, *INSRrs1799817*in the patient group and control group. This indicates that there

was nochange recorded among the two groups, so the polymorphism should not be considered as a major factor for pathogenesis of this disorder.

Keywords: *IRS1*, *INSR*, PCOS, Polymorphism, allele frequency

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LIST OF ABBREVIATIONS

AR: Androgen Receptor Gene

DNA: Deoxyribonucleic Acid

HCG: Human Chorionic Gonadotropin

INR: insulin receptor

IRS: insulin resistant substrate

LHCGR: Luteinizing Hormone Chloriogonadotropin Receptor

PCOS: Polycystic Ovary Syndrome

PCR: Polymerase Chain Reaction

SNP: Single nucleotide polymorphism

VEGF: Vascular endothelial Growth Factors

CHAPTER ONE

1.1Introduction

Polycystic ovary syndrome (PCOS) is one among many under diagnosed and underrated medical condition in females around the globe. PCOS, is a heterogeneous condition that causes intense endocrine reproductive malfunction. It commonly affects females of age 18-44 years, the reproductive age. The gross effects of this condition can lead to hormonal complexities such as a dysfunctional menstrual cycle that may lead to infertility, obesity, excessive hair around the body parts (hirsutism) and acne. Studies have shown that numerous candidate genes are recognizable factors that are of etiological effect, affiliated to this medical condition. Similarly, studies are carried out to identify the ultimate etiology and the genetic correlation (Rao, Manisha et al, 2020). Studies related to the prevalence of PCOS, shows the application of three basic criteria created for clear identification of polycystic ovary syndrome in the United States of America. The first criterion was established in 1992, known as the National Institutes of Health Criteria; second was created in 2003 named as the Rotterdam Criteria and the Androgen Excess Society Criteria in 2006 (Zhou et al, 2018). These criteria include analysis on the morphology of the polycystic ovary using transvaginal ultrasound, clinical and anovulation analysis. Additionally, PCOS is regarded as the most recognized factor that increases high risk of contracting other medical conditions that are of ontological, reproductive, metabolically and perhaps psychological effects on the patient. However, management of this syndrome varies based on observable symptoms. PCOS is usually not diagnosed unless the patient encounters other related medical challenges such as alopecia, hirsutism, androgenic acne or infertility. The first step before diagnosis of PCOS is the presence of observable factors that manifest physically (Speca, S., et al, 2007).

Bozdag et al (Bozdag G, et al, 2016)records that the world prevalence of PCOS is recorded to be between 6% and 26%, respectively (Bozdag G, et al, 2016). A systematic review and meta-analysis data were captured, using a diagnostic criterion. Conclusively, their results show that global prevalence of PCOS was 6%-10%(Liu Z ,2018).

Furthermore, PCOS is diagnosed to cause serious complex conditions among females. It is also recorded that, one in every five females is challenged with infertility and truncated menstrual cycles resulting in PCOS(Bozdag G, et al, 2016).

1.2 Etiology of PCOS:

Environment and lifestyle are factors that affect the developmentPCOS. Furthermore, chemicals may accumulate greatly in individuals with PCOS which may also lead to low hepatic clearance, induce production of androgen and high rejection of insulin. Another factor is intrauterine exposure; which results in development of PCOS-like diseases such as hyperandrogenism, hyperinsulinemia and oligo-ovulation (Soni, N, 2017). Subsequently, androgen exposure may likely impair progesterone and estrogen production which may increase pulse frequency. Progesterone can be classified as the major sex hormone found in females. It is also responsible for regulating female menstrual cycle and pregnancy maintenance. In a situation where the progesterone level in PCOS patients is lower than normal rate, those patients is associated with overstimulation of the immune system (I'd. U.B et al, 2018).

Researchers are still investigating the etiology of autoimmune disease that is related with PCOS.Some causes have been investigated for diseases such as sequestered antigens, molecular mimicry and decrease of T regulatory cells (Tregs) and different autoimmune anomalies are documented in PCOS. Autoimmunity are divided in two such as organic, that leads to formation ofe Grave's disease, Hashimoto's thyroiditis, and IDDM, while nonorganic also leads to formation of rheumatoid arthritis, rheumatic fever(Mobeen et al., 2016).Another research states that estrogen is possibly causing the development of autoimmune diseases since high levels of estrogen normally lead to blood clots, and stroke. Moreover, in females, estrogen is responsible for controlling the reproductive system and it works along with progesterone. Insulin resistance, obesity and lastly androgens are the main causes of autoimmunity in PCOS (Nezos & Mavragani, 2015). The scientists identified that in some cases patients with type 1 diabetes mellitus are giving exogenous insulin for treatment. Interestingly, insulin in this situation plays a role in the development of PCOS. Additionally, low levels of insulin affect sexual function and again exogenous insulin encourages the production of androgens through ovaries. However, the correlation between hyperandrogenism and metabolic hallmark of PCOS is controversial(November, C, 2018;Rosenfield RL, Ehrmann DA, 2016). Furthermore, genetic

background is another factor that increases high chances of PCOS. PCOS is suggested to be heritable and it is proven to cluster in families.

1.3Clinical features of PCOS:

PCOS patients tend to exhibit many cysts of about 8mm and 12 mm in size within the ovaries. This is up to 70% of the causes of infertility(Marilynn Larkin,2018,Sirmans & Pate, 2013). Excessive flow of androgen results in the high level of male hormonal characters such as hirsutism and acne. Furthermore, this condition leads to insulin resistance, obesity and type 2 diabetes mellitus. This also leads to irregularity in the menstrual cycle with consequences of infertility. Other common symptoms experienced by about 20% of patients include sleep apnea, anxiety and depression(Nida Ajmal et al, 2019).The main long-term effects of PCOS are shown in figure 1 (Nida Ajmal et al, 2019). Approximately, 30-75% of PCOS patients are also said to be obese, which exacerbates the clinical features of hyperinsulinemia and hyperandrogenenism Sari (MI, 2019).
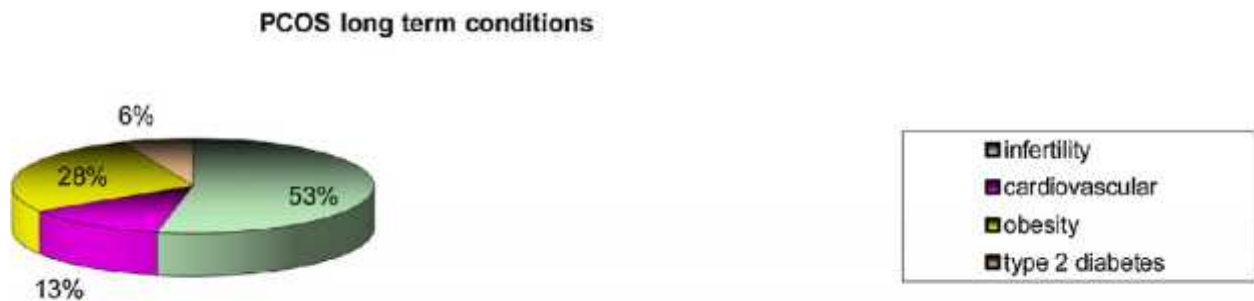
Figure 1. 1: Long-term condition of PCOS (Nida Ajmal et al, 2019)

Treatment of PCOS is focused on the management of the patients who are diagnosed with infertility, hirsutism, obesity and acne. Most importantly, lifestyle and medications are administered for proper management of the condition (Table1.1) (Baba, T et al, 2007).

