NOJAN HAFIZI

EXPRESSION OF GENES IN AKT SIGNALLING PATHWAY IN HUMAN OOCYTES FROM PATIENTS WITH POLYCYSTIC OVARIES

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NEAR EAST UNIVERSITY

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DEPARTMENT OF MEDICAL BIOLOGY AND GENETICS

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January, 2022

Approval

We certify that we have read the thesis submitted by Nojan Hafizi titled "**Expression of Genes in Akt Signalling Pathway in Human Oocytes from Patients with Polycystic Ovaries''** and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Educational Sciences.

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Declaration

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Nojan Hafizi

27/01/2022
Day/Month/Year

Acknowledgments

Since the beginning of my project, world has gone through one of the most difficult times of its history, as a student I have struggled a lot during this pandemic but with the help of family, friends and my peers finally I have reached the end of my postgraduate path.

Without the help and support of my family, I would have not been at this stage of my life, therefore I have to mention my loved ones as the main reason of my success, my mother, Mahparvar Firouzi, and father, Mohammad Hafizi, have been by my side since day one and of course my older sister, Nazgol Hafizi, that has helped me massively in my academic and personal life. I couldn't wish for a more supportive and lovely family and I'm so lucky to have all of you in my life. Also, I would like to thank my dear friend, Marwan Sider, which has helped me enormously during writing of my thesis, he has been more than a friend to me during this time.

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Nojan Hafizi

Abstract

EXPRESSION OF GENES IN AKT SIGNALLING PATHWAY IN HUMAN OOCYTES FROM PATIENTS WITH POLYCYSTIC OVARIES

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Supervisor: Assoc. Prof. Pinar Tulay

Background: Polycystic ovary syndrome (PCOS) is a common disorder among the females, etiology of the diseases is still unknown to the date. PCOS is known as an endocrine disorder and the main reason for the development of the disease is hormonal abnormalities of individuals, mainly androgenic. Studies have shown that prevalence of the disease can get to as high as 26% of female population in some areas. PCOS is categorized as a common complex disease meaning that both environmental and hereditary factors can cause formation of the disease.

mTOR pathway belongs to the family of protein kinase pathways. Main function of this pathway is to sustain cell growth and proliferation in addition to survival of the cell. Any abnormality in the genes of this pathway can lead to crucial diseases, such as cancer or diabetes. Disturbance in mTOR pathway has also been linked to changes in the level of insulin and testosterone production in females which is the main reason for formation of PCOS. This study was designed to investigate the relationship between the expression levels of *IRS1*, *IRS2*, *AKT1* and *AKT2* of mTOR pathway and the development of PCOS.

Material and methods: Oocyte samples were collected at meiosis II stage of development from 13 individuals. Seven samples were from patients with polycystic ovaries (PCO) and the remaining six from healthy individuals with no sign of PCOS, respectively.

RNA was extracted from these samples and cDNA was synthesized from the oocytes to use in RT-PCR. cDNA samples were used to measure the expression level of four candidate genes and the housekeeping gene (*IRS1*, *IRS2*, *AKT1*, *AKT2* and *ACTB*, respectively).

Results: Student's T test was used to measure the significant level of expression of each gene for PCO patients. The results of this study showed that there has been no significant correlation between gene expression level of the candidate genes and PCO since all the obtained values were larger than 0.05.

Conclusion: This study showed no correlation between expression level of *IRS1*, *IRS2*, *AKT1* and *AKT2* between PCO and healthy group samples.

Keywords: mTOR, *IRS1, IRS2, AKT1, AKT2*, PCOS, PCR, oocyte

Table of Content

List of Figures

List of Tables

List of Abbreviation

***ACTB*:**	**Beta Actin**
***AKT*:**	Protein Kinase B
ASRM:	American Society for Reproductive Medicine
BMI:	Body Mass Index
Ct:	Cycle Threshold
FSH:	Follicle Stimulating Hormone
GnRH:	Gonadotropin Releasing hormone
***IRS1*:**	Insulin Receptor Substrate 1
IVF:	*In Vitro* Fertilization
LH:	Luteinizing Hormone
mTOR:	Mammalian Target of Rapamycin
mTORC1:	Mammalian Target of Rapamycin Complex 1
mTORC2:	Mammalian Target of Rapamycin Complex 2
NIH:	National Institute of Health
PCO:	**Polycystic Ovaries**
PCOS:	**Polycystic Ovary syndrome**
PCR:	Polymerase Chain Reaction
***PI3K*:**	Phosphoinositide 3-Kinase
Tm:	Melting Temperature
***TSC2*:**	Tuberous Sclerosis Complex 2

CHAPTER I

Introduction

Polycystic ovary syndrome (PCOS) is one of the most commonly appearing disorders of female reproductive system (Goodman et al., 2015). It is known as an endocrine disorder among the females. Studies has shown that probability of an individual having the disease in some populations can get as high as around 26%, although the rate of the disease is higher in some populations but still the etiology and the main cause of the disease has not been found yet (Muscogiuri, et al., 2017). Disorder has been named after one of the very common symptoms of it which is appearance of cysts structures on the ovarian wall, nevertheless, mentioned cysts are just one of many symptoms of the disorder and should not get confused as the cause of the disease (Dunaif & Fauser, 2013). PCOS belongs to the family of common complex diseases, both heritable factors and environmental factors can lead to the disorder (Diamanti-Kandarakis et al., 2006). Obesity, lack of exercise and bad diet are just some of the lifestyle and environmental factors that are well known as the risk factors of the disease. Main focus of this study was to investigate the genetic side of the disease and finding out about effect of candidate genes and their expression among the patients of PCOS (Sirmans & Pate, 2014). One of the very first appearances of the disease date back to 1721 in Italy. As mentioned previously, PCOS is an endocrine related disorder and among the female adults of age 18 to 44 this is the most common endocrine disorder found (Kovacs & Norman, 2007). Several different causes of infertility have been found, but when infertility is related to inability of ovulation usually PCOS is the main cause (Khan et al., 2019). Patients of the PCOS experience an imbalance in their hormonal secretion which lead to disruption of the menstrual cycle, changing time or skipping cycles, three main hormones which are affected by the disease are androgens, insulin and progesterone. Androgens are mainly known as the male hormones but they can be found in female body in a smaller portion, but due to the imbalance in production of hormones caused by PCOS, level of androgen in PCOS females is higher than normal individuals, insulin production is also disrupted in PCOS patients which lead to changes in blood sugar and in more serious cases diabetes. Finally, progesterone imbalance is the main reason for the females to miss their periods or not being able to predict the time of it,

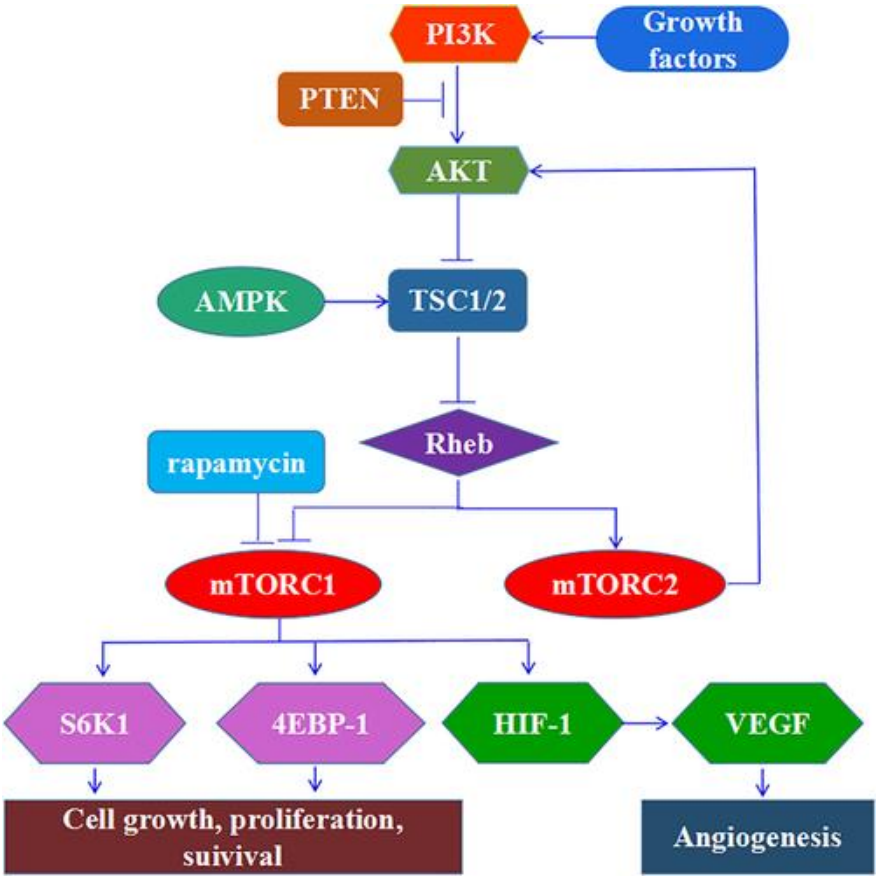
level of this hormone in PCOS patients are usually lower than healthy individuals (Insler & Lunenfeld, 1990).

1.1.mTOR Signaling Pathway

Mechanistic target of rapamycin (mTOR) belongs to the family of protein kinases and is encoded by *mTOR* gene (Feinberg, 2007). mTOR belongs to two protein complexes known as mTORC1 and mTORC2. Each of these complexes of protein pathways function in different bodily processes. Main function of mTOR can be seen during cell growth and proliferation, in addition to motility, survival and protein synthesis (Hanash, 2003). Deregulation of this specific signaling pathway has been correlated with diseases such as cancer and diabetes, due to its effect on the activation level of insulin receptors and insulin growth factor 1 receptors (Webber, et al., 2003). mTORC pathway like many other biochemical pathways of the body is closely interwind with other pathways, activation of Ras signaling pathway by growth factors can directly stimulate mTORC, when these pathways get stimulated *TSC2* gene will get phosphorylated by protein kinase B also known as *AKT*, phosphorylated *TSC2* now is deactivated, therefore mTORC1 gets stimulated and becomes active. In addition to the mentioned pathway, *AKT* expression can directly activate mTORC1 in an independent manner towards *TSC1*/2 (Long et al., 2005). Prior to attachment of insulin to its receptor a series of reactions and activities will occur such as gathering of *INSULIN RECEPTOR SUBSTRATE 1* (*IRS1*) and activation of *AKT*. Promotion of mTORC1 has been connected to repression of PI3K-AKT and phosphorylation of *IRS1* that can lead to instability of this gene (Inoki et al., 2003). Mentioned pathways are auto-regulated in the human body and has been correlated to metabolic disease and tumorigenesis, in comparison to mTORC1 that has shown significant correlation between its function and some diseases, a little has been discovered on the mTORC2 complex and effect of it on biology of individuals. Although with the hard work of scientists and several research groups on this matter, today this complex is related to cell survival, metabolism, proliferation and cytoskeleton organization (Crino et al., 2006). Figure 1.1 provides illustration of part of mTOR pathway.

