**TURKISH REPUBLIC OF NORTHERN CYPRUS**

**NEAR EAST UNIVERSITY**

**INSTITUTE OF HEALTH SCIENCES**

# The expression profile of the *WNT/β -catenin* signalling pathway genes in human oocytes obtained from polycystic ovaries syndrome (PCOS) patients

**Marwan "Mohammad Saeed" Naji Sider**

**Master of Science in Molecular Medicine**

**Thesis Advisors:**

**Assoc. Prof. Mahmut Çerkez Ergören**

**Assoc. Prof. Pinar Tulay**

**2020, Nicosia**

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# THE ETHICAL APPROVAL FOR MASTER'S PROJECT

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BİLİMSEL ARAŞTIRMALAR ETİK KURULU

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Prof. Dr. Rüştü Onur

Yakın Doğu Üniversitesi

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## DECLERATION

I declare that I have no unethical behaviour in all stages from the time I started the laboratory work of the thesis to my writing of the thesis itself, I have gained all the information in this thesis within the academic and ethical guidelines, and I have involved all the information and details in the version and have added these citations to the references part. I hereby have not violated my patents and copyrights while studying and writing this letter.

**Marwan Seder**
**Signature**

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## LIST OF ABBREVIATION

µl Microliter

ng/µl: Nano gram/ Microliter

β: Beta

γ: Gamma

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

PCR: Polymerase chain reaction

PCOS: polycystic ovaries syndrome

F: forward

R: reverse

LH:  Luteinizing Hormone

AR: androgen receptor

LHCGR: Luteinizing hormone/ chorionic gonadotropin receptor

FSH: Follicle Stimulating Hormone

NC: Negative control

GSK3B: Glycogen synthase kinase-3 beta

Ct: Cycle threshold

NA: Nucleic acid

PGCs: precursors of primordial germ cells

GV: germinal vescle

INSR: insulin receptor gene

IRSs: Insulin receptor substrate protein

PPAR- γ: The peroxisome proliferation activated receptor gamma gene

DW: distilled water

## ACKNOWLEDGMENT

As one of the few distinguished students who were able to graduate from the university and overcome the academic and life obstacles due to what happened in the world as a result of the *COVID 19*, I swear that my patience, perseverance and determination to succeed was not enough, but the support of those around me, including family, friends and teachers, in addition to the most important reason which is my support from the Lord was one of the pillars that helped me during this difficult period.

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## ABSTRACT

### The expression profile of the *WNT/β -catenin* signalling pathway genes in human oocytes obtained from polycystic ovaries syndrome (PCOS) patients

**Marwan "Mohammad Saeed" Naji Seder**

**MOLECULAR MEDICINE**

**ADVISOR: Assoc. Prof. Pinar Tulay,**
**Assoc. Prof. Mahmut Çerkez Ergören**

**AIM:**

The main goal of this study was to investigate the expressions of *WNT 1, APC, GSK3B, and β -catenin genes* that are suspected to have a significant impact on development of ovaries and oogenesis with present of polycystic ovarian syndrome.

**BACKGROUND:**

Polycystic ovary syndrome (PCOS) is a disorder that affects females, as it causes menstrual problems. It is considered one of the most frequent forms of endocrine disorders in reproductive aged women with a prevalence of 6% to 15% worldwide. In the previous years, the phenotypic features of the disorder is mainly defined using the 2003 Rotterdam criteria which requires at least two to three of the following features, hyperandrogenism , chronic anovulation , and polycystic ovaries. PCOS is thought to be responsible for 40% of female infertility and also the leading cause of endometrial cancer.

The *WNT* gene family are responsible for cell signalling. The *WNT* signal transduction cascade uses either autocrine or paracrine cell communication to regulates series of biological processes throughout the growth and development of all organisms. In addition to this, the *WNT* signalling pathway is one of the biological components that is responsible for the regulation of cell polarity, cell migration, cell fate and cell proliferation at human embryonic development and adult tissue homeostasis

## MATERIAL AND METHODS:

In this study 13 oocyte samples were collected from Near East University Hospital in vitro fertilization (IVF) Centre, The patients were divided in two groups; the first group consisted of polycystic ovaries donors and the other group were healthy donors (without polycystic ovaries syndrome).

cDNA was synthesized from a single cell oocyte in order to evaluate the gene expression level of by *WNT1, APC, β-catenin,* and *GSK3B* by real time polymerase chain reaction (RT-PCR).

## RESULTS:

When all the genes were compared together (control oocytes and PCO oocytes) with ACTB, there was no significant different in ANOVA summary for the four genes with ACTB gene (p value = 0.0613). However, when each gene was compared separately with *ACTB* (control oocytes and PCO oocytes), there was a significant differences between every gene with *ACTB*.

## CONCLUSION:

Overall, elevated expression of *WNT1* and *GSK3β* regulatory proteins of the WNT/beta-catenin was detected in the oocytes of PCO patient thus it suggests that there is an association between aberrant expression of *WNT1* and *GSK3β* and the pathogenesis of the development of polycystic ovaries. On the other hand, *APC* and *β-catenin* expression were similar in the oocytes of both patients and controls.

**KEYWORDS: *GSK3B, WNT1, APC, β -catenin,* PCOS, oocyte**

# ÖZET


## AMAÇ:

Bu çalışmanın temel amacı, polikistik over sendromu ile yumurtalık ve oogenez gelişimi üzerinde önemli bir etkiye sahip olduğundan şüphelenilen WNT 1, APC, GSK3B ve β -katenin genlerinin ifadelerini araştırmaktı.

## GİRİŞ:

Polikistik over sendromu (PCOS), adet sorunlarına yol açtığı için kadınları etkileyen bir hastalıktır. Dünya genelinde% 6 ila% 15 prevalansıyla üreme çağındaki kadınlarda en sık görülen endokrin bozukluklarından biri olarak kabul edilir. Önceki yıllarda, bozukluğun fenotipik özellikleri temel olarak aşağıdaki özelliklerden en az iki ila üçünü, hiperandrojenizm, kronik anovülasyon ve polikistik yumurtalıklar gerektiren 2003 Rotterdam kriterleri kullanılarak tanımlanmıştır. PCOS'un kadın kısırlığının% 40'ından sorumlu olduğu ve ayrıca endometriyal kanserin önde gelen nedeni olduğu düşünülmektedir.

WNT gen ailesi, hücre sinyallemesinden sorumludur. WNT sinyal iletimi kaskadı, tüm organizmaların büyümesi ve gelişimi boyunca bir dizi biyolojik süreci düzenlemek için otokrin veya parakrin hücre iletişimini kullanır. Buna ek olarak, WNT sinyal yolu, insan embriyonik gelişimi ve yetişkin doku homeostazında hücre polaritesi, hücre göçü, hücre kaderi ve hücre proliferasyonunun düzenlenmesinden sorumlu olan biyolojik bileşenlerden biridir.

## GEREÇ VE YÖNTEM:

Bu çalışmada Yakın Doğu Üniversitesi Hastanesi in vitro fertilizasyon (IVF) Merkezi'nden 13 oosit örneği alındı. Hastalar iki gruba ayrıldı; birinci grup polikistik yumurtalık donörlerinden, diğer grup sağlıklı vericilerden (polikistik over sendromu olmayan) oluşuyordu.

cDNA, gerçek zamanlı polimeraz zincir reaksiyonu (RT-PCR) ile WNT1, APC,-katenin ve GSK3B'nin gen ekspresyon seviyesini değerlendirmek için tek hücreli bir oositten sentezlendi.

## BULGULAR:

ACTB ile tüm genler (kontrol oositleri ve PCO oositleri) birlikte karşılaştırıldığında, ACTB genine sahip dört gen için ANOVA özetinde anlamlı bir fark yoktu (p değeri = 0.0613). Bununla birlikte, her bir gen ACTB (kontrol oositleri ve PCO oositleri) ile ayrı ayrı karşılaştırıldığında, ACTB'li her gen arasında önemli farklılıklar vardı.

## SONUÇ:

Genel olarak, WNT / beta-katenin'in WNT1 ve GSK3β düzenleyici proteinlerinin yüksek ekspresyonu, PCO hastasının oositlerinde tespit edildi, bu nedenle, WNT1 ve GSK3β'nin anormal ekspresyonu ile polikistik yumurtalıkların gelişiminin patogenezi arasında bir ilişki olduğunu gösterir. Öte yandan, hem hastaların hem de kontrollerin oositlerinde APC ve-katenin ekspresyonu benzerdi.

**ANAHTAR KELİMELER: GSK3B, WNT1, APC, β -catenin, PCOS, oocyte**

# CHAPTER I

# INTRODUCTION

## 1.1 INTRODUCTION

Complex disorders are polygenic and multifactorial syndromic diseases affecting a considerable amount of individuals around the world. The aetiology of complex disorders such as, obesity, heart diseases, diabetes mellitus type II and polycystic ovarian syndrome originates from either epistasis or genotype to environmental interactions hence overlapping symptoms manifest in the affected individuals. Although complex diseases cluster in families just like single gene disorders, alternatively the inheritance pattern is extremely heterogenic. Moreover, the genetic susceptibility associated with complex diseases appears to be likely different among affected individuals from or within the same family. Also, due to the complexity and the multifactorial traits associated with complex diseases, they are often difficult to diagnose or treat. For this reason, many studies have tried to identify genetic variations as well as casual variants in genes associated with most of these polygenic disorders to help improve diagnostic, treatments and to understand the pathological pathways underlying the aetiology of these diseases. This chapter will provide basic information about human cell division, gametogenesis, and polycystic

ovarian syndrome as well as the possible causative gene mutations of different biological pathways associated with polycystic ovarian syndrome.

## 1.2 CELL DIVISION

Cell division is the process whereby parental cells go through series of division to produce two or more daughter cells according to the cell type. The process occurs differently among organism. In humans, the processes of cell division usually occur as part of a large cell cycle and it is classified into two distinctive types named mitosis and meiosis (I and II) respectively.

