NEAR EAST UNIVERSITY

INSTITUTE OF HEALTH SCIENCE

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AYA BADEEA ISMAIL

MASTER THESIS MOLECULAR MEDICINE PROGRAM

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Assoc. Prof. MAHMUT ÇERKEZ ERGÖREN

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ACCEPTANCE/APPROVAL

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This work has been adopted as a master thesis in the program of Molecular Medicine by the jury.

Examining Committee in Charge:

Jury Member (Supervisor): Assoc. Prof. Mahmut. C. Ergoren

Jury Member: Prof. Gamze Mocan

Jury Member:Assist. Prof. Özel Yürüker

Approval:

This thesis has been approved by the above jury members in accordance with the relevant articles of the NEU post graduate education, training and examination regulations and has been accepted by the decision of the board of the Institute.

Prof.Dr.Hüsnü Can Başer

Director of Institute of Health and Science

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: AYA .B.ISMAIL

Signature:

Date:

COMPLIANCE AND APPROVAL

YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

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Prof. Dr. Rüştü Onur
Yakın Doğu Üniversitesi
Bilimsel Araştırmalar Etik Kurulu Başkanı

DEDICATION

This thesis is dedicated to:

- My Parent's in low Dr. Saib .M. Zangana and Dr. Hutham.W. Albayatyfortheirunconditionalloveand moral support.

- My Mother Mrs. Kani.Y.Ali for her endless love and constant encouragement.

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LIST OF ABBREVIATION

μl: Microliter

μM: Micromolar

nM: Nanomolar

bp: Base pair

β: Beta

PCOS: Polycystic ovarian syndrome

PCOM: Polycystic ovarian morphology

LH: Luteinizing hormone

ACTH: Adrenocorticotropic hormone

FSH: Follicular stimulating hormone

FSHR: Follicular stimulating hormone receptor

BMI: Body mass index

AE – PCOS: Androgen excess – polycystic ovary society

CYP17: Cytochrome p450 c17

DM: Diabetes mellitus

NIH: National institute of health

ESHRE: European society of human reproductive and embryology

ASRM: American society of reproductive medicine

ROT: Rotterdam Criteria

TTTTA: Promoter penta nucleotide

AXIN2: Axis inhibition Protein

TCF4: Trascriptor Factor 4

FZD4: Frizzled class receptor 4

FZD3: Frizzled classreceptor 3

WNT5A: Wnt family member 5 A

DVL1: Dishevelled 1(homologous to drosophila dsh)

DKK3: Dickkopf related protein 3

cDNA: Complementarydeoxyribonucleic acid

RNA: Ribonucleic acid

PCR: Polymerase chain reaction

qRT- PCR: Quantitative reverse transcriptase – polymerase chain reaction

NTC: No- template control

Ct: Cycle threshold

CVDs: Cardiovascular Diseases

EDCs: Endocrine disturbing chemicals

BPA: Bisphenol

GnRH: Gonadotropin release hormone

AR: Androgen receptor

FTO: Alphaglutate dependent dioxygenase

SNP: Single nucleotide polymorphism

DHEAS: Dehydroepiandrosterone sulfate

FDA: Food and drug administration

TCF: T- cell factor

LEF: Lymphoid enhancing factor

LRP: Lipoprotein receptor- related protein

RYK 7: Atypical receptor related tyrosine kinase

PTK7: Protein tyrosine kinase7

ROR2: Receptor tyrosine like orphan receptor 2

IVF: In vitro fertilization

TBE: Tris borate EDTA.

GSK3: Glycogen synthesis kinase 3

APC: Adenomatous polyposis coli

CKIα: Casein kinase I-α

ABSTRACT

THE EXPRESSION PROFILE OF *AXIN2, FZD4, TCF4,* AND *WNT5A* GENE IN HUMAN OOCYTS OBTAINED FROM POLYCYSTIC OVARIES SYNDROME PATIENTS (PCOS)

AYA BADEEA ISMAIL

MOLECULAR MEDICINE

THESIS ADVISOR

Assoc. Prof. MAHMUT ÇERKEZ ERGÖREN

AIM:

This study was conducted to investigate the expression levels of Wnt–signaling pathway related genes *AXIN2, FZD4,TCF4* and *WNT5A*that were suspected to cause a noteworthy impact in the development of ovaries and oogenesis.

BACKGROUND:

Polycystic ovarian syndrome (PCOS) is a chronic hormonal turmoil that is demonstrated in 2.2%-27% of woman in their pre-menopausal age. It’s due to excessive androgen expression along with genetic susceptibility and environmental influences. This syndrome is exhibited with ovulatory dysfunction, acne, hirsutism and menstral disorder. Other associated disorders of this disease are Type II diabetes mellitus, cardiovascular disease (CVDs),

Endometrial Cancer as well as 40% of female infertility. The diagnosis of the syndrome is reposed on three sets of criteria (NIH) in 1990, (ESHRE/ASRM). Or commonly known as Rotterdam criteria in 2003. And the third set is (AE-PCOS) in 2006. Treatment of the syndrome is based on oral- contraceptives, insulin-sensitizers, or anti-androgens used in off-labeled system.

A Wnt signal transduction pathway is a classical evolutionary pathway that regulates aspects of cell proliferation, migration and cell fate-determination in the tissue along with early embryonic development. It is consisted of a family of lipid modified glycoproteins that help the attachment of Wnt protein to Wntless proteins and transporting them to the plasma membrane for secretion. There are three types of Wnt signaling pathways.Canonical Wnt-signaling pathway (β-catenin dependent pathway), Non-canonical Wnt-signaling Pathway (β-catenin Independent pathway) and Non-canonical Wnt-signaling Pathway/ Calcium pathway. Their activation is through interaction of Wnt protein with different member of the frizzled family receptors. These pathways have been implicated in the development of several types of chronic illnesses.

METHOD:

In this study Human oocyte collection was obtained from the IVF center and laboratory of the Near East University Hospital (NEUH). After the approval of NEU scientific review Board. 13 samples were collected from non-obese and young woman. Seven of these samples were from polycystic ovarian syndrome patients (1, 2, 3, 6, 7, 8, and 11) and were categorized as PCOS group. While the other six samples (4, 5, 9, 10, 12, and 13) were from healthy individuals

and were categorized as Control group. This research was carried out in the Near East University DESAM Institute Molecular Medicine Laboratory. After RNA extraction its concentration and purity were estimated by Nano-drop. Fallowed by cDNA synthesis carried out by trascriptor first strand cDNA synthesis kit. Gradient Polymerase chain reaction was performed by applied bio systems thermal cycler PCR.

Then, RT- qPCR was performed by Rotar Gene-Real Time PCR. For reliable detection and measurement of products generated during each cycle of PCR process. Finally, they were resolved on 2.4% agarose gel electrophoresis.

RESULTS:

A total of 13 oocytes samples acquired from PCOS patients and healthy patients were inspected to observe the expression levels of *AXIN2*, *FZD4*, *TCF4* and *WNT5A* in the oocyte of polycystic ovary and compare it to their expression in the healthy ovary. The results indicated that these genes do not have an expression in the oocyte of both PCOS woman and in healthy woman.

CONCLUSION:

Over all this study displayed the absence of expression of *AXIN2*,*FZD4*, *TCF4* and *WNT5A* genes in PCOS woman and in healthy woman ovaries.

KEYWORDS: PCOS, *AXIN2, FZD4, TCF4, WNT5A*, WNT SIGNALING PATHWAY, OOCYTS.

CHAPTER I: - INTRODUCTION

1.1 Introduction

Polycystic Ovarian Syndrome (PCOS) is a chronic endocrinopathy that is manifested in 2.2%- 27% of woman in their pre-menopausal age which starts usually from 15 to 44 years (Knochenhover et al., 1998). It is believed to be due to excessive androgen hormone expression through the combination of excess LH (luteinizing hormone) secretion, hyperinsulinemia along with genetic susceptibility. (Strauss.,2003; Rebar et al., 1976) This syndrome is represented with a lot of long-term health amplifications such as ovulatory dysfunction, a state of male pattern terminal hair growth known as hirsutism, acne and menstral disorder which is either in state of oligomenorrhea or amenorrhea. (NICHD, 2015; Teede et al., 2010) along with other associated disorders such as Type II diabetes mellitus, cardiovascular disease (CVDs), obesity related Endometrial Cancer as well as 40% of female infertility. (Amowitz and Sobel., 1999; Dokras, 2008; Dahlgram et al., 1991)

The discovery of this disease for the first time was in 1721 by an Italian scientist named Vallisneri who observed the ovaries of a PCOS patient as a white shiny surface with the size pigeon eggs (Kovacs, 2013) and then in the 1935 the first investigation of poly cystic ovarian syndrome was performed by American Gynecologists Irvin - F.Stein and Michal-Leventhal. The name polycystic ovary which is commonly used with this disease is derived from the sight multiple ovarian cysts during Ultrasonography which are actually un-matured follicles arrested in the primordial stage (Richard, 2011; Azziz, 2006).

1.2 Polycystic Ovarian Syndrome Etiology

The exact stimulus provoking polycystic ovary syndrome pathogenicity is unclear up to this date. Some scientists believe it's the result of hereditary factor in combination with environmental influences. Others believe it's a congenital disorder with its first on-set diagnosis being at the age of puberty (Ehrmannet al., 1995; Rosenfield et al., 2000). The genetic base of this disease is reposed on the fact of familial clustering of the cases studies. as most PCOS patient have sisters with either elevated testosterone or suffering from menstral irregularity-(Kahsar-Miller et al.,2001; Legro et al.,1998;Carey et al.1994; Govind et al.1999).