Figure 1.1

Part Of Mtor Signaling Pathway and How It Functions in Processes Such as Cell Growth, Proliferation and Angiogenesis (Jin Liu et al, 2018)



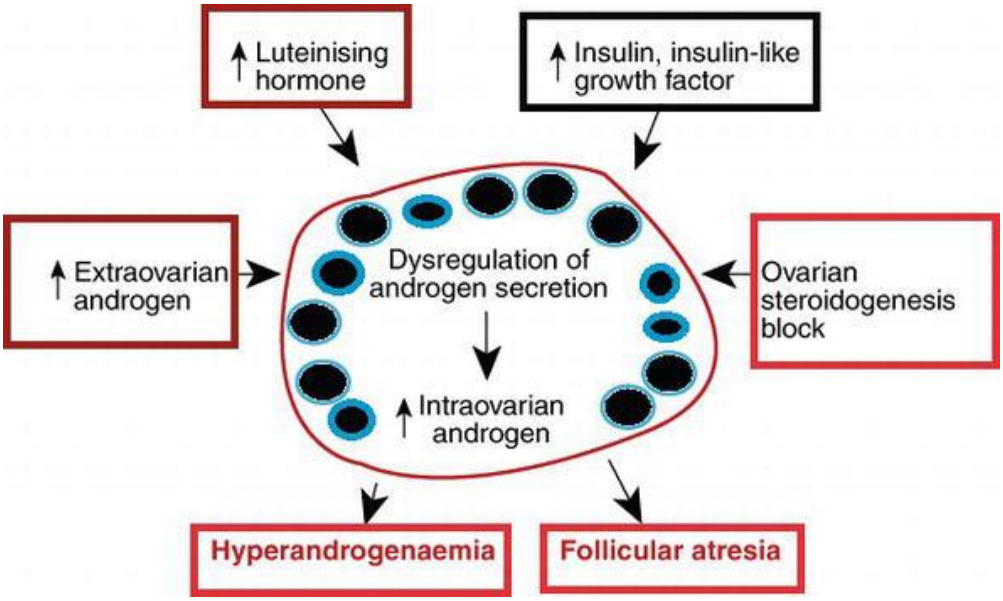
1.2. Pathogenesis of PCOS

Level of androgenic hormones or better known as male hormones among the patients of PCOS is much higher compared to the healthy females. This excess amount of androgen in female body can lead to the formation of polycystic ovaries. Main androgen causing the disorder is testosterone but this hormone is not the only one, excess amount of luteinizing hormone (LH) and hyperinsulinemia (peak of insulin in blood) can also lead to the polycystic ovaries (Strauss, 2003). Figure 1.2 summaries some of hormonal abnormalities among PCOS patients. Female ovaries tend to produce structures known as follicles. Cysts that are formed in this disorder are actually follicles that have stopped growing and maturing and are known as arrested follicles. PCOS patients experience surge of LH and FSH ratio in addition to elevation of GnRH pulses (Hormone Health Network, 2018). Obesity is very common among the PCOS patients due to the formed resistance towards insulin, this can lead to increase in GnRH frequency, dominance of LH over FSH and loss of

follicular maturation. Although it must be mentioned that this resistance towards insulin can be observed in normal weight women as much as overweight ones (Teede et al., 2010). PCOS has been associated with chronic inflammation as well as elevated level of oxidative stress (Murri et al., 2013). An example in which effect of testosterone level on appearance of PCOS can be clearly seen among transgender men that are going through testosterone therapy and prevalence of PCOS among these individuals is much higher than usual, only due to the excess amount of testosterone in their body (Puffer, 2006).

Figure 1.2

The Examples of Hormonal Changes Correlated with Development of PCOS (Bassim Alsadi, 2018)



1.3. Epidemiology of PCOS

The appearance rate of disease can actually vary depending on diagnosis criteria and one country to other, according to World Health Organization (WHO) around 116 million females are suffering from the PCOS that makes up around 3.4% of the female population around the world (Vos et al., 2010). Another study has predicted that about 7% of women are affected after puberty. According to the Rotterdam criteria, around 18% of females are affected and of that 18%, 70% have already been diagnosed. Some countries have a better methodology and resources for their researchers that's why the prevalence rate might differ from a country to another due to lack of reliable research and data. As an example, India is reporting

that 20% of female population is affected by PCOS (Pruthi, 2019). Research studies also shows that between 8-25% of women whom are not affected by PCOS shows presence of cysts in their ultrasonographic tests (Van Santbrink et al., 1997). Another correlation has been found between oral contraceptives and prevalence of PCOS. According to research studies, 14% of females on this drug are affected by the disease. Intrauterine devices are also a common risk factor for appearance of the disorder among young females (Clayton et al., 1992).

1.4. Symptoms

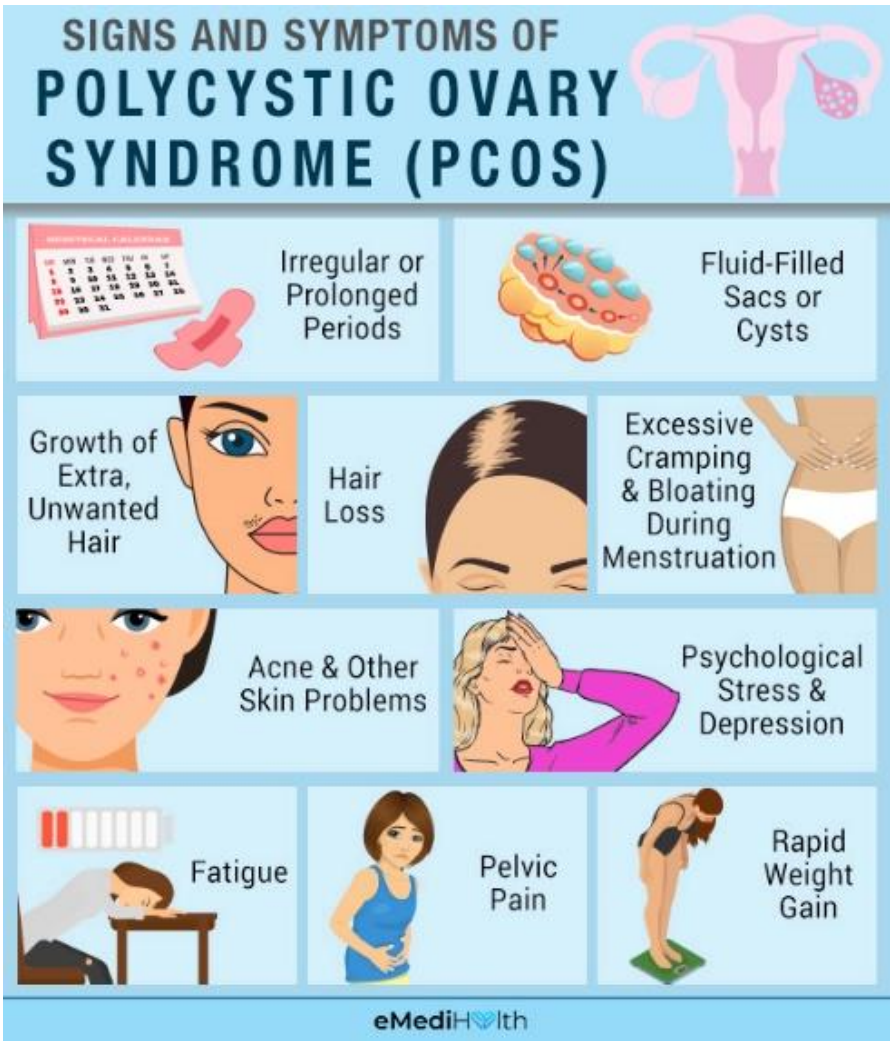
PCOS, like many other disorders, have wide range of symptoms, common symptoms are summarized in figure 1.3. Some of these may not even seem warning, symptoms such as oily skin. Therefore, very commonly individuals are not diagnosed for many years since symptoms are not that troubling and also majority of cases, symptoms are not shown for a long period of time (Nazario, 2020).

Some of the symptoms include, growth of unwanted hair, presence of hair on face, chin and breast can be sign of PCOS, next to presence of unwanted hair, hair on head can get thinner and loss of hair can happen that can get worse among middle age women. PCOS can lead to changes in texture and color of the skin that can be one of the main symptoms and signs of the disease, presence of acne due to hormonal changes and appearance of thick patches of skin in different areas such as neck and breast is another skin abnormality followed by the disorder, though these skin conditions can appear even individual is not affected by PCOS. Ovary is one of the main sections of female reproductive system that function in production and release of mature oocyte, therefore an anomaly in it such as presence of cyst can cause massive problems for reproductive system and menstrual cycle of the females. This can lead to heavy or irregular period as well as trouble getting pregnant. Weight gain and struggling for losing weight is one of the signs of PCOS in females. In addition to psychological effects of gaining weights, being overweight can worsen other symptoms of the disease. Therefore, it is highly recommended to the PCOS patient to have a healthy diet and exercise regularly in order to lose the weight gained due to the disorder to keep other symptoms in a less disrupting level. Also losing weight can help with the changes on the level of cholesterol and blood glucose that can rise due to PCOS (Insler & Lunenfeld, 1990). PCOS can have risks for the individual even after going though treatment and cure of the disorder. One of the risks of later in life

for these patients is type 2 diabetes. In addition to that, depression and mood swing and high cholesterol, stroke and cardiac diseases may be observed in the patients. Sleep apnea due to the gain of weight are couple of more observed symptoms of PCOS patients (Nazario, 2020).

Figure 1.3

Some of the Common Symptoms Among PCOS Patients (Kecia Gaither, 2021, eMedihealth)



1.5. Diagnosis

As mentioned previously, PCOS has adopted its name from one of the main signs of it which is the presence of numerous cysts on the ovary of the patients. However, there are several cases that cysts are not present, likewise in some cases cysts are available but individuals are not diagnosed of PCOS (Marrinan, 2019). There have been two criteria for the diagnosis, first one stablished by the NIH in

1990 and second one by ASRM workshop in Rotterdam in 2003. According to NIH agreement for the PCOS diagnosis, three signs must be present for the person to be diagnosed with the disorder. First, having irregular period cycles, in which normal individuals usually get their period every 25-30 days but PCOS patients may get their period only 8 times per year. This anomaly is known as oligoovulation. Secondly, hormonal changes, specifically changes in androgen hormone and excess amount of androgen must be present. Thirdly, making sure that symptoms are not due to similar diseases (Chang et al., 2004). Similar to NIH agreement, in Rotterdam agreement, the first two criteria are oligoovulation and level of androgen hormones. This agreement also includes the presence of cysts on ovary the (Hart et al., 2004).

