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INVESTIGATION OF THE EXPRESSION OF LNCRNAS IN HUMAN OOCYTES FROM PATIENTS WITH POLYCYSTIC OVARIES

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Approval

We certify that we have read the thesis submitted by Mehmet Aktan titled "**Investigation Of The Expression Of LncRNAs In Human Oocytes From Patients With Polycystic Ovaries**" and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Declaration

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

Mehmet AKTAN

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Mehmet AKTAN

Abstract

INVESTIGATION OF THE EXPRESSION OF LNCRNAS IN HUMAN OOCYTES FROM PATIENTS WITH POLYCYSTIC OVARIES

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Background: Polycystic ovary syndrome (PCOS) is a widespread premenopausal female condition resulting from signs and symptoms of excess androgen and ovarian abnormalities. It affects 6% to 20% of women who have not reached menopause, making it the widespread metabolic and endocrine disease affecting woman who is in the age of reproductive. A high number of women present to fertility clinics for PCOS related problems each year. Altered gene expression levels in ovarian and oocyte samples may be associated with infertility. In this study, the regulatory effects of lncRNAs on changing gene expression in human oocytes donated by patients with polycystic ovaries and patients without polycystic ovaries were examined.

Methods: In total, 13 meiosis II stage (MII) oocytes were collected from patients who applied to *In Vitro* Fertilization (IVF) Clinic at the Near East Hospital, Nicosia, Cyprus. Oocytes were used to examine the expression levels of 3 lncRNAs targeting the *CYP11A1* gene. RNAs were individually extracted from each oocyte. Expression data were obtained for each oocyte using the real-time polymerase chain reaction (PCR) using the cDNA samples obtained from each RNA from each oocyte. Statistical analysis was performed and a P value of 0.05 were considered as statistically significant.

Result: The samples consisted of seven oocytes from patients with polycystic ovaries and six from patients without signs of polycystic ovaries. The real-time PCR results showed that there was no statistically significant ($p>0.05$) difference in the expression levels of RP11-573D15.8, RP11-156E8.1, and lnc-CYP11A1-1 in patients with polycystic ovaries compared to the patients without polycystic ovaries.

Conclusions: The expression of *CYP11A1*, which was previously shown to be upregulated in oocytes obtained from patients with polycystic ovaries, is not implied to be regulated by RP11-573D15.8, RP11-156E8.1 and lnc-CYP11A1-1 since the expression levels of these lncRNAs did not show any variation in oocyte samples between each group. In order to elucidate the up-regulated gene expression in the *CYP11A1* gene associated with the development of polycystic ovary syndrome, different target lncRNA expression should be examined. In this way, more information about the development of polycystic ovary syndrome can be obtained.

***Keywords**:* PCOS, human oocytes, lncRNAs, gene expression

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List of Abbreviations

AFC :	Antral follicle count
BMI :	Body mass index
cDNA :	complementary deoxyribonucleic acid
COS :	Controlled ovarian stimulation
CVD :	Cardiovascular disease
DHEA :	Dehydroepiandrosterone
ESHRE :	European Society For Human Reproduction and Embryology
FSH :	Follicle-stimulating hormone
GC :	Granulosa cell
GnRH :	Gonadotropin-releasing hormone
HA :	Hyperandrogenism
IR :	Insulin resistance
IVF :	In vitro fertilization
LDL :	Low-density lipoprotein
LH :	Luteinizing hormone
LNCRNA:	Long non-coding RNA
MII :	Meioses II stages
MIRNA:	Micro RNA
mRNA :	messenger ribonucleic acid
PCO:	Polycystic ovary
PCOS:	Polycystic ovary syndrome
QPCR :	Real-time quantitative Polymerase chain reaction
RNA-SEQ:	RNA sequencing analysis
T2DM :	Type 2 diabetes mellitus
TC :	Total cholesterol
TG :	Triglyceride

Chapter I

Introduction

Polycystic ovary syndrome (PCOS) is a very common female condition resulting from the combination of signs of excess hormone androgen (hyperandrogenemia) with ovarian dysfunction (polycystic ovarian morphology and/or oligo-ovulation). PCOS patients also face with insulin resistance and insulin excess in the bloodstream, that lead to elevated risk of developing diabetes mellitus (DM). It is also related to various pregnancy-related conditions, endometrial tumors, and cardio-vascular diseases (Lizneva et al., 2018). The prevalence of PCOS in women who have not yet reached menopause is between 6% according to the old criteria and 20% according to the new criteria. This makes it the most common endocrine and metabolic disorder in women of reproductive age (Escobar-Morreale, 2022).

1.1. Pathophysiology of PCOS

PCOS is an endocrine and multifactorial condition, which is characterized by hyperandrogenism, obesity, and insulin resistance. Intraovarian androgen excess stimulates the maturation of small-sized follicles while inhibiting the selection of a dominant follicle. The resulting excess of small follicles that are arrested during development gives the ovaries a "polycystic" appearance. Although PCOS was thought to be a syndrome related to hypothalamic-pituitary gonadotropin secretion disorder at first (Jonard, 2004), it is now proving that PCOS is basically a disorder of ovarian steroidogenesis.

1.1.1 PCOS Symptoms

Hirsutism is a medical name for excessive hair growth. It develops due to high androgen levels. The Ferriman-Gallwey system, which measures hair growth in androgen-sensitive areas, is often used to determine the level of hirsutism. Although it is a common symptom, not every hyperandrogenic juvenile is facing hirsutism, because the pilosebaceous units are insensitive to androgens. Some of the young adolescents whose hyperandrogenism is not fully developed may not have hirsutism. However, the opposite is also possible. The hairiness that occurs when androgen is at normal levels is defined as "idiopathic hirsutism" (Spritzer et al., 2016).

Acne vulgaris is an important problem caused by hyperandrogenism in adolescents. The increased serum level of androgens increases sebum production. Genetic factors, diet, medications, and hyperandrogenism increase skin dysbiosis and increase acne and long-term skin damage (Carmina et al., 2022).

Menstrual irregulations are also common in PCOS patients. The distinction between abnormal and physiological anovulation is often difficult, and clinical examination is often delayed because of this. The absence of a period after the age of 14 or more than three years after the start of breast development is referred to as primary amenorrhea. Secondary amenorrhea is defined as an unnatural ovulation delay lasting more than 90 days, even if menstruation was previously experienced. Oligo-menorrhea is an infrequent delay in ovulation exceeding 39 days outside of the usual ovulation pattern. Studies show that androgen level is associated with long-term infertility or PCOS (Pundir et al., 2020).

Polycystic ovaries are fluid-filled sacs that are formed in the ovaries. Polycystic ovaries are very difficult to detect with ultrasound, especially in adolescents. Ultrasound is nowadays accepted as a tertiary method and now four different phenotypes associated with the syndrome are sought (Carmina et al., 2022).

Obesity and hyperinsulinemia with insulin resistance strongly associated with PCOS although not included in a diagnostic criterion. These are acanthosis nigricans, metabolic syndrome, insomnia like sleeping problems, and fatty liver disease. In the long term, it causes problems such as heart diseases and diabetes mellitus (Ezeh et al., 2013; Pundir et al., 2020). Ten percent of PCOS patients aged 40 years suffer from diabetes mellitus and its complications (Otto-Buczkowska et al., 2018). Insulin resistance exacerbates hyperandrogenemia and aggravates chronic problems (Shaikh et al., 2014).

1.2 Etiology of PCOS

Although familial aggregation suggests that PCOS is genetically based, only a few genetic variants and mutations are associated with PCOS carriers in different populations, with only 10% showing PCOS heritability. PCOS is now recognized being a multigene condition that is characterized by genetic variations that combine with strong environmental variables that cause various PCOS phenotypes. (Escobar-Morreale, 2022). PCOS risk is increased by an unhealthy life or agents of infection.

Insulin resistance and hyperinsulinemia, and its high levels disrupt the ovaries, causing androgen levels to rise, resulting in anovulation (Shaikh et al., 2014).

Polycystic ovaries are associated with the hypothalamus-pituitary junction including hormones like luteinizing hormone (LH) and follicular stimulating hormone (FSH). It is not just related to genetic factors. It is also linked to environmental factors. Related genes and single nucleotide polymorphisms (SNPs) are responsible for disease progression. Databases associate PCOS with over 240 SNPs. Polymorphism, non-coding RNAs and nucleotide mutations affect the expression of related genes (Chen & Fang, 2018).