Endocrine disturbing chemicals (EDCs) are some of the strongest environmental factors inducing PCOS in woman. These chemicals intrude in the hormone homeostasis action starting in fetal development and continue to adulthood(Diamanti-Kandarakis et al., 2009). These EDCs act as agonist or antagonist through attachments to hormone receptors and initiating hormone blockage by increasing the number of receptors and hormone concentration in specialized cells. One the most common EDCs is Bisphenol A (BPA) found in the food packaging containers and plastic bags. The chemical exposure effect of BPA on PCOS woman is lowering antral follicles number hence effecting ovarian function (Zhou et al., 2016). BPA exposure effect the LH:FSH ratio in PCOS woman as well (Vahedi et al .,2016). Another EDC is phthalates, which are derivatives of phthallic acid. Commonly used in fabric softeners, dietary supplements, perfumes and cosmetics. The extensive utilization of this chemical in the environment has led to adverse side effects in the embryonic and adult stages of an individual. This chemical has the ability to stop the ovary in any stage of the embryonic development which results in pre-mature ovarian follicles, infertility, and depletion in steroidogenisis. (Bhattacharya & Keating, 2012) Heavy metals and harmful

industrial chemicals side effects are problematic in both genders reproductive systems. For instance, lead a toxicant found in batteries causes infertility in men and woman (Winder,1993). While chronic expansion of serum copper and lower Zink levels are associated with escalated levels of hormone and insulin-resistance (Spritzer et al., 2017).

1.3 Pathogenicity of Polycystic Ovarian Syndrome

In a study concluded by Horton et al., (1966) Krischer and Barden, (1972) they explained that ovaries and adrenal both excrete equal amounts of Testosterone. On the other hand androgen was secreted again by the same organ in response to LH and ACTH (adrenocorticotropic hormone) hormone stimulus. Since androgen production was not under the control of negative feedback regulation by the endocrine system a slight increase of androgen production would result in disruption of female sex hormones. Apart from this since androgen acts as an intermediated factor in the synthesis of estrogen, it's crucial that androgen and estrogen secretions be co-ordinated for optimum ovulation in the ovaries (Prizent et al., 2014 ; Walters et al., 2008).

Nestler et al., 1998, Munir et al., 2004 , Carmina et al., 1999, Adashi et al., 1981, Diamanti & Dunaif., 2012, Diamanti-Kandarakis., 2006, Marx, 2003 have all established that androgen secretion apart from adrenal gland and LH fluctuation is also facilated by insulin secretion. That acts as a gonadotropin on the surface of the ovary. Hence insulin-resistance or hyperinsulinemia will immediately lead to hyperandroginsm that will result in oligo-ovulation and, disturbance in the LH, FSH, Prolactin and GnRH secretion. Along with causing metabolic dysfunctionin PCOS patients.

Obesity being one of the side effects seen with polycystic ovarian syndrome has been recognized in the past several years more than it was in the past decades. As studies have proven that it's the main reason for insulin-resistance or hyperansulinemia which is either endogenous meaning its caused as a result of mutation in the insulin encoding genes or insulin receptor antibodies either way it leads to a severe case of insulin-resistance as in Type II diabetes mellitus (Alvares et al., 2006;Lo et al., 2006; Conn et al.,2000; Peppard et al., 2001; Musso et al.,2004; Taylor et al., 1982; Satoh etal., 2001; Murray et al., 2000; Stancio et al.,2003).Orexogenous as in the case of Type I diabetes mellitus (Escobar, 2016). Grundy et al., (2004) and Eckel et al.,(2005) both explained in their studies that metabolic dysfunction syndrome is the outcome of insulin-resistance, obesity in abdominal and visceral areas of the body recurring together with age and it is reflected in 1/3 of adolescent polycystic ovarian syndrome patients and in about 1/2 of adult patients.

As mentioned before current understanding of this syndrome in the health care society indicates that it is seen as a complex multigenic disorder. This means that it is the result of interaction between hairidetry factors and environmental influences as diet or lifestyle for instance that trigger PCOS phenotype. In a study carried out by Escobar et al.,(2005) showed that environmental factors vary between different populations. Which means based on the population that is being studied different genes maybe shown in association with PCOS. In the past few years many attempts have been made for the purpose of understanding the hereditary aspect of this disease. The proclaimed results revealed that this disorder is inherited in an autosomal dominant manner. As statistical analysis reported that 3-35% of adult female individuals with PCOS had mothers or a female sibling (about 2%) with the same syndrome. While most of the adolescent female individuals with polycystic ovarian morphology(PCOM) had either an asymptomatic mother or a father with metabolic syndromes (Lebiel et al., 2006; Sam et al., 2005 ; Covillo et al., 2009).

1.4 Genetic susptibility and polycystic ovarian Syndrome

Countless attempts and efforts have been made for the complete understanding of the mode of actions and the hereditary mechanisms used in the PCOS transmission from one generation to the others. Many scientists seeked out for pinpointing the precise genes underling the fundamental causes of PCOS. As a result of those studies many gene variants have been uncovered that are directly linked or associated with polycystic ovarian Syndrome (Ehrmann., 2005; Escobar et al., 2005; urbanek et al., 1999; Goodarzi et al., 2011).

One of these genes that are associated with PCOS is *AR* gene or (androgen receptor) gene which is an X- linked gene located on chromosome Xq10. The study conducted by Urbanek, (2014) showed that the X- inactivation of this gene resulted in an unsettled androgen signaling pathway. While the NCBI Human Database showed results of *FSHR* (follicular stimulation hormone receptor) gene which is located on chromosome 2p16.3 linked to PCOS. It is a well-known fact that this receptor plays a significant role in Gonad-development in humans. And since FSH is encoded by FSHR any malfunction in the follicular stimulation hormone receptor will end in follicular and ovary dysfunction (Aysha et al., 2017).

Rizwan et al (2018) stated, another gene that is linked to PCOS is *FTO* It is located on chromosome 16q12.2. This gene is originally associated with Type II diabetes mellitus and obesity. However, a study conducted in Pakistani population showed that PCOS patients having the rs9939609 SNP of the intronic variant had different BMI (Body mass Index) in comparison to healthy individuals.

Since insulin- resistance and Type II diabetes mellitus are associated with polycystic ovarian syndrome as we had mentioned earlier any abnormality in the *Caplain10* gene on

chromosome 2q36.3 will lead to PCOS manifestation. This gene is involved in insulin action and secretion (Margret, 2006).

Other well-known genes with their connection to PCOS are the Aromatase genes which are steroidogenisis enzymes that belong to the Cytochrome p450 family and are contributed in the process of androgen conversion to estrogen. Harada et al., (1992) showed in their study that any defect in these enzymes functions caused obstruction in the conversion pathway utilized in the change of androgen to estrogen.This family is consisted of seven genes. The first being *CYPA1A* is located on chromosome 15q24.1. That encodes cytochrome p450 proteins presented in the Endoplasmic reticulum. A study conducted by Ibrahim et al., (2008) on *CYPA1A* and polycystic ovary syndrome illustrated that the individuals carrying polycystic ovarian syndrome had higher isoleucine /valine rates than free polycystic ovarian syndrome individuals. And then it was further elucidated that isoleucine was replaced by valine, and PCOS carrying individuals actually showed Valine Phenotypes.

The second gene of this family is *CYP11A1* which is located on chromosome 15q24.1. This plays an important role in the steroid synthesis pathway. In the Ranjith et al., (2014) study done in south India on the polycystic ovary syndrome showed that the group of people that were studied had (TTTTA)n or promoter penta nucleotide polymorphism which lead them to having the disease.

Another gene is *CYP11b2* which is located on chromosome 8q24.3and is responsible for delivering orders of aldosterone synthesis in the adrenal gland. The study performed by Zhao et al., (2003) for understanding the association between polymorphism in the aldosterone synthetize gene and the pathology of polycystic ovarian syndrome. The results were polycystic ovary patients carried higher rates of aldosterone and testosterone in comparison to normal individuals tested.

CYP17A1 is reported as a causative gene for PCOS and it is located on chromosome 10q24.32. This gene encodes 17-α hydroxylase enzyme that is mostly expressed in the theca cells. The 17- hydroxylase enzyme regulates the conversion of pregnenolon to 17- hydroxyl pregnenolon and progesterone to hydroxyl progesterone for limiting androgen expression (Gilep et al., 2011). Studies by Carey et al., (1993) and Diamanti-Kandarakis., (1999) showed that rs743572 polymorphism of the *CYP17A1* gene is associated with hyper expression of androgen in PCOS patients in Greek population. On the other hand, Mohammed et al., (2015) and Techatraisak et al., (2016) denied this association in Iraqi and Thai populations respectively. The preliminary study by Barbra et al., (2008) on *CYP21A2* gene in the woman with polycystic ovary syndrome concluded that it was associated with disease progression, Insulin- resistance along with increasing Body weight. The *CYP21A2* gene it is located on the chromosome 6p21.33. This gene encodes 21-hydroxylase enzyme that is responsible for the production of steroids. This enzyme regulates the conversion of 17- hydroxyprogesterone to 11-deoxycortisol. Hence any deficiency in the 21-hydroxylase enzyme results in increased level of 17-hydroxyproegsterone in PCOS woman (Prapas et al., 2009).*CYP21A2* gene heterozygous mutation results in the increase of the diseases progression through reduction of aldosterone and cortisol which leads to excess production of testosterone and dihydrotestosterone (Trapp& Oberfield, 2012;Barnhart, 2012; Settas, 2013).The last two genes of the family are *CYP3A7* and *CYP19A1*. The first one is located on the chromosome 7q22.1. And its main expression is in the liver where it assesses in the metabolism of DHEAS (dehydroepiandrosterone sulfate). A study by Mark et al., (2012) on the serum DHEAS levels in woman with polycystic ovary syndrome showed that variants of this gene causes reduction of serum DHEAS in the female carriers of PCOS. As for the second one which is located chromosome 15q21.2. Is also involved in the estrogen pathway and has two single nucleotide polymorphism(SNP) rs700519 (C/T) in exonic region and

rs710059 (C/T) of its intronic region. Polycystic ovary syndrome statistical analysis of this gene revealed its strong connection to protein Arg264Cys.Along with provoking endometrial cancer and other types of metastatic cancers(Norhiko et al., 2006; Sun et al., 2010).

1.5 Diagnosis of PCOS and Its Criteria

The diagnosis of the syndrome is reposed on three sets of criteria that have been assembled for the accurate determination of PCOS presence in an individual (Barbeiri et al., 1986; Hernandez et al., 1988; Cara et al., 1988).