1.6. Causes

PCOS is a complex disease, therefore the exact cause of the disease is still a mystery to the scientists, although changes in the hormonal level of individuals have been related to the development of PCOS. One of the main hormones that help body to keep the blood sugar level in control is insulin. An imbalance in the production of this hormone has been noticed among PCOS individuals. This hormone has the function of carrying glucose as energy source from blood to the body cells to provide them with the source of energy (Legro & Strauss, 2002). PCOS patients usually develop a resistance towards this hormone. As a result, tissue repel the hormone and body automatically try to produce more to keep up with the demand of cell for energy. Excess amount of this hormone in our circulation can directly affect the production of testosterone in the ovaries. Testosterone is usually found in male body and very little of it can be found in females. Therefore, increase in the level of this hormone can interfere with ovulation in females. Insulin is not the only hormone related to PCOS. Increase in luteinising, prolactin and testosterone hormones, low amount of sex hormone-binding globulin has all shown correlations with development of PCOS (Filippou & Homburg, 2017).

At the genetics point of view, genes with mutations and/or variation can be inherited form either of parents. In male newborns the gene with the mutation and/or variation does not lead to the disease so basically, they are carrier or in some cases early baldness can be observed among males. But if the gene with the mutation and/or variation is inherited to the female newborn, then child may develop PCOS to some extent. Next to hormonal problems or genetic abnormalities, environmental

factors can increase the risk of disease as well. Exposure to different chemicals and drugs at different stages of the life can increase the risk of PCOS development (Draper et al., 2003). Exposure to endocrine disruptors, most famously bisphenol A that can alter the normal function of endocrine system of the human body are one of the most dangerous environmental factors for appearance of PCOS. These chemicals act in the body as estrogen hormone therefore bodily production of estrogen hormone drops massively and can damage the reproductive system of the male leading to PCOS (Faghfoori et al., 2017).

1.7. Treatment

PCOS is a non-curable disease but with some lifestyle changes, certain drugs and surgeries, symptoms can be controlled and life quality of patients can be improved. Treatment techniques vary from patient to patient due to the different levels of expressivity in individuals. In some patients, symptoms might be much worse compared to another patient. Main lifestyle changes that can help PCOS patients massively is regular exercise and having healthy diet. Due to the imbalance of hormones, such as insulin among these patients, increase in bodyweight is very common that can worsen the symptoms and health problems. Therefore, having regular exercise and healthy diet may avoid excessive weight gain and improve the health of the patient. Different drugs have been developed for each symptom of PCOS, for example for irregular menstrual cycle, contraceptive pills or methods such as intrauterine system might be prescribed to patients. For fertility problems drugs such as clomiphene, metformin or in vitro fertilization (IVF) treatment is suggested to patients. Additionally, laparoscopic ovarian drilling may be used to eliminate the malfunctioning and producing male hormones, therefore this procedure will lead to decrease level of testosterone and luteinising hormone as well as an increase in the follicle-stimulating hormone in the patients. As a result, hormonal balance of the body will be restored so the ovulation process can occur normally and fertility is achieved Another symptom of PCOS is unwanted hair growth or loss. To treat these issues, certain drugs such as cyproterone acetate, spironolactone, flutamide and finasteride are developed. For patients where drug treatment is not working, surgical methods is used to treat fertility problems (Van santbrink, 1997).

1.8. Complications

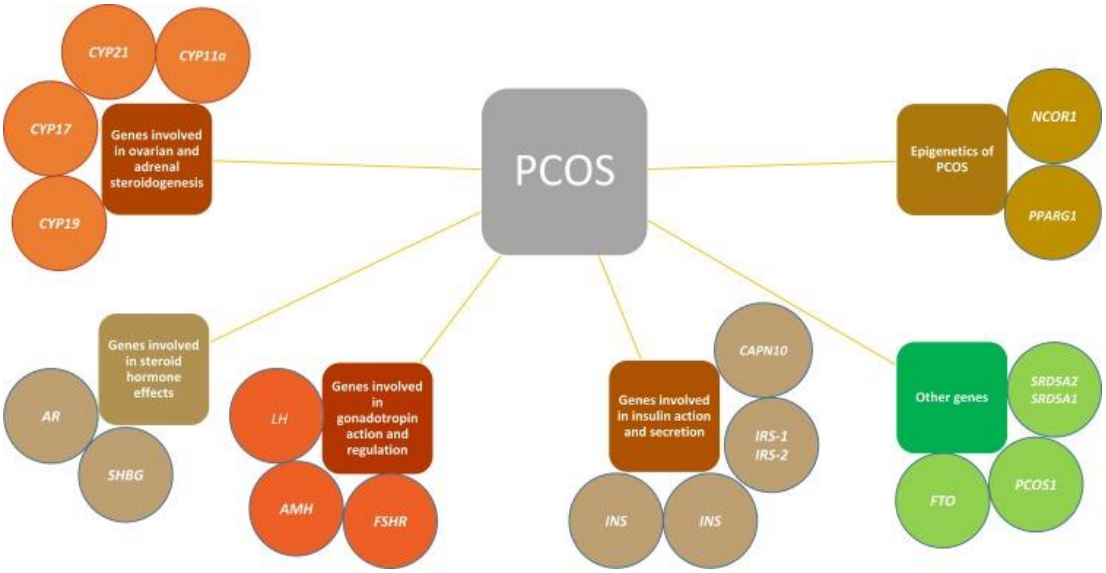
PCOS can bring about couple of different complications with it to the individuals, these must not be confused as the symptoms of the disease. As mentioned previously, changes in hormonal level and appearance of cysts at ovary can lead to troubles for pregnancy of the female due to malfunction of ovaries in releasing eggs during ovulation. Another complication due to PCOS is the development of diabetes due to resistance toward insulin blood sugar. PCOS also has been related to several cardiovascular diseases. The etiology of them is due to increase in triglyceride and high-density lipoprotein of blood as well as high blood pressure that can lead to atherosclerosis. Other complications include depression, anxiety, bleeding from uterus that can increase risk of uterine cancer, sleep problem and inflammation of the liver (Pathak, 2021).

1.9. Genetics of PCOS

Relating a single gene to complex diseases such as PCOS is impossible. Thus, genetic aspect of PCOS is under study for different pathways and different genes. Therefore, in order to be able to find a link between a certain gene and the disease genome-wide association studies must be done (Hamosh, 2011). Due to multifactorial characteristics of the disease multiple pathways with each having specific genes can be related to PCOS. CYP11a, CYP21 and CYP17 are some of the genes that are related to PCOS due to their function in production of androgen hormones (Franks et al., 2000). Any genes that play a role in the gonadotropin regulation pathway can lead to PCOS as well. These genes may include *LHR* gene and *FSHR* gene (Gromoll Simoni, 2005). One of the most important genes for this study *INSULIN RECEPTOR SUBSTRATE* genes (*IRS1* and *IRS2*) that function in regulation and production of insulin in the body. According to Petermann and colleagues (2001), presence of Arg92 *IRS1* is higher in PCOS patients (Sir-Petermann et al.,2001). On the contrary, El Mkadem and colleagues (2001) reported no correlation and change between the control and the PCOS group for the same genes (El Mkadem et al., 2001). Another study illustrated that there is presence of Gly972Arg in *IRS1* Turkish females with the disease (Dilek et al., 2005). These variations in the results of different studies are actually proving the complexity of the disease and how environmental and epigenetic factors may play a role in the development of PCOS.

Figure 1.4

Candidate Genes for PCOS and How They Can Lead to Development of Disorder (Khan et al, 2019)



1.10. PCOS and Weight Gain

Due to the abnormalities on the level of certain hormones, such as insulin in body of PCOS patients weight gain is a very common symptom, in spite of appearance concerns for females about gaining weight, this issue can lead to severe health problems such as diabetes, heart problems and uterine cancer. Due to insulin resistance of these females, blood glucose level increase which cause increase on production of male hormones. High level of androgens in the body is the main reason behind the weight gain of PCOS patients. Since the weight gain is mostly due to male hormones, majority of the weight gain appears in abdomen which is the most dangerous fat deposit since its correlated with heart disease. Research studies have shown that only 10% cut on the bodyweight can bring back normal periods for females of PCOS and help with other issues such as insulin sensitivity. In addition to medicines that can aid to control the hormonal problems, lifestyle choices can be of huge help such as having a healthy diet rich with fiber, increasing number of meals per day instead of eating huge meals, exercising regularly for at least half an hour daily (Pagano, 2020).

1.11. PCOS and Infertility

Hormonal abnormalities followed by PCOS can lead to infertility of the females, PCOS is known as one of the most common reasons for fertility problems

among the females. Due to PCOS, ovaries enlarge in size that carry several numbers of cysts. High level of androgen in females can interfere with ovulation since there is no oocyte released by time of ovulation. Therefore, there is no chance of fertilization and fertility cannot be achieved. In addition to problems with maturity of the oocytes, one of the very first signals on presence of PCOS can be missing or having irregular menstrual cycles. Medicines such as birth control pills can aid with irregular menstrual cycles and for ovulation problems drugs such as metformin and clomiphene can be helpful (Traci, 2021).

1.12. Objectives

Aim of this study is to investigate mTOR and AKT pathway genes and their effect on the development of PCOS in females. Several genes belonging to these pathways have been connected as candidate genes for PCOS, including AKT1, AKT2, IRS1 and IRS2 that has been studied in this research. Samples have been divided into two groups of PCOS patients and control in order to study differences in gene expression of mentioned genes in these two groups to find correlation between mutation in genes and development of PCOS.

1.13. Significance

To investigate the molecular basis of the PCOS can help medical doctors and scientists to detect the disease earlier and improve the chances of successful treatment. Unfortunately, there have not been wide area of studies on this matter, therefore studies like this can be of importance.

CHAPTER II

Materials and Methods

Ethical approval was granted by the Near East University Institutional Review Board (YDU/2019/75-920). The samples were collected from Near East University Hospital IVF Clinic and RNA was already obtained from these samples.

The aim was to investigate the relation between expression level of the genes of AKT pathway (*IRS1, IRS2, AKT1* and *AKT2*) collected from two different groups of samples, one group of oocytes obtained from PCO patients and the oocyte samples obtained from healthy individuals as the control group. Oocyte samples have been collected from IVF Center located in NEU Hospital in Nicosia, Cyprus. Experimental procedures, including cDNA synthesis and real time PCR, were performed in DESAM Research Institute laboratory.