Table 1. 1: Treatment of PCOS (Markler et al, 2020)

Lifestyle: The individual's lifestyle modification helps to reduce the risk of infertility.	Exercise, weight loss and improved diet help manage PCOS and helps in reducing metabolic abnormalities associated with PCOS
Clomiphene citrate: A medication that	Clomiphene is an estrogen receptor

induces ovulation.	modulation, used in inducing ovulation through interfering through increased FSH release.
Estrogen and progestin: Contraceptive therapy is applicable in treatment of acne, irregular menstrual cycles and hirsutism	Applied to normalize androgen levels as well as to regulate menstrual cycle. Helps in reducing risk of hemorrhage.
Metformin: Applicable in treatment of glucose intolerance, anovulation and hyperinsulinemia	Glucose intolerance is reduced by metformin and decreasing hepatic gluconeogenic
IVF: Infertility in women can be treated using IVF	The retrieval of oocytes from the ovaries is done using IVF. It is also done in vivo by combination with sperm for embryos
Ovarian drilling: Surgery process of treating clomiphene citrate-resistant anovulation	Drilling in the using laparoscopic procedure is done. however, surgical complications may arise such as adhesion

1.4 Diagnosis of PCOS

PCOS diagnosis is performed in different ways systematically by physician. The first process involves the pelvic examination visually and manually, inspecting of reproductive organs to examine growth or other abnormalities. Secondly, the blood tests are analyzed to measure the level of hormones (Shi X, 2016). Glucose and cholesterol levels are also measured. The ultrasound is performed by checking the appearance of ovaries to examine the thickness of the uterus.(Bani Mohammad, M., & Majdi Seghinsara, A.,2017).

1.5 PCOS and Pregnancy:

The reproductive system is one of the vital systems in the body. The ovaries produce the female egg cells, while the uterus serves as the host by which the fetus develops and aids in the passage of male sperm cells *via* the fallopian tubes. The reproductive process is aided by dispatch of the

egg molecules to sperm cells, which subsequently allow the surface of the egg to attach to the sperm surface, ready for fertilization.The fertilization takes place in the oviduct. The moving zygote gets implanted to the surface of the uterus; where the embryogenesis and morphogenesis start. Furthermore, when the zygote develops adaptation to the outside of the vagina, the cervix opens and contracts to allow the uterus to propel through the delivery canal (vagina). The vagina subsequently meets the outside organs, such as the vulva, urethra, labia, and clitoris, respectively. The ovum is dispatched by the ovaries at some point, which allows the passage via the fallopian tube to the uterus. However, during the fertilization processes, body abnormalities are observed in the system which leads to ovarian cancer and polycystic ovarian syndrome among others (Chen and Fang, 2018).

In PCOS patients, although there are large numbers of primordial germ cells and early follicules, these get arrested at the pre-antral stage. Thus, majority of the patients experience anovulation(Sirmans & Pate, 2013).

1.6 PCOS in Relation to Diseases

PCOS is recorded as one of the major common cause of infertility in women of reproductive age, which are recorded in U.S 6-10 women. Managing this condition is difficult, due to its change to other complicated conditions that are detrimental to the body. Heart diseases and high risk of diabetes type 2 is also experienced. Various possible treatments are administered to female with this condition. Weight loss is one difficult task for the obese females. For this reason, diabetes medications prove to be promising as the therapy for PCOS. Various research studies show that insulin resistance is a complex part of polycystic ovarian syndrome, which is associated with diabetes and heart failures from metabolism (Escobar Morreale, 2018).

Comparative studies, relating to females having normal menstrual cycle and women with PCOS of same age bracket have shown an increased prevalence of cardiovascular risk factors among the women, which may also lead to increased prevalence of dyslipidemia and hypertension. This is also found to be associated with a subclinical atherosclerosis and the endothelial dysfunction. Furthermore, more findings suggest that females with PCOS are at high risk to experience cardiovascular disease. The high prevalence of PCOS in female population, also account for a high proportion of atherosclerotic heart disease recorded among young women (Franks,S, 2002).

Another most common problem in the PCOS is the dyslipidemia and the prevalence of a low-density lipoprotein (LDL). Many studies also show increasing number of women with PCOS, affected with high incidences of hypertension with increased systolic blood pressure. The two most important anatomic markers related to subclinical cardiovascular disease in PCOS, are the coronary calcifications and carotid intima-media thickness, using both an electron beam tomography and an ultrasonography. Hence, having a high prevalence of cardiovascular risk among PCOS patients is not surprising, because of the prevalence of metabolic syndrome (MBS) among women with PCOS (Shen, L.X,1999;Sirmans & Pate, 2013).

1.7 Genetics of PCOS

PCOS is shown to be clustered in families as discussed previously. Thus, this appears to exhibit complex multifactorial etiology. A number of candidate genes with specific alleles at single nucleotide polymorphisms (SNPs) are shown to be associated with PCOS. Similarly, twin studies show PCOS to be 70% hereditary and 30% environmental(Unluturk et al., 2007).

All the genes that function in oogenesis and ovulation are believed to play a role in the development of PCOS. Some of these genes are*LUTEINIZING HORMONE/CHORIOGONADOTROPIN RECEPTOR*(*LHCGR*), *ESTROGEN RECEPTOR (ER)* geneand ANDROGEN *RECEPTOR* (*AR*) gene. In addition to these genes involved in gametogenesis, genes involved in insulin homeostasis, such as *INSULIN RECEPTOR SUBSTRATE 1* (*IRS1*) and *INSULIN RECEPTOR* (*INSR*) genehave also been proposed as PCOS-associated genes (Crespo et al., 2018).

1.8SNPs associated with PCOS:

IRS1 is a protein coding gene that is located on chromosome 2q36, it also be called as a signaling adapter protein. *IRS1* gene plays a role in insulin signaling pathway(Baba et al., 2007), while insulin receptor(*INSR*) rs1799817 is protein coding gene which belong to tyrosine kinase family of protein, that is located on chromosome 19q. *INSR* it is responsible during the formation of certain subunit such as alpha and beta subunit through heterotetrameric receptor(Lee et al., 2008). *IRS1* rs1801278 and *INSR* rs1799817 polymorphisms have been associated with PCOS previously(Ruan et al., 2012).

1.9SNP analysis using PCR:

Polymerase chain reaction (PCR) is one of the most important techniques used in molecular study. PCR allows amplification of nucleic acid sequences that is even present at low concentrations. PCR has become important within ten years of its existence serving in fields of biomedical sciences and molecular biology. One importance of PCR is its ultimate sensitivity using single DNA molecule detected and analyzed in sequence constant. Detection of allelic frequencies at SNP sites occurs from single base (skrgatic L; et al, 2013).

1.10OBJECTIVE OF THE RESEARCH

The main goal of this research was to investigate the allelic frequencies of polymorphic genes of *IRS1rs1801278*and *INSR*rs1799817 in PCOS patients relative to the control patients.