The first set of Criteria which are assembled by the national institute of health (NIH) in the 1990 international conference on polycystic ovary syndrome. States that the individuals suspected for carrying the syndrome should have signs of 1- oligo-ovulation, 2- androgen excess biochemically / physiologically,3- the exclusion of other factors that result in menstral irregulation and hyperandroginsm (Richard, 2011).

The second set of criteria was by European Society of Human reproductive and Embryology and the American Society of Reproductive Medicine (ESHRE/ ASRM) oras commonly known Rotterdam Criteria was assembled in 2003. This criteria state that the individual suspected for carrying the syndrome should have signs of 1-oligo-ovulation or anovulation,2- biochemical or physical excess androgen activity and 3-polycystic ovary diagnosis through ultrasound (Teede et al., 2010; Azziz, 2006; Rotterdam ESHRE/ASRM IN 2004).

The third set of criteria was assembled by the Excess Androgen- polycystic ovary Society (AE- PCOS) in 2006. Require that the main aspect to be taken into consideration for the correct and accurate diagnosis of PCOS patients is the biochemical and physiological signs of excess androgen.

The most common criteria utilized for the diagnosis of polycystic ovary syndrome by the scientist and healthcare society is the 2003 Rotterdam criteria. First, because it requires only two out the three rules. And second, the use of the new and developed sonography devices makes the diagnosis more accurate. Yet the prevalence rate and consistency are still challenging to determine up till now as these criteria are constantly debated and changed(Amato et al., 2008). Nonetheless, accurate evaluation of the utilized method is no less important of the diagnosis. Therefore, all means should be provided for the selection of the proper method.

1.5.1- Clinical and Biochemical Hyperandroginsm

Clinical trials and studies have showed that hirsutism is the most valid marker for hyperandroginsm as acne is not connected to menstral de-regulations or reproductive out turns.(Escobar et al., 2012; Sanchon et al., 2012; Schmdit et al., 2016). The Ferriman-Gallway method of hirsutism rate quantification should be utilized. This is assigning the score of 1-4 to 9 areas of the body. The total score of less than eight being normal, 8-15 is mild hirsutism and finally the score of more than 15 being sever(Yildiz et al., 2010).As for biochemical hyperandroginsm, serum concentration of free testosterone measurement is the most sensitive method(Rosner et al., 2007; Rosner, 2001).

1.5.2- Ovulatory Dysfunction

Since hirsutism and ovulation irregularity starts early after menarche in the form of sever oligo-amenorrhea or anamenorrhea that rises in the pre-menopausal years in woman with polycystic ovarian syndrome. Hence, it is necessary that pregnancy tests are taken before

assuming that PCOS is the cause of missing cycles (Azziz, 2009). In the case of PCOM, if the patient has already been diagnosed with hyperandroginsm and ovulatory dysfunction ovarian morphology sonography is not required (Escobar, 2010).

1.5.3 Secondary Etiological Factors

Life threatening tumors of adrenal and ovaries are considered as secondary etiological causes that need to be excluded. There characteristic diagnostic marker is the initiation of hyperandroginsm before puberty. Along with de-feminization which needs an immediate adrenal and ovarian imaging luckily though theses tumors are rarely manifested (Azziz, 2009).

1.6 Treatment of PCOS

Owing to the fact of poor comprehension of this syndrome, rather it was being in patients, medical practitioners and even scientist lead to the unfavorable lack of interest of pharmaceutical companies and healthcare organizations in obtaining a specified treatment for this disease. Most of the pharmaceutical drugs that are used for treating the symptoms of the disease rather it being oral- contraceptives, insulin–sensitizers,or anti- androgens all are used in off- labeled system. As neither the FDA nor the European medicine agency have ever approved them or any other drug for that matter as a specific medicine for the treatment of PCOS (Radosh, 2009; Dokras et al., 2017; Padmanabhan, 2009).

1.7 Wnt signal transduction pathway

In this progressively advanced generation of molecular medicine, a great deal of effort has been made for the investigation of molecular mechanisms that lead the developmental process of an organism. One of the major mechanisms that scientist have tried to represent is the Wnt signal transduction pathways. This is a classical evolutionary pathway that regulates aspects of cell proliferation, migration and cell fate-determination in the tissue along with early embryonic development (Gilbert, 2010). The name Wnt is derived from joining wingless (Drosophila segment polarity gene) with Int-1(vertebrate homologue Integrated -1)(Wodarz et al., 1998). The discovery of Wnt signal pathway was during research on oncogenic retrovirus conducted by Roel Nuss and Harold Vamus in 1982 (Nusse, 2005; Nusse et al., 1984).The Wnt protein is consisted of a family of lipid modified glycoproteins, (palmitoleoylation modification) which helps the attachment of Wnt protein to Wntless proteins and transporting them to the plasma membrane for secretion. (Cardigan and Nusse ., 1997; Yu et al., 2014). Up to this date only three types of Wnt signal pathways have been recognized and their activation is through interaction of Wnt protein with different member of the frizzled family receptors.

1.7.1Canonical Wnt –signal pathway (β-catenin dependent Pathway)

Canonical Wnt–signal pathway (β-catenin dependent pathway)is where β-catanine accumulation takes place in the cytoplasm. And then it is translocated to the nucleus where it assesses in the co-activation of transcriptional factors of the (TCF/LEF) T-cell factor/Lymphoid enhancing factor. (Minde et al., 2011; Minde et al., 2013) without Wnt protein β-catenin accumulation is not possible. As the cytoplasmic destruction complex would immediately depredate it through phosphorylation and Ubiquintation.

1.7.2- Non-canonical Wnt-signal Pathway (β-catenin Independent pathway)

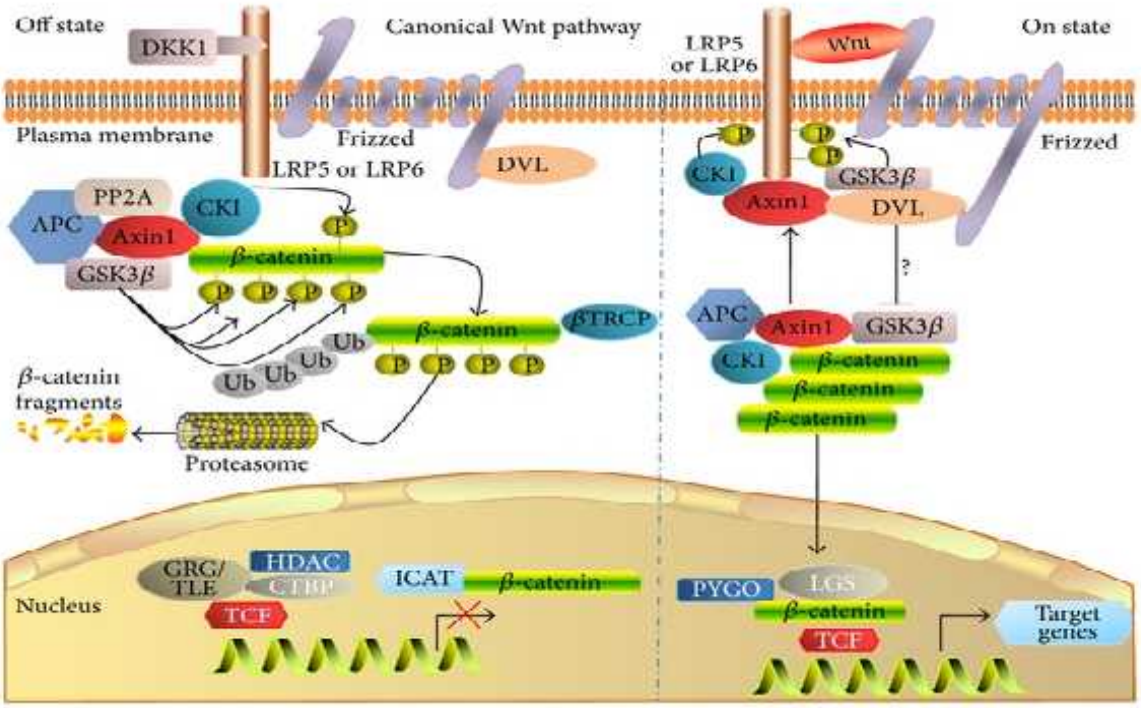
This pathway isknown as planar cell pathway that doesn't utilize B-catenin or LRP 5/6 as its co-receptor. Instead it uses NRH-1, RYK7, PTK7, ROR2. It isbest known for its role in epithelial cell polarization and arrangement of cell-migration (Nusse, 2005).

1.7.3-Non-canonical Wnt-signal Pathway/Calcium pathway

This pathway is also known for its unused properties of β-catenin. However, it does regulate calcium release from endoplasmic reticulum in order of its controlling intracellular levels of Ca^{+2}(Komiya and Habas, 2008).

1.8 Mechanism of Action in Wnt signal pathway

Initiation of Wnt signal is through interaction between Wnt-Protein and N-terminal domain of frizzled receptor(Rao and khul, 2010). Which span the plasma membrane 7 turns. Activation of co-receptors such as LRP-5/6, RTK, ROR2 fallows and the signal is send to the Dsh(disheveled) protein that is in the cytoplasm and contain three domains amino terminal DIX domain, central PDZ and carboxy- terminal DEP domain. These form three different combinations every time they interact with different Wnt- signaling pathway (Habas and Dawid, 2005). Activation of disheveled protein leads to the inhibition of GSK-3β that consequence in the destructing of multiprotein complex. This stabilizes intracellularaccumulation of β-catenin in the cytoplasm; and then translocated to the



nucleus, where it takes place as a transcriptional co-activator of TCF/LEF to trigger Wnt-target genes.

Figure 1.1 Canonical Wnt signaling pathway (β-catenin-dependent). (Adapted from Juan Shi et al., 2016)

Wnt signaling pathways have been implicated in the development of several types of chronic illnesses such as cancer as it was stated that *WNT-1*, *WNT-2, WNT-7A* genes are involved in development of glioblastoma, esophagus cancer, and ovarian cancer susceptibility in respective order (Anastas and Moon, 2013). Another study demonstrated that *WNT5b* over-expression increases insulin- sensitivity due to its role in the adepogenesis.