### 1.2.1 MITOSIS

Mitotic division in human results in the production of two daughter cells genetically identical to the cell of origin, the process is separated into five different phases; interphase, prophase, metaphase, anaphase and telophase. The first stage of mitotic cell division is the interphase which consists of three sub stages; GI, S, and G2. At the GI phase, human cells undergo specialized cellular functions and growth in order to prepare for DNA replication. Subsequently, the duplication of chromosomes occurs at S phase and the cell continues to grow at G2 phase before advancing to M phase where the spindle fibers of the chromatids are synthesized. At prophase, the chromatids condense to form shorter visible strands called chromosomes with the microtubules fibers attach at the kinetochore of the centromere. During metaphase, the centromere

of the chromosome aligns at the equatorial plate of the cell by the microtubules organizing centers. In anaphase, the chromatids separate apart with each moving abruptly towards opposite poles and a certain spindle fibers begin to elongate, this process is controlled by the anaphase promoting complex. During telophase, new nuclear envelop form around the newly produced sister chromatids.

### 1.2.2 MEIOSIS

Meiosis is the division of cells that consequently leads to the production of haploid number of cells called gametes. The process is ramified into two types: meiosis (meiosis I and meiosis II). Meiosis I is segmented into four stages, prophase I, metaphase I, anaphase I and telophase I. The first stage of meiosis I which is the prophase I is further separated into five stages; leptotene, zygotene pachytene, diplotene and diakinesis. During leptotene, identical copies of chromatids become closely associated and are visually indistinguishable. At zygotene, the chromosomes line up next to each other in homologous pairs this enables the central element of the synaptonemal complex to initiate synapsis. In pachytene, homologous chromosomes exchange genetic information between sister chromatids forming a region called chiasmata. At diplotene and diakinesis, the synaptonemal complex degrades, allowing autologous chromosomes to detach from each other before entering metaphase 1 where they align at the metaphase plate and are independently assorted until there

transition to anaphase I. During anaphase I, the kinetochore of the chromosomes shortens pulling sister chromatids to the opposite side of the poles and upon reaching telophase, the microtubules of the chromosomes disappear and new nuclear envelop forms surrounding each haploid cells. The cells ingress into a cycle of rest known as interkinesis, therefore, meiosis I effectively ends when the centromeres of the chromosome arrived at the pole (Mazia, 1961).

The second meiotic division is comparable to mitosis and it is divided into prophase II, metaphase II, anaphase II and telophase II. In prophase II, the nuclear envelop begins to disintegrate in to vesicle and the meiotic spindle forms. During metaphase II, the chromosome line up at the equatorial plate with each centromeres containing threes kinetochore attached to the spindle fibers from the centrosomes on each side. At anaphase II, the centromeres of the chromosome get cleaved and the microtubules begin to shorten pulling the chromosomes to opposite poles. The process ends at telophase II where the chromosomes uncoils and lengthen and a new nuclear envelop forms around the four daughter cells produced.

### 1.3 Gametogenesis

Gametogenesis is the process of producing mature gametes and it involves the reduction of the genome content of a diploid germ line precursor cell into haploid cells. The process utilizes two meiotic divisions to produce mature gametes. Though, the process occur differently in male and females; in such

spermatogenesis in male results in the production of sperm cells (Manku & Culty, 2015), while oogenesis produces oocyte in females (Arkoun & Livera, 2018). Previous studies using gastrulating mice embryo indicates that the precursor of primordial germ cells (PGCs) dwell at the epiblast region which later separates to form the three primary germ layers: (i) ectoderm, (ii) endoderm and (iii) extraembryonic mesoderm (Manku & Culty, 2015). The PGCs move from the extraembryonic mesoderm back into the embryo through the allantois (Gilbert., 2000). Thereafter, they migrate adjacently to the yolk sac where they separate into two populations of cells which locomote to either the left or right side of the genital ridge. The PGCs advance caudally from the yolk sac along the hindgut towards the dorsal mesentery before arriving to the genital ridge.

Comparably to gastrulating mice embryo, gametogenesis in human embryo initiates through the migration of human PGCs (hPGCs) at the dorsal mesentery of the yolk sac, the hPGCs by pass the hindgut and progress to the gonadal ridge. Upon reaching the gonadal ridge, the hPGCs go through meiosis I and meiosis II to produce haploid number of daughter cells which further differentiates into sperm cells in males and oocyte in females (Gilbert., 2000).

Spermatogenesis in males begins at the pubertal stage of life between the ages of 13 to 16 when the germ line epithelial cells of the seminiferous tubules divides by mitosis to produce spermatogonia which later undergo proliferation

to become spermatocytes. Subsequently, the produced spermatocytes undergo two meiotic divisions to produce four haploid daughter cells called spermatids which differentiate into functional sperm cells called spermatozoa. In addition, spermatogenesis in human takes approximately 74 days with an average sperm production ranges of 150 to 275 million spermatozoa (Ibtisham et al., 2017). Also the process persist throughout lifetime until death although the production rate decreases due to paternal aging (Xiao et al., 2013).

### 1.3.1 OOGENESIS

Oogenesis is a highly specialized process of differentiation of the female ovum. It is linked to the synthesis and accumulation of RNAs and protein. The mechanism of oogenesis varies in every species depending on their specific reproductive pattern. In humans, oogenesis begins prior to birth (Arkoun & Livera, 2018) through the migration of hPGCs from the yolk sac to the outermost layer of organ called the cortex of the embryo and at this point they are referred as oogonia. The oogonia go through mitotic division to produce seven million germ cells at approximately two to seven months of gestation. A number of this germ cells experience atresia reducing the peak to two million cells named primary oocytes (Rojas et al., 2015).

The primary oocytes each containing a nucleus called the germinal vesicle (GV) proceeds to meiosis I where they are surrounded by follicular cells a layer of cuboidal cells of the epithelium however, they are arrested at the diplotene stage

of meiosis I until puberty. At adolescence, the entrapped primary oocytes resume prophase I, where they undergo periodic atresia decreasing the number of oocytes to 40,000. This is followed by metaphase I where the GV stage oocytes breakdown and the follicular cells surrounding the oocytes release glycoprotein forming translucent layer called the zona pellucide, which differentiate to form the follicule luteinizing hormone (LH) responsive layer responsible for androgen secretion (Johnson et al., 2005), also homologous chromosomes align at the metaphase plate. At anaphase I, the metaphase spindle migrates to the fringe of the cell. The process ends with telophase I where the nuclear envelop reform and a space filled with fluid called the antrum forms in between the follicular cells. At the end meiosis I, two daughter cell of unequal size are produced. The smaller daughter cell becomes the first polar body while the larger cell called secondary follicle which contains nearly the entire volume of the constituents becomes the mature oocyte under the effects of follicle stimulating hormone, luteinizing hormone and oestrogen secreted by the anterior pituitary gland of the hypothalamus. (Sánchez & Smitz, 2012).

The cells progress to the meiosis II where they go through similar cytokinese to produce haploid cells of unequal sizes. At the beginning of menstrual cycle, a number of follicles gets recruited under the influence of elevated FSH to form secondary follicle (Sánchez & Smitz, 2012). On the ninth day of the cycle, a mature secondary follicle is retained while the remaining follicle undergo atresia, On the 14th day of the cycle a positive feedback is triggered by

luteinizing hormone and the secondary oocyte is ovulated 24 -36 hours.

The ovulated oocyte released by the ovaries gets picked up by the fimbriae into the fallopian tube a structure where fertilization takes place in the presence of sperm cells (Jodar et al., 2013), whereas in the absence of sperm cells the ovulated oocyte degenerates (Coticchio et al., 2012). The emptied follicle forms a temporary endocrine structure called corpus luteum which produces progesterone needed to maintain the endometrium until further menstrual cycle.

### 1.3.2 The morphology of the ovum

The female egg is approximately 0.2mm in diameter implanted within the follicles of the ovaries, each follicle often contains a single ovum. However, sometimes it contains two or more and by subsequent enlargement and disruption of the follicle it is released by the ovaries. The ovum is conveyed through the uterine tube into the uterus. The structure of the ovum is similar to that of an ordinary cell although each part of the cell has a distinctive name as discussed below.

The egg is covered by a thick membrane consisting of a stratified layer of numerous connective tissues called the zona pellucida which play a role in cell segmentation. The outer layer of the egg also consists of the corona radiata or follicular cells which adhere to the surface of the zona strata after the egg is released from the ovaries. The cell substance of the female egg is referred to as the yolk or ooplasm which comprises of the cytoplasm with hyaloplasm,

spongioplasm and also several roundly shaped granules of albuminoid and fatty substance imbedded inside the cytoplasm. Within the ovum is a balled shaped structure called germinal vesicle located at the centre of the egg cell and a nucleolus named the germinal spot. Additionally, the egg structure also consists of a reticulum with meshes filled with nucleoplasm and fewer number of chromosomes arrange in form of a rod.

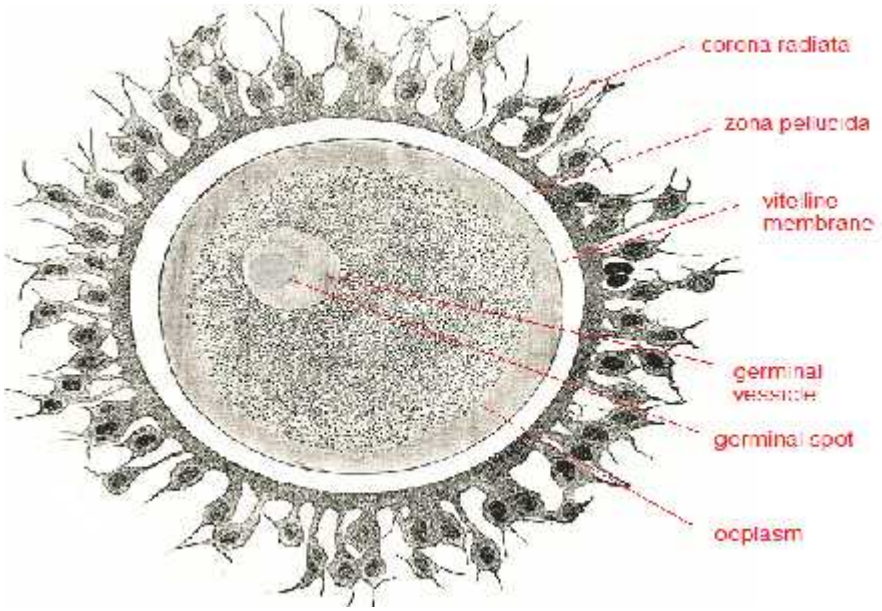



**Figure 1.1: A close diagram of the female egg.**

### 1.4 Polycystic ovarian syndromes

Polycystic ovarian syndrome (PCOS) is an eminent type of endocrinal disorder in the breeding age of women with a ubiquity of 6% to 15% worldwide. In previous years, the phenotypic features of the disorder is mainly defined using the 2003 Rotterdam criteria which requires at least two to three of the adherent attributes, this includes (i) chronic anovulation (ii) polycystic ovaries and (iii)

hyperandrogenism (Trikudanathan, 2015). PCOS is speculated to be culpable for 0.4% of female infertility and also, it is the leading cause of endometrial cancer (Crespo et al., 2018). Although, in some mild PCOS cases, these patients may have hyperandrogenism and polycystic ovaries with normal ovulations and higher risks of cardiovascular events or type 2 diabetes mellitus (He et al., 2012).