2.1. Sample Collection and Size

Thirteen individuals were selected for this research. Seven of which included individuals with the PCO and six from healthy females, respectively. From each individual, oocytes were collected at meiosis II stage of development in the embryology laboratory of the IVF Center. Individuals were divided into two groups of PCO positive and control group (no sign of PCOS). Variables such as age and BMI were controlled in order to get a more reliable result. All the individuals were between 20 to 30 years of age and non-obese. Gene expression on these oocytes' samples were investigated for a selected group genes of AKT pathway. First stage of laboratory work included RNA extraction followed by cDNA synthesis. Duplicate real-time PCR had been performed for each of the 13 samples for *IRS1, IRS2, AKT1* and *AKT2* genes, respectively. In order to minimize the error and make sure there was no contamination during each PCR procedure a negative control with no cDNA was performed.

2.2. *In Vitro* Fertilization (IVF)

Antral follicle count was decided by performing ultrasound on the third day of menstrual cycle of candidates. Accepted range on size of follicles were set between 2-9mm and anything equal or more than 10mm was excluded from experiment. Beginning of the first ovarian stimulation techniques were started for four days till day 5 of menstrual cycle. First stage of stimulation, FSH hormone was given to individuals, considering the antral follicle count, the differences of age and BMI. To

follow up with the growth of follicle from day four onward ultrasound was performed daily. Following up on FSH treatment, GnRH hormone was given after observation of minimum 3 follicles with size of 14mm in diameter. Oocytes were collected for the experiment after around 35 hours into the ovulation cycle.

2.3. Laboratory Methods and Machineries

After collecting the oocytes, the first step was RNA extraction, for this stage Norgen's purification kit was used by following the given instruction by the manufacturers. After RNA extraction, in order to check the quality of extracted RNA samples, Nanodrop was used. In order to synthesize the cDNA reverse transcription method was performed on the RNA samples, for this stage Norgen's transcript first strand synthesis kit was used.1µl of oligo primer was added to 2 µl of hexamers in addition to 10µl of our extracted RNA. Prepared sample was centrifuged. Reverse transcriptase was added to mix with RNase inhibitors to deactivate RNase enzyme from cutting the RNA. For cDNA synthesis machine has been set to10 minutes at 25 degrees, 60 minutes at 50 degrees and last 5 minutes at 85 degree. Gene expression level of selected genes were measured by using real time PCR using the cDNA samples. Expression levels of four genes were studied in this experiment (IRS1, IRS2, AKT1 and AKT2). Primers that were used for PCR were designed by Assoc. Prof. Pinar Tulay. The sequences of the primers are shown in table 2.1 below and PCR condition have been listed in table 2.2. For master mix of the PCR, light cycler 480 SYBR was used in addition to 0.2 µl of final concentration of the forward and reverse primers. Comparative methodology was set to be ΔΔCt method. These measurments were collected with the help of the Rotar Gene Software. Houskeeping gene, ACTB, was used during the experiments.

Table 2.1

Sequence of Designed Primers and Their Melting Temperature

GENES	**PRIMERS SEQUENCES FORWARD**	**PRIMERS SEQUENCES REVERSE**
AKT1	GCTGGAGGACAATGACTACG	TTCTTGAGCAGCCCTGAAAG
AKT2	GCTGGAGGACAATGACTATGG	GAAGCGGATCTCTTCCATGA
IRS1	GGCCACCACTCTCATGTCTT	CTTGTGCTGGGGGTCCTC
IRS2	ACAAGCGCTTCTTCGTGCT	TTGATGTTCAGGCAGCAGTC

Table 2.2

PCR Stages and Conditions

	PCR Steps	**Temperature C^0 \ time (second)**	**Cycles**
Stages	Denaturing	95 / 30 sec	1
	Annealing	58 / 30 sec	50
	Elongation	72 / 30 sec	

2.4.Data and Statistical Analysis

GraphPad prism software has been used to prepare the graphs and do statistical analysis of this experiment. Anova test and t-test has been performed to gather the data.

CHAPTER III

Results

A total of thirteen oocytes were collected for this study. Seven were collected from patients of PCO and six from healthy individuals, respectively. Patients were given ID numbers and details of each individual are summarized in table 3.1. All the selected genes for this experiment were shown to be expressed in human oocytes. Table 3.2 illustrate the Nanodrop results for each candidate participated in the experiment. 260/280 ratio test can assess the purity level of nucleic acids, this value is different for DNA or RNA. A ratio around 2 is usually considered as pure RNA and if the results are considerably lower, it can illustrate the presence of contamination during the experiment.

Table 3.1

Information of Participants in the Study

Patient's ID	**PCOS**	**Maternal Age**	**BMI**
1	Yes	22	27
2	Yes	29	22
3	Yes	26	19
4	Yes	23	21
5	Yes	21	19
6	Yes	27	16
7	Yes	28	34
8	No	23	22
9	No	21	19
10	No	21	19
11	No	25	18
12	No	29	18
13	No	27	23

Table 3.2

Concentration of RNA Samples and the Purity of the Samples

Sample ID	**Concentration**	**260/280**
1	10	1.52
2	11	1.48
3	12.7	1.46
4	11	1.5
5	9.7	1.51
6	9.9	1.52
7	12.5	1.53
8	10.9	1.56
9	10.3	1.53
10	10	1.52
11	10.9	1.56
12	11.5	1.51
13	10	1.52

Real time PCR was used to measure the level of expression of our four genes in addition to the housekeeping gene from a total of 13 oocyte samples. Observed Ct values of genes is summarized in table 3.3.

Table 3.3

Mean Ct Values of Target and Housekeeping Gene (Continue)

Genes	**ACTB**	**IRS1**	**IRS2**	**AKT1**	**AKT2**
ID	Ct	Ct	Ct	Ct	Ct
1	34.96	31.74	26.23	26.26	26.28
2	36.63	28.49	26.30	26.10	24.08
3	33.52	26.88	25.62	25.65	25.14
4	34.42	29.69	25.29	25.66	25.74
5	31.42	31.59	25.50	25.69	26.57
6	35.98	30.48	25.67	25.81	26.63
7	28.85	30.75	25.38	25.92	25.77
8	34.78	30.48	26.18	25.85	26.19
9	35.63	30.76	25.68	25.75	25.78

10	39.35	29.85	25.90	25.80	27.77
11	37.60	28.66	39.22	25.58	25.70
12	36.64	30.36	30.60	25.70	25.97
13	33.89	32.50	28.34	25.72	25.54

The student's T-test was used to find the significancy of the results obtained by the experiment for PCO patients. For statistical investigation 2^ (-ΔΔCt) values of each gene was calculated that is shown in table 3.4.

Table 3.4

Individual 2^ (-ΔΔCt) of Target Genes and Housekeeping Gene

Genes	***IRS1***	***IRS2***	***AKT1***	***AKT2***
ID	2^ (-ΔΔCt)	2^ (-ΔΔCt)	2^ (-ΔΔCt)	2^ (-ΔΔCt)
1	1.981647012	90.30287407	88.4444678	87.22682667
2	59.99207388	273.7471245	314.4528716	1275.36988
3	21.21040113	50.79800733	49.75259946	70.85019899
4	5.643929629	119.1553567	92.20032941	87.22682667
5	0.189031688	12.87678027	11.28785926	6.13345509
6	9.624422657	269.9783669	245.0105395	138.784246
7	0.056983972	2.356588726	1.62079317	1.798382622
8	4.189273283	82.52160637	103.7307629	81.951588
9	6.219074932	210.3575808	200.3946246	196.2705629
10	153.9907625	2379.924349	2550.739761	651.0840563
11	104.4522674	0.069189647	883.2634482	812.7681173
12	16.52639328	13.99364604	417.8090696	392.5411258
13	0.557367196	9.96382719	61.25263487	69.39212298

2^ (-ΔΔCt) values were used to calculate the p value of each gene that can estimate the significance of the results and correlation between the expression level of the genes and PCOS. For calculation of p value Graph Pad Prism 9.3.1 was used and the results are summarized in table 3.5.

Table 3.5

P Value of Each Candidate Gene and Level of Significance.

Gene	**P Value**	**Statistical Significance**
IRS1	0.2421	NO
IRS2	0.3742	NO
AKT1	0.1317	NO
AKT2	0.5721	NO

According to table 3.5 and data given for p value, it can be observed that there is no significant difference in results of control and PCO group for all the target genes since the calculated p values are all larger than 0.05.

Figure 3.1 and 3.2 illustrate the fold change value of each candidate gene between two groups of control and PCO patients. Values and graph have been obtained from Graph Pad Prims software *via* Anova test.

Figure 3.1

Anova Results Showing the Fold Change Values for Target and Housekeeping Genes Among PCO Patients.

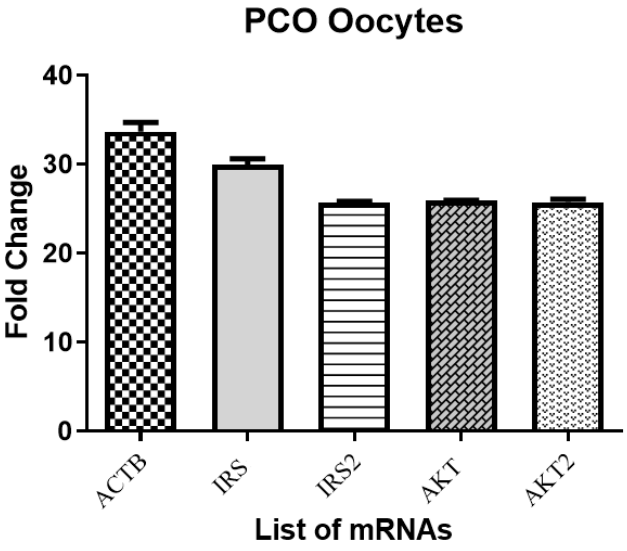
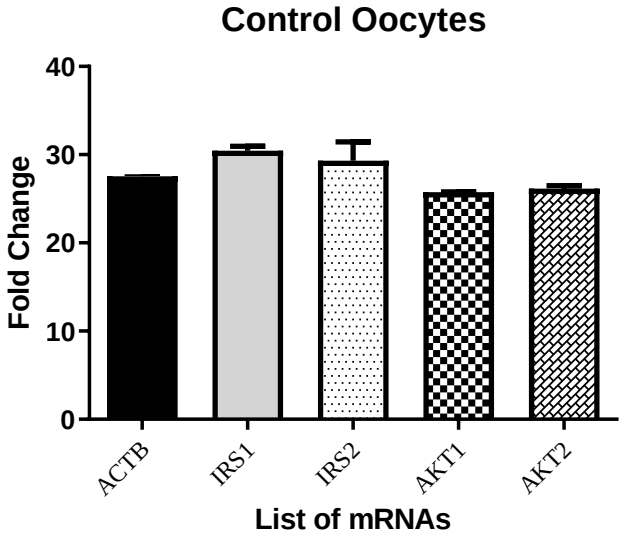


Figure 3.2

Anova Results Showing the Fold Change Values for Target and Housekeeping Genes Among Control Group



CHAPTER IV

Discussion and Conclusion

4.1. Discussion

PCOS can be categorized as one of the complex diseases among the female of childbearing age. PCOS can lead to difficulties in pregnancy and disturb metabolic reactions of the body. Main cause of PCOS is the abnormalities in hormonal levels, mostly androgenic hormones of females. Etiology of the disease is still a mystery to the day, though there are a number of research studies performed to find the genetic side of the PCOS.