In the present study we aimed to inspect the expression levels of four Wnt-signal transduction pathway related genes named as *AXIN2*, *WNT5A*, *TCF4*, and*FZD4* in to group of PCOS woman in comparison to control healthy woman. The *AXIN2* gene is located on chromosome 17q23-q24 and encodes axin2 protein or else known as conductin which acts as

a scaffold for β-catenin stabilization in Wnt signaling pathway (May, 1999).This gene and its identical isoform *AXIN1* are important components of a complex located in the cell cytoplasm. Along with APC, CKI, GSK3 all together target β-catenin degradation in the cell in case of a ligands absence (Gluecksohn-Schoenheimer, 1949; Zeng et al., 1997). The *AXIN1* and *AXIN2* genes might vary according to their expression none the less their actions in the un-stabilization and nuclear translocation reluctance of β-catenin is the same when they are over expressed in the cell (Mao et al., 2001;Zeng et al., 2008; Behrens et al., 1998). Studies investigating induced expression effects of *AXIN2* gene by canonical signaling pathway showed sever malformation presentations in mice lacking *AXIN1*(Gluecksohn-Schoenheimer, 1949; Zeng et al., 1997; Jho et al., 2002; Lustig et al., 2002).The *WNT5A* gene is a member of a large Wnt family that possess a significant role in the embryonic development and adult tissue homeostasis (Nishita et al., 2010; Yamaguchi et al., 1999). This gene is located on chromosome 3p14.3 and encodes wnt5a a lipid-modified glycoprotein that is implicated in oncogenesis and cell fate-regulation (Clark et al., 1993). Abnormal expression of this gene has been associated with many malignancies ranging from proinflammation to lung and hepatic fibrosis (Iozzo et al., 1995;Xiong et al., 2012).Katoh., (2009) studied the pathological disorders associated with lacking both copies of *WNT5A* gene in a mice the result was pre-natal death due to respiratory failure.*WNT5A* has the ability of functioning as an inhibitor and as an inducer of β-catenin. As studies showed mice lacking *WNT5A* gene have higher levels of β-catenin (Sato et al., 2010). While transgenic mice with induced *WNT5A* expression in primary embryonic development suffered from prenatal death due to deformations (Bakker et al., 2012). The *TCF4*is atranscriptional factor in human encoded by *TCF7L2* or as formally known (*TCF4*) gene (Castrop et al., 1992). It holds 19 axons and it is located on chromosome 10q25.2–q25.3 (Henthornetal., 1990). *TCF4* influences the transcription of many genes along with biological pathways. It has a part in

the activation of Wnt- targeted genes, pro-glucagon regulation through Wnt signaling pathway and metabolic glucose balancing in liver cells instead of pancreatic B- cells (Jin & Liu., 2008;Facchinello et al., 2017). Studies have shown that rs7903146 SNP of TCF4 is associated with type II diabetes (Vaquero et al., 2012). And the last gene which is *FZD4* encodes a 537- amino acid receptor that is a member of the frizzled family receptors on the plasma membrane controlling cell polarity and proliferation in the embryonic development (Peifer, 1999).This gene is associated with β-catenin canonical signaling pathway and it is located on chromosome 11q14.2 (Kirikoshi et al., 1999). Abnormal activation of this gene has been associated with cellular malfunction and exudative vitreoretinopathy which is an inherited form of retinal degradation (Robitailleet al., 2010; Toomes et al., 2004; Milhem et al., 2014). A study by Wanget al., (2001) showed that mice lacking *FZD4* gene suffered from esophagus and auditory dysfunctions.

1.9 The Aim of this study

This study was conducted to investigate the expression levels of Wnt – signaling pathway related genes such as *AXIN2*, *TCF4*, *FZD4*, and *WNT5A* if they were suspected to cause a noteworthy impact in the development of ovaries and oogenesis.

1.10 The significance of this study

Lateststudy conducted by Wu et al., (2017) showed that Wnt /β-catenin pathway was involved in the granulose cell apoptosis. *WNT4* aggravated the canonical signal pathwayalong with decreasing the levels of β-catenin in the granulose cells of polycystic ovary syndrome patients of north china. The expression of these genes will be investigated which will have a clinical and basic research impact as the transcriptional profile of these

genes might lead to novel critical information regarding molecular basis of ovarian dysfunction in PCOS patients.

CHAPTER II: - Materials and Methods

2.1Materials

2.1.1 Suppliers

Thermo-scientific marker (Pittsburg, USA),Nano-drop (Thermo-scientific, Pittsburg, USA),cDNA Synthesis Kit (Basel, Switzerland).Applied bio-systems thermal cycler PCR, (Waltham, Massachusetts, USA), Eppendorf Scientific(Hamburg, Germany),RotarGene Real-Time PCR (Qiagen,Hilden, Germany), Bio-Rad Electrophoresis instrument (Hemel Hemstead,UK), Ultraviolent Trans-Illuminator (DNR Bio-Imaging System, Neve Yamin, Israel).

2.1.2 Chemical Reagents

2.1.2.1 Molecular Wight Markers

Thermo-scientific 50 bp – 1000 bp (Pittsburg, USA) DNA ladder was utilized as a molecular weight marker.

2.1.2.2 Oligonucleotides

Utilized primers were from Oligomer Company (Turkey)

2.1.2.3 Human Oocyte Collection

Human oocyte collection was obtained from the IVF center and laboratory of the Near East University Hospital (NEUH) after the approval of Near East University Scientific Review Board (registration number: YDU, 2020/80-1120). 13 samples were collected from non-obese and young woman. Seven of these samples were from polycystic ovarian syndrome patients (1, 2, 3, 6, 7, 8, and 11) and were categorized as PCOS group. While the other six

samples (4, 5, 9, 10, 12, and 13) were from healthy individuals and were categorized as control group. The trial investigation of this project was carried out in the Near East University DESAM Institute Molecular Medicine Laboratory, Nicosia, North Cyprus. All the reagents, pipettes and tubes used in the experiment were UV treated to prevent any risk of contamination happening.

2.1.2.4Standard Solutions

10X Tris-borate/ EDTA (TBE) electrophoresis buffer was prepared as marked out by Sambrook et al.1989. And then was further diluted to 1X (100 ml from 10X TBE + 900 ml Distilled water). The dilution of the 10X TBE buffer is necessary as it is too concentrated and delays the bands movements.

The second solution was Thermo-Scientific 2x Master Mix.This solution contains 0.05 U/µlTaq DNA polymerase, reaction buffer, 4 nM $MgCl_2$, 0.4 nM of each dNTP (dATP, dCTP, dTTP, dGTP)

Third solution was Wiz-pure qPCR SYBR green (Seongam, South-Korea) This SYBR green contains antibody mediated hot star, Taq DNA polymerase, ultrapure dNTPs, $MgCl_2$, SYBR green I with enhancers and stabilizers.

2.1.2.5 Other chemical agents

Agarose biomax 100mg, Ethidium Bromide (Serva, Heidelberg, Germany)

2.1.3 Computers

Software packages were used to store data and precede imaging.

2.2 Methods

2.2.1 RNA Extraction from Oocytes

The RNA from the PCOS group and Control group had already been extracted by Assoc. Prof. Pinar Tulay (Near East University).

2.2.2 Measuring RNA concentration

RNA concentration and purity was estimated through measuring optical density at 260/ 280 nm wave length by Nano-drop (Thermo-scientific, Pittsburg, USA) optimum purified density of the RNA is about 2.1ng/µl.

2.2.3 Complementary DNA (c DNA) synthesis

Component of the kit	For 1X
Rxn buffer	2µl
Random hexamer	2 µl
dNTPs	1µl
RTase	1 µl
RNAse free water	3.5 µl
Total	10 µl

cDNA synthesis was carried out by using trans-criptoror first strand cDNA-synthesis kit (Basel, Switzerland).This kit contained trascriptor reverse transcriptase, trascriptor RT reaction buffer, 5x concentrated RNAse inhibitor, dNTPs mix ,and anchored oligo dt18 primer. Also random hex-amer primer with Forward and Reverse primers. These entire components were mixed with 2µl RNA which was stored in -15 – 25℃.

Table 2.1 the table shows the necessary calculations done for cDNA synthesis

2.2.4 Primer Optimization for Gradient PCR

The primer optimization phase of this experiment started with preparing oligomer stock primers for four genes. This is by adding specified amount of distilled water for each specific gene primer to form 100 µM. This is further diluted to 10 µM working solution by taking 10µl of stock primer and mixing it with 90µl of distilled water.

Oligo Name	**Base sequence 5' – 3'**	**100µM stock-µl TE**
TCF4- F	GCATCACCAACAGCGAATGG	759
TCF4- R	TGTCTGTACCTCCATGGCAC	613
WNT5A- F	TCGCTGATGGACGTTGGAAA	578
WNT5A-R	CCAATGGACTTCTTCATGGCG	826
AXIN2- F	CCCGAGAGCCGGGAAATAAA	653
AXIN2-R	CTCCTCTCTTTTACAGCAGGGC	504
FZD4- F	CAGCTGCAGTTCTTCCTTTGT	759
FZD4- R	TGTGGTTGTGGTCGTTCTGT	743

Table 2.2shows the stock primers of the four genes.

Gradient PCR was performed by the applied bio systems vertiti 96 well thermal cycler PCR (Waltham, Massachusetts, USA). For distinguishing, the optimum temperature condition for qRT-PCR. This step was done for all four genes. The temperature range selected wasbetween 55℃ to 64℃. The conditions used for gradient PCR are listed in (**Table2.4**) the whole analyzing process took about 1hour 30 minutes. All the reactions for both PCRs Thermal cycler and RT- qPCR were carried out in a category II laminar flow hood to limit the risk of contamination; furthermore,all the reagents and plastic ware and pipettes were sterilized anddesignated to PCR.

Component	**1X**	**14X**
PCR Master mix	12.5 µl	175 µl
Forward primer	1.25 µl	17.5 µl
Reverse primer	1.25 µl	17.5 µl

Distilled water	9 µl	126 µl

Table 2.3 Gradient PCR Master Mixture calculations

24 µl from the final mixture + 1µl of cDNA (making the volume of the reaction 25µl) were put in (Hamburg, Germany) Eppendorf Scientific PCR tubes for analysis. These calculations were for all 13 samples + 1 Negative control (ntc). The measurements were repeated four times for 4 different genes. Each time with a different set of primers.