Presently, there are four clinical characterized phenotype associated with PCOS: i) hyperandrogenism and chronic anovulation, ii) polycystic ovarian morphology and hyperandrogenism iii) polycystic ovarian morphology and chronic anovulation iv) hyperandrogenism and chronic anovulation and polycystic ovarian morphology (Crespo et al., 2018). Moreover, aside reproductive dysfunctions, PCOS has also been linked to distinctive types of metabolic anomalies, this includes, glucose intolerance, type II diabetes mellitus, dyslipidemia, hepatic steatosis, hypertension and cardiovascular disease (A. L. Liu et al., 2017). Females with PCOS are usually obese with prominent central or gastric obesity, which sequentially antagonize insulin resistance and increased susceptible of developing debilitated glucose tolerance (IGT), cardiovascular disorders, diabetes mellitus type 2, high blood pressure and dyslipidaemia.

### 1.4.1 Causes of the PCOS

The PCOS originates from hormonal variation as well as erratic menstrual cycle with cystic and enlarged ovaries, hirsutism or amenorrhea in reproductive females. The disease is mainly a spring of sterility alongside social imbalance.

In healthy adult females, a mature oocyte is released by the ovaries during ovulation under the influence of oestrogen, LH and FSH. Contrarily, in women with PCOS one or both ovaries may carry numerous immature or mature ovarian follicles that are cystic in nature. These changes observed were associated with subcellular aberrations in the granulosa and theca cells of women with PCOS which was caused by prominent androgen and elated serum anti-mullerian hormone released as a result of inherent activation of theca cell steroidogenesis despite the lack of gonadotrophic factors such as FSH and LH (Nelson et al., 1999). Also, more number of pre-antral and lower number of antral follicles (Webber et al., 2003) as well as defective apoptotic process of maturing follicles (Das et al., 2008) were described as causative agents of PCOS. Furthermore, aberration in the insulin signalling pathway and gene expression independent of obesity have also been expressed as an elementary cause of PCOS (Cortón et al., 2007). Additionally, the glycol oxidative stress pathway was reported as causative factor for PCOS (Khan et al., 2019). Even so, due to the complexity of the disorder, the causative mechanisms stated as causes of PCOS are still not fully understood.

### 1.4.1. Genetics and Pathogenesis of Polycystic Ovarian Syndrome

PCOS is a multifactorial disorder with genetic background which originates from the combination of both genetic and environmental factors. The hereditary cause of PCOS is distinct among families but shares predominant pathway. Moreover, owing to the heterogeneous nature of the disease, no single gene or affiliated genes in a particular family have been reported so far. However, the pathogenesis of the disease appears to have several inherited genes and environmental factors which can lead to or increase the risk of developing the disorder. Besides, the predominant genetic factors associated with PCOS are mainly alterations in genes responsible for the production of androgen, insulin and other hormones that directly or indirectly play key important roles in female ovulation (Khan et al., 2019). Other genetic variant related to PCOS includes genes associated with immune response to injury, energy production, and Wnt pathways involved in the production of fat. The efforts to identify these genetic predispositions have been based on linkage or association studies of some suspected functional genes. Still and all, only few studies have confirmed this suspicion and most of the effective findings attain with PCOS phenotypes has not been reproduced in more than one population. In spite, these genetic polymorphisms and their potential roles in pathogenesis of PCOS are further discussed below in details.

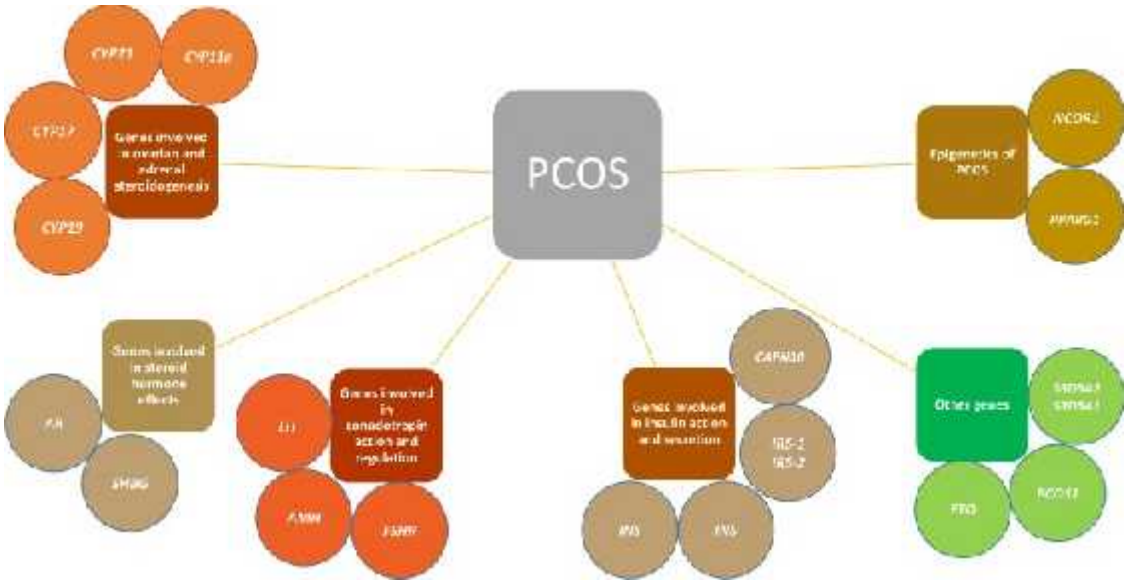



**Figure 1.2: A clear illustration of the multiple genes involved in the pathogenesis of PCOS as retrieved from (This illustration was adapted from Khan et al., 2019)**

### 1.4.2 Genes Correlated with Female Adrenal and Ovarian Steroidogenesis

Hyperandrogenemia is one of the most common features observed in women with PCOS. Currently four (*CYP11a, CYP21, CY917 and CYP19*) relevant genes associated with steroidogenesis have been reported as genes responsible for PCOS however till date contrasting results have been published. (Unluturk et al., 2007)

The *CYP11a* is a gene located on chromosome15q24 which codes for a cytochrome side chain cleavage (P450) enzyme that is responsible for transforming cholesterol into progesterone at the outset of adrenal and ovarian steriodogenesis (Unluturk et al., 2007). Numerous reviews have reported

*CYP11a* polymorphism as risk factor for hyperandrogenemia and PCOS. An association was reported between the *CYP11a* alleles and increased serum testosterone level in PCOS patients (S et al., 2000), PCOS in Chinese women (Y. Wang et al., 2005) and in Greek women (Diamanti-Kandarakis et al., 2000). Diversely, in a subsequent study using a large scale sample size from Finland and the United Kingdom no statistical association was found between *CYP11a* polymorphism and excessive androgen production PCOS women (Gaasenbeek et al., 2004).

The *CYP21* gene encodes for 21- hydroxylase enzyme which converts 17-hydroxyprogesterone into 11- deoxycortisol during adrenal and ovarian steriodogenesis. Genetic alteration in the *CYP21* gene was allied with elevated serum 17-hydroxy-progesterone response to adrenocorticotrophic stimulation (R. Azziz et al., 1995). Witchel et al (2000) reported an association between *CYP21* polymorphism and PCOS in women heterozygous for hyperandrogenism (Witchel & Aston, 2000). Contrary, a number of studies failed to establish a link between *CYP21* polymorphism and increased level of androgen in women with PCOS. Glintborg et al (2005) found no statistical association between *CYP21* alleles and PCOS in women with idiopathic hirsutism (Glintborg et al., 2005) and also in case control study of 114 women with PCOS (Sf et al., 2005).

The *CYP17* gene on chromosome 10q24.3 encodes for the P450c17α enzyme that is responsible for the conversion of 17-hydroxyprogesterone and 17-hydroxypregnenolone into androstenedione and dehydro-epiandrosterone, respectively. The enzyme has the activities of both 17,20-lyase and 17 α-hydroxylase (Picado-Leonard & Miller, 1987). Variations in the *CYPc17α* gene was significantly associated with increase androgen level in women with hyperandrogenism and PCOS (Rhoda et al) thus several studies have supported this finding. Wickenheisser et al (2004) found a correlation between increased enzymatic activity of *CYPc17α* (P450c17) and high androgen level in the theca cells of women with PCOS (Wickenheisser et al., 2004). Similarly, *CYPc17α* polymorphism was significantly association with increased androgen secretion in the ovaries and adrenal sites of women with PCOS and hyperandrogenism (H. Escobar-Morreale et al., 1994). Wickenheisser J and colleagues (2005) reported dysregulation in the expression of the *CYPc17* gene in the theca cell of women with PCOS (Wickenheisser et al., 2005).

The *CYP19* gene located on the short arm of chromosome 15q21 which provides instruction for making an aromatase P450 enzyme responsible for the conversion of androgen to oestrogen. The enzyme consists of two cytochrome complex the NADPH cytochrome P450 reductase and cytochrome aromatase (P450arom). Deficiency of the P450 enzyme significantly led to lower levels of aromatase stimulating bioactivity and lower estradiol in women PCOS when

compared to the reference group (Jakimiuk et al., 1998). This finding implies that a decrease in aromatase activity might contribute to the abnormal follicular development seen in women with PCOS.