In this study, four genes (*IRS1*, *IRS2*, *AKT1*, and *AKT2*) were selected from mTOR pathway to study the expression levels in oocytes obtained from the individuals with PCO. A total of 13 samples were collected which seven of them belongs to PCO patients and six to normal females, the control group. Results found in this research have shown no significant relationship between the expression levels of mentioned four genes and PCOS. Although there have been number of studies which has found some correlation between these genes and PCOS.

In a drug trial study done by a group of Chinese scientists on the effect of Rhizoma coptidis (extract of the medicinal plant Ranunculaceae) on development of PCOS, number of key targets were selected. Among these key targets *AKT1* and AKT2 can be observed. Results has shown that drug which has shown potential effect in clinical practice have very good binding ability with these key targets, therefore from this study it can be summarized that *AKT1* and *AKT2* can be actually correlated with development of PCOS (Duan et al., 2021). In another similar project, rat model was used to investigate effect of Rhizoma Curculiginis on PCOS. Morphology of the ovary was compared before and after the treatment in addition to Western blot and PCR to investigate *AKT1* gene as the candidate gene for PCO. Results of this study also have shown that expression level of the *AKT1* gene has changed in the rat model after the treatment which can show the relation between activity of this gene and PCOS (Liu et al., 2021).

As mentioned before one of the main reasons for development of PCOS is disturbance in normal level of androgenic activities in female bodies as well as insulin resistance in these individuals. In a study performed by Michael C Allemand and his team in 2009, effect of change in testosterone level on insulin activity of

body has been investigated. In this study, phosphorylation of serine amino acid on *IRS1* gene was studied. Myotubules of rats have been used as samples of studies. According to the findings of this project, relationship between elevated level of androgen and insulin with development of PCOS has been discovered, as well as involvement of IRS1 serine in development of insulin resistance among PCO patients (Allemand et al., 2009).

AKT2 has always been one of the candidate genes when it comes to the study of PCOS. Reason behind this persistency is that *AKT2* gene product is used for glucose metabolism which is therefore related to insulin resistance. Additionally, this gene is related to the cell survival in the ovary. In 2008, a study was performed to illustrate the relation between SNPs of *AKT2* gene and PCOS. Genotyping technique was used two study four different SNPs on the gene. Two SNPs on *AKT2* were found to be responsible for the increase risk of PCOS among the patients (rs3730051 and rs8100018). Each of this SNPs had p value less than 0.05 that shows their significance in the development of PCOS. A haplotype (T-G-C-T) was found to be responsible for even further enhancing the risk of PCOS development (Goodarzi et al., 2008).

In year 2012, a meta-analysis study had been performed on the relationship between SNPs of *IRS1* and *IRS2* gene and PCOS. The results showed that two SNPs, Gly972Arg and Gly1057Asp, were involved in PCOS (Ruan et al., 2012).

4.2. Conclusion

This project was performed to study the expression level of four selected genes from mTOR pathway. Collected oocytes from PCO patients and control individuals have been used to measure the mentioned gene expression level.

There have not been large number of studies done on the relationship between mTOR pathway genes and development of PCOS and this study can be mentioned as one of few articles on this matter. During this study *IRS1*, *IRS2*, *AKT1* and *AKT2* expression levels were compared between healthy and PCO patients. Results of this study after calculating student's T test showed that there is no significant correlation between expression of mentioned genes and development of PCOS. Although this does not mean that further study and research on these genes is not necessary. By

having some literature review studies, some correlation between these genes and PCOS have been observed that have been mentioned previously.

There have been certain limitations to this study that must be mentioned, since this There have been certain limitations to this study. Since this study was performed on oocytes obtained from MII stage of development, collecting eggs at this stage is very difficult and rare. Therefore, having a large sample size in order to increase reliability of this research seems highly unlikely. Therefore, having 13 samples only may be a limitation but taking this factor into account can overcome this issue. Another limitation to this study can be the lack of measurements of hormonal levels of each patient before initiation of the study and sample collection. In addition to controlling other variables such as diet, ethnicity and lifestyle choices such as level of exercise and smoking which can affect chances of development of PCOS.

In conclusion, after studying the target genes and the expression levels among the control and PCO patients, it has been observed that there is no significant relationship that can link these genes to development of the PCOS.

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ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi : 19.12.2019
Toplantı No : 2019/75
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Yakın Doğu Üniversitesi Tıp Fakültesi öğretim üyelerinden Doç. Dr. Pınar Tulay'ın sorumlu araştırmacısı olduğu, YDU/2019/75-920 proje numaralı ve **"Investigation of steroidogenesis related gene expression in human oocytes obtained from patients with polycystic ovaries"** başlıklı proje önerisi kurulumuzca değerlendirilmiş olup, etik olarak uygun bulunmuştur.

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T.R.N.C NEAR EAST UNIVERSITY INSTITUTE OF HEALTH SCIENCES EXPRESSION OF GENES INVOLVED IN AKT SIGNALLING PATHWAY IN HUMAN OOCYTES OBTAINED FROM PATIENTS WITH POLYCYSTIC OVARIES (PCOS) BY NOJAN HAFIZI Master of science in Medical Biology and Genetics Supervisor: Assoc.Prof.Pinar Tulay Nicosia, North Cyprus 2022 January T.R.N.C NEAR EAST UNIVERSITY INSTITUTE OF HEALTH SCIENCES EXPRESSION OF GENES INVOLVED IN AKT SIGNALLING PATHWAY IN HUMAN OOCYTES OBTAINED FROM PATIENTS WITH POLYCYSTIC OVARIES (PCOS) BY NOJAN HAFIZI Master of science in Medical Biology and Genetics Supervisor: Assoc.Prof.Pinar Tulay Nicosia, North Cyprus 2022 January Approval Abstract EXPRESSION OF GENES INVOLVED IN AKT SIGNALLING PATHWAY IN HUMAN OOCYTES OBTAINED FROM PATIENTS WITH POLYCYSTIC OVARIES (PCOS) NOJAN HAFIZI Master of science in Medical Biology and Genetics Supervisor:Assoc.Prof.Pinar Tulay AIM: This study has been designed to investigate the relationship between expression level of IRS1, IRS2, AKT1 and AKT2 of mTOR pathway with development of PCOS. BACKGROUND: PCOS is a common disorder among the females, etiology of the diseases is still unknown to the date. PCOS is known as an endocrine disorder and main reason for development of diseases is hormonal abnormalities of individuals, mainly androgenic. Studies has shown that prevalence of the disease can get to as high as 26% of female population in some areas. PCOS is categorized as a common complex disease meaning that both environmental and hereditary factors can cause formation of the disease. mTOR pathways belong to the family of protein kinase pathways. Main function of this pathway is to sustain cell growth and proliferation in addition to survival of cell. Any abnormality in the genes of this pathway can lead to crucial diseases such as cancer or diabetes. Disturbance in mTOR pathway has also been linked to changes in level of insulin and testosterone production in females which is the main reason for formation of PCOS. MATERIAL AND METHODS: Oocyte samples were collected at meiosis II stage of development from 13