1.11SIGNIFICANCE OF THE STUDY

PCOS is considered as a multifactorial condition that affects female of reproductive age. Hence, the molecular regulation of this disease is not well understood. Genetic factors are thought to play important roles in the development due to hereditary observed in families around the globe. Furthermore, recent studies revealed that SNPs may be important for evaluating PCOSsusceptibility. Therefore, this study investigates the allelic frequencies of SNPs within genes associated with PCOS in PCOS patients and the control group.

CHAPTER TWO

2.0 MATERIALS AND METHOD

2.1 Sample collection:

The samples required for this study were obtained from patients of Near East University Hospital, Department of Obstetrics and Gynecology. Ethical approval was granted by the Near East University Ethical Commission (YUD/2019/67-784). Informed consent was taken from each patient. Clinical information of the patient was collected and body mass indexes were reported. The samples to be studied were divided into two groups. The control group was consisting of normal ovulation and non-obese women and the patient group included non-obese PCOS patients. Blood samples were collected from 55 women in the control and 65samples from the patient group for DNA isolation in EDTA tubes and the experiments were performed in DESAM laboratory. DNA from the whole blood was obtained. The allelic frequencies of SNPs in two genes associated with PCOS was determined using real time PCR.

2.2 Materials:

The kits and equipment used in this study is as follows;

Invitrogen by thermo fisher scientific kit(Pure LinkGenomic DNA Mini kit, USA),proteinase K(supplied with the kit),RNase A(supplied with the kit),genomic lysis/ binding buffer(supplied with the kit), incubator(DESAM laboratory), ethanol(supplied with the kit)water bath or heat block(DESAM laboratory), spin column(supplied with the kit),wash buffer 1 and wash buffer 2(supplied with the kit),pure link genomic elution buffer(supplied with the kit), Nano Drop 2000/2000c spectrophotometers(DESAM laboratory), primers both forward and reverse, light cycler SYBR Green 480 high resolution melting $MgCl_2$25 Mm(USA, light cycler SYBR Green 480 H_2O(USA 001),light cycler SYBR Green 480 high resolution melting master mix 2x concentration,laminar flow hood, and thermal cyclerPCR,statistical packages for the social sciences (SPSS).

2.3 Methods

2.3.1 DNA Extraction from Blood Samples

DNA from each sample wasextracted using Invitrogen pure link genomic DNA mini kit(USA). Frozen blood samples were put at room temperature (15-20), respectively. Water bath was set at 55 and 200µl of defrosted blood also pitted into a 1. 5ml micro centrifuge tube, then 20 µlof proteinase K was added into it. About 20 µl of RNase A was added. 200 µl of pure link genomic lysis/binding buffer was added, and then all the samples were mixed by vortexing to obtain a homogenous solution for 10seconds and the samples were incubated at 55$^{\text{o}}$C for 10 minutes to promote protein digestion. After the incubation 200 µl 96-100% of ethanol was added into each tube by vortexing for 5 seconds to yield a homogenous solution. Six thousand four hundred µl of the samples weretransferred into fresh pure link spin column and centrifuged at 10,000xg for 1 minute at room temperature; the flow-through and the filtrate/collection tube were discarded. The spin column was placed into a new collection tube and 500 µl of wash buffer 1 was added and centrifuged at 10,000xg for one minute at room temperature, then the collection tube was discarded.Five hundred µl of wash buffer 2 was added and centrifuged at high speed for three minutes at room temperature. Lastly the spin column was transferred into a new 1.5-mL micro centrifuge tube, then 200 µl of Pure Link Genomic Elution Buffer was added plus incubation at room temperature for one minute then centrifuged the column at maximum speed for one minute at room temperature to obtain the eluted DNA.

2.3.2 DNA concentration

The concentration of the DNA was measured using Nano drop (thermo scientific, pittsburg, USA) at wavelength of 260 nm (OD_{260}). The purity and quality were evaluated by the 230:260 ratio.

2.3.3 Statistical Analysis

Statistical packages for the social sciences (SPSS version 10, Chicago USA) was used in this study.Descriptive statistics and independent sample test of Mann Whitney Test was performed. The results were considered statistically significant if p 0.05.

2.3.4 PCR amplification

Real time PCR was conducted in order to identify the allelic frequencies at the particular SNP sites of polymorphic genes, *IRS1* and *INSR*,which are associated with PCOS. These primers were designed by Assoc.Prof. Pinar Tulay, Near East University (Intron, Turkey). Five µl of master mix,0.8 µl of both forward and reverse primer, 0.6 µl of $MgCl_2$sand 0.8 µl of H_2Owere included in the reaction mixture, respectively. Two µl of the extracted DNA were added to each reaction. All the PCR's were set up were in laminar flow hood in order to avoid contamination. The allelic frequencies of the two SNPs within two genes were analyzed by high resolution melting method(HRM) and thermal cycler software were used to obtain the cycle of threshold (Ct)and melting temperature (Tm)values. The conditions of the PCR, for the amplification is shown in table 2.1.

Table 2. 1: The PCR cycling conditionsused in the amplification of IRS and INSR sites

PCR Conditions	Denaturation	Annealing	Extension	HRM
Temperature/ time (second)	95 for 10minutes	95 for 10seconds	72 for 25secons	95 for 1hour 40 for 1hour 65 for 1seconds 97 for 1seconds

Cycle	1	40		

3.0RESULTS

This study was designed to investigate the allelic frequencies for the polymorphic variant genes that are associated with PCOS. A total number of 120 blood samples were collected. Of these, 65 were diagnosed with PCOS and 55 were included inthe control group, whom did notpresentany signs of PCOS. The demographic details of each patient are represented in table 3.1 and 3.2. The average age was 20 and the average body mass index for all the patients and the control group was 17.The PCOS patients were evaluated by the hormonal levels as well as the vaginal ultrasonography to determine the groups, respectively.

For each amplification, the cycle of threshold (Ct) was recorded. Ct indicates the total amount of cycle required for the fluorescent signal to cross the threshold Likewise, for each amplification, melting temperatures (Tm) values were recorded. Tm represents the melt curve when the DNA is 50% double-stranded and 50% single-stranded. In HRM analysis, following PCR amplification, the amplicons produced is melted gradually. This enables emission of fluorescence that is detected by the real time PCR equipment. These melt curves have different shape due to the differences in the Tm values.

The results of the real-time PCR-HRM analysis were presented graphically and in tabular formats. The statistical significance was determined using SPSS.Descriptive statistics and independent sample test of Mann Whitney Test were used to identified the significant difference between each polymorphic gene such as for *IRS1* and *INSR*.Descriptive statistics is a type of analysis in research that is used in order to identified the significant interval between two or more variables, and also it gives a summary of the result data during statistical analysis so that to be mean full during interpretation.