### 1.4.3 Genes responsible for Steroid Hormone Effects

The androgen receptor *(AR)* gene is cytogenetically located on chromosome Xq11-12. The gene is enclosed with four clinical domains, this includes; (i) the ligand binding domain, (ii) the transactional domain and (iii) the DNA binding domain. The transactional domain of gene contain variable number of tandem repeats (VNTR) of CAG repeats about 11to 38 in lengths on exon -1of the gene which code for a polyglutamine protein chain positioned at the N-terminal of the *AR* gene (Carson-Jurica et al., 1990). In addition, the transcriptional activity of the *AR* gene is conversely proportional to the expression of the CAG repeats. Therefore, variation in the number of the CAG repeats even within the normal range has been associated with decrease or increase in androgenic activities (Unluturk et al., 2007). Although a proportion of PCOS patients with lower number of CAG repeats still express higher androgen activity, thus this could explain why some women with PCOS have hyperandrogenism symptoms as well as normal androgen levels (Mifsud et al., 2000). Hickey et al (2002) reported significant increase in androgen levels as a result of variation of CAG repeats (22 repeats or more) in infertile women with PCOS (Hickey et al., 2002). On the contrary, Jääskeläinen and colleagues (2005) found no

correlation between the CAG repeats of AR gene and the testosterone levels or body mass index of PCOS patients (Jääskeläinen et al., 2005).

The sex hormone binding globulin (*SHBG*) gene consists of a homodimeric glycoprotein produced by hepatocytes in the liver which modulate androgen access to specific tissues. The *SHBG* gene is encoded by the 4kb gene on chromosome 17p12-p13. Additionally, the *SHBG* gene also regulates the levels of various sex hormones by binding to androgen, comprehensively oestrogen or testosterone. Aberration in the *SHBG* gene significantly led to lower serum *SHBG* level which in turn contributes to increase tissue androgen availability observed in hyperandrogenism patients and primarily in corporation with PCOS (Unluturk et al., 2007). Moreover, the pentanucleotide repeats of (TAAAA) nucleotides at the promoter region of the *SHBG* gene has also been reported as a risk factor for PCOS (Ünlütürk et al., 2016). Various studies have investigated the association between the *SHBG* (TAAAA) *n* repeats and PCOS. Xita et al (2003) analysed the frequency of the *SHBG* (pentanucleotide TAAAA) repeats in Greek women with PCOS and those without, in such they found higher number of *SHBG* repeats in PCOS women when compared to women without (Xita et al., 2003). Similarly, Cousin and colleagues (2004) found decreased levels of serum *SHBG* as a result of longer (TAAAA)*n* repeats in women with hirsutism as well as the involvement of a single nucleotide

polymorphism of (Asp327Asn) on exon 8 of the *SHBG* gene and PCOS (Cousin et al., 2004).

### 1.4.4 Aberration in genes that regulates Insulin Action and Secretion

Insulin resistance and hyperinsulimea are common pathophysiological features observed in most PCOS patients. A significant number of researches have investigated the association of genes responsible for insulin secretion and action in relation to PCOS this includes; (i) insulin receptor gene (ii) insulin substrate receptor gene *(INRS)* (iii) the insulin gene and (iv) calpain-10 gene (Khan et al., 2019). However, to date controversial results have been published.

The insulin (*INS*) gene is situated in amidst the genes that code for the insulin-like growth factor 2 and tyrosine hydroxylase located on chromosome 11p15.5. The 5' regulatory region of the *INS* partly contains variable number of tandem repeats (*VNTR*) which controls the transcriptional rate of *INS* receptor gene and IGFII gene (Ünlütürk et al., 2016). The number of VNTR repeats ranges from 26 – 200 and they are grouped into three classes; class -1 consist of shorter VNTR of 40 repeats length. Class II alleles have a minimum of 80 repeats while class-III has the longest length with an average of 157 repeats (Unluturk et al., 2007). MichaelMore and colleagues (2001) reported a statistical association between paternal class III of the *INS* polymorphism and reduced insulin sensitivity alongside increase PCOS features in PCOS patient (Michelmore et al., 2001).

The insulin receptor gene (*INSR*) encodes for a turmeric protein consisting of two alpha and beta chains, the gene is located on chromosome 19. Several analyses have described the relationship between the *INSR* genetic polymorphism and the observed insulin resistance different patient with PCOS. Tucci et al (2001) reported an association between the dinucleotide repeat marker (D195884) of the *INRS* polymorphism in Caucasian women with PCOS (Tucci et al., 2001). In another study, Urbanek and associates (2005) evaluated the association of *INRS* gene in 367 families from Europe with PCOS, in this study they investigated a large part of chromosome 19p13.2 and found an association between the D195884 marker of the insulin receptor gene in women with PCOS (Urbanek et al., 2005).

Insulin receptor substrate protein (IRSs) secrete a protein responsible for the autophosphorylation of the beta subunit of the insulin receptor substrates (IRS-I and IRS-2) which trigger proximate effectors such as phosphoinositide 3-kinase. The tyrosine kinase activity of the IRSs regulates the metabolic secretion of insulin and variation on this gene is directly connected to the rate of abnormal insulin metabolism detected with PCOS disorder. Villuendas et al (2005) evaluated the association between IRS-1and IRS-2 genes in women with PCOS using the (Gly972Arg) polymorphism for IRS-1 and (Gly1057Asp) for IRS-2, in which they detected a statistical difference in the ratio of Gly972Arg of IRS-

1 in Turkish women with PCOS when compared to those without (Villuendas et al., 2005).

The Calpain 10 *(CAPN10)* is a gene located on chromosome 2 which encodes for calcium dependent cysteine protease a heterodimeric protein responsible for insulin metabolism and secretion. Variations in the *CAPN10* gene, results to higher insulin levels in most PCOS patients. Ehrmann et al (2002) found statistical correlation for the 112/121 haplotypes of the *CAPN10* gene in respect to extreme insulin concentration in black American women with PCOS (Ehrmann et al., 2002), and the (SNP-44) of the *CAPN10* gene in Spanish women with PCOS (Unluturk et al., 2007).

### 1.4.5 Genes associated with human Gonadotropin modulation and action

Luteinizing hormone/ chorionic gonadotropin receptor *(LHCGR)* gene codes for receptor protein which binds to two ligands; the luteinizing hormone and chorionic gonadotropin. In females *LH* stimulates the release of ova from the ovaries at ovulation. Most women with PCOS develop common features such as hyperandrogenism, chronic anovulation and hirsutism as a result of excess androgen or other sex hormones such as *LH* and *FSH* (Ricardo Azziz, 2018). Since women with PCOS have either low or high levels of LH as well as altered *LH* action, the β subunit of the *LH* gene has been part of the genes explored in women with PCOS. In previous years, two Variations (Trp8Arg and I1g15Thr) was discovered at the β -subunit of the *LH* gene in two sisters with PCOS (Furui

et al., 1994) and were later demonstrated as common polymorphism with 15% prevalence globally. Additionally, Takahashi et al (2003) reported several redundant SNPs at the regulatory region of the *LH* (beta-subunit) in the PCOS patients group when compared to controls (Takahashi et al., 2003).

**1.4.6 Follicular Stimulating Hormone Receptor *(FSHR)* gene** provides instruction for making a G coupled receptor protein required for the gonadal development. Variation in the gene *FSHR* gene has been associated with abnormal ovarian morphology, ovarian hyperstimulation syndrome and hormonal imbalance in North Iraq women with PCOS (Baban et al., 2018).

**1.4.7 Anti Müllerian hormone *(AMH)* gene** is a gene found on chromosome 19p13.3 which modulates the production of a protein that is involved in male sex differentiation at the foetal stage of growth and the development. The protein secreted by this gene binds to a surface receptor on the Müllerian ducts an organ present in both male and female embryo. In males, *AMH* protein and receptor complex induces apoptosis and regression of the Müllerian duct whereas in females the Müllerian duct differentiates into various female organs. Consequently aberration in the *AMH* gene has been associated with infertility and PCOS disorder. In an objective whole genome sequencing studies by Lidija et al (2017), in such they reported several deleterious variants in the *AMH* gene which led to significant decrease in *AMH* signalling levels in PCOS patient

when compared to the controls (Gorsic et al., 2017) indicating an association between *AMH* polymorphisms and PCOS.

**Follistatin gene** (*FST*) encodes for the activin-binding protein, the gene is located on chromosome 5. It is anatomically not related to the TGF- β gene superfamily. However, it acts as a ligand for activin a dimeric glycoprotein of the (TGF- β) gene family which partakes in the induction of follicogenesis, FSH, ovulation, insulin secretion and the inhibition of LH stimulated androgen production. Genetic alteration in the *FST* gene is associated with increased level of follistatin in the body which in turn leads to excessive activin synthesis and decreased FSH concentration and impaired insulin secretion. Since these changes are predominantly seen in women with PCOS the FST gene has been examined as a candidate gene for the pathology of PCOS disorder. Urbanek et al (1999) reported an association between follistatin gene polymorphism and PCOS (Urbanek et al., 1999).

### 1.4.8 The genes involved in human homeostasis

The adipose tissue is an active endocrine organ that is responsible for the secretion of adipocytokines such as leptin and adiponectic. Since a significant ratio of women with PCOS are either weighty or highly obese, these genes have also been studied as causative genes for PCOS disease (Unluturk et al., 2007).

**Leptin protein** is a hormone produced by the *ob* gene which is mostly expressed in adipose tissue, the gene belongs to a non-glycosylated peptide of 146 amino acid chain that is responsible for the regulation of energy production, body weight and also the regulation of FSH, LH, ACTH and cortisol. Merhi et al (2013) demonstrated how aberration in the leptin resulted in the decrease in mRNA expression of AMH through the Jak2/STAT3 pathway (Merhi et al., 2013)

**Adiponectin** is a protein hormone that is encoded by *ADIPOQ* gene which modulate several metabolic processes in the human body this include glucose regulation and fatty acid oxidation. Mutations in the *ADIPOQ* gene was reported as a risk factor for insulin resistance discerned in women affected with PCOS (Benrick et al., 2017) although the causal relationship between adiponectin levels, infertility and metabolic dysfunction in PCOS is not fully understood. Several studies have explored the (T45G) in exon 1 and (G276T) in intron 2 of the *ADIPOQ* gene as causative agents in genetics of PCOS, due to their association with insulin resistance, obesity and type 2diabetes. Panidis et al (2004) studied the feasible association of the T45G polymorphism in women with PCOS, in such they found similar frequency rates of the GG, TT and TG genotypes in both PCOS affected and unaffected groups. However, when they analysed the frequency of the (GG) and (TG) genotypes together, a statistical difference was detected between women with PCOS and controls. In their

findings, women who were carriers of the G alleles significantly showed lower serum adiponectin concentration in comparison to those women without the G allele (Panidis et al., 2004). Also, two other studies have confirmed the association of the T45G and G276T polymorphism with PCOS in Spanish women (H. F. Escobar-Morreale et al., 2006) and Greece (Xita et al., 2005).