Stage	**Temperature**	**Time**	**Cycles**
Initial denaturation	95 ℃	5 minutes	1 cycle
Denaturation	95 ℃	15 seconds	35 cycles
Annealing	55℃ - 64℃	30 seconds	
Extension	72 ℃	45 seconds	
Termination	72℃	5 minutes	1 cycle

Table 2.4 Shows condition utilized for gradient PCR.

2.2.5 Primer Optimization for qRT- PCR

Real-Time quantitative reverse transcription–polymerase chain reaction (RT-qPCR) was performed by RotarGene Real Time PCR (Qiagen,Hilden, Germany). This machine was utilized to enables reliable detection and measurement of products generated during each

cycle of PCR process. Just like gradient PCR a number of necessary calculations were performed for figuring out the exact measurements need for 56 samples + 4 ntc for all 4 genes.19 µl from the final mixture + 1 µl of cDNA (making the volume of the reaction 20 µl) were put in (Hamburg, Germany) Eppendorf Scientific PCR tubes for quantitative analysis. The process took about 1 hour and 15 minutes. The conditions used for qRT- PCR are listed in**Table 2.6**

Component	**1X**	**14X**
SYBR green	10µl	140 µl
Forward primer	2 µl	28 µl
Reverse primer	2µl	28 µl
Distilled water	5 µl	70 µl

Table 2.5RT-qPCR Master Mixture calculations

Stage	**Temperature**	**Time**	**Cycles**
Initial denaturation	95 ℃	5 minutes	1 cycle
Denaturation	95 ℃	15 seconds	35 cycles
Annealing	57℃	30 seconds	
Extension	72 ℃	45 seconds	1 cycle

Table 2.6 Quantitative real time PCR conditions.

2.2.6 Agarose gel Electrophoresis

After the gradient PCR had completed the yielded products were passed on gel electrophoresis. A 2% concentrated gel was prepared by using Sigma agarose (Merck KgaA, Darmstadt, Germany). 2.4 grams of agarose were mixed with 120 ml of TBE buffer. The mixture was put in to the microwave on the highest power for 30 seconds then removed, swirled and put back again. The procedure was repeated several times till the mixture reached boiling point and became clear. Then it was put aside for 1-2 minutes so it can cool down a bit. Before pouring the mixture in to a 20 cm x 20 cm size tray, 0.25µl of Ethidium Bromide was added to the mixture and mixed very well. The gel mixture was poured in to the tray and left till it solidified. 6µl of each PCR product was mixed with 2µl of loading dye ((Thermo Scientific, Pittsburg, USA) and then was loaded in to the well. Lastly, 2µl of a ladder with known size was loaded alongside the samples. The samples were run at 130-140 volts by Bio-Rad electrophoresis devise (Hemel Hemstead,UK). The process took about 1 hour and 30 minutes.Visualization of the bands was through an ultraviolent trans-illuminator (DNR Bio Imaging system, Neve Yamin, Israel).

CHAPTER III: - RESULTS

3.1 Introduction

Reproduction in female adults is highly dependent on functional ovary production and normal hormonal secretion. Oogenesis is the process of ovum differentiation to cell components for further development after fertilization (Balen and Michelmore, 2002). The process is initiated in the intra-uterine life of humans with the differentiation of primordial germ cells to oogonia which undergo meiotic division and are known as primary oocytes and are surrounded by primordial follicles. These two (primary oocyte and primordial follicles) are arrested in the prophase of first meiotic division until puberty. At the adolescent age they both mature to form Graafian follicle which is consisted of two layers first one is theca cells that is responsible for estrogen, androgen and progesterone production and the second one granulosa cells which produce a portentous liquor containing estrogen (Goodman et al., 2015). At the reproductive age monthly several primordial follicles development and gap junction between granulosa cells and oocyte occur as response to FSH stimulation. Only one follicle grows enough to produce FSH receptor and estrogen. This stimulates LH receptors in theca cells and leads to FSH reduction. The dominant follicle goes into ovulation process while all the others are broken down (Dokshin et al., 2013;Nagaoka, 2012). In PCOS patients the same process is quite different as it is usually arrested in pre-antral follicular stage even though FSH stimulation is available (Omar, 2020).the abnormal FSH secretion induces androgen conversion to estrogen causing the environmental status of the follicle to be androgenic rather than estrogenic. This causes dominant follicle suppression and small follicle apoptosis blockage (Willis et al., 1998). Studies have been conducted to investigate the expression and regulation levels of the *WNT* gene and Wnt signal transduction pathway in the follicular development of immature rats, mice and humans (Harwood et al., 2008; Wang et al., 2009; Gupta et al., 2014). The first study pinpointing the significance Wnt signaling in the female ovary was by Vaino et al., (1999) as they showed that female mice lacking the*WNT4* gene in there early embryonic development expressed genes that are associated with testicular

development. Another study by Ricken et al., (2002) showed that the expression of *WNT2* is regulated by FSH mediated β-catenin in all the stages of follicular development of immature rats. While, Wang et al., (2013) observed regulated gab junction in mice granulosa cells by *WNT2*. On the other hand female adult mice lacking *FZD4* are sterile as result of failed embryo implantation (Hsieh et al., 2005). Another study showedWNT3A induced expression of β-catenin resulted in down regulation of FSH leading to decreased level of estrogen and progesterone production (Stapp et al., 2014). In the present study we assessedthe expression levels of Wnt signaling pathway genes *AXIN-2*, *TCF4*, *FZD4*, and *WNT5A* in the oocyte obtained during IVF fertilization from female donors with PCOS and compared them with those found in the healthy woman ovary as a control group.

3.2 Extracted RNA Measurement

Extremely pure RNA have a 260/280 ratio of about 2.1ng/µl.

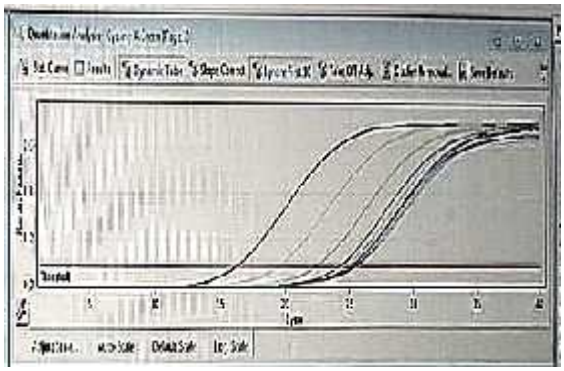
Sample number	RNA concentration (ng/µl)	260/280
1	10	1.52
2	11	1.48
3	12.7	1.46
4	11	1.50
5	9.7	1.51
6	9.9	1.52
7	12.5	1.53
8	10.9	1.56
9	10.3	1.53
10	10	1.52
11	10.9	1.56
12	11.5	1.51
13	10	1.52

Table 3.1 shows the results of the RNA Purification extracted from PCOS and healthy oocytes that were investigated by Nano drop

3.3Gene expression analysis

Gene expression analysis by synthesized cDNA was carried out for four Wnt/beta-catenin genes (*AXIN2, WNT5A, FZD4,* and *TCF*). The gene expression analysis was conducted by RT-qPCR which a positive reaction observation is done through accumulation of fluorescent signal. Cycle threshold or Ct is the number of cycles required for the fluorescent signal to cross the threshold. Another thing is that Ct levels are almost always reversely scaled to the quantity of nucleic acid in the sample. Which means, the lesser the Ct the higher the nucleic acid amount in the sample. RT-q PCR analysis was conducted for all 13 samples PCOS and control group the experiment was performed utilizing optimum annealing temperature of 57℃ for 1 hour and a half. Resulted Ct values are listed below in **Table 3.2**

Sample IDs	***WNT5A***	***TCF4***	***AXIN2***	***FZD4***
1	20.65	19.46	24.21	22.90
2	23.41	19.64	25.00	23.55
3	24.57	19.60	25.11	23.87
4	24.28	19.14	25.10	24.60
5	25.23	19.75	25.06	24.72
6	24.21	19.00	25.58	25.19
7	23.90	19.71	25.40	25.33
8	25.89	19.51	25.31	25.79
9	25.65	17.96	25.09	25.58
10	23.34	17.68	24.78	25.78
11	19.94	18.54	16.25	27.59
12	24.53	19.29	22.46	25.88
13	24.44	18.69	19.50	26.54
NTC	23.15	18.75	24.76	26.03



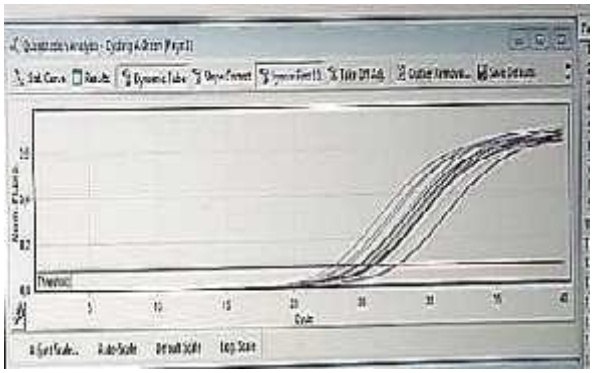
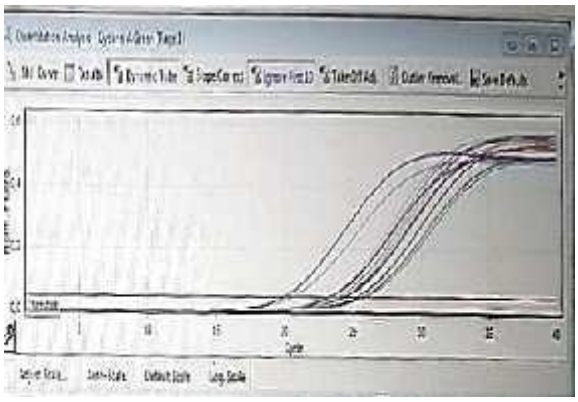


Table 3.2 Expression levels of four genes in all 13 samples.