Table 3.1: Details of each patient information

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 3. 2: Describe the details for control group

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

A total of 79.3 %, of the patients were shown to be homozygous and10.3% was heterozygous for *IRS1 rs1801278*, respectively (Figure 3.1, table 3.3). The primer-dimer was present in a number of the patients with distinctive melting temperature relative to the products melting temperature (Figure 3.1, b).
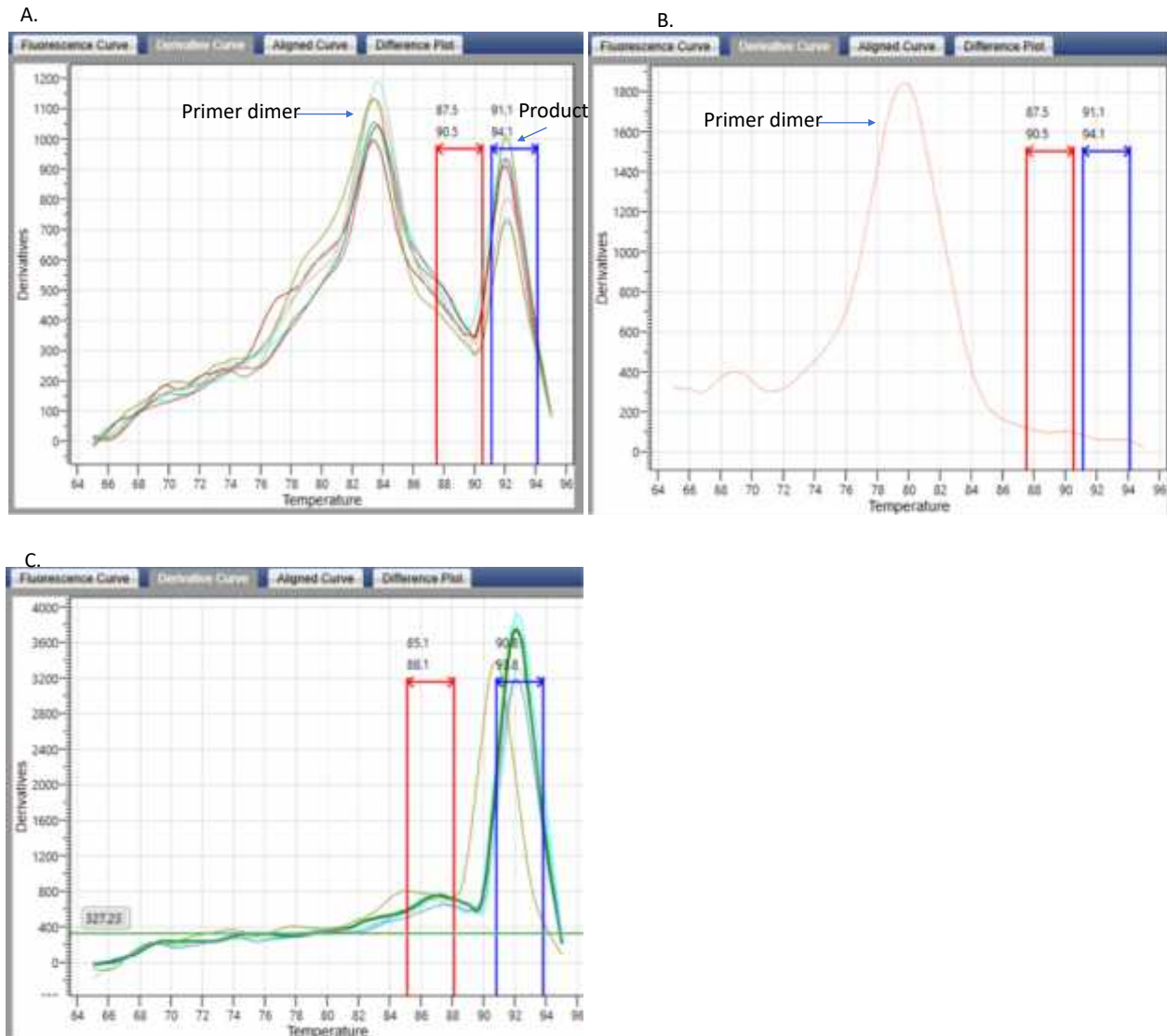
Figure 3. 1: a) PCR-HRM image showing melting curve analysis of the PCR products of the homozygote samples for *IRS1 rs1801278*b) PCR-HRM image showing the negative control with thepresence of primer dimer and no product. c) PCR-HRM image showing melting curve analysis of the PCR products of the homozygote samples for different alleles of*IRS1* rs1801278

Table 3.3 summarizes the *IRS1rs1801278* heterozygosity status in the PCOS patients. The homozygous recorded high percentage of about 79.3 %, while the heterozygous recorded low percentage with about 10.3%.

Table 3. 3 Summary table showing the percentages of heterozygosity of*IRS1* rs1801278in PCOSpatients

	Number of patients	Percentage
Homozygous	46	79.3
Heterozygous	6	10.3
Total	52	89.7
No result	6	10.3
Total	58	100.0

Table 3.4 summarizes the *INSR* heterozygosity status in the PCOS patients. The homozygous recorded high percentage of about 94.8 %, while the heterozygous recorded low percentage with about 3.4%.

Table 3.4Summary table showing the percentages of heterozygosity of*INSRrs1799817*

Genetic conditions	Number of patients	Percentage
Homozygous	55	94.8
Heterozygous	2	3.4
Total	57	98.3
No amplification	1	1.7
Total	58	100.0

Table 3.5 summarizes the *IRS1 rs1801278*heterozygosity status in the control group. The homozygous recorded high percentage of about 85.1%, while the heterozygous recorded low percentage with about 6.4%.

Table 3. 5*IRS1* rs1801278 status for PCOS control group

		percentage
Homozygous	40	85.1
Heterozygous	3	6.4

Total	43	91.5
System	4	8.5
TOTAL	47	100.0

Table, 3.6 summarizes the *INSR rs1799817* heterozygosity status in the control group. The homozygous recorded high percentage of about 83.0%, while the heterozygous recorded low percentage with about 10.6%.

Table 3. 6*INSR rs1799817*homozygosity status for control group

	Number of patients	Percentage
Homozygous	39	83.0
Heterozygous	5	10.6
Total	44	93.6
System	3	6.4
Total	47	100.0

The figure 3.2 represents *IRS1* rs1801278 heterozygosity status for PCOS patients with homozygous displayed higher allelic frequency than the heterozygous bar chart (table 3.3).
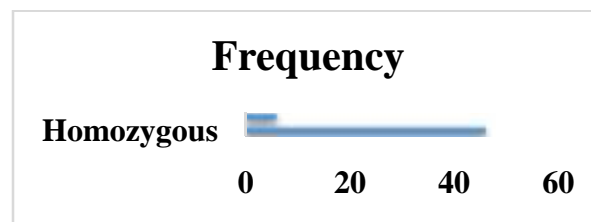
Figure 3. 2**:***IRS1* rs1801278heterozygosity status for PCOS patients

The figure 3.3 for *INSR* rs1799817 heterozygosity status for PCOS patients, in which homozygosity state was presented with higher frequency, than heterozygous state at this SNP site (table 3.4).

Frequency

Heterozygous ▪
Homozygous ▬▬▬▬▬▬▬

0   20   40   60

Figure 3. 3:*INSR* rs1799817 heterozygosity status for PCOS patients

Figure 3.4 represents the bar chart for the *IRS1rs1801278* heterozygosity status for control group, in which homozygosity state was presented with higher frequency than heterozygous state at this SNP site (Table 3.5).