**The peroxisome proliferation activated receptor gamma *(PPAR- γ)* gene:** is a type-II receptor encoded by the *PPARG* gene that is cytogenetically located on chromosome 3p25 whose molecular function is to regulate tissue metabolism and adipogenesis. Besides, it functions in the regulation of insulin sensitivity, lipid metabolism and energy storage. Additionally, the gene also has functional receptors for Thiazolinediones (T2Ds) an insulin sensitizing agents. Genetic variation on the *PPARG* gene was significantly associated with up regulated *PPARG- γ* protein in the ovaries of women with PCOS (Khan et al., 2019b). Additionally, Hahn et al (2005) reported a statistical association between proline12alanine (Pro12Ala) missense variant of the *PPAR- γ* and increase insulin hypersensitivity as well as lesser hirsutism count in women with PCOS (Hahn et al., 2005). Similarly, Tok el al (2005) reported that women who were heterozygous for the Pro12Ala variant significantly expressed less insulin resistance compared to controls (Tok et al., 2005).

### 1.4.9 Other genes and proteins associated with Polycystic Ovarian Syndrome:

The plasminogen activator inhibitor 1 gene (*PAI-1*) or Serpine 1 gene encodes for a serine protease inhibitor enzyme which play an important role in the conversion of inactive protein plasminogen into plasmin during fibrinolysis. Abnormalities in the *PAI-1* lead to disruption in coagulation and fibrinolysis which as a result add to the risk of cardiovascular events in PCOS patients (Sahay et al., 2017). Also, Diamant et al (2004) analysed the role of *4G/5G* polymorphism of the *PAI-1* gene in Greek women with PCOS and control, in which they also reported higher levels of *PAI-1* protein in PCOS patient due to the presence of 4G allele (Diamanti-Kandarakis et al., 2004). In accordance to this findings, the PAI-1 4G/5G polymorphism was also reported as a susceptible gene for type 2 diabetes and myocardial event in some PCOS patients (Sahay et al., 2017).

**Tumor necrosis factor alpha *(TNF)*** is a proinflammatory cytokine produced by adipose tissue which induce a negative inotropic effect in the heart and also play a role in insulin resistance. Recently, changes in the expressing level of the *TNF* protein has also been reported as a genetic risk factor PCOS (Younis et al., 2014).

***PCOS1:***

Gene also referred to as the *PCO* gene is located on p13.2 of chromosome 19, though the molecular function of the gene is still not fully understood.

Aberration in the PCO gene was associated with polycystic ovarian syndrome (Urbanek et al., 2005 and Khan et al., 2019 ).

Although, there are inconsistent data on most of the polymorphisms involved with PCOS, the above mentioned polymorphisms remain at least in part as potential candidate genes for the underlying cause of PCOS. Though, additional research studies needs to be conducted in order to further confirm most of the controversial results.

### 1.5 The WNT Signalling Pathway:

Since the discovery of the WNT pathway in 1982, the roles of the *WNT* signalling and mechanisms have been studied on a large scale. The gene was named after a gene called Int-1 was first discovered in mice and in subsequent years, the wingless (Wg) gene, a homolog of the int-1 was discovered in drosophila (Rijsewijk et al., 1987). Hence, both genes were formally combined to form the WNT gene family which is composed of 19 cysteine amino acid and glycoprotein structures whose primary goal is to pass signals in to a cell by binding to cell surface receptors (Ilyas, 2005). The WNT signal transduction cascade uses either autocrine or paracrine cell communication to regulates series of biological processes throughout the growth and development of all organisms. In addition to this, WNT signalling pathway is one of the biological component that is responsible for the regulation of cell polarity, cell migration, cell fate and cell proliferation at human embryonic development and adult tissue

homeostasis (Clevers & Nusse, 2012). Indeed, the etiology of most complex diseases especially cancer and some birth defects are thought to be caused by disruptions in genes that encode for proteins in the signalling pathway, and correspondingly aberration that can lead to an uncontrolled activation of the Wnt signalling pathway.

### 1.5.1 A Review of the WNT Signalling Pathway

The WNT signalling pathway is activated through the binding of WNT ligand to the N-terminal auxiliary cysteine rich domain of the frizzled receptor (a transmembrane span receptor). The frizzed receptors (Fz) spans the plasma membrane to form a family of seven (pass) trans membrane domain receptor also known as (G-protein coupled receptors). At this point, the WNT protein and Fz receptors interact with other co receptors this includes, receptor tyrosine kinase (RTK), tyrosine protein kinase transmembrane receptors (ROR2) and protein receptor related protein (LRP), which as a result, convey transductional signals to the Phosphosprotein Dishevelled (Dsh) protein, a highly evolutionarily conserved protein consisting of three distinctive domains; (i) the central-PDZ-domain (ii) a carboxyl terminal-DEP-domain and (iii) the amino terminal-Dix-domain which facilitates the distribution of the Wnt conducted signals to the three different types of the WNT signalling pathway; the WNT/ β -catenin ( canonical ) signalling pathway, WNT/planar cell polarity ( non-

canonical ) signalling pathway, and WNT/Ca+2 signalling pathway (Huelsken & Behrens, 2002).

### 1.6 Overview of the Functional Regulation of WNT/β-Catenin Signalling

The WNT β- catenin operates in an on or off state as illustrated in (**Figure 3)**. In the absence of a WNT ligand (the OFF state), β-catenin protein is persistently broken down by the Axin mediated β-catenin phosphorylation destruction complex which consist of the tumour suppressor adenomatous-polyposis-coli (APC), the Axin-scaffolding protein, the glycogen-synthase-kinase 3 (GSK3) and casein kinase-1 (CKI). During the process, GSK3 and CK1 of the Axin complex subsequently phosphorylate the amino-terminal-region of the β catenin, hence allowing it to be recognized by the E3-ubiquitin-ligase subunit (Sanchez et al., 2014a). The β-catenin protein undergo sequential unbiquitination and proteosomal degradation which prevents it from been translocated into the nucleus of the cell thusly restraining the transcription of the Wnt targeted genes (MacDonald et al., 2009). However, in the presence of a ligand, the Wnt-β-catenin pathway is activated (ON state) when the WNT-ligand binds to a frizzled (Fz) receptor with a co receptor related protein 6 (LRP6) or LRPS belonging to the family of low density lipoproteins (LDL) receptor, this results to the formation of a (WNT and Fz) like complex which with the recruitment of the dishevelled protein (Dsh) leads to the phosphorylation of the LRP6 and the disruption of axin destruction complex

(MacDonald et al., 2009). And with the complete inhibition of the Axin mediated phosphorylation, β-catenin secretion is stabilized thus allowing it to accumulate and translocate into the nucleus to form a complex with the regulatory factors (TCF or LEF) in order to initiate WNT targeted gene transcription (Huelsken & Behrens, 2002).

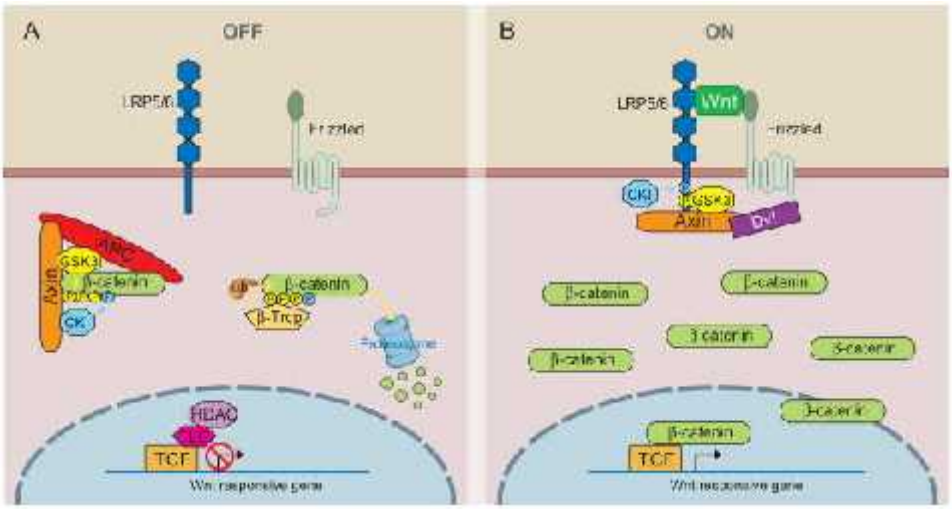



**Figure 1.3: The OFF and ON functional switch of the β-catenin pathway adapted from ( Adapted from MacDonald et al., 2009).**

## 1.7 Work in this thesis

Chapter one offers background information about this research. In this chapter, general information of cell division, gametogenesis, oogenesis and ovum morphology was discussed. Furthermore, polycystic ovarian syndrome and its causes with genetics side were conferred. In addition, there are some genes and pathways like WNT B-catenin pathway was argued.

The second chapter provides information about the methodology that used for data collection and the statistical analysis to analyse the results for the study.

Chapter three discusses the results that obtained from this research.

The fourth chapter describes the results in detailed and associate the data with literature review, the conclusions and dissection of this study is included in this chapter.

# CHAPTER II

# Materials and Methods

## 2.1 Materials

### 2.1.1: Suppliers and sample collection

Oocytes samples were collected from Near East Hospital IVF Center, the samples were divided into two groups according to the present or absence of the polycystic ovaries in the females who donated oocytes for the project.

The experiments were performed in Molecular Medicine Laboratory, DESAM Institute, Near East University, Nicosia, North Cyprus.

Seven samples among 13 oocyte samples were collected from patients with polycystic ovaries and the other six samples were collected from females without polycystic ovaries. The criteria for collecting the samples from women patients included non-obese and young patients and it also included two groups; control group without polycystic ovaries syndrome, and PCOS group with polycystic ovaries syndrome.