FSH receptors, androgen hormone receptors, LH receptors, and leptin receptors are the most common genes involved. A polymorphism within a gene may disrupt the metabolic process, resulting in ovarian abnormalities (Xita et al., 2002). *StAR* polymorphisms, *FSHR* polymorphisms, *FTO* polymorphisms, *VDR* polymorphisms, *IR* and *IRS* polymorphisms, and *GnRHR* polymorphisms have all been linked to PCOS. The progression and severity of PCOS worsen when insulin levels and androgen levels rise. Theca cells of the ovary are affected by hyperinsulinemia, which raises androgen levels. SHBG and IGFBP-1 production in the liver are reduced as a result of this disease. Increased testosterone levels, on the other hand, encourage the production of free fatty acids (FFAs) in the visceral adipose tissue (VAT), which causes increased insulin resistance (Chen & Fang, 2018; Gambineri et al., 2022). PCOS patients have numerous 8mm cycts and about 70 percent patients face with infertility and acne caused by elevated level of testosterone (Ye et al., 2020). Twenty percent of patients suffers from sleep apnea. Elevated levels of insulin hormone cause irregularity in the menstrual cycle which increases risk of infertility in older ages. Depression and anxiety are also common psychological disorders in patients.

Aromatase, an one of the members of the complex Cytochromes P450 (CYPs), is a steroidogenesis enzyme (in Steroidogenic pathway) that generally plays a key part in steroid conversion. It enables the body to convert testosterone to estrogen. Aromatase insufficiency causes a malfunction in the pathway, which prevents it from being converted. Because C19 does not convert to C18, this defect affects ovarian function and raises androgen levels. *CYP1A1, CYP11A, CYP11B2, CYP17, CYP19A, CYP21,* and *CYP3A7* are aromatase genes that have been reported in PCOS datasets. Any cytochrome P450 abnormality increases the risk of PCOS

development (Joseph et al., 2015; Gambineri et al., 2022). Importantly, this study shows that the expression of *CYP11A1* was significantly different in human oocytes donated by PCO patients (Al-Omar et al., 2020).

1.3 Diagnostic criteria

In 1935, Dr. Stein and Dr. Leventhal published the first complete description of Stein-Leventhal Syndrome, or another name known as PCOS. Due to its diverse clinical presentations, uncertain genesis, complex pathophysiology, and poor diagnosis, it has led to significant scientific controversy. U.S. National Institute of Health (NIH) criteria were established in 1990 in attempt to produce a comprehensive and detailed characterization to diagnose PCOS. Then, in 2003, an atelier in Rotterdam, Netherlands, built up the Rotterdam criteria, a next diagnostic criterion for PCOS (Franks, 2006). Out of the three criteria, this criterion needs the presence of two; atypical ovulation, hyperandrogenism, and/or polycystic ovarian morphology (>11 follicles in each ovary measuring between 2 and 8.99 mm) (Pundir et al., 2020).

The diagnostic criteria for of Androgen Excess and Polycystic Ovary Syndrome Society (AES) were changed in 2006. The presence of hyperandrogenism in association with either oligo-ovulation or polycystic ovaries is required for the AES. The process of diagnostic standardization has certain challenges. First, ovulation is frequently erratic during early menarche. As a result, anovulation cannot be regarded conclusive evidence of the syndrome's occurrence. Second, in youths, transvaginal ultrasonography is not frequently performed, limiting ovary visibility, and hence excluding any invasive diagnosis of polycystic ovarian morphology. Third, there is just no agreement on the normal androgen levels, and there are little studies for normal androgen levels for females with ages 10-20. As a result, detecting androgen abnormalities is a difficult undertaking. Finally, cystic ovaries are difficult to separate from multi follicular ovaries, which are common in females with ages of 10-20 years. As a result, the Pediatric Endocrinology Society has issued recommendations for the differential diagnosis of PCOS in both adults and adolescents. In 2012 and one year later, a new two set of adult and adolescent PCOS criteria have recently been proposed, one by an ESHRE and ASRM working group another by the Endocrine Society's clinical practice recommendations committee. While PCOS is still not openly visible by person of mature age standards,

serologically elevated androgen levels and progressively condensed hirsutism, in combination with abnormal ovulation for at least 24 months after menstrual cycles after age 15 years, or an ovarian volume>10 c m3, may be considered in adolescents. It is important to highlight, however, that none of the proposed criteria has been verified (Pundir et al., 2020).

Table 1 summarizes the acceptable consensus (persistent hyperandrogenic oligoanovulation) for early PCOS diagnosis based on age and stage appropriate norms (Rosenfield et al., 2015; Liznevaet al., 2016).

Table 1.

Diagnostic criteria for PCOS

Parameter/age group	Adult	Adolescence
NIH 1990	Hyperandrogenism Ovaries Abnormality	Abnormal Uterine Bleeding Persistent Symptoms For 1-2 Years Abnormal For Age
Rotterdam 2003	Hyperandrogenism Ovarial Abnormality Polycystic Ovary	Hyperandrogenism Moderate-To-Severe Hirsutism Moderate-To-Severe Acne Vulgaris
Androgen Excess & PCOS Society 2006	Hyperandrogenism Ovarial Abnormality And/Or Polycystic Ovary	
ESHRE/ASRM 2012	Hyperandrogenism Ovaries Abnormality Polycystic Ovary	Clinical Or Biochemical Hyperandrogenism Ovaries Abnormality Polycystic Ovary

Table
1(continued)

Endocrine Society2013	Clinical Or Biochemical Hyperandrogenism Ovaries Abnormality	Clinical Or Biochemical Hyperandrogenism Ovaries Abnormality

ESHRE: European Society for Human Reproduction and Embryology, ASRM: American Society for Reproductive Medicine; (Rosenfield, 2015; Lizneva. 2016).

1.4 The association of the *CYP11A1* gene and PCOS

Cytochrome P450 in the family-11 with the subfamily-A, polypeptide 1(*CYP11A1*) is a human protein encoded by the *CYP11A* gene. *CYP11A1* catalyzes the cholesterol to pregnenolone, which is the first action in the biosynthesis of steroids. Many studies show the association of *CYP11A1* polymorphisms and the development of PCOS, but other factors (such as lncRNA regulation) may affect transcription of this gene as well that may lead to pathophysiology of PCOS (Heidarzadehpilehrood et al., 2022; Zhang et al., 2012).

1.5 LncRNAs

Long non-coding RNAs and microRNAs (miR or miRNAs) are two of the most common types of non-coding/non-structural RNAs. They used to be classified as "junk" or "noise" but these days they are being discovered to have many critical roles. Both appear to have mostly regulatory functions, while much remains unknown about their functions. LncRNAs are at least 200 bases long and have a unique three-dimensional structure. They have a critical role in a variety of biomolecular processes, including epigenetic modifications, transcription, post-transcriptional and post translational regulation of target genes (Mohr et al., 2021).

Gene expression is linked to mRNA stability in all living things. mRNAs can remain functional for a few minutes to several days in eukaryotes. Its stability and operational time help maintain cell homeostasis. The dynamics of mRNA lifespan

and quantity help to respond to changes in environmental factors, regulation of cell development, and gene regulation at the stage of cell differentiation. Levels of mRNA are maintained by the balance between production and decay. Across most eukaryotic cells, mRNA molecule decay begins with adenylation and capping of the messenger RNA, continued with exonuclease-mediated degrading (Bicknell & Ricci, 2017).

However, the control of mRNA stability is primarily dependent on how regulatory variables influence multiple processes (deadenylation, decapping, and degradation), hence these aspects should be considered when studying mRNA stability regulation. Studies show that miRNAs can promote mRNA degradation by aiding endonucleases (Eulalio et al., 2008; Iwakawa & Tomari, 2015).

Previously published studies showed that non-coding RNAs are associated with mRNA stability (Sebastian-delaCruz et al., 2021). Non-coding RNAs act as enhancer RNAs signaling RNAs, guide, decoy, scaffold. LncRNAs guide the target and bind directly to specific mRNAs, specific RNA-binding proteins, or microRNAs (miRNAs). LncRNAs can bind to miRNAs or miRNA-messenger RNA junctions, block miRNAs and additionally promote gene expression (Tu et al., 2021).

RNA binding proteins (RBPs) are proteins that are critically important in the regulation of gene expression (Corley et al., 2020). LncRNAs can accelerate their degradation by binding RBPs to mRNA (Noh et al., 2018). However, it can also stabilize mRNA by preventing them from binding (Briata & Gherzi, 2020). It supports modulation of target genes by interacting with m6a machinery to lncRNAs (Huang et al., 2018; Wang et al., 2013)

1.5.1 Biogenesis of the lncRNAs

LncRNAs, like other RNAs, are transcribed by RNA polymerase 2. They have a 3' poly a tail and a 5′ methyl-cytosine cap (Zhang et al., 2019). They can be classed in a variety of ways based on their characters. It can be characterized as sense, anti-sense, bi-directional, intronic, or intergenic based on its genetic origin (Lanzafame et al., 2018). They are classed as nuclear, cytoplasmic, or mitochondrial based on their subcellular location. LncRNAs can be capped or polyadenylated, and they can also be created via splicing (Gourvest et al., 2019). Ribonuclease P can cut them to create 3' ends and turn them into a circular structure. This prevents them from degrading (Alessio et al., 2020; Chen, 2016).