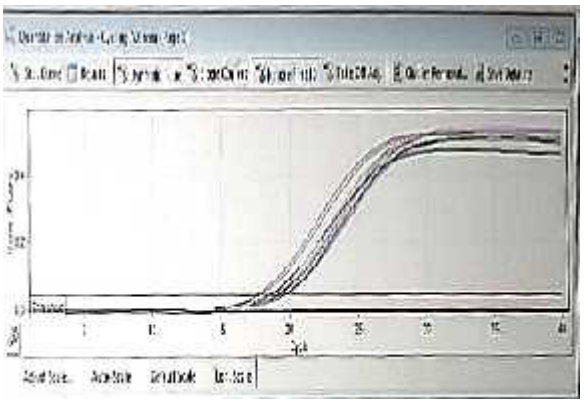


Figure3.1 RT-qPCR reaction curve for *AXIN2* **Figure3.2** RT-qPCR reaction curve for *FZD4*

Figure3.3 RT-qPCR reaction curve for *WNT5A* **Figure3.4** RT-qPCR reaction curve for *TCF4*

As it is shown in the table and in the figures in every experiment the genes had signals in their no template controls (NTC) producing false positive results. The NTC is usually utilized for monitoring contamination and primer dimers.

3.4 Gradient PCR and Agarose Gel Electrophoresis Results

The first gradient PCR analysis was conducted to determine the optimum annealing temperature for the*AXIN2* gene and the *FZD4* gene. The *AXIN2* gene were expected to display 101 base pairs (bp) bands on the gel electrophoresis (Figure 3.5) once visualized under the UV light while *FZD4*gene should be detected at 193bp (Figure 3.6). The ranges of temperatures chosen in the experiment were from 55℃ to 64℃ .The *AXIN2* gene displayed bands at approximately 61 bp as dimers.

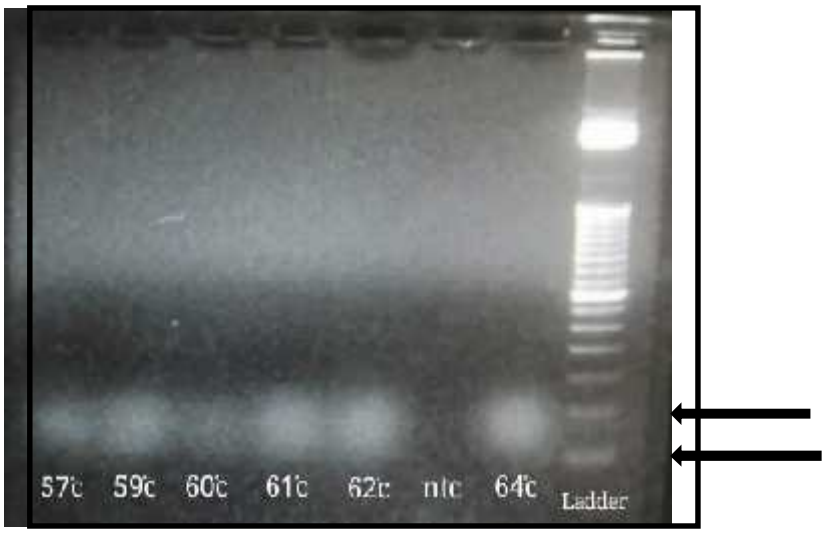


Figure 3.5 Agarose gel showing results of first gradient PCR for *AXIN2* gene

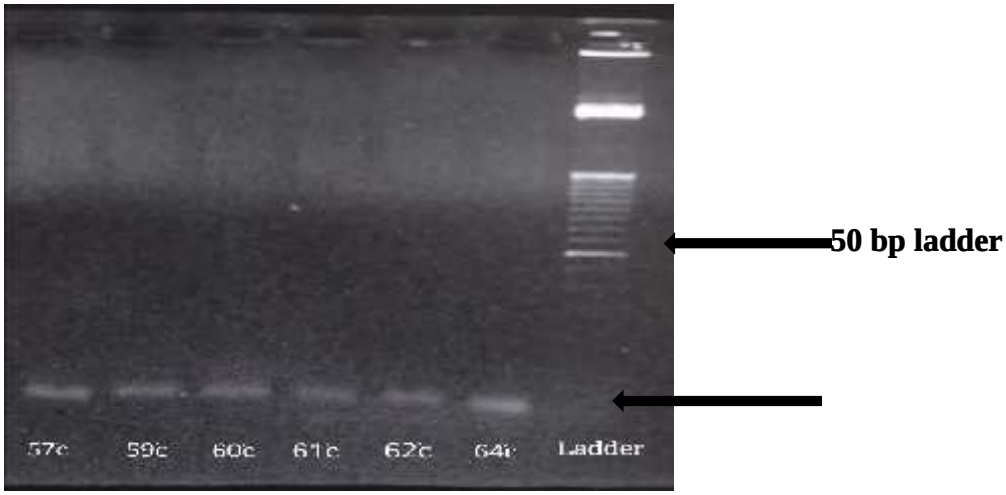


Figure3.6 Agarose gel showing first gradient PCR for *FZD4* gene

After observing a Ct value on negative controls for each experiment a second gradient PCR analysis was conducted for the four genes to check primer sensitivity and efficiency. A new set of cDNAs were synthesized along with utilizing new SYBR green and distilled water. The selected temperatures for this experiment were 55℃, 58℃, 61℃ and 64℃ respectively. As *WNT5A* and *TCF4*observed primer dimers at 55bp (Figure 3.9 and Figure 3.10). The *WNT5A* gene PCR products were supposed to be at 508 bp.