PCOS samples were coded as 1, 2, 3, 6, 7, 8, and 11. Control samples were coded as 4, 5, 9, 10, 12, and 13.

## 2.1.2: ANALYSIS OF OOCYTES

The procedures were performed with three main steps, the RNA extraction from the samples, then cDNA synthesizes was performed, and the last step was real-time PCR amplification for the samples. It is worth to mention that duplicated RT-PCR was done by using Real Time PCR (Qiagen,Hilden, Germany).

Therefore, the total PCR samples were 27 samples with one sample as negative control for each analyzed gene, the negative control contains master mix contents without adding any cDNA sample, unlike the other 26 samples which consists of master mix contents and cDNA samples.

The expression levels were investigated in a total of four genes: *WNT1, APC, B-catenin, and GSK3B* genes.

## 2.2 RNA concentration

RNA concentrations were measured by using NanoDrop Spectrophotometers (Thermo scientific, Pittsburg, USA). The equipment was used to assess and quantify purity of RNA.

The 260/280 ratio of pure RNA is expected to be around 2ng/µl

### 2.2.1: cDNA Synthesis

Transcriptor First Strand cDNA Synthesis Kit (Thermo scientific, Pittsburg, USA) was used for cDNA synthesis, the kit should be stored at -15 to -25 °C. The kit is used to generate cDNA.

There are three different types of priming methods for cDNA synthesis, anchored-oligo dT primer (oligonucleotide), random hexamer primer.

**Table 2.1 Template-Primer Mix**

| Component | Volume | Final conc |
|---|---|---|
| Total RNA or mRNA | - | 1 µg total RNA |
| Anchored-oligo dT | 1 µl | 2.5 µM |
| Random hexamer | 2 µl | 60 µM |
| Water | Variable | To make total volume = 13µl |

The table (2.2) was also used for generating cDNA

**Table 2.2 cDNA synthesis**

| Component | For (1 X) |
|---|---|
| Rxn buffer | 2 µl |
| Randome hexamer | 2 µl |
| Dntp | 1 µl |

| | |
|---|---|
| RTase | 1 µl |
| RNAse inhibition | 0.5 µl |
| RNAse free water | 3.5 µl |
| Total | 10 µl |

### 2.2.2: Real Time PCR (Master Mix)

WizPure qPCR Master (SYBR) kit (Wizbiosolutions Inc, South Korea) was used in order to evaluate the expression level used by RT-PCR.

Certain calculations have been made in order to know what the appropriate amount for the mixture is. Reverse and forward primers was used for four genes according to the Figure 4.1and 4.2, the primers were designed for *WNT1, APC, B-catenin, GSK3B.*

### Table 2.3: oligonucleotide synthesis

| Oligo Name | Sequence of the Primer | Purification Type | Synthesis Scale (nmol) |
|---|---|---|---|
| Beta Catenin (CTNB1).F | AGACGGAGGAAGGTCTGAGG | Desalting | 50 |
| Beta Catenin (CTNB1).R | TTCAAATACCCTCAGGGGAACA | Desalting | 50 |
| GSK3B .F | ACAGCAGCGTCAGATGCTAA | Desalting | 50 |
| GSK3B .R | TGACCAGTGTTGCTGAGTGA | Desalting | 50 |
| WNT1.F | CCGATGGTGGGGTATTGTGA | Desalting | 50 |
| WNT1.R | ACGATCTTGCCGAAGAGGTG | Desalting | 50 |
| GAPDH(CDH).F | CAAATTCCATGGCACCGTCAAG | Desalting | 50 |
| GAPDH(CDH).R | GCAAATGAGCCCCAGCCTTC | Desalting | 50 |

| Oligo Name | Tm ° C | GC (%) | MW (g /mol) | nmol | A 260 | 100 µM stock- ul TE |
|---|---|---|---|---|---|---|
| Beta Catenin (CTNB1).F | 61 | 60 | 6296 | 62 | 48 | 621 |
| Beta Catenin (CTNB1).R | 58 | 45 | 6712 | 56 | 47 | 563 |
| GSK3B .F | 57 | 50 | 6135 | 36 | 27 | 359 |
| GSK3B .R | 57 | 50 | 6188 | 70 | 54 | 700 |
| WNT1.F | 59 | 55 | 6244 | 65 | 50 | 653 |
| WNT1.R | 59 | 55 | 6182 | 68 | 52 | 684 |
| GAPDH(CDH).F | 60 | 50 | 6688 | 67 | 55 | 668 |
| GAPDH(CDH).R | 61 | 60 | 6047 | 72 | 54 | 717 |

The table (2.3) shows how master mix for the components and the calculations were done

**Table 2.4: Master Mix**

| **Final concentration** | **1X** | **28X** |
|---|---|---|
| **QPCR Master (SYBR)** | 5µl | 140µl |
| **FORWARD PRIMER** | 0.5µl | 14µl |
| **REVERSE PRIMER** | 0.5µl | 14µl |
| **Water,RNase-free** | 3.5µl | 98µl |
| **Cdna** | **0.5µl** | |

The component volumes have been multiplied to 27 times to be enough for the 13samples that were doubled and the one negative control.

Then the samples were run by PCR machine, according to the PCR conditions.

**Table 2.5: PCR Conditions**

| **Step** | **Temp(°C)** | **Time** | **cycle** |
|---|---|---|---|
| Initial denaturation | 95 | 5 min | 1 |
| Denature | 95 | 10-30 sec | 40 |
| Anneal | 55-68 | 10-60 sec | |
| Melting curve analysis | 65-96 | 2-5 sec/step | 1 |

After running the samples on PCR machine for about an hour and half, the results were obtained and measured by CT values.

### 2.2.3: Statistical Analysis

Statistical analysis was performed by using ANOVA test (GraphPad Prism 8.4.3) and student's T-test. The student's T-test was used to find the correlation analysis between each gene and PCO.

All the samples were separated into two types: study group (PCO, oocytes collected from females with polycystic ovaries), and the control group. PCO samples are 1, 2, 3, 6, 7, 8, and11. Control samples are 4, 5, 9, 10, 12, and 13.

P value was used to compare the results between the genes; if the result for P value was less than 0.05 then the result will be considered as significant, and if the result was higher than 0.05, it was considered as not significant.

The expression levels were performed using the comparative individual ΔΔCt. ΔΔCt means the difference or the expression level between the *ACTB* gene and the target genes (*WNT1, GSK3B, APC, and B-catenin* genes).

Individual 2^ (-ΔΔCt) was used for the Student's T-test analysis, the Ct values were used for ANOVA, to analyze the results.

*ACTB* was used for expression analysis in the four genes, which is considered as a housekeeping gene for normalization.

# CHAPTER III

# RESULT

## 3.1 Introduction:

A total of 13 oocyte samples were included in this study, seven samples among 13 oocytes were obtained from females with polycystic ovaries and the other six samples were obtained from females without polycystic ovaries. Following a through literature review, it was noticeable that there are a few articles talking about these genes with PCOS. On the contrary, there are some genes that there is no article that discusses their relationship with PCOS. To our knowledge; this is the first study that shows the possible role and relationship of *APC* gene in the development of polycystic ovaries. As far as we have noticed, this is the second study that discusses the relationship between *WNT1* with *PCOS* in general because the first study was by way of a blood sample. However, it can be consider as the first study that shows the relationship between *WNT1* and *PCOS* by oocyte sample in specific. The aim of this study was to investigate the expression levels of *WNT1, GSK3β, β-CATENIN* and *APC* functioning in the *WNT/β-catenin* signaling pathway in oocytes obtained from seven patients with polycystic ovaries and six females with no polycystic ovaries.

The RNA concentrations were measured by NanoDrop Spectrophotometers. Nucleic acid concentrations and 260/280 results were obtained from it.

**Table 3.1:** RNA concentrations

| sample ID | NA conc | 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 |

# 3.2: Ct Values results

The expression levels of these genes were investigated in human oocytes by RT-PCR using RNA samples obtained from female donors. In total, 13 patients were included in this study. The Ct values for the four genes were as shown in Table 3.2

**Table 3.2**: Ct values for WNT1, APC, Beta-catenin, and GSK3B genes.

| sample nu | APC gene | WNT1 gene | B-cat gene | GSK3B |
|---|---|---|---|---|
| 1 | 31.39 | 38.48 | 33.96 | 36.41 |
| 2 | 36.42 | 34.34 | 31.85 | 33.28 |
| 3 | 35.9 | 35.67 | 34.42 | 34.2 |
| 4 | 37.35 | 34.12 | 30.46 | 33.59 |
| 5 | 32.57 | 34.23 | 32.69 | 35.9 |
| 6 | 36.2 | 32.57 | 32.66 | 43.49 |
| 7 | 34.71 | 31.8 | 32.63 | 41.19 |
| 8 | 32.51 | 30.06 | 31.47 | 33.44 |
| 9 | 33.89 | 29.04 | 31.38 | 31.5 |
| 10 | 33.15 | 30.51 | 31.61 | 32.12 |
| 11 | 38.28 | 30.67 | 32.96 | 31.88 |
| 12 | 34.8 | 31.25 | 30.38 | 32.25 |
| 13 | 32.6 | 32.32 | 30.45 | 31.79 |

## 3.3: 2^ (-ΔΔCt) results

By using student's T-test with 2^ (-ΔΔCt), analysis was used for the statistical investigation using the Student's T-test. The results which aimed to detect the presence of significant values between the four genes and PCO syndrome were obtained.