LncRNA biogenesis can be regulated by various mechanisms. Epigenetic changes can affect lncRNA expression; for example, acetylation of the *H3K56ac* and *CAF-1* can enhance the antisense of *H3K56* lncRNA (Quinn & Chang, 2015). Exosomes, as well as slc22a2 and slc22a3, are capable of degrading them. In fact, their degradation involves Nrd1, Nrd3 like complexes inside the nuclear membrane, and protein XRN1 (5'-3' Exoribonuclease 1) in the cytoplasm (Quinn & Chang, 2015). The UPF1 protein inhibits these processes as well (Sleutels et al., 2002). Although epigenetic factors seem to play a role in regulation, we are still at the beginning of understanding their detailed mechanisms. It seems that more research on lncRNA biogenesis needs to be study in the future (Liu et al., 2021).

1.6 The statement of the problem

PCOS is one of the most problematic phenotypes in women of reproductive age. Pathophysiology of the syndrome increases the risk of being infertile. The studies suggest that expression of *CYP11A* gene which has a key role in steroidogenesis pathway is related with development of PCOS. The expression of *CYP11A* may be regulated by lncRNAs or epigenetic mechanism which finally affect the development of PCOS. Understanding gene regulation and the relationship of lncRNAs can help to develop early diagnosis and treatments.

1.7 Significance of the study

To date, there has not been any studies investigating the lncRNA expression levels in human oocytes donated by patients with polycystic ovaries. This study can provide information about lncRNA expression in oocytes and how it may be regulating *CYP11A* expression. Thus, the result of this study is important to understand the underlying mechanism of PCOS.

1.8 Study Hypothesis and Goals

LncRNAs are nucleotides that have a regulatory effect on gene expression. This study aims to examine the expression levels of lncRNAs that regulates *CYP11A* in human oocytes from PCO patients and oocytes from patients with no polycystic ovaries (control group). It was hypothesized that there would be a significant variation in the expression levels of lncRNAs in oocytes from patients with polycystic ovaries relative to the control group.

CHAPTER II

Literature Review and Related Research

2.1 Noncoding RNA Expression in the Ovaries and the Oocytes

Studies show that miRNAs have a high expression in the mammalian ovaries (Tripurani et al., 2010; Ro et al., 2007; Ahn et al., 2010; Choi et al., 2007). A previously published study showed that microRNAs are involved in important processes in development of the ovary (Fitzgerald et al., 2015). It even showed a high expression in the ovary compared to organs such as the brain, hypothalamus, small intestine, and kidney (Fitzgerald et al., 2015).

Studies are showing an association between miRNA expression and polycystic ovaries, for example over 120 miRNAs were overexpressed in PCOS patients, but only miR-320 showed a significantly lower expression profile (Sang et al. 2013). This study also showed that microRNA-93, microRNA-133, and microRNA-223 targeting the insulin receptor have a high expression profile in PCOS patients (Chen et al., 2013).

Although micro-RNA expression has been studied for a long time, lncRNAs in the ovaries have only recently been investigated. However, the results show that lncRNAs are associated with PCOS, in such lncRNA NEAT1 had increased expression in the ovaries of PCOS patients (Sang & Zhang, 2020). It also shows the elevated expression of lncRNAs such as ZFAS1 (Zhu et al., 2020), MALAT1 (Zhang et al., 2020), and PVT1 (Liu et al., 2020) in granulosa cells. Regarding the steroidogenesis pathway, silenced OC1 long non-coding RNA promotes aromatase mRNA expression and increases estrogen production (Wu et al., 2020). In addition, the HUPCOS has been shown to affect the expression of CYP11 (Che et al., 2020). However, there is no studies investigating the expression of lncRNAs in human oocytes.

2.2 LncRNA associated with *CYP11*

RP11-573D15.8 is a lncRNA with a length of 1129 bases located on chromosome 3. According to the genomic origin, it is an antisense RNA and has three exons. It interacts with *CYP11* mRNA with a high energy of -46.16 kcal/mol (lnc-HNRNPU-5:3, LNCipedia 2018).

RP11-156E8.1 is a 1739 base-long antisense lncRNA. It is found in chromosome 1 and can interact with *CYP11* at -36.57 kcal/mol (lnc-TBCCD1-4:21, LNCipedia 2018).

Lnc-CYP11A1-1 is a 612 bases long lncRNA that is antisense of *CYP11A1* and is located on chromosome 15. There is no direct interaction with *CYP11*. But similar sequence can establish a competitive relationship with ncRNAs targeting *CYP11* (lnc-CYP11A1-1:1, LNCipedia 2018), and finally affect *CYP11* gene expression level Competitive lncRNAs have similar sequences to target genes. In this way, they can bind to ncRNAs targeting the target gene and suppress the regulation of ncRNAs on the target gene (Dong et al., 2019).

Chapter III

Materials and Methods

CYP11A has an important role in steroidogenesis pathway. Previous studies had shown that statistically significant changes in the expression of this gene was detected in human oocytes from patients with polycystic ovaries. The objective of this research was to investigate the expression levels of lncRNAs that have possible regulatory effects on the expression pattern of *CYP11A* gene in human oocytes obtained from PCO patients and the control group.

Oocytes were collected from Near East University Hospital *In Vitro* Fertilization (IVF) Center, Nicosia, Cyprus. Ethical approval was provided by the Near East University Institutional Review Board with the decision numbered YDU/2019/75-920.

3.1 The sample collection and sample size

A total of 13 oocytes were obtained at meiosis II stages (MII) from the IVF center. Seven oocytes were donated by PCO patients with polycystic ovaries, while six oocytes were donated by females without signs of polycystic ovaries. The samples were included in the control group if the females are non-obese (within the age range of BMI) with normal ovarian morphology and antral follicle count. The sample group included young females with polycystic ovaries with typical morphology and within the normal range of BMI (non-obese). These obtained human oocytes were then used to study expression of selected lncRNAs which possibly have regulatory roles on *CYP11* gene. The main steps included extraction of RNA and synthesis of cDNA. Real-time PCR was performed for each sample in duplicates. For each PCR, a negative control with no cDNA sample was used.

3.2 *In Vitro* Fertilization (IVF)

In the first three days of the menstrual period, transvaginal ultrasound was applied to the patients and antral follicle count (AFC) was assessed. The follicles were expected to have a diameter of 2-9 mm. Patients who have different follicle diameter were not included in the study. Controlled ovarian stimulation (COS) was initiated between the first and the fifth day following short antagonist protocol. Considering the age, BMI and AFC data, different doses of FSH were applied to each patient. Ultrasound controls were performed on the fourth and sixth days.

Gonadotropin-releasing hormone (GnRH) antagonist was applied daily to patients to obtain a follicle with 14mm diameter. After the ovulation trigger, the oocytes were collected after an average of 35.5 hours.

3.3 Analysis of oocytes: RNA extraction, cDNA synthesis and real time PCR

The study group included 13 oocytes, of which seven oocytes obtained from females with the polycystic ovaries and six oocytes from the control group. The experiments were performed at NEU DESAM Research Institute's Laboratory, Nicosia, North Cyprus. RNA purification was carried out using Norgen's RNA purification kit (Canada). NanoDrop ND-1000 was used to assess the quality of the RNA obtained following manufacturer's protocol.

Norgen's Transcript First Strand cDNA Synthesis kit (Norgen, Canada) was used for reverse transcription from RNA to synthesize cDNA following manufacturer's protocol with no modifications. Real time polymerase chain reaction (PCR) was carried out using cDNA samples to investigate the expression levels of selected lncRNAs. The expression levels were investigated in a total of three lncRNA genes. The target lncRNAs were selected using the LncRRIsearch database which is a bioinformatics tool that calculates the possible relationship between lncRNAs and messenger RNAs and other lncRNAs using human and mouse genome data, and ranks the results from high to low. The Incipedia database was used to obtain RNA sequences. Primers were designed for RP11-573D15.8, RP11-156E8.1, lnc-CYP11A1-1. The primers were designed to flank the exon-exon boundaries to avoid amplification of any DNA that may be contaminating the samples. The designed primer sequences for each lncRNAs are shown in table 2.

The LightCycler® 480 SYBR Green I Master kit was used for the real time PCR. The manufacturer's protocol with no modification and the final concentration of 0.2 μM of the primers were used in the reaction mixture. The PCR conditions are listed in table 3. 2^-ΔΔCt values were obtained to analyze expression levels and log10 values were used to calculate the significance level. In the experiment conducted with the Insta Q96™ Real Time Machine, Ct values were obtained for each sample with melting curve data. *ACTB* was used as housekeeping gene.

Table 2.