Frequency

Heterozygous ▪
Homozygous ▬▬▬▬▬▬▬

0   10   20   30   40   50

Figure 3. 4*: IRS1 rs1801278* heterozygosity status for Controlgroup

Figure3.5 The bar charts *INSR rs1799817* heterozygosity status for control group, in which homozygosity state was present with higher frequency than heterozygous at this SNP site. (see table 3.6).
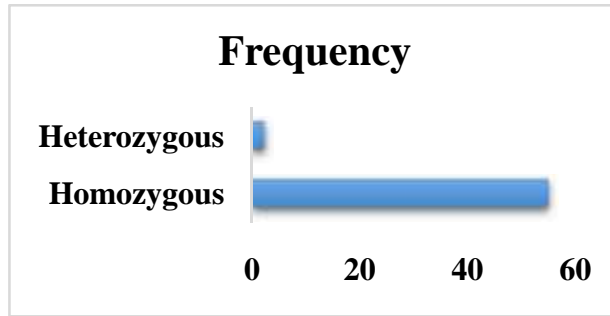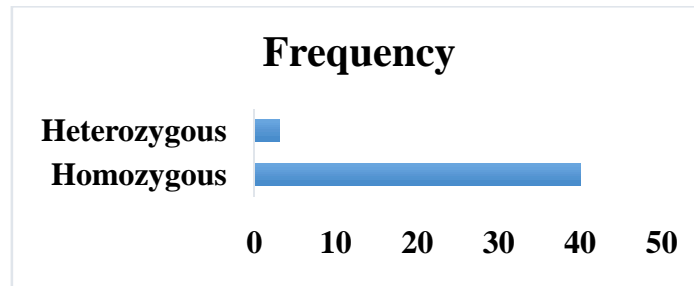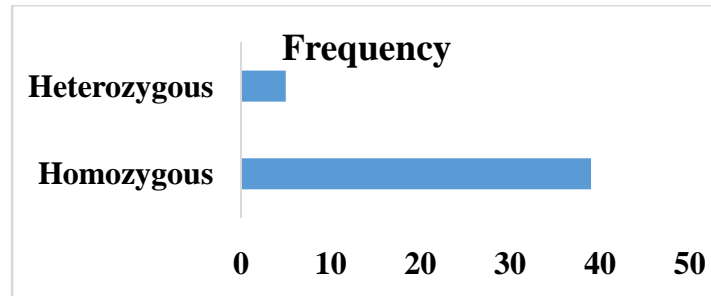
Figure 3. 5:*INSR rs1799817* heterozygosity status for control group

3.1 Independent sample t-test for patients' data *IRS1* rs1801278 gene

The statistic result for *IRS1 rs1801278* Tm using normality test for parametric and non-parametric statistics. Based on the result data in table 3.7 and 3.8, the p-value (0.073) which is less than alpha values (0.05) means that there was no significant between *IRS1* rs1801278 Tm and its heterozygosity when compared with the control group, so this variant does not have any contribution in the pathogenesis of PCOS (Table 3.7).

Table 3. 7Descriptive statistics for *IRS1 rs1801278* gene Tm for Patients conditions

Genetics condition	N	Mean	S.D	Median	Min	Max
Homozygous	46	86.1613	6.07256	91.0500	76.50	92.20
Heterozygous	6	91.5483	.66964	91.5500	90.70	92.30

Table 3. 8Mann Whitney Test value and P-value

Mann Whitney test	P-value	Alpha value
75.000	0.073	0.05

The assumptions for both parametric and non-parametric statistics normality assumptions, centering on the outcome of normality, the data were not normally distributed, therefore alternative using Mann Whitney test was conducted, these tools where used in order to find the significant interval between different variables. The *INSR* rs1799817 Tm patients' showed a p-value (0.059) greater than alpha value (0.05) compared with its heterozygosity in both patients

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and control group. means there was no significant difference between *INSR rs1799817* Tm value among patients' heterozygosity and control group so this variant does not have any affect in the pathogenesis of the syndrome (Table 3.9, 3.10).

Table 3. 9Descriptive statistics for *INSR*rs1799817 Tm for Patients Heterozygosity

Genetics condition	N	Mean	S.D	Median	Min	Max
Homozygous	55	84.6829	5.65289	86.000	61.30	87.40
Heterozygous	2	88.2000	2.68701	88.2000	86.30	90.10

Table 3. 10Mann Whitney Test value and P-value

Mann Whitney test	P-value	Alpha value
72.000	0.059	0.05

3.2 Independent sample t-test for control data

The statistical analysis provides the record for normality statistics both parametric and non-parametric statistics, which the result for the normality showed the data, were not normally distributed. The next alternative analysis conducted was Mann Whitney test. Based on p-value (0.736) there was statistically significant difference between *IRS1 rs1801278* Tm value among the control and the PCOS patient groups, respectively (Table 3.11, 3.12).

Table 3. 11Descriptive statistics for *IRS1 rs1801278* Tm for Control Heterozygosity

Genetics condition	N	Mean	S.D	Median	Min	Max
Homozygous	40	88.6702	5.23150	91.4850	77.90	99.90
Heterozygous	3	87.7333	3.90171	87.6000	83.90	91.70

Table 3. 12Mann Whitney Test value for control *IRS1 rs1801278*Tm

Mann Whitney test	P-value	Alpha value
52.000	0.736	0.05

The statistical provides the record values for the executed parametric and non-parametric assumptions, which result in dropping the earlier analysis of independent sample test and performing the present analysis of Mann Whitney test. Based on the *INSR* rs1799817 Tm in control and the PCOS patients, there was no statistical significant difference(p-value=0.472)(Table 3.13, 3.14).

Table 3. 13Descriptive statistics for INSR Tm ControlHomozygosity

Genetics condition	N	Mean	S.D	Median	Min	Max
Homozygous	38	84.2750	6.47176	85.9000	62.54	87.62
Heterozygous	5	73.5480	0.14237	63.9000	62.80	90.94

Table 3. 14Mann Whitney Test value for control *INSR rs1799817* Tm

Mann Whitney test	P-value	Alpha value
75.500	0.472	0.05

The table 3.15 is a summary for the cumulative results used in statistical analysis in this study. Tentatively, the majority of the results revealed that the p-value is significantly higher than the alpha value. However, the results show different data value in *IRS1* rs1799817 Tm, *INSRrs1799817*Tm, with the p-value (0.073), (0.059) and the alpha value (0.05), respectively. Also, there was no statistical difference between the *IRS1* Tm gene values among the patient's and control group. P-value is greater than the alpha value, this means there was no significant change occurred which may contribute to pathogenesis of PCOS among the polymorphic population.

Table 3. 15**:** Summary of the descriptive statistics for polymorphic genes

Gene	*IRS1* Tm gene	*INSR* Tm	*IRS1* Tm control	*INSR* Tm control
Mann Whitney test	75.00	72.000	52.000	75.500
P-value	0.073	0.059	0.736	0.472
Alpha	0.05	0.05	0.05	0.05

CHAPTER FOUR

4.0 DISCUSSION

PCOS is one of the most common endocrine disorder found in female population; which is a leading factor for infertility. Genetic and environment factors tend to influence the complexity of PCOS.Studies have shown that PCOS patients have high resistance to insulin. Furthermore, insulin resistor proteins are considered as the most important factor in its role in insulin action relating to molecular polymorphism in genes.Insulin receptor genes, such as *IRS1* and*INSR,* are among the key genes that can be associated with the pathogenesis of PCOS.*IRS1*and *INSR* polymorphisms have tendencies of participating in problems of insulin signaling(Thangavelu et al., 2017). Due to the possible association of SNPs within these two genes with the development of PCOS, this study aimed to analyze two insulin receptor variants of PCOS, performed using SNP analysis.