**Table 3.3**: results of 2^ (-ΔΔCt) by student's T-test

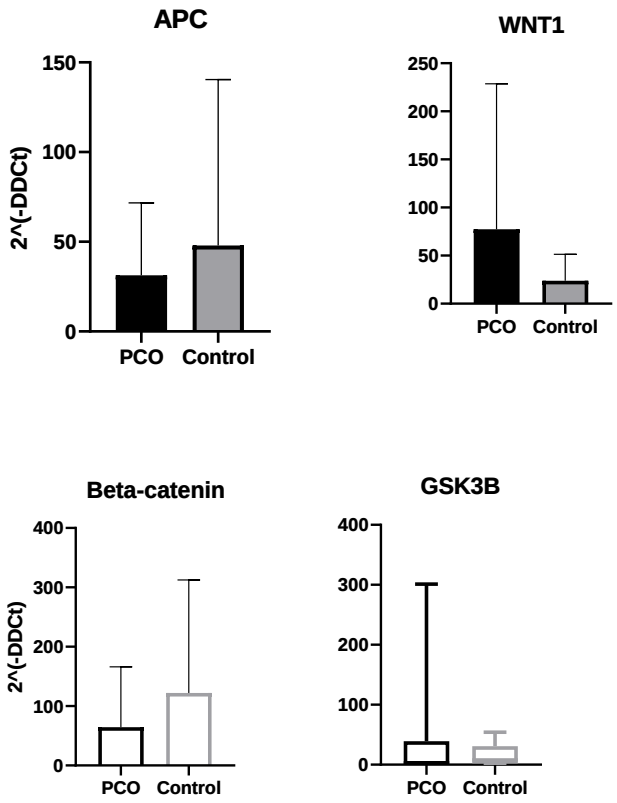
| | | APC gene | WNT1 gene | Beta-catenin gene | GSK3B gene |
|---|---|---|---|---|---|
| | Patient Code | Individual 2^(-DDCt) | Individual 2^(-DDCt) | Individual 2^(-DDCt) | Individual 2^(-DDCt) |
| PCO | 1 | 49.3507464 | 0.36223554 | 8.31087283 | 1.52097875 |
| PCO | 2 | 0.50437938 | 3.82378127 | 21.48116 | 7.9723221 |
| PCO | 3 | 92.73229 79 | 108.75968 | 258.67557 | 301.288125 |
| PCO | 6 | 0.13360818 | 1.65290064 | 1.55293775 | 0.0008531 |
| PCO | 7 | 1.57461595 | 11.8351001 | 6.65758988 | 0.01764007 |
| PCO | 8 | 74.8017439 | 408.728774 | 153.809414 | 39.2603193 |
| PCO | 11 | 0.03528014 | 6.89236928 | 1.40932076 | 2.97935493 |
| control | 4 | 46.0458746 | 37.4008 72 | 472.771743 | 54.004211 |
| control | 5 | 233.131477 | 73.7719262 | 214.524706 | 23.1830745 |
| control | 9 | 0.5340327 | 15.401511 1 | 3.04195751 | 2.79917173 |
| control | 10 | 0.21389876 | 1.33329868 | 0.62200583 | 0.43678645 |
| control | 12 | 0.49827013 | 5.83608138 | 10.6663891 | 2.91801069 |
| control | 13 | 7.23503502 | 8.7847425 1 | 32.111096 | 12.6845462 |

By using student's T-test, it was noted that there was no significant difference for *Beta-catenin* gene (p value = 0.4995) and *APC* gene (p value = 0.6744).

But for the other genes (*WNT1* and *GSK3B* genes) it was noted that p value was less than 0.05 which indicates that there is a significant difference between *WNT1* gene and PCOS (p value = 0.0018), and between *GSK3B* gene with PCOS (p value = 0.0019).

It is clear from figure 3.1 that only in *WNT1* & *GSK3B* the PCO has higher rates than control rates.

**Figure 3.1:** Graphs for each gene with student's T-test by using 2^(-ΔΔCt) values.




When all the genes were compared together (control oocytes and PCO oocytes) with *ACTB*, there was no significant difference in ANOVA summary for the four genes with *ACTB* gene (p value = 0.0613) as shown in Figure 3.2. However, when each gene was compared separately with *ACTB* (control oocytes and PCO oocytes), there was a significant difference between every gene with *ACTB* as shown in Table 3.4.

**Figure 3.2:** graphs for PCO & Control samples for the genes with fold change by using Ct values.

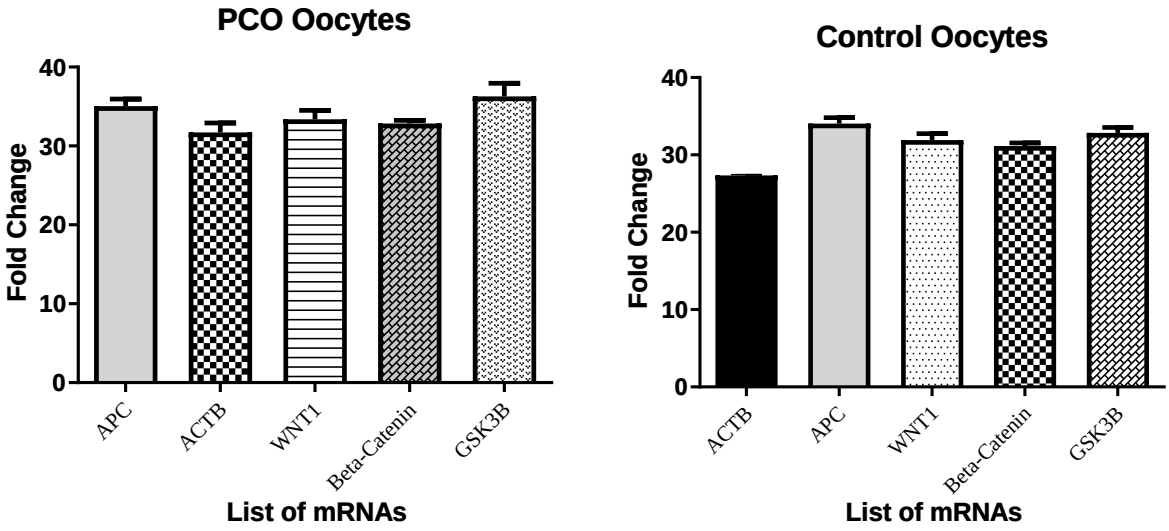



**Table 3.4:** Separate multiple comparisons among each gene.

| Multiple Cmparisions test | significant | adjusted P value |
|---|---|---|
| **ACTB vs WNT1** | yes | <0.0001 |
| **ACTB vs β -CAT** | yes | <0.0001 |
| **ACTB vs GSK3B** | yes | <0.0001 |
| **ACTB vs APC** | yes | <0.0001 |

# CHAPTER IV

## Discussion

This research was designed to investigate the expression levels of *WNT I*, *GSK3β*, *β-catenin* and *APC* components of the *WNT/β-catenin* signalling pathway genes in oocytes obtained from seven patients with PCOS and six controls without PCOS. Until today, there are limited number of functional studies that have analysed the expression levels of *β-catenin*, *WNT1*, *APC* and *GSK*3β genes of the canonical pathway in the oocytes of patients with PCOS. Generally, the *WNT/β*-catenin signalling pathway, in the presence of a *WNT* protein act as a mainspring which controls the quantity of cytosolic catenin protein in the cytoplasm along with it displacement into the cell nucleus where they act as transcriptional co-regulators by binding to an activator protein *(TCF/LEF)* in order to control the transcription of *WNT* targeted genes such as *cyclin D* and *c-myc* (Clevers & Nusse, 2012). Contrastingly, in the absence of a *WNT* ligand, dephosphorylated *GSK3β* forms a multimeric complex with *AXIN1* and A*PC* component of the *WNT* pathway to facilitate the phosphorylation of the N-terminal of the *CTNNB1* (beta-catenin) targeting it for degradation by proteasome and inhibiting it ability to regulate the expression levels of *CTNNBI* regulated genes (Wang et al., 2013). Although, the mechanism by which *WNT/β-catenin* family of proteins and it associated component molecules such as *APC* and *GSK3β* regulates ovarian folliculogenesis in human adults is still

not fully understood. However, the distinctive role of various component of the pathway in early embryonic development and gametogenesis have been reported by several studies( Bernard & Harley, 2007, Heikkilä et al., 2002 and MacDonald et al., 2009a) Viano *et al* (1999) described the role of *WNT-4* ligand of the *WNT/ β-catenin* signalling pathway in normal ovarian development in mice. In this study, they reported that the inactivation of *WNT-4* in female mice resulted in a partial female to male reversal and oocytes depletion at birth. However, male mice with mutant *WNT4* had normal gonadal growth at birth (Vainio et al., 1999). In a subsequent study, Heikkilä *et al* (2002) demonstrated the role of *WNT-4* in the sorting of adrenal and gonadal cells at fetal stage of development as well as in cell migration (Heikkilä et al., 2002) and in female gonadal development at birth (Pellegrino et al., 2010). Also, Bernard *et al* (2007) showed how aberrant expression of *WNT4* ligand (β-catenin signalling pathway) significantly led to Müllerian duct regression as well as the inhibition of steriodogenesis and sex specific migration events in fetal mice (Bernard & Harley, 2007). Likewise, Liu *et al* (2010) demonstrated how normal expression of *WNT4* active protein prevented germ cell loss in the ovaries of *WNT4O*-fetal mouse (Liu et al., 2010).

Additionally, few studies have evaluated the role of *WNT*/beta catenin signalling in the development of PCOS. Wang and colleagues (2013) described a mechanism by which *WNT2* regulates adheren gap junction signalling

pathway that is crucial for ovarian folliculogenesis. In their study, they showed how the knocking down of *CTNNBI* restrained the mobilization of CX43 in to the adheren gap junction in mouse granulosa cells ( Wang et al., 2013). Also, Wu *et al* (2017) reported an association between higher transcript regulatory activity of *WNT1*, *WNT3* and *WNT4* (protein component of the *(WNT)*) canonical pathway and granulosa cell apoptosis in North Chinese women with PCOS ( Wu et al., 2017) and lower *mRNA* expression of *WNT7a*, *WNT8a* and *WNT3* ligands of the *WNT/β-catenin* signalling pathway in endometrial dysfunctions in PCOS patients (Mehdinejadiani et al., 2018). Furthermore, Sanchez et al 2014 found lower levels of *WNT1* and higher levels of *WNT4* and *WNT5* independent component of beta catenin in the luteinized granulosa cells of patient with PCOS. They reported lower concentration of Beta catenin and its dephosphorylation as well as the abnormal expression of surviving and *BMP4* downstream targets (Sanchez et al., 2014b). Likewise, genetic aberration in *FZD3/WNT2/β-catenin* pathway lowered oestrogen synthesis in the cumulus cells of women with PCOS (Qiao et al., 2017). Wang *et al* (2010) reported an accumulated level of *GSK3β* and lower expression of beta catenin in the cytoplasm after *WNT2* knockdown. The overexpression of *GSK3β* induced beta catenin translocation from cell membrane into the nucleus while *CTNNB1* knockdown restrained DNA synthesis in the granulosa cells of women with PCOS(Wang et al., 2010). Furthermore, Mehdinejadiani *et al* (2018) demonstrated how letrozole activation of the *WNT*-canonical pathway improved

adequate proliferation in the endometrium of PCOS patients receiving letrozole treatments (Mehdinejadiani et al., 2018).