Primers sequences

Gene ID(Gene name)	Transcript ID	Forward Primer	Reverse Primer
ENSG00000197099(RP11-573D15.8)	ENST00000627551	TGATCATCCAGGAAGCCAACC	GAAGCCACTAAGACGGTGAGT
ENSG00000272195(RP11-156E8.1)	ENST00000607453	TGCCTAACGATAACCTCGGC	GACGACGCAATGTTTGTGCT
ENSG00000277749(lnc-CYP11A1-1; RP11-60L3.6	ENST00000611006	CATGACTCCTTGGTATTGG	AGAGTGGTGTTGTGAATGAC

Table 3.

PCR Conditions

	PCR Steps	**Temperature C^0 \ time (second)**	**Cycles**
Steps	Initial Denaturation	95 / 10 min	1
	Denaturing	94 / 10 sec	40
	Annealing	57-64 / 10-20 sec	
	Elongation	72 / 30 sec	
	High Resolution Melting analysis		1

3.4 Statistical analysis

The obtained data were evaluated by using SPSS and GraphPad Prism v.8 applications.

Chapter IV

Findings and Discussion

A total of 13 oocytes used to investigate the expression levels of lncRNAs in this study. Seven oocytes were collected from PCO patients and six oocytes were collected from the control group for this study. The details of the samples are listed in the table 4. The details of NanoDrop showing the concentration of each RNA extracted and the purity of these samples are shown in table 5.

Table 4.

Details of oocytes donors used in study

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

Table 5.

Concentration and absorbance details of the RNA samples used in study

Sample	Concentration (ng/µl)	260/280
1	10.0	1.52
2	11.0	1.48
3	12.7	1.46
4	11.0	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	10.0	1.52
11	10.9	1.56
12	11.5	1.51
13	10.0	1.52

4.1 LncRNA expression levels in oocytes obtained from the PCO patients

The Ct, ΔCt and ΔΔCt values for each oocyte are shown in table 6. The fold change values and student's T-test results for each lncRNAs obtained from the PCO patients and control group are shown in table 7.

Expression levels of lncRNAs were analyzed using one-way Anova statistical analysis method. The results showed that *RP11-573D15.8* and *lnc-CYP11A1* have elevated expression levels as shown in figure 1, however *RP11-156E8.1* has decreased expression in human oocytes obtained from the study group (PCO) compared to the control group as shown in figure 1. However, these results were not statically significant ($p>0.005$).

Table 6.

Ct values for each gene investigated in each oocyte sample.

Patient ID\GENE ID	**ACTB**	**RP11-573D15.8**			**RP11-156E8.1**			**lnc-CYP11A1-1**		
	Ct	Ct	ΔCt	ΔΔCt	Ct	ΔCt	ΔΔCt	Ct	ΔCt	ΔΔCt
1	32.4	19.2	-13.20	1.63	22.80	-9.60	4.27	26.70	-5.70	2.57
2	35.6	19.9	-15.70	-0.87	23.10	-12.50	1.37	22.80	-12.80	-4.53
3	34.7	19.2	-15.50	-0.67	24.00	-10.70	3.17	25.60	-9.10	-0.83
4	35.4	18.3	-17.10	-2.27	21.50	-13.90	-0.03	25.50	-9.90	-1.63
5	38.5	19.8	-18.70	-3.87	21.90	-16.60	-2.73	26.00	-12.50	-4.23
6	33.79	20.3	-13.49	1.34	21.60	-12.19	1.68	25.00	-8.79	-0.52
7	34.8	22.4	-12.40	2.43	22.10	-12.70	1.17	24.40	-10.40	-2.13
8	37.6	20.9	-16.70	-1.87	22.70	-14.90	-1.03	27.70	-9.90	-1.63
9	36.7	22.7	-14.00	0.83	24.00	-12.70	1.17	28.90	-7.80	0.47
10	35.7	21.1	-14.60	0.23	18.90	-16.80	-2.93	28.60	-7.10	1.17
11	32	22	-10.00	4.83	22.20	-9.80	4.07	27.50	-4.50	3.77
12	35.1	21.5	-13.60	1.23	22.60	-12.50	1.37	26.50	-8.60	-0.33
13	38.2	18.1	-20.10	-5.27	21.70	-16.50	-2.63	26.50	-11.70	-3.43

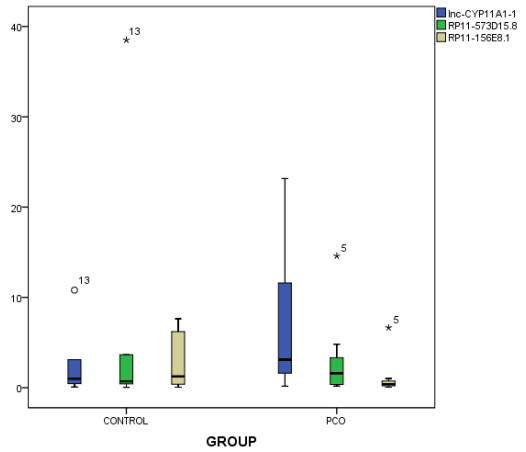
Table 7.

Fold changes and student's T-test result for each lncRNA

lncRNAs and fold changes	RP11-573D15.8	RP11-156E8.1	lnc-CYP11A1-1
Average PCO oocytes 2^-ΔΔCt	3.39	1.28	7.55
Average control oocytes 2^-ΔΔCt	7.33	2.89	2.73
Fold change PCO/Control	0.46	0.46	2.76
Student's T-Test	0.85	0.38	0.26
p<0.05	Non-significant	Non-significant	Non-significant

Figure 1

Expression levels (2^-ΔΔCt) of all lncRNAs in oocytes obtained from PCO patients and control group.



4.2 The analysis of lncRNA expression levels in oocytes

This study, as the first lncRNA research in oocytes, showed that lncRNAs are expressed more than the housekeeping gene in oocytes. However, the expression levels of lncRNAs in PCO patients did not differ statistically from the control group. Thus, these three selected lncRNAs do not show statistical association in oocytes obtained from PCO patients compared to the control group.

Chapter V

Discussion

PCOS is of the leading phenotypes causing abnormalities in reproduction, metabolism, and physiology in females. Although PCOS has been known for a long time, its etiology is still not well understood. Studies show that the genetic abnormalities are associated with PCOS. The regulatory roles of lncRNAs in gene expression have proven to be important in the recent years. Thus, it is a possibility that abnormal gene expression levels regulated by lncRNAs may be involved in the development of PCOS.

Previously published studies have shown that *CYP11A1* gene is expressed significantly different in the oocytes of PCO patients (Al-Omar et al., 2020). *CYP11A1* has an important role in the steroidogenesis pathway, which provides the production of estrogen and androgens from cholesterol. While the association of hyperandrogenism with PCOS is evident, it is not surprising that in oocytes, *CYP11* is overexpressed in PCO patients, unlike healthy individuals. In this study, a hypothesis was developed on the regulatory effects of lncRNAs on gene expression (Sebastian-delaCruz et al., 2021) causing *CYP11A1* to be overexpressed. However, the results of this study showed that there was no significant difference in the expression patterns of these three lncRNAs in the PCO samples and the control group. Thus, it does not seem that these lncRNAs are involved in the regulation process of *CYP11A1* gene in human oocytes. Despite this, there are still numerous lncRNAs associated with *CYP11A1*, and as more studies are conducted and the number of samples increased, the statistical significance of the change may occur.

Two lncRNAs, *RP11-573D15.8* and *RP11-156E8.1*, which were examined in this study, have a strong interaction with *CYP11A1* and may affect mRNA stability. In addition, although lnc-CYP11A1-1 does not directly interact with CYP11A1, it can indirectly affect CYP11A1 gene expression by binding to ncRNAs that strongly target CYP11A1 such as RP11-573D15.8, MIR6820-001 and RP3-323A16.1-001 (Fukunaga et al., 2019).

Previously published studies showed an association between altered expression of *PVT1* and *microRNA-17-5p* in the development of PCOS (Liu et al., 2020). Increased expression of *NEAT1* lncRNA, which also interacts with *CYP11A1*, in mouse and human granulosa cells were associated with PCOS (Zhen et al., 2021). This is the first lncRNA that has been associated with *CYP11A1* and may be

associated with increased *CYP11A1* in oocytes as well. Over-expression of *lnc-MAP3K13-7* was shown to be associated with the development of PCOS (Geng et al., 2021). Although there is no lncRNA study in oocytes with PCO/PCOS yet, two studies in this field showed that circular RNAs are important in the development of oocytes, which can indicate that expression changes or junction anomalies can cause PCOS development (Dang et al., 2016; Cheng et al., 2017). A number of lncRNAs were examined in human oocytes and neighboring cells, and a different lncRNA expression pattern between the oocytes and neighboring cells was observed (Bouckenheimer et al., 2018). In healthy oocytes, lncRNAs such as, *TUNAR, LINC01118, C3orf56, CASC8* and *BCAR4* appear to be higher expression levels than in cumulus cells. In addition, expression analysis of 943 lncRNAs in cumulus cells were shown to be associated with mRNA expression in the oocyte, suggesting that the varying expression levels may originate from neighboring cells in the ovary (Bouckenheimer et al., 2018). This situation seems to create difficulties in understanding the mechanism of PCOS development. On the other hand, it can be concluded that more ncRNA studies are required in this field.