IRS1 gene is a protein coding gene that is located on chromosome 2q36 with 1242 amino acid protein. *IRS1* is identified as a candidate gene that leads to different diseases, such as type two diabetes which has an association with pathogenesis of PCOS. Thus, thispolymorphisms may play a role in the occurrence of insulin-resistance, cholesterol metabolism and lastly metabolic perturbations(Ruan et al., 2012).

INSR gene is a protein coding gene which belongs to tyrosine kinase family of protein. *INSR* is located on chromosome 19p13.2 with allelic variation of G/A.This SNP consists of a minor allele frequency of MAF:0.29(A) and highest population MAF:0.49. *INSR* gene functions in the formation of certain subunit such as alpha and beta subunits through heterotrimeric receptor. Insulin receptor can be seen in many types of cells; the alpha subunit binds to insulin which allows the beta subunit to activate the signaling pathways around the cell that may alter cell functions. Peripheral insulin resistance in PCOS may lead to deactivation of many receptor kinase, that may lead to reduction in tyrosine auto phosphorylation. Certain genetic changes in insulin receptor might result to abnormalities such as donohve syndrome, rabson-mendenhall syndrome, type A insulin resistance syndrome, and lastly PCOS (Bozdag et al., 2016).

In this study, the homozygosity and heterozygosity status of the SNPs within *IRS1* and *INSR* genes were analyzed using melting temperature (Tm). The statistical differences were investigated using theMann Whitney test.The results of this study showed that the *INSR* theheterozygosity and homozygosity show significantly no difference between the *INSR Tm* values among the patients and the control group, respectively.Similarly, this result agrees with other studies after evaluation for the *IRS1 TM and INSR TM* for both the homozygous and heterozygous polymorphism. A clear example is a study by Rashidi and colleagues (2011). In this study, forty-eight Iranian females diagnosed with PCOS with fifty-two non-diabetic and women without PCOS enrolled as their control subjects. The researchers investigated the variations within the *IRS1* and *IRS2*genes, respectively. Their results showed no significant differences in the allelic frequencies of *IRS1* and *IRS2* in the control relative to PCOS patients (p<0.02). The same research group reported no association of*IRS1*polymorphism in PCOSpatients and control group in Spain (Rashidi et al., 2012). Similarly, genome wide association studies have failed to show the link between the polymorphism of *IRS1* and PCOS in Han Chinese population (Galusha, 2013). Moreover,further studies reported no association between SNPs within *INS, INSR, IRS1, IRS2, PPAR-G* and *CAPN10* genes and PCOS (Thangavelu et al, 2017).

Opposing studies had been published, in such Rashidi and colleagues found out that the control subjects *IRS1Gly972Arg* p-value *(P > 0.02)*washigher between the control and patient when compared with those with Gly/Gly genotype(P=0.037)(Rashidi B, Azizy L,2012).Furthermore, a study reported in Turkey revealed significantly different frequencies of IRS1 Gly972Arg polymorphism in PCOS and the control subjects of 8.3% and 23.3%m respectively which may contribute to the pathogenesis of this syndrome (Dilek S, Ertunc ,2005).Furthermore, another study was conducted to determine the frequency of polymorphism for the *IRS1* at codon 972 in women with PCOS in South Italy. The authors assessed *rs1801278* polymorphic variants in *IRS1* gene; in such Gly972Gly as wild-type;Gly972Arg as heterozygote; Arg972Arg as homozygote, respectively. The study was performed in 65 women with PCOS and 27 age- matched healthy women. The results showed a significant difference with Gly972Arg present in PCOS patients with 77% and 18% in controls, respectively. Also, compared to wild type PCOS, heterozygous PCOS women had three significantly different results means among the result some variants are significantly associated with PCOS while some variants might not contribute to the pathogenesis

of PCOSFurthermore, Tang and colleagues used a comprehensive meta-analysis in more than four thousand subjects. They reported that *IRS1* rs1801278G polymorphism is associated with PCOS. In results by Tang et al, the variant A allele (AA/GA) of the *IRS1* polymorphism increased the susceptibility of PCOS when compared to the homozygote GG. Similarly, Ryan Yuan and Ma Joshua (2012) investigated the association of SNPs within*IRS1* and *IRS2* genes in relation to PCOS using a meta-analysis method. They gathered data through different databases such as PubMed and EMBASE databases. They used a Q-statistic test to assess heterogeneity and Begg's test and Eggers test to evaluate publication bias. Their results indicated that A allele of Gly972Arg significantly increased risk of PCOS compared with G allele; with Gly1057Asp polymorphism. Using the meta-analysis their results suggested that *IRS1* Gly972Arg polymorphism could be considered significant risk for PCOS. They also recorded no significant association observed in *IRS2*Gly1057Asp polymorphism (Ryan Yuan and Ma Joshua, 2012).

Additionally, the studies by Skrgatic and colleagues (2013) aimed to determine some selected genes of *INS*, *INSR* and *IRS1* and their potentials related to the development of PCOSin Croatian population. There was a significant difference in clinical and biochemical characteristics of the studied groups except for BMI and fasting glucose levels, moreover no significant differences was observed in the genotype and allele distribution of the *VNTR INS, C/T INSR, GLY792Arg IRS*-1 variants between patients and control group. The diagnosis was based on Rotterdam consensus criteria with BMI evaluation, menstrual cycle problems, LH, FSH and testosterone.Research study by Dakshinamoorthy *et al* (2020) evaluated two *INSR* variants (*rs2059809 and rs1799817*) in 253 PCOS patients and 308 age-matched controls in Indian population. Their result revealed that, the minor allele frequency of one of the genes *rs2059807* recorded an odd ratio of 13.5 while the *rs1799817* recorded 11.8 with different representation of the hormonal patterns(Dakshinamoorthy et al., 2020).

In Zhengling Liu study, they try to find the association between *ADIPOQ* polymorphisms that is related with PCOS formation. Two polymorphic variants were used such as rs1501299 and rs2241766 to see the significant among Caucasians population. *ADIPOQ* it belongs to adipocytokine family that is secreted by adipocytes it helps in regulating energy and metabolic materials. Expression level of adiponectin was significantly lower in patients that are associated with certain metabolic disorders such as obesity, diabetes and lastly insulin resistance. which

means that adiponectin might contribute in the formation of the diseases mentioned above*(Dobrzyn K, Smolinska N. 2018)*. Previous studies mentioned that the expression level of adiponectin and its receptor which occur in female reproductive organs such as ovary and uterus differs in oestrous cycle, this mean adiponectin play a role in pathogenesis of PCOS (Achari AE, Jain SK.2017). Adiponectin is responsible in the production of ADIPOQ gene, that is located chromosome 3q27 with two common polymorphic variants with alter serum concentration in adiponectin, because these two polymorphism were set have a link with genetic biomarkers of many metabolic disorders including PCOS. (Dahlman I, Arner P.2010). In this study meta-analysis was used to identified the roles of *ADIPOQ* polymorphisms that is related in PCOS, because the result in previous studies were controversial. The statistical analysis was done using Review Manager Version 5.3.3. fifteen studies were done using rs1501299 variant while seventeen studies using rs2241766 a significant association related to PCOS was achieve in both rs1501299, rs2241766 polymorphism with a p-value less than alpha value($p=0.002$), ($p=0.001$) mean this polymorphism have a strong significant correlation in PCOS risk, both in Caucasians and east Asians population *(*Al Hannan FA.2016).