individuals. 7 samples were from PCOS patients and the remaining 6 from healthy individuals with no sign of PCOS. First stage of process cDNA was obtained from the oocytes to use in RT-PCR. cDNAs were used to measure the expression level of four candidate genes (IRS1, IRS2, AKT1 and AKT2). RESULTS: Student's T test was used to measure the significant level of expression of each gene for PCOS patients. Sensitivity have been $p<0.05$. values gathered showed that there has been no significant correlation between gene expression of our candidate genes and PCOS since all the obtained values have been larger than 0.05. CONCLUSION: This study shows no correlation between expression level of IRS1, IRS2, AKT1 and AKT2 between PCOS and healthy group samples. KEYWORDS: mTOR, IRS1, IRS2, AKT1, AKT2, PCOS, PCR, oocyte Declaration I hereby declare that during this project, at all stages, including the laboratory work, there have been no unethical conducts. During the experimental process no living organism has been harmed and all sample collections have been done with fully consent of the candidates. All the gathered information from other sources have been fully cited and there are no misconducts of copyright while writing this article. Nojan Hafizi TABLE OF CONTENTS Cover page _____ Title page _____ Approval _____ Abstract _____ Declaration _____ Table of content _____ List of figures _____ List of tables _____ List of abbreviation _____ Acknowledgment _____ Chapter 1 _____ 1.1 Introduction 1.2 mTOR signaling pathway 1.3 Pathogenesis of PCOS 1.4 Epidemiology of PCOS 1.5 Symptoms 1.6 Diagnosis 1.7 Causes 1.8 Treatment 1.9 Complications 1.10 Genetics of PCOS 1.11 PCOS and weight gain 1.12 PCOS and infertility 1.13 Objectives 1.14 Significance Chapter 2_____ 2.1 Sample collection and size 2.2 In Vitro Fertilization (IVF) 2.3 Laboratory methods and machineries 2.4 Data and statistical analysis Chapter 3 _____ 3.1 Results Chapter 4 _____ 4.1 Discussion 4.2 Conclusion References _____ List of figures Figure 1.1: mTOR signaling pathway and functions Figure 1.2: Hormonal changes correlated with PCOS Figure 1.3: Common symptoms of PCOS Figure 1.4: Candidate genes of PCOS Figure 3.1: fold change values for candidate genes and housekeeping genes among PCO patients. Figure 3.2: fold change values for candidate genes and housekeeping genes among control group. List of tables Table 2.1: Sequence of primers and their melting temperature Table 2.2: PCR conditions Table 3.1: Detail of participants Table 3.2: RNA absorbance and concentration Table 3.3: Ct value of candidate genes and housekeeping gene Table 3.4: individual $2^{-\Delta\Delta Ct}$ of candidate genes and housekeeping gene Table 3.5: p value of each candidate gene and level of significancy. List of abbreviations PCOS: Polycystic Ovarian syndrome mTOR: Mammalian Target of Rapamycin mTORC1: Mammalian Target of Rapamycin Complex 1 mTORC2: Mammalian Target of Rapamycin Complex 2 TSC2: Tuberous Sclerosis Complex 2 AKT: Protein Kinase B IRS1: Insulin Receptor Substrate 1 PI3K: Phosphoinositide 3-Kinase LH: Luteinizing Hormone GnRH: Gonadotropin Releasing hormone NIH: National Institute of Health ASRM: American Society for Reproductive Medicine PCR: Polymerase Chain Reaction IVF: In Vitro Fertilization ACTB: Beta Actin BMI: Body Mass Index FSH: Follicle Stimulating Hormone Tm: Melting Temperature Ct: Cycle Threshold SNP: Single Nucleotide Polymorphism Acknowledgments Since the beginning of my project, world has gone through one of the most difficult times of its history, as a student I have struggled a lot during this pandemic but with the help of family, friends and my peers finally I have reached the end of my postgraduate path. Without the help and support of my family, I would have not been at this stage of my life, therefore I have to mention my loved ones as the main reason of my success, my mother, Mahparvar Firouzi, and father, Mohammad Hafizi, have been by my side since day one and of course my older sister, Nazgol Hafizi, that has helped me massively in my academic and personal life. I couldn't wish for a more supportive and lovely family and I'm so lucky to have all of you in my life. Also, I would like to thank my dear friend, Marwan Sider, which has helped me enormously during writing of my thesis, he has been more than a friend to me during this time. I need to show my outmost appreciation to my dear supervisor Assoc. Prof. Pinar Tulay that have showed me the path to success and without her help I wouldn't be able to succeed in my education. She has been more than just an advisor during this difficult time to me, she has always been a friend to me to help me get through this difficult time of pandemic and I personally will never forget her help in my life. At the end I need to thank Dr. Burcu Ozbakir for providing me with samples collected to make this research possible. CHAPTER 1 introduction 1.1 Introduction Polycystic ovarian syndrome or better known as PCOS is one of the most commonly appearing disorders of female reproductive system (Goodman et al., 2015). It is known as an endocrine disorder among the females. Studies has shown that probability of an individual having the disease in some populations can get as high as around 26%, although the rate of the disease is higher in some populations but still the etiology and the main cause of the disease has not been found yet (Muscogiuri, et al., 2017). Disorder has been named after one of the very common symptoms of it which is appearance of cysts structures on the ovarian wall, nevertheless, mentioned cysts are just one of many symptoms of the disorder and should not get confused as the cause of the disease (Dunaif & Fauser, 2013). PCOS belongs to the family of common complex diseases, both heritable factors and environmental factors can lead to the disorder (Diamanti-Kandarakis et al., 2006). Obesity, lack of exercise and bad diet are just some of the lifestyle and environmental factors that are well known as the risk factors of the disease, main focus of this study going to be on the genetic side of the disease and finding out about effect of candidate genes and their expression among the patients of PCOS (Sirmans & Pate, 2014). One of the very first appearances of the disease date back to 1721 in Italy. as mentioned previously, PCOS is an endocrine related disorder and among the female adults of age 18 to 44 this is the most common endocrine disorder found (Kovacs & Norman, 2007). several different causes of infertility have been found, but when infertility is related to inability of ovulation usually PCOS is the main cause (Khan et al., 2019). Patients of the PCOS experience an imbalance in their hormonal secretion which lead to disruption of the menstrual cycle, changing time or skipping cycles, three main hormones which are affected by the disease are androgens, insulin and progesterone. Androgens are mainly known as the male hormones but they can be found in female body in a smaller portion, but due to the imbalance in production of hormones caused by PCOS, level of androgen in PCOS females is higher than normal individuals, insulin production is also disrupted in PCOS patients which lead to changes in blood sugar and in more serious cases diabetes. Finally, progesterone imbalance is the main reason for the females to miss their periods or not being able to predict the time of it, level of this hormone in PCOS patients are usually lower than healthy individuals (Insler & Lunenfeld, 1990). 1.2 mTOR signaling pathway mechanistic target of rapamycin (mTOR) belongs to the family of protein kinases and is encoded by a gene with same name (Feinberg, 2007). mTOR belongs to two protein complexes known as mTORC1 and mTORC2. Each of these complexes of protein pathways function in different bodily processes, main function of mTOR can be seen during cell growth and proliferation, in addition to motility, survival and protein synthesis (Hanash, 2003). Deregulation of this specific signaling pathway has been correlated with diseases such as cancer and diabetes, due to its effect on the activation level of insulin receptors and insulin growth factor 1 receptors (Webber, et al., 2003). mTORC pathway like many other biochemical pathways of the body is closely interwind with other pathways, activation of Ras signaling pathway by growth factors can directly stimulate mTORC, when these pathways get stimulated TSC2 gene will get phosphorylated by protein kinase B also known as AKT, phosphorylated TSC2 now is deactivated therefore mTORC1 will get stimulated and becomes active, in addition to the mentioned pathway on activation of mTORC1, AKT activation also can directly activate mTORC1 in an independent manner towards TSC1/2 (Long et al., 2005). Prior to attachment of insulin to its receptor a series of reactions and activities will occur such as gathering of insulin receptor substrate 1 (IRS1) and activation of AKT. Promotion of mTORC1 has been connected to repression of PI3K-AKT and phosphorylation of IRS1 that can lead to instability of this gene (Inoki et al., 2003). Mentioned pathways are auto-regulated in the human body and has been correlated to metabolic disease and tumorigenesis, in comparison to mTORC1 that has shown significant correlation between its function and some diseases, a little has been discovered on the mTORC2 complex and effect of it on biology of individuals. Although with the hard work of scientists and

several research groups on this matter, today this complex is related to cell survival, metabolism, proliferation and cytoskeleton organization (Crino et al., 2006). Figure 1.1: part of mTOR signaling pathway and how it functions in processes such as cell growth, proliferation and angiogenesis (Jin Liu et al, 2018) 1.3 pathogenesis of PCOS level of androgenic hormones or better known as male hormones among the patients of PCOS is much higher compare to the healthy females, this excess amount of androgen in female body can lead to the formation of polycystic ovaries. Main androgen causing the disorder is testosterone but this hormone is not the only one, excess amount of luteinizing hormone (LH) and hyperinsulinemia (peak of insulin in blood) can also lead to the polycystic ovary (STRAUSS, 2003). Female ovaries tend to produce structures known as follicles. cysts that are formed in this disorder are actually follicles that have stopped growing and maturing and are known as arrested follicles. PCOS patients experience surge of LH and FSH ratio in addition to elevation of GnRH pulses (Hormone Health Network, 2018). Obesity is very common among the PCOS patients due to the formed resistance towards insulin, this can lead to increase in GnRH frequency, dominance of LH over FSH and loss of follicular maturation. Although it must be mentioned that this resistance towards insulin can be observed in normal weight women as much as overweight ones (Teede et al., 2010). PCOS has been associated with chronic inflammation as well as elevated level of oxidative stress (Murri et al., 2013). An example in which effect of testosterone level on appearance of PCOS can be clearly seen among transgender men that are going through testosterone therapy and prevalence of PCOS among these individuals is much higher than usual, only due to the excess amount of testosterone in their body (Puffer, 2006). Figure 1.2: some of the hormonal changes correlated with development of PCOS (Bassim Alsadi, 2018) 1.4 epidemiology of PCOS the appearance rate of disease can actually vary depending on diagnosis criteria and one country to other, according to WHO around 116 million females are suffering from the PCOS disorder, that makes up around 3.4% of the female population around the world (Vos et al., 2010). Another study has predicted that about 7% of women after puberty are affected, in comparison according to the Rotterdam criteria around 18% of females are affected and of that 18%, 70% have already been diagnosed. Some countries have a better methodology and resources for their researchers that's why the prevalence rate might differ from a country to another due to lack of reliable research and data, as an example India is showing 20% of female population is affected by the PCOS (Pruthi, 2019). Researches also shows that between 8-25% of women whom are not affected by PCOS shows presence of cysts in their ultrasonographic tests (Van Santbrink et al., 1997), another correlation has been found between oral contraceptives and prevalence of PCOS, according to researches 14% of females on this drug are affected by the disease, intrauterine devices are also a common risk factor for appearance of the disorder among young females (Clayton et al., 1992). 1.5 Symptoms PCOS like many other disorders have wide range of symptoms and some of those may not even seem warning, symptoms such as oily skin. Therefore, very commonly takes many years for individuals to diagnose the disease, cause symptoms are not that troubling and also majority of cases, symptoms are not shown for a long period of time (Nazario, 2020), here we are going to mention some of the common signs and symptoms that can be alarming and pointing towards the disease, but must be mentioned that having these symptoms is not a fact on presence of disease since some of them are very common among different disorders. Growth of unwanted hair is one of the symptoms of the disease, for female's presence of hair on face, chin and breast can be sign of PCOS, next to presence of unwanted hair, in addition, hair on head can get thinner and loss of hair can happen that can get worse among middle age women. PCOS can lead to changes in texture and color of the skin that can be one of the main symptoms and signs of the disease, presence of acne due to hormonal changes and appearance of thick patches of skins in different areas such as neck and breast is another skin abnormality followed by the disorder, although mentioned skin conditions can appear even individual is not affected by PCOS. Ovary is one of the main sections of female reproductive system that function in production and release of mature oocyte, therefore an anomaly in it such as presence of cyst can cause massive problems for reproductive system and menstrual cycle of the females. This can lead to heavy or irregular period as well as trouble getting pregnant. Weight gain and struggling for losing weight is one of the signs of PCOS in females, next to psychological effects of gaining weights, being overweight can worsen other symptoms of the disease, that's why it is highly recommended to the PCOS patient to have a healthy diet and exercise regularly in order to lose the weight gained due to the disorder to keep other symptoms in a less disrupting level. Also losing weight can help with the changes on the level of cholesterol and blood glucose that can rise due to PCOS (Insler & Lunenfeld, 1990). PCOS can have risks for the individual even after going though treatment and cure of the disorder, one of the risks of later in life for these patients is type 2 diabetes, in addition to that, depression and mood swing, high cholesterol that can end up with stroke and cardiac diseases and sleep apnea due to the gain of weight are couple of more available symptoms of PCOS patients (Nazario, 2020). Figure 1.3: some of the common symptoms among PCOS patients (Kecia Gaither, 2021, eMedihealth) 1.6 Diagnosis as mentioned before, disorder has adopted its name from one of the main signs of it which is the presence of numerous cysts on the ovary of the patients but there are several cases that cysts are not present, likewise in some cases cysts are available but individuals are not diagnosed of PCOS (Marrinan, 2019). There have been two criteria for the diagnosis, first one stablished by the NIH in 1990 and second one by ASRM workshop in Rotterdam in 2003. According to NIH agreement on criteria of diagnosis, 3 signs must be present for the person to be diagnosed with the disorder, 1. Having irregular period cycles, normal individuals usually get their period every 25-30 days but female of PCOS may get their period only 8 times per year this anomaly is known as oligoovulation 2. Hormonal changes, specifically changes in androgen hormone and excess amount of it 3. Making sure that symptoms are not due to similar diseases (Chang et al., 2004). Like NIH agreement first two criteria are oligoovulation and level of androgen hormones, Rotterdam agreement include presence of cysts on ovary as one of the diagnosis signs as well (Hart et al., 2004). 1.7 Causes PCOS is a complex disease therefore the exact cause of the disease is still a mystery to the scientists, although changes in the hormonal level of individuals have been related to the development of PCOS. One of the main hormones that help body to keep the blood sugar level in control is insulin. An imbalance in the production of this hormone has been noticed among PCOS individuals. This hormone has the function of carrying glucose as energy source from blood to the body cells to provide them with the source of energy, (Legro & Strauss, 2002) PCOS patients usually develop a resistance towards this hormone, as a result, tissue repel the hormone and body automatically try to produce more to keep up with the demand of cell for energy. Excess amount of this hormone in our circulation can directly affect the production testosterone hormone in ovaries of females. Testosterone is usually found in male body and very little of it can be found in females, therefore increase in level of this hormone can interfere with ovulation in female bodies. Insulin is not the only hormone related to the disease. Increase in luteinising, prolactin and testosterone hormone, low amount of sex hormone-binding globulin has all shown correlations with development of PCOS (Filippou & Homburg, 2017). At the genetics point of view, PCOS has been categorized as an autosomal dominant disease with high expressivity among the female individuals (Hamosh, 2011). Faulty gene can be inherited form either of parents, but in male newborns faulty gene does not lead to the disease so basically, they are carrier or in some cases early baldness can be observed among males. But f the faulty gene is inherited to the female newborn then child will develop PCOS to some extent (nhs, 2019). Next to hormonal problems or genetic abnormalities, environmental factors can increase the risk of disease as well. Exposure to different chemicals and drugs at different stages of the life can increase risk (Draper et al., 2003). Exposure to endocrine disruptors most famously bisphenol A that can alter the normal function of endocrine system of the human body are one of the most dangerous environmental factors for appearance of PCOS. These chemicals act in the body as estrogen hormone therefore bodily production of estrogen hormone drops massively and can damage the reproductive system of the male leading to PCOS (Faghfoori et al., 2017). 1.8 Treatment PCOS is a non-curable disease but with some lifestyle changes, certain drugs and surgeries symptoms can be controlled and life quality of patients can be improved. Treatment techniques vary from patient to patient due to the different levels of expressivity in individuals, in some patient's symptoms might be much worse compare to next patient. Main lifestyle changes that can help PCOS patients massively is regular exercise and having healthy diet, due to the imbalance of hormones such as insulin among these patients, increase in bodyweight is very common that can worsen the symptoms and health problems, therefore having regular exercise and healthy diet will avoid massive weight gain and improve health of patient. Different drugs have been developed for each symptom of PCOS, for example for irregular menstrual cycle, contraceptive pills or methods such as intrauterine system might be prescribed to patients. For fertility problems drugs such as