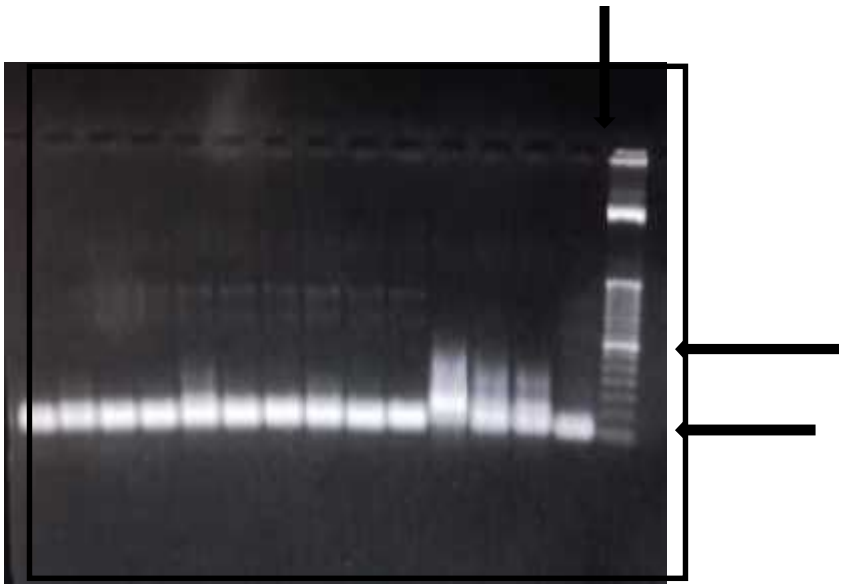


Figure 3.7 Agarose gel showing results of second gradients PCR for *AXIN2* gene

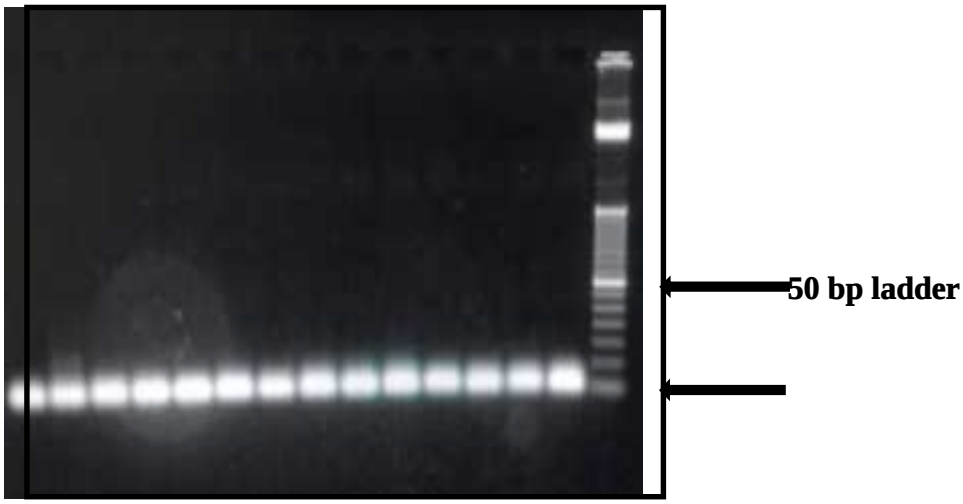


Figure3.8 Agarose gel showing results of second gradient PCR for *FZD4*

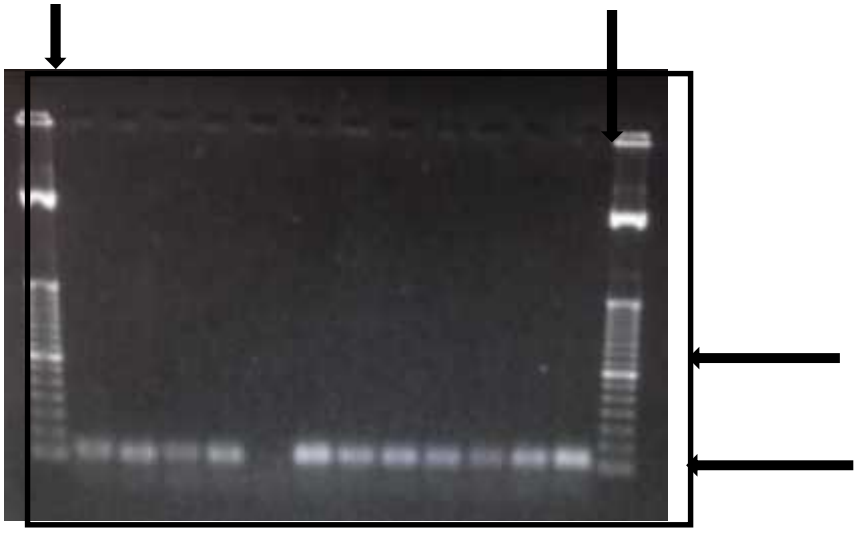


Figure 3.9 Agarose gel showing results of second gradients PCR for *WNT5A* gene

NTC

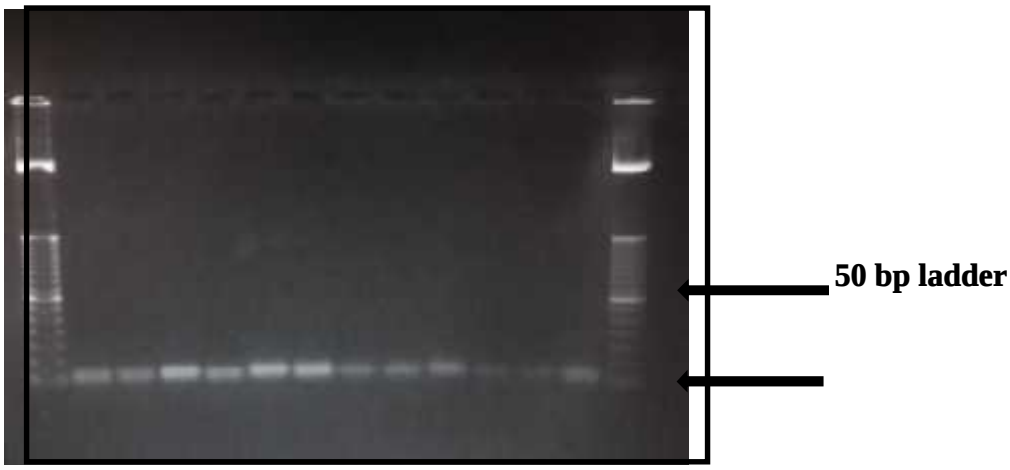


Figure3.10 Agarose gel showing results of second gradient PCR for *TCF4* gene

Although four different temperatures were set for the four genes respectively as its shown in the images none the less all of them produced primer dimers. Hence, we utilized the previously set template to perform another RT-qPCR. Unfortunately the CT values second timer around for *AXIN2, FZD4, WNT5A* and *TCF4* were not much different along with observing signals in the no template controls again. We also loaded the RT-qPCR products on the gelfor band observation under the UV light. Dimers were seen again (Figure 3.11 and Figure 3.12).

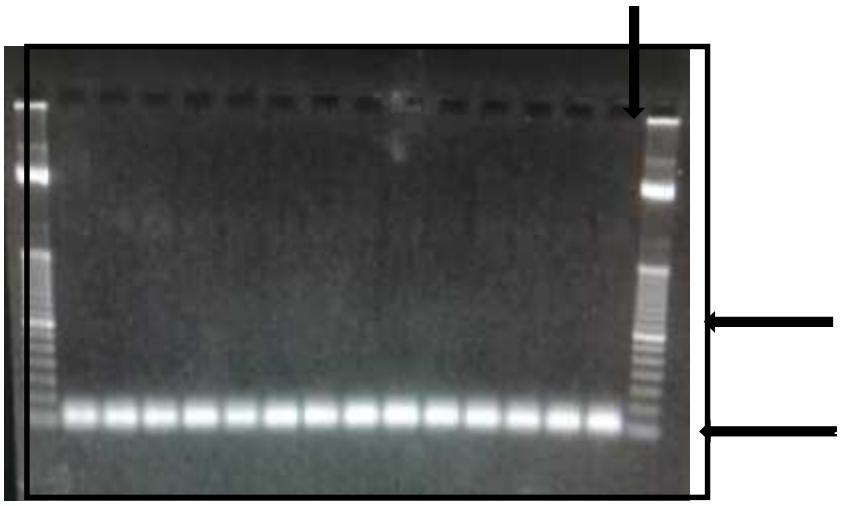


Figure3.11 Agarose gel showing results of RT-qPCR for the *AXIN2* gene

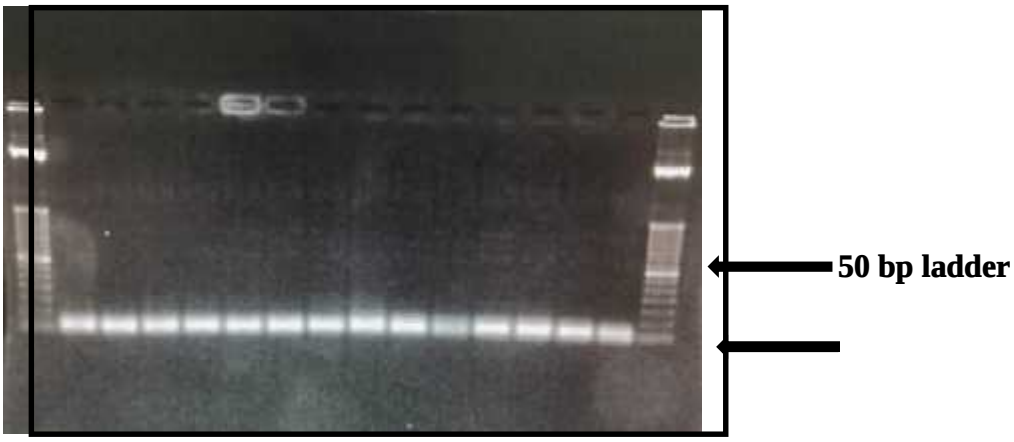


Figure 3.12 Agarose gel showing results of RT-qPCR for the *FZD4* gene

3.5 Conclusion

A total of 13 oocytes samples acquired from PCOS patients and healthy patients were inspected to observe the expression levels of *AXIN2, FZD4, WNT5A* and *TCF4* in the oocyte of polycystic ovary and compare it to their expression in the healthy ovary.The results indicated that these genes do not have an expression in the oocyte of both PCOS woman and in healthy woman.

CHAPTER IV: - DISCUSSION

4.1 Introduction

Polycystic ovarian syndrome (PCOS) has remained a major health challenge and infertility trigger in woman for the past few decades. Common heterogeneous clinical characters of the disease are hirsutism, hyperandroginsm, ovulatory dysfunction, obesity, CVDs and type II diabetes mellitus (Rebar et al., 1976; Dokras, 2008;Dahlgram et al., 1991). The complete patho-physiological effects of the syndrome are still unclear. None the less, scientists believe it is due to androgen excess, environmental effects associated with genetic inheritance of the disease. Depending on different diagnostic criteria prevalence statistics varies in between different populations. Based on a study conducted by Amato et al., (2008) indicated that PCOS prevalence diagnosed by National Institute of Health (NIH) criteria were about 51%, 83% for Rotterdam and 70% by AE-PCOS criteria. And when they were all combined

together it only reached 49%. No specific treatment for the syndrome has been manufactured so far. Physicians rely on oral –contraceptives for the treatment of the symptoms in off-labeled manner (Radosh, 2009; Dokras et al., 2017; Padmanabhan, 2009).

Wnt signal transduction pathway regulates cell proliferation, migration and cell fate-determination in the early embryonic development (Gilbert, 2010). The activation of this pathway is through attachment of Wnt ligand to frizzled receptor and LRP5/6 co-receptors. This triggers DVL protein to block β-catenin degradation through obstruction of GSK-3β and destruction of cytoplasmic protein complex. Cytoplasmic β-catenin is stabilized and translocated to nucleus were its helps TCF/LEF activation of responsive target genes (Komiya and Habas, 2008). However, if Wnt ligands are absent β-catenin is phosphorylated by cytoplasmic multiprotein complex of Axin, adenomatous polyposis coli (APC), the glycogen synthase kinase 3β (GSK3β), and casein kinase 1α (CK1α). The phosphorylated β-catenin is recognized by E3 ubiquitin and targeted to proteasomal degradation, causing reduction incytoplasmic β-catenin level (Moon, 2002). A vast range of clinical studies have elucidated the significance of Wnt signal transduction pathway in the developmental process of the body and maintaining tissue homeostasis. Having said that, it is only in the recent history that scientist came upon the fact that any transformation in the pathway factors might play a part in the progression of human chronic illnesses (Peiferand Polakis, 2000).

4.2 Wnt signaling in the follicular development

The existence of Wnt in the normal ovarian activity is not much of shocker given the diversity of physiological systems regulated by the family. It's evidently clear that Wnt (canonical and non- canonical) signaling pathways control the proper activation of female reproductive system along with regulating hormone activity in the ovary's granulosa cells (Miller et al., 1998, Castanon et al., 2012). Majority of studies investigated Wnt ligands in the folliculogenesis processes that addressed *WNT2* and *WNT4* genes with the addition of *WNT3A* gene recently in mice, rats and human embryos (Li et al., 2002; Wang et al., 2010). *WNT2* expression has been detected through all the stages of follicular development in rat ovaries with the highest level in the cumulus and granulosa cells (Ricken et al., 2002; Wang et al., 2010). Wang et al., (2013) observed regulated Gab-junction in the mouse granulosa cells by *WNT2*. The importance of the *WNT2* gene in the granulosa cell maturation is not lost upon us none the less Wang et al., (2010) and Finnson et al., (2012) showed that over expression of

the *WNT2* gene resulted in the cytoplasmic and nucleic accumulation of β-catenin of mice and rat granulosa cells in the early embryonic development. In a study by Monkley et al.,(1996) they noticed that mutant adult female mice lackingthe*WNT2* gene are still fertile indicating the presence of more than one Wnt ligand in the process. As mentioned before the second most studied ligand is *WNT4* just like *WNT2* this gene has been detected in the Granulosa cells and cumulus cells of all the stages of folliculogenesis (Hsieh et al., 2002; Hernandez-Gonzalez et al., 2006). However, the expression of the *WNT4* gene has not been detected in adult human cumulous cells of oocytes prior toIVF (Wang et al., 2009). Boyer et al., (2010) studied the effect of the *WNT4* gene deletion in a mouse granulosa cells and compared it to normal *WNT4*obtaining mouse. The results were supfertile female with much smaller ovaries and follicle number compared to control group. This group also inspected the overexpression effect of the *WNT4* gene on steroidogenic enzymes in eCG treated mice the results were elevated levels of *CYP11A1* and *CYP19A1* (Boyer et al., 2010). As for *WNT3A*, Stapp et al., (2014) revealed in their study that minimum exposure of rat granulosa cells to this gene caused induced expression of *AXIN2* and stimulation of β-catenin /TCF promoter. This lead to induction in the canonical Wnt pathway and resulted in down regulation of FSH-mediated expression of *AR, CYP11A1* and *CYP19A1*. The bulk of data collected on Wnt ligand and their involvement in the oocyte development is based on embryonic investigations. The number of researches addressing Wnt ligands expression in adult's oocyte is quite humble.