Further studies also investigated *INSR*rs1799817 associates with susceptibility to the PCOS on a given population of women from Saudi Arabia. Thus, 126 PCOS women and 118 controls were used in the study. Also, demographic data was included, including plasma levels of glucose, lipids, lepton and insulin. Similar to our work, the allele frequencies of *rs1799817* were evaluated in PCOS and control subjects. In addition, PCR was also used in amplifying *INR* gene. The SNPs (C to T) were found at locus 10923 (His1058) or *rs1799817*. The results revealed that in the PCOS group, the mutant homozygous allele was significantly higher in frequency when compared to the control group. Also, it indicates dormant effects and risk of PCOS in the heterozygous (RR=2.82). When evaluating the subjects into body mass index, the frequency of T allele was significantly higher among the lesser body patients with PCOS, when compared to the control subjects. However, the authorsreported that, the obese PCOS had a higher frequency than the obese control with no significant difference. Evaluating the different literatures used in this research study, one can establish the knowledge on the fact that, PCOS is strongly determined by the person's body mass, age, environment and diet. C/T single nucleotide polymorphism frequency at exon 17 of *INSR* in patients with PCOS was significantly higher than that in normal female (41% vs. 12.5%, $P < 0.01$).Chen and his team alternatively found out that, there was a

significant difference between the frequency in women that are not obese with PCOS was recorded lower than that with C allele (26+/-4, P<0.05). (Chen ZJ, Shi YH, Zhao YR, et al.,2004)

Particularly, our study is distinctly unique because of the ethnicity and statistical analysis technique used. The statistical packages for the social sciences (SPSS version 10, Chicago USA), the descriptive statistics and independent sample test of Mann Whitney Test were all performed similar to Thangavelu and colleagues.

Conclusion

In this study, allelic frequencies of genes *IRS1* rs1801278 and *INSR*rs1799817involved in PCOS recorded no significant difference on the given population. One of the limitations of this study was the small size. It is a possibility that with increased number of PCOS patients and control group, the heterozygosity status may have shown significant differences. The next stage of this study is to genotype alleles specifically. In this study, we just determined the heterozygosity status. Further studies will also include investigation of more SNPs within these genes to have a more comprehending understanding of the involvement of these SNPs with the pathophysiology of PCOS.

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Appendix

Table showing the heterozygosity status with the Ct and Tm values for *IRS1* rs1801278 and
INSR rs1799817 in PCOS patients

Patients code	*IRS1* Ct	*IRS1* Tm	Heterozygosity	*INSR* Ct	*INSR* Tm	Heterozygosity
1	27.13	91.5	Homozygous	23.7	85.8	Homozygous
2	25.22	91.4	Homozygous	23.04	86.2	Homozygous
3	25.7	92.1	Homozygous	25.8	86	Homozygous
4	30.98	78.9	Homozygous	27.2	86	Homozygous
5	31.37	78	Homozygous	27.8	87.4	Homozygous
6	24.99	91.4	Homozygous	26.1	84.6	Homozygous
7	29.44	83.6	Homozygous	29.94	87.36	homozygous
8	28.22	83.3	Homozygous	36.7	86.3	Heterozygous
9	22.8	91.2	Heterozygous	22.5	85.7	Homozygous
10	32.75	92	Homozygous	28.9	86.7	Homozygous
11	29.7	78.7	Homozygous	24.26	86.4	Homozygous
12	25.85	91.8	Homozygous	23.1	86.1	Homozygous
13	29.33	91.2	Homozygous	23.1	85.4	Homozygous
14	25.48	91.3	Homozygous	23.49	85.8	Homozygous
15	31.31	76.5	Homozygous	22.75	86.1	Homozygous

16	28.02	91	Heterozygous	23.4	86	Homozygous
17	22.27	92.3	Heterozygous	23.58	85.9	Homozygous
18	31.99	77.9	Homozygous	22.1	90.1	heterozygous
19	30.29	79	Homozygous	26.3	85.8	Homozygous
20	23.93	91.6	Homozygous	24.2	86.5	Homozygous
21	30.52	78.1	Homozygous	24.05	86.1	Homozygous
22	22.2	92.19	Heterozygous	23.6	85.7	Homozygous
23	30.47	79.1	Homozygous	23.36	86.1	Homozygous
24	29.36	78.9	Homozygous	23.7	85.9	Homozygous
25	26.5	92.02	Homozygous	22.4	85.6	Homozygous
26				35.4	86.4	Homozygous
27	31.96	78.4	Homozygous	24.77	85.6	Homozygous
28	29.85	79.1	Homozygous	23.35	86.1	Homozygous
29	No result	No result	No result	22.3	86	Homozygous
30	No result	No result	No result	28.1	86.2	Homozygous
31	29.48	78.5	Homozygous	27.7	85.9	Homozygous
32	31.8	78.7	Homozygous	27.5	86	Homozygous
33				23.5	86	Homozygous
34	30.91	78.5	Homozygous	26.6	85.7	Homozygous

35	29.65	91.7	Homozygous	26.9	86.2	Homozygous
36	32.07	78.4	Homozygous	26.7	86.5	Homozygous
37	31.99	85.62	Homozygous	23	86.3	homozygous
38	31.68	86.04	Homozygous	25.8	87.3	homozygous
39	30.42	91.6	Homozygous	25.77	87.1	homozygous
40	24.47	83.6	Homozygous	23	85.9	homozygous
41				24.1	85.7	homozygous
42	27.2	91.74	Homozygous	22.63	85.7	homozygous
43	25.85	91.6	Homozygous	23.5	85.8	homozygous
44	25.15	91.5	Homozygous	23	86	homozygous
45				23.7	86.6	homozygous
46	25.85	90.7	Heterozygous	23	84.9	homozygous
47	27.4	91.6	Homozygous	24.3	84.6	homozygous
48	31.77	82	Homozygous	29.34	87.1	homozygous
49	23.88	91	Homozygous	23.3	84.9	homozygous
50	24.15	91.9	Heterozygous	24.1	84.8	homozygous
51	26.35	91.6	Homozygous	28.89	87.1	homozygous
52	24.04	83.8	Homozygous	23.15	86	homozygous
53	25.7	91.6	Homozygous	26.41	61.3	homozygous
54	25.98	91.5	Homozygous	25.36	61.7	homozygous

55	25.83	91.6	Homozygous	27.72	61.5	homozygous
56	26.6	91.1	Homozygous	22.8	85.9	homozygous
57	25.65	92.1	Homozygous	23.4	85.9	Homozygous
58	26.36	92.2	Homozygous	22.9	85.9	Homozygous

Table showing the heterozygosity status with the Ct and Tm values for *IRS1* rs1801278 and*INSR* rs1799817 in control group