There are a number of studies examining the relationship between *CYP11A1* and ncRNAs. In the first study, the expression relationship between various miRNAs and *CYP11A1* was examined in the corpus luteum (CL) obtained from cattle, and no correlation was found between the selected miRNAs and the target gene (Donadeu et al., 2020; Andreas et al., 2021). Another ncRNA study on granulosa cells in sheep showed that microRNA-150 has negative regulation on the *CYP11A1* gene. A study in Leydig cells of goats also showed that miR-1197-3p increases testosterone synthesis by promoting *CYP11A1* (An et al., 2019). MiR-628-5p obtained from the serum of pregnant women was associated with *CYP11A1* expression level and lncRNA-*miR-628-5p-CYP11A1* networks indicating that it may play a role in increasing the risk prenatal abnormalities and post-natal abnormalities in PCOS patients (Martinez-Fierro et al., 2019). Whether miR-628-5p and its associated lncRNAs play a role in PCOS development in oocytes remains to be clarified.

CHAPTER VI

Conclusion and Recommendations

The current study investigated the gene expression levels of the selected lncRNAs targeting the *CYP11A1* gene involved in the steroidogenesis pathway in oocytes donated by PCO patients and the control group, respectively.

6.1 Conclusion

In this study, no statistically significant results could be obtained between the lncRNAs with transcript IDs ENST00000627551, ENST00000607453, ENST00000607453, and upregulated *CYP11A1* gene expression in human oocytes obtained from PCO patients. However, studies show that ncRNAs have a strong relationship with PCOS and the regulation of related genes.

No ncRNA study has yet been performed in human oocytes obtained from PCOS/PCO patients. This study shows that the selected lncRNAs are highly expressed compared to the housekeeping gene. Although the functions of lncRNAs in oocytes are not well known, miRNAs are known to be important in oocyte development, and this study may show that they may have important roles with the high expression levels.

In conclution, this study shows that there is no regulatory relationship between selected ENST00000627551, ENST00000607453, ENST00000607453, and *CYP11A1*. It can be concluded that the three selected lncRNAs are not associated with PCOS, but it would not be wrong to conclude that they also show a high expression in human oocytes.

6.2 Recommendations

Altered expression levels of genes such as *CYP17*, *HSD17B1*, and *CYP11A1*, which have important roles in the steroidogenesis pathway, may be associated with hyperandrogenism. This can cause the arrest of follicular development. Future studies may examine ncRNAs and epigenetic mechanisms associated with genes involved in this pathway. In addition, by examining the changing ncRNA expression levels in the neighbouring tissues of oocytes, a relationship can also be established.

Understanding the developmental mechanism of PCOS seems promising to develop treatment strategies for PCOS and other reproductive disorders.

The major limitations for this study were the very few number of samples and the difficulty of primer design because lncRNAs are mostly composed of repeat regions. However, the difficulty of collecting human oocytes at the MII stage proves that studies with low number of oocytes were sufficient for the study. In addition, not performing hormonal checks from patients donating oocytes for this study was another limitation. In the future studies, it may be useful to use RNA-seq analysis for miRNAs where primer design is almost impossible and to identify high numbers of lncRNAs.

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BİLİMSEL ARAŞTIRMALAR ETİK KURULU

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

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Yakın Doğu Üniversitesi Tıp Fakültesi öğretim üyelerinden Doç. Dr. Pınar Tulay'ın sorumlu araştırmacısı olduğu, YDU/2019/75-920 proje numaralı ve **"Investigation of steroidogenesis related gene expression in human oocytes obtained from patients with polycystic ovaries"** başlıklı proje önerisi kurulumuzca değerlendirilmiş olup, etik olarak uygun bulunmuştur.

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Appendix X

Turnitin Similarity Report

Tez

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Abstract

EXAMINATION OF THE EXPRESSION OF LNCRNAS IN HUMAN OOCYTES FROM PATIENTS WITH POLYCYSTIC OVARIES

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Background: Polycystic ovary syndrome (PCOS) is a widespread premenopausal female condition resulting from signs and symptoms of excess androgen and ovarian abnormalities. It affects 6% to 20% of women who have not reached menopause, making it the widespread metabolic and endocrine disease affecting woman who is in the age of reproductive. A high number of women present to fertility clinics for PCOS related problems each year. Altered gene expression levels in ovarian and oocyte samples may be associated with infertility. In this study, the regulatory effects of lncRNAs on changing gene expression in human oocytes donated by patients with polycystic ovaries and patients without polycystic ovaries were examined.

Methods: In total, 13 meiosis II stage (MII) oocytes were collected from patients who applied to *In Vitro* Fertilization (IVF) Clinic at the Near East Hospital, Nicosia, Cyprus. Oocytes were used to examine the expression levels of 3 lncRNAs targeting the *CYP11A1* gene. RNAs were individually extracted from each oocyte. Expression data were obtained for each oocyte using the real-time polymerase chain reaction (PCR) using the cDNA samples obtained from each RNA from each oocyte. Statistical analysis was performed and a P value of 0.05 were considered as statistically significant.

Result: The samples consisted of seven oocytes from patients with polycystic ovaries and six from patients without signs of polycystic ovaries. The real-time PCR results showed that there was no statistically significant ($p>0.05$) difference in the expression levels of RP11-573D15.8, RP11-156E8.1, and lnc-CYP11A1-1 in patients with polycystic ovaries compared to the patients without polycystic ovaries.

Conclusions: The expression of *CYP11A1*, which was previously shown to be upregulated in oocytes obtained from patients with polycystic ovaries, is not implied to be

regulated by RP11-573D15.8, RP11-156E8.1 and lnc-CYP11A1-1 since the expression levels of these lncRNAs did not show any variation in oocyte samples between each group. In order to elucidate the up-regulated gene expression in the *CYP11A1* gene associated with the development of polycystic ovary syndrome, different target lncRNA expression should be examined. In this way, more information about the development of polycystic ovary syndrome can be obtained.

***Keywords**:* PCOS, human oocytes, lncRNAs, gene expression

Chapter I
Introduction

Polycystic ovary syndrome (PCOS) is a very common female condition resulting from the combination of signs of excess hormone androgen (hyperandrogenemia) with ovarian dysfunction (polycystic ovarian morphology and/or oligo-ovulation). PCOS patients also face with insulin resistance and insulin excess in the bloodstream, that lead to elevated risk of developing diabetes mellitus (DM). It is also related to various pregnancy-related conditions, endometrial tumors, and cardio-vascular diseases (Lizneva et al., 2018). The prevalence of PCOS in women who have not yet reached menopause is between 6% according to the old criteria and 20% according to the new criteria. This makes it the most common endocrine and metabolic disorder in women of reproductive age (Escobar-Morreale, 2022).

1.1. Pathophysiology of PCOS

PCOS is an endocrine and multifactorial condition, which is characterized by hyperandrogenism, obesity, and insulin resistance. Intraovarian androgen excess stimulates the maturation of small-sized follicles while inhibiting the selection of a dominant follicle. The resulting excess of small follicles that are arrested during development gives the ovaries a "polycystic" appearance. Although PCOS was thought to be a syndrome related to hypothalamic-pituitary gonadotropin secretion disorder at first (Jonard, 2004), it is now proving that PCOS is basically a disorder of ovarian steroidogenesis.

1.1.1 PCOS Symptoms

Hirsutism is a medical name for excessive hair growth. It develops due to high androgen levels. The Ferriman-Gallwey system, which measures hair growth in androgen-sensitive areas, is often used to determine the level of hirsutism. Although it is a common symptom, not every hyperandrogenic juvenile is facing hirsutism, because the pilosebaceous units are insensitive to androgens. Some of the young adolescents whose hyperandrogenism is not fully developed may not have hirsutism. However, the opposite is also possible. The hairiness that occurs when androgen is at normal levels is defined as "idiopathic hirsutism" (Spritzer et al., 2016).

Acne vulgaris is an important problem caused by hyperandrogenism in adolescents. The increased serum level of androgens increases sebum production. Genetic factors, diet,

medications, and hyperandrogenism increase skin dysbiosis and increase acne and long-term skin damage (Carmina et al., 2022).