clomiphene, metformin or IVF treatment is suggested to patients. Another symptom of disease is unwanted hair growth or loss, to treat these issues certain drugs such as cyproterone acetate, spironolactone, flutamide and finasteride is developed. For patients that drug treatment is no working, surgical methods is used to treat fertility problems, this techniques is known as laparoscopic ovarian drilling, laparoscope which is a tiny microscope will be entered to the ovary through a cut on the stomach and with help of heat or laser tissues that are malfunctioning and producing male hormones will be destroyed, therefore this procedure will lead to decrease level of testosterone and luteinising hormone as well as an increase in the follicle-stimulating hormone in the patients. As a result, hormonal balance of the body will be restored so the ovulation process can occur normally and fertility is achieved (nhs, 2019,b). 1.9 Complications PCOS can bring about couple of different complications with it to the individuals, these must not be confused as the symptoms of the disease. As mentioned before changes in hormonal level and appearance of cysts at ovary can lead to troubles for pregnancy of the female due to malfunction of ovaries in releasing eggs during ovulation. Another complication due to PCOS is development of diabetes in these patients due to resistance toward insulin blood sugar increase significantly and can lead to diabetes. PCOS also has been related to several cardiovascular diseases, etiology of them is due to increase in triglyceride and high-density lipoprotein of blood as well as high blood pressure that can lead to atherosclerosis. If we want to mention couple of other complications of disease we can mention about depression, anxiety, bleeding from uterus that can increase risk of uterine cancer, sleep problem and inflammation of liver (Pathak, 2021). 1.10 Genetics of PCOS Relating a single gene to complex diseases such as PCOS is impossible that why when genetic aspect of PCOS is under study different pathways and different genes must be considered. Genome screening or linkage analysis usually led to failure for very complex diseases such as PCOS that's why in order to be able to find a link between a certain gene and the disease genome-wide association studies must be done (Hamosh, 2011). Due to multifactorial characteristics of the disease multiple pathways with each having specific genes to them can be related to PCOS. CYP11a, CYP21 and CYP17 are some of the genes that related to the disease due to their function in production of androgen hormones (Franks et al., 2000). Another pathway that mutation in its genes can lead to PCOS is regulation of gonadotropin, LH receptor gene and FSHR gene are examples of it (Gromoll Simoni, 2005). One of the most important genes for this study insulin receptor substrate proteins (IRS1 and IRS2) that function in regulation and production of insulin in body, according to Petermann et al level of Arg92 IRS1 is higher in PCOS patients (Sir-Petermann et al.,2001), in contrary, El Mkadem et al result shows no correlation and change between the control and PCOS group (El Mkadem et al., 2001). Another study done on relation of Gly972ARG in IRS1 by Dilek et al illustrate that there is a higher level of this matter among Turkish females with the disease (Dilek et al., 2005). These changes in result are actually proving the complexity of the disease and how environmental and epigenetic factors play role in the disease. Figure 1.4: candidate genes for PCOS and how they can lead to development of disorder (Khan et al, 2019) 1.11 PCOS and weight gain Due to the abnormalities on the level of certain hormones such as insulin in body of PCOS patients weight gain is a very common symptom, in spite of appearance concerns for females about gaining weight, this issue can lead to severe health problems such as diabetes, heart problems and uterine cancer. Due to insulin resistance of these females, blood glucose level increase which cause increase on production of male hormones. High level of androgens in body is the main reason behind weight gain of PCOS patients and since its due to male hormones most of the weight gain appears in abdomen which is the most dangerous fat deposit since its correlated with heart disease. Research has shown only 10% cut on the bodyweight can bring back normal periods for females of PCOS and help with other issues such as insulin sensitivity. In addition to medicines that can aid to control the hormonal problems, lifestyle choices can be of huge help such as having a healthy diet rich with fiber, increasing number of meals per day instead of eating huge meals, exercising regularly for at least half an hour daily (Pagano, 2020). 1.12 PCOS and infertility Hormonal abnormalities followed by PCOS can lead to infertility of the females, PCOS is known as one of the most common reasons for fertility problems among the females. Due to the disease ovaries enlarge in size that carry several numbers of cysts. High level of androgen in females can interfere with ovulation, if there is no egg released by time of ovulation therefore there is no chance of fertilization between egg and sperm so fertility cannot be achieved, in addition to problem with production of eggs, one of the very first signals on presence of PCOS can be missing or having irregular menstrual cycles. Medicines such as birth control pills can aid with irregular menstrual cycles and for ovulation problems drugs such as metformin and clomiphene can be helpful (Traci, 2021). 1.13 Objectives Aim of this study is to investigate mTOR and AKT pathway genes and their effect on development of PCOS in females. Several genes belonging to these pathways have been connected as candidate genes for PCOS such as AKT1, AKT2, IRS1 and IRS2 that has been studied in this research. Samples have been divided into two groups of PCOS patients and control in order to study differences in gene expression of mentioned genes in these two groups to find correlation between mutation in genes and development of PCOS. 1.14 Significance Finding the out about the molecular basis of the PCOS can help doctors and scientists to detect the disease earlier and improve the chances of successful treatment. Unfortunately, there have not been wide area of studies on this matter, therefore studies like this can be of importance. This study has been done with the hope to shed some light on these dark areas of the disease and help patients prevent the development of disease by finding candidate genes correlated to PCOS. CHAPTER 2 Material and methods The samples were collected from Near East University Hospital and RNA was already obtained from these samples (Ethical approval was granted YDU/2019/75- 920). Research has been designed with the aim to find the relation between expression level of the genes of AKT pathway (IRS1, IRS2, AKT1 and AKT2) collected from two different groups of individuals, one group of PCOS patients and the other healthy individuals as control group. Oocyte samples have been collected from IVF center located in NEU hospital in Nicosia, Cyprus. Experimental procedures such as cDNA synthesis and real time PCR, DESAM laboratory have been used. 2.1 sample collection and size 13 individuals have been selected for this research, 7 of which are individuals with the PCOS and 6 are healthy females. From each individual an oocyte has been collected at meiosis II stage of development in IVF center. Individuals have been divided into two groups of PCOS positive and control group (no sign of PCOS). Variables such as age and BMI have been controlled in order to have get a more reliable result, all sample are between 20 to 30 years of age and non-obese BMI range. Gene expression on these oocytes' samples have been investigated for certain genes of AKT pathway. First stage of laboratory work includes RNA extraction followed by cDNA synthesis. Duplicate real-time PCR had been done for each of the 13 samples for IRS1, IRS2, AKT1 and AKT2 genes. In order to minimize the error and make sure there is no contamination in the research during each PCR procedure negative control with no cDNA has been performed. 2.2 in vitro fertilization (IVF) Antral follicle count was decided by performing ultrasound at third day of menstrual cycle of candidates. Accepted range on size of follicles were set between 2-9mm and anything equal or more than 10mm was excluded from experiment. Beginning of the first ovarian stimulation techniques were started for four days till day 5 of menstrual cycle. First stage of stimulation, FSH hormone was given to individuals, taking into account their differences of age and BMI. To follow up with the growth of follicle from day four onward ultrasound was performed daily. Following up on FSH treatment, GnRH hormone was given after observation of minimum 3 follicles with size of 14mm in diameter. Oocytes were collected for the experiment after around 35 hours into the ovulation cycle. 2.3 laboratory methods and machineries 13 individuals were selected as the study group for this experiment, 7 of which have been diagnosed with PCOS and 6 normal healthy individuals as the control group. Near East University DESAM lab have been used to perform the experiments. First step of experimental procedure after collecting the oocytes has been RNA extraction, for this stage Norgen's purification kit has been used by following the given instruction by the manufacturers. After RNA extraction, in order to check the quality of extracted RNAs, Nanodrop method was used. In order to synthesize the cDNA reverse transcription method was performed on the RNA samples, for this stage Norgen's transcript first strand synthesis kit was used. Gene expression level of selected genes were measured by using real time PCR machine and cDNAs that have been transcribed on the previous stage. expression of four genes have been studied in this experiment (IRS1, IRS2, AKT1 and AKT2) Primers that have been used for PCR were designed by Assoc. Prof. Pinar Tulay. The sequence of the primers can be found in table 1 below and PCR condition have been listed in table 2. For master mix of the PCR method light cycler 480 SYBR was used in addition to 0.2 μM of designed primers. Comparative methodology was set to be ΔΔCt method, these measurments were collected with the help of the Rotar Gene Software. Houskeeping gene during the experiment has been set