The purpose of the current research was to assess and analyze the expression levels of the fallowing four genes *AXIN2, WNT5A, TCF4* and *FZD4* in the oocytes obtained from PCOS group then compare them to healthy group.

4.3 Previously published data on *AXIN2, FZD4, TCF* and *WNT5A* genes

The gene *AXIN2* is considered as a negative regulator of Wnt signaling pathway as it restricts the action of β- catenin and leads to complete shutdown of TCF gene transduction (Jho et al., 2000).The *AXIN1* and *AXIN2* genes might vary according to their expression none the less theirover activation in the cell gives the same results, un-stabilization and nuclear translocation reluctance of β-catenin (Mao et al., 2001; Zeng et al., 2008; Behrens et al., 1998).Gluecksohn-Schoenheimer, (1949), Zeng et al., (1997), Jho et al., (2002) and Lustig et al., (2002)have all investigated the effect of induced expression of the*AXIN2* gene by canonical signaling pathway in mice lacking the*AXIN1* gene the results were sever

malformations and death. In a study investigating the role of the *APC2* gene on the Wnt signal transduction pathway by Mohamed et al., (2019) they reviled that the ovaries with concealed *APC2* expression exhibited higher levels of *AXIN2* compared with normal ovaries. Another study conducted on PCOS woman with ovarian carcinoma reviled that carcinogenic ovaries with de-regulated β-catenin had higher *AXIN2* expression than normal ovaries (Leung et al., 2002).

The *WNT5A* gene is a member of a large family composed of 19 Wnt proteins in humanranging in length between 350-400 amino acids (Cadigan & Nuss, 1997; Clevers & Nuss, 2012). Abnormal expression of the *WNT5A* gene has been associated with lung and hepatic fibrosis (Iozzo et al., 1995; Xiong et al., 2012). Pathological disorders associated with lacking both copies of the*WNT5A* gene in mice resultedin pre-natal death due to respiratory failure(Katoh, 2009).Sato et al., (2010) stated that*WNT5A*acts as an inhibitor β-catenin as their study showed mice lacking the*WNT5A* gene had elevated β-catenin level. WhileBakker et al., (2012) proved that *WNT5A* over activation in transgenic mice triggered deformed embryonic development and resulted in death.

Recently scientists involved pro-inflammation as progression factor of PCOS pathogenesis. Zhao et al., (2015) stated in their study that PCOS patients ovary and granulosa cells showed signs of pro inflammation and oxidative stress as the expression of *WNT5A* gene raised. In a study investigating the expression levels of Wnt family genes in the granulosa cells of polycystic ovary patients and healthy ovary patient, the results showed *WNT1*, *WNT3* and *WNT4* had higher expression in PCOS ovary than in healthy ovary while *WNT5A* had no significant difference what so ever (Wu t al., 2017). Another study by Sanchez et al., (2014) implicated that over expression of *WNT4,WNT5A*is directly correlated to inhibition and deduction of β-catenin levels in granulosa cells.

Just as Wnt protein standard expression has been of a significant importance in the normal ovulation and folliculogenesis there are frizzled receptors that do the same action. Frizzled family is a family of trans-membrane receptors responsible for cell polarity and proliferation during embryonic development (Peifer, 1999). They aremostly associated with β-catenin canonical signaling pathway.Robitailleet al., (2010), Toomes et al., (2004), and Milhem et al., (2014) have studied the abnormal expression of *FZD4* and its associationwith cellular malfunction and exudative vitreoretinopathy. Studies observing mutant mice with deletion of the *FZD4* gene resulted in mice suffering from esophagus and auditory dysfunctions(Wanget

al., 2001). Many studies have revealed that ovarian follicular response to gonadotropin hormone is under the control regulation of Wnt signaling members and FSH hormone (Boyer et al., 2010; Lapointe and Boerboom, 2011). According to a study by Owens et al., (2002) genetically altered mice with excessive LH production develop granulosa cells tumors with increased *FZ10* expression. A study on rodent ovaries demonstrated LH over expression promoted *FZD1* and *FZD4* elevation (Gupta et al., 2014). On the other hand, a study on adult female mice granulosa cells with germ line deletion of the *FZD4* gene showed normal ovulation and production of fertilized oocyte however they were still sterile as a result of failed embryo implantation. This outcome was due to abnormal corpora lutea formation and progesterone reduction (Hsieh et al., 2005).

Transcription factor-4 in human is encoded by *TCF7L2* or as formally known *(TCF4)* gene (Castrop et al., 1992). *TCF4*regulated expression influences biological pathways along with the activation of Wnt- targeted genes, pro-glucagon management through Wnt signaling pathway and metabolic glucose balancing in liver cells instead of pancreatic B- cells (Jin & Liu., 2008; Facchinello et al., 2017). *TCF4* genes most studied single nucleotide polymorphism is rs7903146 which is associated with type II diabetes (Vaquero et al., 2012). Scientists were dis-joined on the basis of this genes association to the PCOS pathogenicity. Some of them have observed the correlation of this gene to the syndrome, while others have not. For instance, in a study conducted on 283 individual with PCOS in Greek population Christopoulos et al., (2006) observed the rs7903146 polymorphism of *TCF4* gene in the PCOS group. While other like (Xu et al., 2010; Kim et al., 2012 and Ben- Salem et al., 2014) deny the association due to absence of the *TCF4* gene polymorphisms in Chinese population, Korean population and Tunisian population respectively. In a recent study performed by Prabhu et al., (2018) they utilized polymerase chain reaction – restriction fragment length polymorphism(PCR-RELP) analysis to validate the fact that *TCF4*gene polymorphisms are not associated to PCOS pathology by any means.

4.4 The results of this study

Oocytes obtained from seven patients with PCOS and six controls without PCOS were inspected to observe the expression level of *AXIN2, FZD4,TCF4* and *WNT5A* in the adult ovaries. While carrying out our experiments on these four genes it was noted that the RT-qPCR results of the mentioned genes exhibited false positive signals in their non-control

templates. As we thought it might have been due to a contamination in the process of sample preparation or cDNA synthesis. All the materials were changed new cDNAs were synthesized and RT-qPCR analysis was repeated. None the less, the results were the same. When the RT-q PCR products were runned on the gel electrophoresis and visualized under the UV light absence of bands for all four genes in all thirteen samples was observed. On the contrary only dimers were seen. Suggesting failed expression of all four genes in both groups. Upon seeing those results we decided to re do the experiment once more for further confirmation however, this time with different primers. The three added primers were *DKK3, FZD3*and *DVL1* these three genes were also found to be associated with Wnt signal transduction pathway. *DKK3*is a member of the Dickkopf family proteins that are activated through photolytic cleavage (Niehrs, 2006). It is located on chromosome11p15.3 and encodes dickkopt related protein that highly involved in embryonic development (Krupnik et al., 1999). Tada et al., (2002) explained in their study that *DKK3* is considered as a negative regulator of Wnt signaling pathway because of its function in the inhibition of Planner cell polarity. While Mao and Niehrs, (2003), Liang et al., (2015) showed that their inhibition is through binding to LRP5/6 and breaking them. In a study investigating methylation status of reduced expression of immortalized cells/ Dickkopf 3 (REIC/*Dkk3*) in human malignancies by Hayashi et al.,(2012) they showed that epigenetic silencing or methylation of *DKK3* associated with β- catenin deregulation and apoptosis disruption. Higher incidence has been observed in Granulosa cell tumors in comparison with healthy tissue (Xu et al., 2016).

*FZD3*which encode another member of the frizzled family (FZD3 Protein)is associated with B-catenin canonical signaling pathway; it is located on 8p21.1 (Kirikoshi et al., 2000). In a usually balanced cumulus cell of the ovary FSH binding to FSHR facilities activation of *CYP19A1* through β-catenin, which results in enhanced estrogen synthesis. However, studies conducted on polycystic ovary cumulus cells with elevated expression of *FZD3* showed, FSH inactivating the steroidogenesis process of cumulus cells through accumulation of β-catenin (Qiao et al., 2017). And the last gene which was disheveled homolough-1 a protein involved in cell proliferation it is encoded by *DVL1* gene located on chromosome 1p36.33 (Pizzuti et al., 1997).In a study onserous ovarian carcinomas carried out by Karen et al., (2019) it was noted that the expression levels of *DVL1* were higher in low grade serious carcinoma cells.in contrary to normal ovary or high grade carcinoma cells. The real time analysis for the three later genes showed the same results as the former genes (signals in ntc). Our findings in the first four genes of *AXIN2*, *FZD4*, *TCF4*, and *WNT5A* along with the three added ones of

DKK3, *FZD3* and *DVL1* suggest that all of these genes have higher expression in the embryonic stages of human development oocyte than in adult stages.

4.5 CONCLUSION

PCOS remains as the most predisposed disorder among woman of reproductive age with excruciating concomitant as infertility. With the contemporary treatment prototypes hardly addressing the direct grounds of the disease a lot of effort is needed for diminishing reproduction infertility. All together, the results of this study displayed the absence of expression of *AXIN2, FZD4, TCF4* and *WNT5A* genes in PCOS woman and in healthy woman ovaries.One of the limitations of the study was the extracted RNA purity from oocyte. Pure RNA has a 260/280 ratio of 2.1ng/µl however, our samples yielded less due to a delayed extraction.Another limitation was the small sample size of utilized oocytes. As obtaining a large number of oocytes is a rather difficult task as most women use it for pregnancy purposes. Never the less, confirmation of these finding is in need of furthers study using wider rang data.

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