Patients code	IRS1 Ct	IRS1 Tm	Genetic conditions	INSR Ct	INSR Tm	Genetic conditions
1				23.5	87.5	Homozygous
2	27.72	83.8	homozygous	24.5	85.4	Homozygous
3	29.94	82.6	homozygous	22.7	86.3	Homozygous
4	30.74	78.6	homozygous	23.2	85.9	Homozygous
5	31.1	78.4	homozygous	24	90.94	Heterozygous
6	25.88	92.1	homozygous	29.29	87.23	Homozygous
7	26.5	92.26	homozygous	23.2	85.9	Homozygous
8	32.27	78.5	homozygous	24.1	85.9	Homozygous
9	27.3	91.47	homozygous	22.83	85.8	Homozygous
10	27.2	92	homozygous	23.01	85.9	Homozygous

11	26.09	92	homozygous	33.15	87.3	Homozygous
12	26.21	91.4	homozygous	28.76	87	Homozygous
13	30.35	82.38	homozygous	27.1	87.56	Homozygous
14	25.92	91.6	Homozygous	25.69	62.8	Heterozygous
15	21.91	91.4	Homozygous	25	84.8	Homozygous
16	24.4	91.7	homozygous	24.7	85	Homozygous
17	25.3	92.4	Homozygous	24.9	84.5	Homozygous
18	24.5	92.2	homozygous	24.1	85.6	Homozygous
19	24.86	91.9	homozygous	22.4	85.7	Homozygous
20	35.17	77.9	homozygous	23	84.8	Homozygous
21	24.67	91.4	homozygous	25	84.7	Homozygous
22	23.42	91.3	homozygous	23.3	84.7	Homozygous
23	25.92	91.4	homozygous	23.9	84.2	Homozygous
24	28.74	91.6	homozygous	23.2	85	Homozygous
25	26.01	91.7	homozygous	25.91	62.54	Homozygous
26	25.02	91.8	homozygous	25.36	63.9	Heterozygous
27	25.11	99.9	homozygous	26.7	63	Homozygous
28	25.38	91.6	homozygous	24.95	62.9	Heterozygous
29	26.8	91.7	homozygous	26.41	63.3	Homozygous
30	29.35	83.2	Homozygous	24.96	85.6	Homozygous

31	30.33	83.2	homozygous	27.88	87.56	Homozygous
32	20.99	87.6	Heterozygous	29.41	86.84	Homozygous
33				29.5	87.36	Homozygous
34	30.13	83.9	heterozygous			Homozygous
35	26.8	91.8	Homozygous	28.59	87.3	Homozygous
36	25.85	91.8	Homozygous	31.16	87.2	Homozygous
37	25.46	91.6	Homozygous	26.47	86.9	Homozygous
38	22.55	91.7	Heterozygous	23.61	62.6	Homozygous
39	26.8	91.4	Homozygous	28.17	86.9	Homozygous
40	25.63	91.5	Homozygous	29.22	86.7	Homozygous
41						
42	25.55	91.6	homozygous	29.06	87.1	Homozygous
43	30.63	82.8	homozygous	28.7	87.62	Homozygous
44	29.51	82.8	homozygous	23.6	87.2	Heterozygous
45	21.49	84.1	homozygous	22.99	86	Homozygous
46	29.96	83.3	homozygous	23.27	86.1	Homozygous
47	22.97	83.9	homozygous	22.32	85.8	Homozygous

APPENDIX

Table: Showing the concentration and absorbance details of the DNA

Patients code	DNA Concentration (ng/μl)	DNA (260/280)
1	34.3	1.69
2	23.7	1.89
3	31.7	1.84
4	39.7	1.88
5	23.6	1.92
6	22.7	1.94
7	12.4	1.42
8	4.2	2.08
9	3.3	2.57
10	2.5	1.80
11	18.1	1.24
12	7.1	1.56
13	3.2	2.18
14	7.0	2.28
15	10.1	2.09
16	3.1	2.31
17	24.6	1.90
18	39.9	1.77
19	49.6	1.89
20	33.9	1.73
21	31.2	1.90
22	31.3	1.81
23	15.8	1.99
24	22.2	1.84
25	24.5	1.90
26	39.9	1.89
27	26.4	1.88
28	22.4	1.92
29	31.7	1.80
30	19.9	1.79
31	22.0	1.88
32	47.9	1.84
33	21.2	1.93
34	30.5	1.91
35	22.6	1.89

36	37.9	1.90
37	18.8	1.89
38	23.1	1.59
39	55.8	1.85
40	15.6	1.51
41	26.2	1.72
42	120.9	1.47
43	36.5	1.87
44	14.5	1.84
45	15.6	1.86
46	34.7	1.41
47	21.1	1.89
48	24.0	1.49
49	97.7	1.49
50	16.2	1.98
51	185.7	1.45
52	37.6	1.91
53	50.0	1.64
54	224.6	1.48
55	31.6	1.77
56	145.9	1.51
57	158.2	1.54
58	130.6	1.49
59	120.0	1.57
60	12.8	1.94
61	9.5	1.89
62	15.0	1.74
63	80.3	1.51
64	9.2	1.86
65	20.0	1.57
66	48.0	1.56
67	11.8	1.88
68	28.6	1.52
69	16.9	1.47
70	16.5	1.65
71	12.3	1.71
72	6.9	2.01
73	7.0	2.19
74	18.3	1.95
75	33.7	1.86
76	9.2	1.91
77	15.3	2.11
78	19.9	1.87
79	15.5	1.87
80	45.9	1.71

81	27.5	1.82
82	25.2	1.69
83	46.2	1.77
84	17.2	1.90
85	23.0	1.75
86	30.0	1.84
87	15.0	1.78
88	27.1	1.76
89	25.1	1.68
90	21.6	1.85
91	114.4	1.49
92	91.0	1.51
93	112.8	1.56
94	292.3	1.44
95	17.8	1.86
96	27.5	1.86
97	34.3	1.88
98	16.0	1.86
99	35.6	1.86
100	36.9	1.87
101	124.3	1.49
102	17.2	1.73
103	65.0	1.55
104	137.2	1.42
105	48.2	1.78
106	27.9	1.76
107	82.6	1.55
108	51.9	1.65
109	135.1	1.45
110	90.4	1.53
111	42.1	1.67
112	125.5	1.48
113	259.9	1.47
114	25.1	1.80
115	15.2	1.89
116	14.5	1.89
117	23.6	1.92
118	19.5	1.77
119	15.7	1.85
120	22.0	1.79
121	49.7	1.57
122	18.2	1.78
123	21.0	1.90
124	14.2	1.91

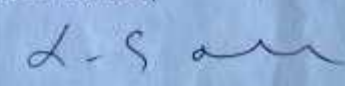|		
125	26.5	1.75
126	26.5	1.87
127	27.8	1.60
128	17.6	2.00
129	23.6	1.86
130	25.0	1.85
131	26.7	1.78
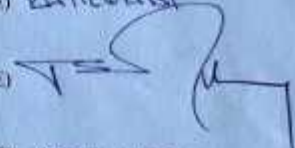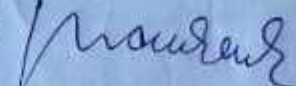
YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

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)

4. Prof. Dr. Şahan Saygı   (ÜYE) KATILMADI

5. Prof. Dr. Sanda Çalı   (ÜYE)

6. Prof. Dr. Nedim Çakır   (ÜYE)

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)