Menstrual irregulations are also common in PCOS patients. The distinction between abnormal and physiological anovulation is often difficult, and clinical examination is often delayed because of this. The absence of a period after the age of 14 or more than three years after the start of breast development is referred to as primary amenorrhea. Secondary amenorrhea is defined as an unnatural ovulation delay lasting more than 90 days, even if menstruation was previously experienced. Oligo-menorrhea is an infrequent delay in ovulation exceeding 39 days outside of the usual ovulation pattern. Studies show that androgen level is associated with long-term infertility or PCOS (Pundir et al., 2020).

Polycystic ovaries are fluid-filled sacs that are formed in the ovaries. Polycystic ovaries are very difficult to detect with ultrasound, especially in adolescents. Ultrasound is nowadays accepted as a tertiary method and now four different phenotypes associated with the syndrome are sought (Carmina et al., 2022).

Obesity and hyperinsulinemia with insulin resistance strongly associated with PCOS although not included in a diagnostic criterion. These are acanthosis nigricans, metabolic syndrome, insomnia like sleeping problems, and fatty liver disease. In the long term, it causes problems such as heart diseases and diabetes mellitus (Ezeh et al., 2013; Pundir et al., 2020). Ten percent of PCOS patients aged 40 years suffer from diabetes mellitus and its complications (Otto-Buczkowska et al., 2018). Insulin resistance exacerbates hyperandrogenemia and aggravates chronic problems (Shaikh et al., 2014).

1.2 Etiology of PCOS

Although familial aggregation suggests that PCOS is genetically based, only a few genetic variants and mutations are associated with PCOS carriers in different populations, with only 10% showing PCOS heritability. PCOS is now recognized being a multigene condition that is characterized by genetic variations that combine with strong environmental variables that cause various PCOS phenotypes. (Escobar-Morreale, 2022). PCOS risk is increased by an unhealthy life or agents of infection. Insulin resistance and hyperinsulinemia, and its high levels disrupt the ovaries, causing androgen levels to rise, resulting in anovulation (Shaikh et al., 2014).

Polycystic ovaries are associated with the hypothalamus-pituitary junction including hormones like luteinizing hormone (LH) and follicular stimulating hormone (FSH). It is not just related to genetic factors. It is also linked to environmental factors. Related genes and single nucleotide polymorphisms (SNPs) are responsible for disease progression. Databases associate PCOS with over 240 SNPs. Polymorphism, non-coding RNAs and nucleotide mutations affect the expression of related genes (Chen & Fang, 2018).

FSH receptors, androgen hormone receptors, LH receptors, and leptin receptors are the most common genes involved. A polymorphism within a gene may disrupt the metabolic process, resulting in ovarian abnormalities (Xita et al., 2002). *StAR* polymorphisms, *FSHR* polymorphisms, *FTO* polymorphisms, *VDR* polymorphisms, *IR* and *IRS* polymorphisms, and *GnRHR* polymorphisms have all been linked to PCOS. The progression and severity of PCOS worsen when insulin levels and androgen levels rise. Theca cells of the ovary are affected by hyperinsulinemia, which raises androgen levels. SHBG and IGFBP-1 production in the liver are reduced as a result of this disease. Increased testosterone levels, on the other hand, encourage the production of free fatty acids (FFAs) in the visceral adipose tissue (VAT), which causes increased insulin resistance (Chen & Fang, 2018; Gambineri et al., 2022). PCOS patients have numerous 8mm cycts and about 70 percent patients face with infertility and acne caused by elevated level of testosterone (Ye et al., 2020). Twenty percent of patients suffers from sleep apnea. Elevated levels of insulin hormone cause irregularity in the menstrual cycle which increases risk of infertility in older ages. Depression and anxiety are also common psychological disorders in patients.

Aromatase, an one of the members of the complex Cytochromes P450 (CYPs), is a steroidogenesis enzyme (in Steroidogenic pathway) that generally plays a key part in steroid conversion. It enables the body to convert testosterone to estrogen. Aromatase insufficiency causes a malfunction in the pathway, which prevents it from being converted. Because C19 does not convert to C18, this defect affects ovarian function and raises androgen levels. *CYP1A1, CYP11A, CYP11B2, CYP17, CYP19A, CYP21*, and *CYP3A7* are aromatase genes that have been reported in PCOS datasets. Any cytochrome P450 abnormality increases the risk of PCOS development (Joseph et al., 2015; Gambineri et al., 2022). Importantly, this study shows that the expression of *CYP11A1* was significantly different in human oocytes donated by PCO patients (Al-Omar et al., 2020).

1.3 Diagnostic criteria

In 1935, Dr. Stein and Dr. Leventhal published the first complete description of Stein-Leventhal Syndrome, or another name known as PCOS. Due to its diverse clinical presentations, uncertain genesis, complex pathophysiology, and poor diagnosis, it has led to significant scientific controversy. U.S. National Institute of Health (NIH) criteria were established in 1990 in attempt to produce a comprehensive and detailed characterization to diagnose PCOS. Then, in 2003, an atelier in Rotterdam, Netherlands, built up the Rotterdam criteria, a next diagnostic criterion for PCOS (Franks, 2006). Out of the three criteria, this criterion needs the presence of two; atypical ovulation, hyperandrogenism, and/or polycystic ovarian morphology (>11 follicles in each ovary measuring between 2 and 8.99 mm) (Pundir et al., 2020).

The diagnostic criteria for of Androgen Excess and Polycystic Ovary Syndrome Society (AES) were changed in 2006. The presence of hyperandrogenism in association with either oligo-ovulation or polycystic ovaries is required for the AES. The process of diagnostic standardization has certain challenges. First, ovulation is frequently erratic during early menarche. As a result, anovulation cannot be regarded conclusive evidence of the syndrome's occurrence. Second, in youths, transvaginal ultrasonography is not frequently performed, limiting ovary visibility, and hence excluding any invasive diagnosis of polycystic ovarian morphology. Third, there is just no agreement on the normal androgen levels, and there are little studies for normal androgen levels for females with ages 10-20. As a result, detecting androgen abnormalities is a difficult undertaking. Finally, cystic ovaries are difficult to separate from multi follicular ovaries, which are common in females with ages of 10-20 years. As a result, the Pediatric Endocrinology Society has issued recommendations for the differential diagnosis of PCOS in both adults and adolescents. In 2012 and one year later, a new two set of adult and adolescent PCOS criteria have recently been proposed, one by an ESHRE and ASRM working group another by the Endocrine Society's clinical practice recommendations committee. While PCOS is still not openly visible by person of mature age standards, serologically elevated androgen levels and progressively condensed hirsutism, in combination with abnormal ovulation for at least 24 months after menstrual cycles after age 15 years, or an ovarian volume>10 c m3, may be considered in adolescents. It is important to highlight, however, that none of the proposed criteria has been verified (Pundir et al., 2020).

Table I summarizes the acceptable consensus (persistent hyperandrogenic oligoanovulation) for early PCOS diagnosis based on age and stage appropriate norms (Rosenfield et al., 2015; Liznevaet al., 2016).

Table 1.
Diagnostic criteria for PCOS

Parameter/age group	Adult	Adolescence
NIH 1990	Hyperandrogenism Ovaries Abnormality	Abnormal Uterine Bleeding Persistent Symptoms For 1-2 Years Abnormal For Age
Rotterdam 2003	Hyperandrogenism Ovarial Abnormalit y Polyc ystic Ovary	Hyperandrogenism Moderate-To-Severe Hirsutism Moderate-To-Severe Acne Vulgaris
Androgen Excess & PCOS Society 2006	Hyperandrogenism Ovarial Abnormality And/Or Polycystic Ovary	
ESHRE/ASRM 201 2	Hyperandrogenism Ovaries Abnormalit y Polyc ystic Ovary	Clinical Or Biochemical Hyperandrogenism Ovaries Abnormalit y Polycystic Ovary

Table

1(continued)

Endocrine Society2013	Clinical Or Biochemical Hyperandrogenism Ovaries Abnormality	Clinical Or Biochemical Hyperandrogenism Ovaries Abnormality

ESHRE: European Society for Human Reproduction and Embryology, ASRM: American Society for Reproductive Medicine; (Rosenfield, 2015; Lizneva. 2016).

1.4 The association of the *CYP11A1* gene and PCOS

Cytochrome P450 in the family-11 with the subfamily-A, polypeptide 1(*CYP11A1*) is a human protein encoded by the *CYP11A* gene. *CYP11A1* catalyzes the cholesterol to pregnenolone, which is the first action in the biosynthesis of steroids. Many studies show the association of *CYP11A1* polymorphisms and the development of PCOS, but other factors (such as lncRNA regulation) may affect transcription of this gene as well that may lead to pathophysiology of PCOS (Heidarzadehpilehrood et al., 2022; Zhang et al., 2012).