Glycogen synthase kinase (*GSK*) is a multifactorial serine/threonine kinase enzyme that is encoded by the *GKS3* gene. The gene plays a crucial role in *WNT/β-catenin* signalling pathway, it also acts as a mediator of glycogen synthesis alongside insulin signalling (Doble & Woodgett, 2003, Grimes & Jope, 2001).There are two distinctive isoforms of *GSK3* gene in mammals (*GSK3α* located at 19q13 and *GSK3β* at 19q13.2), respectively ( Ali et al., 2001, Doble & Woodgett, 2003). Both isoforms are 85% identical to one another (Doble & Woodgett, 2003). However, they have different functions (Lee & Kim, 2007).

*GSK3α* is an active protein kinase enzyme which acts as a regulator of glucose metabolism in the liver as well as a mediator of insulin resistance through the modulation of various transcription factors (Lee & Kim, 2007, Nikoulina et al., 2000). In *WNT* signalling, *GSK3α* regulate the level and transcriptional activity of *CTNNB1*(Asuni et al., 2006). However, *GSK3β* regulate glucose metabolism in the muscle and also, it acts as a negative regulator of *WNT* signalling pathway by forming a multimeric complex with *AXIN1* and *APC* which in turn influences the phosphorylation of the N-terminal of *CTNNB1* alongside it degradation by the proteasomes (Luo, 2009). Hence, *GSK3β* also regulates other cellular processes outside of the *WNT* signalling pathway and can mediate

signalling events initiated by insulin receptors and several growth factors (Grimes & Jope, 2001). A handful of studies have investigated the function of GSK3 in insulin signalling pathway. White (2002) described how the activation of insulin receptor led to the phosphorylation of insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) (White, 2002). And upon glycogen synthesis, phosphorylated *IRS* protein activates phatidylinositol-3 kinase *(P-13)* cascade which is the main pathway associated with glycogen synthesis and glucose transport. *P13* kinase undergoes series of chemical reaction to produce phosphatidylinositol- (3, 4, 5)-triphosphate *(PIP3)* which activates protein kinase 1 (*PDK1*) and in turn activates protein kinase B (*PKB/AKT*) (Myers et al., 1992). This data indicates that the activity of glycogen synthase *(GS)* is constitutively regulated by the activity of glycogen synthase -3 *(GSK-3)* which upon activation by insulin signalling cascade (PKB) a serine component of the insulin-pathway, *GSK-3* is inhibited thus resulting in the increased activity of *GS*. Likewise, Pearce *et al* (2004) described how elevated levels of *(GSK3β)* in the muscle of transgenic mice led to hyperlipidemia with constant increased insulin resistance and glucose intolerance (Pearce et al., 2004) and specific inhibition of *GSK-3* enhances insulin action on glucose transport in skeletal muscle of the insulin-resistant Zucker type 2 diabetic fatty rats (ZDF) (Henriksen et al., 2003). Liberman *et al* (2005) showed a mechanism by which aberrant GSK3β function increased insulin resistance through the phosphorylation of *IRS-1* on *Ser332* which significantly reduced tyrosine

phosphorylation of *IRS-1* by insulin receptor protein and limited signalling to downstream components (Liberman & Eldar-Finkelman, 2005). Additionally, Nikoulina and colleagues (2002) demonstrated how selective *GSK3* inhibition impacted both GS and glucose uptake as well as on insulin action in cultured human skeletal muscle (Nikoulina et al., 2002).

Likewise, other studies have investigated the role of *GSK3β* in relation to the impaired insulin-stimulated glycogen synthesis observed in women with PCOS. Wu et al (2003) reported lower levels of glycogen synthesis in cultured ovarian granulosa cells of women with PCOS as a result of insulin resistance caused by *GSK3* deficiency (Wu et al., 2003) and in the fibroblast of with women PCOS (Book & Dunaif, 1999). Similarly, Chang *et al* (2008) reported higher tyrosine phosphorylation and lower insulin stimulated serine phosphorylation of *GSK3* in adipocytes of PCOS patient (Chang et al., 2008). Furthermore, Goodarzi and colleagues (2007) found a common haplotype of the *GSK3β* gene among white (CACCGGAGG) and black (TTTATAAAG) that was significantly associated with the increase risk of PCOS (Goodarzi et al., 2007).The findings of these studies ( Book & Dunaif, 1999,Wu et al., 2003 and Chang et al., 2008 ) suggest that aberrant regulation of *GSK3* contributes to the impaired insulin resistance seen in women with PCOS. Although, it may not be the only factor that is associated with the insulin resistance in women PCOS, owing to the complexity of the insulin signalling cascade.

Additionally, few studies have reported the involvement of *GKS3* in androgen secretion and action in prostate cancer cell lines (Liao et al., 2004). Wang et al (2004) indicated that the *AR* gene might be a substrate of *GSK3*, suggesting that aberrant expression of *GSK3* could possibly be the source of the hyperandrogenism in *PCOS* patient (Wang et al., 2004). Likewise, Munir *et al* (2006) demonstrated how elevated level of *GSK3β* triggered the 17-hydroxylase activity of P450C17 in the theca cells of women with *PCOS* (Munir et al 2006).

Adenomatous polyposis coli (*APC*) gene is a tumour suppressor gene that is encoded by the *APC* gene and it is cytogenetically located at 5q21-22 (Frayling & Arends, 2017). In mammals, an additional form of this gene is a product of *APC2/APCL* (Ferreira et al., 2014). Functionally, *APC* is an antagonist regulatory component of the *WNT/β-catenin* pathway which promotes the degradation of B-catenin protein (Tannapfel, 2005). In addition, *APC* also regulates cell migration, spindle assembly, cell adhesion, neural differentiation and apoptosis (Frayling & Arends, 2017). During *WNT* signalling, *APC* influence the recruitment of *β-catenin* protein to other Axin destruction protein such as *β-catenin*/*GSK3* which facilitates it subsequent ubiquitination by ubiquitin ligase *(β -Trcp*) and degradation by the proteasome (Coleman, 2018). Also, the protein enhances the enzymatic activity of *GSK3* during the phosphorylation of β-catenin protein. Structurally, *APC* is composed of several domains; (I) N-terminal is an armadillo repeat, (II) an axin binding motifs in the

middle and, (III) a b-catenin binding motifs consisting of 15 amino acid and seven repeats of 20 amino acids of *APC* protein which interacts with *β-catenin* and is required to mediate it degradation (Hankey et al., 2015).

Genetic aberration in the *APC* gene have been associated with familiar Adenomatous polyposis (*FAP*) and other sporadic cancers (for example prostate and colon cancers) most of which are caused by a truncated protein lacking most or all of the 15 amino acids repeats or particularly the 20 amino acid repeats thereby preventing the ability of APC protein to interact and facilitate the degradation of Beta-catenin hence when *APC* is mutated, *β-catenin* accumulates in the cytoplasm and ultimately translocate to the nucleus where it associates with *TCF* family of transcript factors (Myers et al., 2019). Currently there are no studies demonstrating the role of *APC* in normal ovarian development. However, several studies have reported the role of *APC* in ovarian tumours. Henderson *et al* (2000) described APC as a nuclear-cytoplasmic shuttling protein and chaperone of β-catenin (Henderson, 2000). Additionally, Mohamed *et al* (2019) showed how *APC2* deficient mouse developed tumours that had similar histological features and signatures to that of human adult granulosa cell tumours (GCTs) (Mohamed et al., 2019). Likewise, Karbova *et al* (2002) analysed the expression level of *APC, E-cadherin* and *β-catenin* in 113 patients with primary and metastatic serous ovarian carcinoma. They found a significant association for both *E-cadherin* and *β-catenin* in both the primary

and metastatic group. However, there was no significant association for APC expression level in both groups (Karbova et al., 2002).

The result of this study showed that there was a significant increase in the expression of both G*SK3β* and *WNT1* component proteins of the *WNT/ β -catenin* signalling pathway in the oocytes of all seven PCO patients when compared to the oocytes of women without the syndrome (a control group of (n=6)), p-values were 0.0019 and 0.0018 for *GSK-3β* and *WNT1*, respectively. Contrastingly, no significant difference was detected in the expression of both *APC* and *β-catenin* proteins in the oocytes of both groups (PCO and control).

Further evaluation showed that all four genes *(WNT1, β-catenin, GSK3β and APC)* had similar expression to the *ACTB*-reference gene included in this study (P-value = 0.0613) in *PCO* oocytes. Likewise, the expression of *WNT1*, *APC*, β-*catenin* and *GSK3β* genes were similar to that of the *ATCB*-sample gene in the control oocytes as well (p-value = 0.0613). However, when the expression level of each gene *(WNT1, APC, β-catenin and GSK3β)* was independently compared to the expression level of *ACTB*, a statistical difference was detected.

## Conclusion

The exact role of Wnt/Beta catenin signalling molecules such as *APC, β-catenin, GSK3β* and *WNT1* in normal ovarian development is still not fully understood. However in this research, elevated expression of *WNT1* and *GSK3β* regulatory proteins of the *WNT/beta-catenin* was detected in the oocytes of PCO patient thus suggesting an association between aberrant expression of *WNT1* and *GSK3β* and the pathogenesis of PCOS in these data sets. On the other hand, *APC* and *β-catenin* expression were similar in the oocytes of both patients and controls.

Furthermore, there was no significant difference in the expression level of all genes (*WNT1, APC, β-catenin* and *GSK3β*) when compared with the *ACTB*-reference-gene. Contrastingly, when the expression of each gene (WNT1, *β-catenin, GSK3β* and *APC*) was independently compared with *ACTB*- reference gene, a significant difference was observed thus indicating that all genes were active at the stage and might have a significant role in the development of the disease. These conflicting results might have arisen due to contamination or errors during sample preparation or circle run, however these findings needs to be further evaluated using large data.

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