to be ACTB gene. TABLE 2.1: sequence of designed primers and their melting temperature Genes Primers sequences Forward Primers sequences Reverse Tm (C0) AKT1 AKT2 IRS1 IRS2 TABLE 2.2 : PCR stages and conditions PCR Steps Temperature C0 time (second) \ Cycles Denaturing 95 / 30 sec 1 Stages Annealing Elongation 58 / 30 sec 72 / 30 sec 50 2.4 data and statistical analysis GraphPad prism software has been used to prepare the graphs and do statistical analysis of this experiment. CHAPTER 3 RESULTS 13 oocytes have been collected for investigation, 7 from patients of PCOS and 6 from healthy individuals. Patients have been given ID numbers and details of each individual is summarized in table 3 below. All the selected genes for this experiment have shown level of expression in human oocyte. Table 4 illustrate the nanodrop results for each candidate participated in the experiment. TABLE 3.1: information of participants in the study Patient's ID PCOS Maternal Age BMI 1 Yes 22 27 2 Yes 29 22 3 Yes 26 19 4 Yes 23 21 5 Yes 21 19 6 Yes 27 16 7 Yes 28 34 8 No 23 22 9 No 21 19 10 No 21 19 11 No 25 18 12 No 29 18 13 No 27 23 TABLE 3.2: level of absorbance of RNA and their concentration level Sample ID concentration 260/280 1 10 1.52 2 11 1.48 3 12.7 1.46 4 11 1.5 5 9.7 1.51 6 9.9 1.52 7 12.5 1.53 8 10.9 1.56 9 10.3 1.53 10 10 1.52 11 10.9 1.56 12 11.5 1.51 13 10 1.52 Real time PCR have been used to measure the level of expression of our 4 candidate genes in addition to our housekeeping gene from total of our 13 samples oocytes. Observed Ct values of genes is summarized in table 3.3. TABLE 3.3: Ct value of candidate genes and housekeeping gene Genes ACTB IRS1 IRS2 AKT1 AKT2 ID Ct Ct Ct Ct Ct 1 34.96 31.74 26.23 26.26 26.28 2 36.63 28.49 26.30 26.10 24.08 3 33.52 26.88 25.62 25.65 25.14 4 34.42 29.69 25.29 25.66 25.74 5 31.42 31.59 25.50 25.69 26.57 6 35.98 30.48 25.67 25.81 26.63 7 28.85 30.75 25.38 25.92 25.77 8 34.78 30.48 26.18 25.85 26.19 9 35.63 30.76 25.68 25.75 25.78 10 39.35 29.85 25.90 25.80 27.77 11 37.60 28.66 39.22 25.58 25.70 12 36.64 30.36 30.60 25.70 25.97 13 33.89 32.50 28.34 25.72 25.54 The student's T-test was used to find the significancy of the results obtained by the experiment for PCO patients. For statistical investigation 2^ (-ΔΔCt) values of each gene was calculated that can be find in table 3.4. TABLE 3.4: individual 2^ (-ΔΔCt) of candidate genes and housekeeping gene Genes IRS1 IRS2 AKT1 AKT2 ID 2^ (-ΔΔCt) 2^ (-ΔΔCt) 2^ (-ΔΔCt) 2^ (-ΔΔCt) 1 1.981647012 90.30287407 88.4444678 87.22682667 2 59.99207388 273.7471245 314.4528716 1275.36988 3 21.21040113 50.79800733 49.75259946 70.85019899 4 5.643929629 119.1553567 92.20032941 87.22682667 5 0.189031688 12.87678027 11.28785926 6.13345509 6 9.624422657 269.9783669 245.0105395 138.784246 7 0.056983972 2.356588726 1.62079317 1.798382622 8 4.189273283 82.52160637 103.7307629 81.951588 9 6.219074932 210.3575808 200.3946246 196.2705629 10 153.9907625 2379.924349 2550.739761 651.0840563 11 104.4522674 0.069189647 883.2634482 812.7681173 12 16.52639328 13.99364604 417.8090696 392.5411258 13 0.557367196 9.96382719 61.25263487 69.39212298 2^ (-ΔΔCt) values have been used to calculate the p value of each gene that can estimate the significancy of the results and correlation between expression of the gene and PCOS. For calculation of p value Graph Pad Prism 9.3.1 has been used and the results have been summarized in table 3.5. TABLE 3.5: p value of each candidate gene and level of significancy. Gene P Value significancy IRS1 0.2421 NO IRS2 0.3742 NO AKT1 0.1317 NO AKT2 0.5721 NO According to table 3.5 and data given for p Value it can be observed that there is no significant difference in results of control and PCOS group for all our candidate genes since the calculated p values are all larger than 0.05. Figure 3.1 and 3.2 illustrate the fold change value of each candidate gene between two groups of control and PCO patients. Values and graph have been obtained from Graph Pad Prims software. PCO Oocytes 40 Fold Change 30 20 10 0 A C T B I RS! IRS2 T ! A K K T 2 A List of mRNAs Figure 3.1: fold change values for candidate genes and housekeeping genes among PCO patients. Control Oocytes 40 Fold Change 30 20 10 0 C T B IRS1 IRS2 T 1 T 2 A A K A K List of mRNAs Figure 3.2: fold change values for candidate genes and housekeeping genes among control group. Chapter 4 Discussion and conclusion 4.1 discussion PCOS can be categorized as one of the complex diseases among the female of childbearing age, it can lead to difficulties in pregnancy and disturb metabolic reactions of the body. Main cause of the disease is abnormalities in hormonal levels mostly androgenic hormones of females. Etiology of the disease is still a mystery to the day. Although there have been number of researches done trying to find the genetic side of the disease. In this study, four genes (IRS1, IRS2, AKT1, AKT2) were selected from mTOR pathway to study their expression level among the individuals with PCOS. 13 samples were collected which 7 of them belongs to PCO patients and 6 to normal females known as control group. Results found in this research have shown no significant relationship between expression level of mentioned four genes and development of PCOS. Although there have been number of studies which has found some correlation between these genes and the disease. In a drug trial study done by a group of Chinese scientists on the effect of Rhizoma coptidis (extract of the medicinal plant Ranunculaceae) on development of PCOS, number of key targets were selected. Among these key targets AKT1 and AKT2 can be observed. Results has shown that drug which has shown potential effect in clinical practice have very good binding ability with these key targets, therefore from this study it can be summarized that AKT1 and AKT2 can be actually correlated with development of PCOS (Duan et al., 2021). In another similar project, rat model was used to investigate effect of Rhizoma Curculiginis on PCOS. Morphology of the ovary was compared before and after the treatment in addition to Western blot and PCR to investigate AKT1 gene as the candidate gene for PCOS. Results of this study also have shown that expression level of the AKT1 gene has changed in the rat model after the treatment which can show the relation between activity of this gene and PCOS (Liu et al., 2021). As mentioned before one of the main reasons for development of PCOS is disturbance in normal level of androgenic activities in female bodies as well as insulin resistance in these individuals. In a study done by Michael C Allemand and his team in year 2009, affect of change in testosterone level on insulin activity of body has been investigates, during this study phosphorylation of serine amino acid on IRS1 gene have been studied. Myotubules of rats have been used as samples of studies. According to the findings of this project, relationship between elevated level of androgen and insulin with development of PCOS as well as involvement of IRS1 serine in development of insulin resistance among PCOS patients (Allemand et al., 2009). AKT2 has always been one of the candidate genes when it comes to the study of PCOS, reason behind this persistency is that AKT2 gene product is used for glucose metabolism which is therefore related to insulin resistance, also this gene is related to the cell survival in ovary. In 2008 a study was done to illustrate the relation between SNPs of AKT2 gene and PCOS. During this study candidate have been divided in to two groups of PCO patients (287 patients) and control group (187 healthy females). Genotyping technique was used two study four different SNPs on the gene. Two SNPs on AKT2 were found to be responsible for the increase risk of PCOS among the patients (rs3730051 and rs8100018). Each of this SNPs had p value less than o.o5 that shows their significancy in the experiment. During research a haplotype (T-G-C-T) was found to be responsible for even further enhancing risk of PCOS development among patients (Goodarzi et al., 2008). In year 2012 a meta-analysis study had been done on the relationship between SNPs of IRS1 and IRS2 gene and PCOS, 21 PubMed and 26 EMBASE published articles were used for this literature review project. From these articles only 16 were selected which were related to IRS1 and IRS2 and PCOS. During the research effect of two SNPs were studies, firstly Gly972Arg and secondly Gly1057Asp polymorphisms. As a conclusion of the results, it can be observed that Gly972Arg is correlated with PCOS and it is a significant risk of disease. On the other hand, due to small number of studies on the second polymorphism as well as limited number of different ethnicities that have been studied, no significant relationship was observed between this polymorphism and PCOS. Further studies must be done on this polymorphism in order to be able draw a linkage for it with PCOS (Ruan et al., 2012). 4.2 conclusion This project has been done with the aim to study the expression level of four selected genes from mTOR pathway. Collected oocytes from PCOS patients and control individuals have been use to measure the mentioned gene expression level. There have not been large number of studies done on the relationship between mTOR pathway genes and development of PCOS and this study can be mentioned as one of few articles on this matter. During this study IRS1, IRS2, AKT1 and AKT2 expression level have been compared between healthy and PCOS patients. Results of this study after calculating student's T test have shown that there is no significance correlation between expression of mentioned genes and development of PCOS. Although this does not mean that further study and research on this gene is not necessary. By having some literature review studies, some correlation between these genes and PCOS have been observed that have been mentioned in discussion part of article. There have been certain limitations to this study that must be mentioned, since this study has been done on oocytes obtained from MII stage of development, collecting eggs at this stage is very difficult and rare therefore having a large sample size in order to increase reliability of this research seems an impossible job, therefore having 13 samples only may be a limitation but taking this factor

into account can overcome this issue. Another limitation to this study can be lack of measurements of hormonal levels of each candidate before initiation of study and sample collection, in addition to controlling other variables such as diet, ethnicity and lifestyle choices such as level of exercise and smoking which can affect chances of development of PCOS. In conclusion, after studying the candidate genes and their expression among control and PCOS patients, it has been observed that there is no significant relationship that can link these genes to development of the PCOS. References Allemand, M. C., Irving, B. A., Asmann, Y. W., Klaus, K. A., Tatpati, L., Coddington, C. C., & Nair, K. S. (2009). Effect of testosterone on insulin stimulated IRS1 Ser phosphorylation in primary rat myotubes—a potential model for PCOS-related insulin resistance. PloS one, 4(1), e4274. Chang, J., Azziz, R., & Legro, R. (2004). Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group (2004). 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Patents

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9. Administrative designations

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11. Awards and grants

12. Courses taught over the last two academic years

Academic Year	Term	Course Name	Hours/week		Number of Students
			Theoretical	Applied	
	Fall				
	Spring				