1.5 LncRNAs

Long non-coding RNAs and microRNAs (miR or miRNAs) are two of the most common types of non-coding/non-structural RNAs. They used to be classified as "junk" or "noise" but these days they are being discovered to have many critical roles. Both appear to have mostly regulatory functions, while much remains unknown about their functions. LncRNAs are at least 200 bases long and have a unique three-dimensional structure. They have a critical role in a variety of biomolecular processes, including epigenetic modifications, transcription, post-transcriptional and post translational regulation of target genes (Mohr et al., 2021).

Gene expression is linked to mRNA stability in all living things. mRNAs can remain functional for a few minutes to several days in eukaryotes. Its stability and operational time help maintain cell homeostasis. The dynamics of mRNA lifespan and quantity help to respond to changes in environmental factors, regulation of cell development, and gene regulation at the stage of cell differentiation. Levels of mRNA are maintained by the balance between production

and decay. Across most eukaryotic cells, mRNA molecule decay begins with adenylation and capping of the messenger RNA, continued with exonuclease-mediated degrading (Bicknell & Ricci, 2017).

However, the control of mRNA stability is primarily dependent on how regulatory variables influence multiple processes (deadenylation, decapping, and degradation), hence these aspects should be considered when studying mRNA stability regulation. Studies show that miRNAs can promote mRNA degradation by aiding endonucleases (Eulalio et al., 2008; Iwakawa & Tomari, 2015).

Previously published studies showed that non-coding RNAs are associated with mRNA stability (Sebastian-delaCruz et al., 2021). Non-coding RNAs act as enhancer RNAs signaling RNAs, guide, decoy, scaffold. LncRNAs guide the target and bind directly to specific mRNAs, specific RNA-binding proteins, or microRNAs (miRNAs). LncRNAs can bind to miRNAs or miRNA-messenger RNA junctions, block miRNAs and additionally promote gene expression (Tu et al., 2021).

RNA binding proteins (RBPs) are proteins that are critically important in the regulation of gene expression (Corley et al., 2020). LncRNAs can accelerate their degradation by binding RBPs to mRNA (Noh et al., 2018). However, it can also stabilize mRNA by preventing them from binding (Briata & Gherzi, 2020). It supports modulation of target genes by interacting with m6a machinery to lncRNAs (Huang et al., 2018; Wang et al., 2013)

1.5.1 Biogenesis of the lncRNAs

LncRNAs, like other RNAs, are transcribed by RNA polymerase 2. They have a 3' poly a tail and a 5′ methyl-cytosine cap (Zhang et al., 2019). They can be classed in a variety of ways based on their characters. It can be characterized as sense, anti-sense, bi-directional, intronic, or intergenic based on its genetic origin (Lanzafame et al., 2018). They are classed as nuclear, cytoplasmic, or mitochondrial based on their subcellular location. LncRNAs can be capped or polyadenylated, and they can also be created via splicing (Gourvest et al., 2019). Ribonuclease P can cut them to create 3' ends and turn them into a circular structure. This prevents them from degrading (Alessio et al., 2020; Chen, 2016).

LncRNA biogenesis can be regulated by various mechanisms. Epigenetic changes can affect lncRNA expression; for example, acetylation of the *H3K56ac* and *CAF-1* can enhance the

antisense of *H3K56* lncRNA (Quinn & Chang, 2015). Exosomes, as well as slc22a2 and slc22a3, are capable of degrading them. In fact, their degradation involves Nrd1, Nrd3 like complexes inside the nuclear membrane, and protein XRN1 (5'-3' Exoribonuclease 1) in the cytoplasm (Quinn & Chang, 2015). The UPF1 protein inhibits these processes as well (Sleutels et al., 2002). Although epigenetic factors seem to play a role in regulation, we are still at the beginning of understanding their detailed mechanisms. It seems that more research on lncRNA biogenesis needs to be study in the future (Liu et al., 2021).

1.6 The statement of the problem

PCOS is one of the most problematic phenotypes in women of reproductive age. Pathophysiology of the syndrome increases the risk of being infertile. The studies suggest that expression of *CYP11A* gene which has a key role in steroidogenesis pathway is related with development of PCOS. The expression of *CYP11A* may be regulated by lncRNAs or epigenetic mechanism which finally affect the development of PCOS. Understanding gene regulation and the relationship of lncRNAs can help to develop early diagnosis and treatments.

1.7 Significance of the study

To date, there has not been any studies investigating the lncRNA expression levels in human oocytes donated by patients with polycystic ovaries. This study can provide information about lncRNA expression in oocytes and how it may be regulating *CYP11A* expression. Thus, the result of this study is important to understand the underlying mechanism of PCOS.

1.8 Study Hypothesis and Goals

LncRNAs are nucleotides that have a regulatory effect on gene expression. This study aims to examine the expression levels of lncRNAs that regulates *CYP11A* in human oocytes from PCO patients and oocytes from patients with no polycystic ovaries (control group). It was hypothesized that there would be a significant variation in the expression levels of lncRNAs in oocytes from patients with polycystic ovaries relative to the control group.

CHAPTER II

Literature Review and Related Research

2.1 Noncoding RNA Expression in the Ovaries and the Oocytes

Studies show that miRNAs have a high expression in the mammalian ovaries (Tripurani et al., 2010; Ro et al., 2007; Ahn et al., 2010; Choi et al., 2007). A previously published study showed that microRNAs are involved in important processes in development of the ovary (Fitzgerald et al., 2015). It even showed a high expression in the ovary compared to organs such as the brain, hypothalamus, small intestine, and kidney (Fitzgerald et al., 2015).

Studies are showing an association between miRNA expression and polycystic ovaries, for example over 120 miRNAs were overexpressed in PCOS patients, but only miR-320 showed a significantly lower expression profile (Sang et al. 2013). This study also showed that microRNA-93, microRNA-133, and microRNA-223 targeting the insulin receptor have a high expression profile in PCOS patients (Chen et al., 2013).

Although micro-RNA expression has been studied for a long time, lncRNAs in the ovaries have only recently been investigated. However, the results show that lncRNAs are associated with PCOS, in such lncRNA NEAT1 had increased expression in the ovaries of PCOS patients (Sang & Zhang, 2020). It also shows the elevated expression of lncRNAs such as ZFAS1 (Zhu et al., 2020), MALAT1 (Zhang et al., 2020), and PVT1 (Liu et al., 2020) in granulosa cells. Regarding the steroidogenesis pathway, silenced OC1 long non-coding RNA promotes aromatase mRNA expression and increases estrogen production (Wu et al., 2020). In addition, the HUPCOS has been shown to affect the expression of CYP11 (Che et al., 2020). However, there is no studies investigating the expression of lncRNAs in human oocytes.

2.2 LncRNA associated with *CYP11*

RP11-573D15.8 is a lncRNA with a length of 1129 bases located on chromosome 3. According to the genomic origin, it is an antisense RNA and has three exons. It interacts with *CYP11* mRNA with a high energy of -46.16 kcal/mol (lnc-HNRNPU-5:3, LNCipedia 2018).

RP11-156E8.1 is a 1739 base-long antisense lncRNA. It is found in chromosome 1 and can interact with *CYP11* at -36.57 kcal/mol (lnc-TBCCD1-4:21, LNCipedia 2018).

Lnc-CYP11A1-1 is a 612 bases long lncRNA that is antisense of *CYP11A1* and is located on chromosome 15. There is no direct interaction with *CYP11*. But similar sequence can establish a competitive relationship with ncRNAs targeting *CYP11* (lnc-CYP11A1-1:1, LNCipedia 2018), and finally affect *CYP11* gene expression level Competitive lncRNAs have similar sequences to target genes. In this way, they can bind to ncRNAs targeting the target gene and suppress the regulation of ncRNAs on the target gene (Dong et al., 2019).

Chapter III

Materials and Methods

CYP11A has an important role in steroidogenesis pathway. Previous studies had shown that statistically significant changes in the expression of this gene was detected in human oocytes from patients with polycystic ovaries. The objective of this research was to investigate the expression levels of lncRNAs that have possible regulatory effects on the expression pattern of *CYP11A* gene in human oocytes obtained from PCO patients and the control group.

Oocytes were collected from Near East University Hospital *In Vitro* Fertilization (IVF) Center, Nicosia, Cyprus. Ethical approval was provided by the Near East University Institutional Review Board with the decision numbered YDU/2019/75-920.

3.1 The sample collection and sample size

A total of 13 oocytes were obtained at meiosis II stages (MII) from the IVF center. Seven oocytes were donated by PCO patients with polycystic ovaries, while six oocytes were donated by females without signs of polycystic ovaries. The samples were included in the control group if the females are non-obese (within the age range of BMI) with normal ovarian morphology and antral follicle count. The sample group included young females with polycystic ovaries with typical morphology and within the normal range of BMI (non-obese). These obtained human oocytes were then used to study expression of selected lncRNAs which possibly have regulatory roles on *CYP11* gene. The main steps included extraction of RNA and synthesis of cDNA. Real-time PCR was performed for each sample in duplicates. For each PCR, a negative control with no cDNA sample was used.

3.2 *In Vitro* Fertilization (IVF)

In the first three days of the menstrual period, transvaginal ultrasound was applied to the patients and antral follicle count (AFC) was assessed. The follicles were expected to have a diameter of 2-9 mm. Patients who have different follicle diameter were not included in the study. Controlled ovarian stimulation (COS) was initiated between the first and the fifth day following short antagonist protocol. Considering the age, BMI and AFC data, different doses of FSH were applied to each patient. Ultrasound controls were performed on the fourth and sixth days. Gonadotropin-releasing hormone (GnRH) antagonist was applied daily to patients to obtain a

follicle with 14mm diameter. After the ovulation trigger, the oocytes were collected after an average of 35.5 hours.

3.3 Analysis of oocytes: RNA extraction, cDNA synthesis and real time PCR

The study group included 13 oocytes, of which seven oocytes obtained from females with the polycystic ovaries and six oocytes from the control group. The experiments were performed at NEU DESAM Research Institute's Laboratory, Nicosia, North Cyprus. RNA purification was carried out using Norgen's RNA purification kit (Canada). NanoDrop ND-1000 was used to assess the quality of the RNA obtained following manufacturer's protocol.

Norgen's Transcript First Strand cDNA Synthesis kit (Norgen, Canada) was used for reverse transcription from RNA to synthesize cDNA following manufacturer's protocol with no modifications. Real time polymerase chain reaction (PCR) was carried out using cDNA samples to investigate the expression levels of selected lncRNAs. The expression levels were investigated in a total of three lncRNA genes. The target lncRNAs were selected using the LncRRIsearch database which is a bioinformatics tool that calculates the possible relationship between lncRNAs and messenger RNAs and other lncRNAs using human and mouse genome data, and ranks the results from high to low. The Incipedia database was used to obtain RNA sequences. Primers were designed for RP11-573D15.8, RP11-156E8.1, lnc-CYP11A1-1. The primers were designed to flank the exon-exon boundaries to avoid amplification of any DNA that may be contaminating the samples. The designed primer sequences for each lncRNAs are shown in table 2.

The LightCycler® 480 SYBR Green I Master kit was used for the real time PCR. The manufacturer's protocol with no modification and the final concentration of 0.2 µM of the primers were used in the reaction mixture. The PCR conditions are listed in table 3. $2^{-\Delta\Delta Ct}$ values were obtained to analyze expression levels and log10 values were used to calculate the significance level. In the experiment conducted with the Insta Q96™ Real Time Machine, Ct values were obtained for each sample with melting curve data. *ACTB* was used as housekeeping gene.

Table 2.

Printer.s .sequent e.s

Gene ID(Gene name)	Transcript ID	Forward Primer	Reverse Primer
ENSG00000197099(RP11-573D15.8)	ENST00000627551	TGATCATCCAGGAAGCCAACC	GAAGCCACTAAGACGGTGAGT
ENSG00000272195(RP11-156E8.1)	ENST00000607453	TGCCTAACGATAACCTCGGC	GACGACGCAATGTTTGTGCT
ENSG00000277749(lnc-CYP11A1-1; RP11-60L3.6	ENST00000611006	CATGACTCCTTGGTATTGG	AGAGTGGTGTTGTGAATGAC

Table 3.

PCR Conditions

	PCR Steps	Temperature C' \ time (second)	Cycles
Steps	Initial Denaturation	95 / 10 min	1
	Denaturing	94 / 10 sec	40
	Annealing	57-64 / 10-20 sec	
	Elongation	72 / 30 sec	
	High Resolution Melting analysis		1

3.4 Statistical analysis

The obtained data were evaluated by using SPSS and GraphP and Prism v.8 applications.

Chapter IV

Findings and Discussion

A total of 13 oocytes used to investigate the expression levels of lncRNAs in this study. Seven oocytes were collected from PCO patients and six oocytes were collected from the control group for this study. The details of the samples are listed in the table 4. The details of NanoDrop showing the concentration of each RNA extracted and the purity of these samples are shown in table 5.

Table 4.

Details of oocytes donors used in study

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

Table 5.

Concentration and absorbance details of the RNA samples used in study

Sample	Concentration (ng/µl)	260/280
1	10.0	1.52
2	11.0	1.48
3	12.7	1.46
4	11.0	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	10.0	1.52
11	10.9	1.56
12	11.5	1.51
13	10.0	1.52

4.1 LncRNA expression levels in oocytes obtained from the PCO patients

The Ct, ΔCt and ΔΔCt values for each oocyte are shown in table 6. The fold change values and student's T-test results for each lncRNAs obtained from the PCO patients and control group are shown in table 7.

Expression levels of lncRNAs were analyzed using one-way Anova statistical analysis method. The results showed that *RP11-573D15.8* and *lnc-CYP11A1* have elevated expression levels as shown in figure 1, however *RP11-156E8.1* has decreased expression in human oocytes obtained from the study group (PCO) compared to the control group as shown in figure 1. However, these results were not statically significant ($p>0.005$).

Table 6.

Ct values for each gene investigated in each oocyte sample.

Patient ID\GENE ID	22 TB	RP11-573D15.8			RP11-156E8.1			lnc-CYP11A1-1		
	Ct	Ct	ΔCt	ΔΔCt	Ct	ΔCt	ΔΔCt	Ct	ΔCt	ΔΔCt
1	32.4	19.2	-13.20	1.63	22.80	-9.60	4.27	26.70	-5.70	2.57
2	35.6	19.9	-15.70	-0.87	23.10	-12.50	1.37	22.80	-12.80	-4.53
3	34.7	19.2	-15.50	-0.67	24.00	-10.70	3.17	25.60	-9.10	-0.83
4	35.4	18.3	-17.10	-2.27	21.50	-13.90	-0.03	25.50	-9.90	-1.63
5	38.5	19.8	-18.70	-3.87	21.90	-16.60	-2.73	26.00	-12.50	-4.23
6	33.79	20.3	-13.49	1.34	21.60	-12.19	1.68	25.00	-8.79	-0.52
7	34.8	22.4	-12.40	2.43	22.10	-12.70	1.17	24.40	-10.40	-2.13
8	37.6	20.9	-16.70	-1.87	22.70	-14.90	-1.03	27.70	-9.90	-1.63
9	36.7	22.7	-14.00	0.83	24.00	-12.70	1.17	28.90	-7.80	0.47
10	35.7	21.1	-14.60	0.23	18.90	-16.80	-2.93	28.60	-7.10	1.17
11	32	22	-10.00	4.83	22.20	-9.80	4.07	27.50	-4.50	3.77
12	35.1	21.5	-13.60	1.23	22.60	-12.50	1.37	26.50	-8.60	-0.33
13	38.2	18.1	-20.10	-5.27	21.70	-16.50	-2.63	26.50	-11.70	-3.43

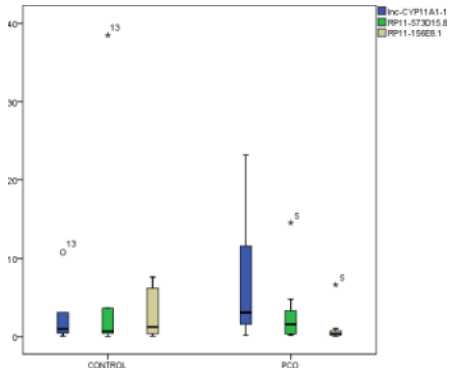
Table 7.

Fold changes and student's T-test result for each lncRNA

lncRNAs and fold changes	RP11-573D15.8	RP11-156E8.1	lnc-CYP11A1-1
Average PCO oocytes 2^-ΔΔCt	3.39	1.28	7.55
Average control oocytes 2^-ΔΔCt	7.33	2.89	2.73
Fold change PCO/Control	0.46	0.46	2.76
Student's T-Test	0.85	0.38	0.26
p<0.05	Non-significant	Non-significant	Non-significant

Figure 1

Expression levels (2^-ΔΔCt) of all lncRNAs in oocytes obtained from PCO patients and control group.



4.2 The analysis of lncRNA expression levels in oocytes

This study, as the first lncRN A research in oocytes, showed that lncRN As are expressed more than the housekeeping gene in ocic ytes. However, the expression levels of lncRN As in PCO patients did not differ statistically from the control group. Thus, these three selected lncRN As do not show statistical association in cinc ytes obtained from PCO patients compared to the control group.

Chapter V

Discussion

PCOS is of the leading phenotypes causing abnormalities in reproduction, metabolism, and physiology in females. Although PCOS has been known for a long time, its etiology is still not well understood. Studies show that the genetic abnormalities are associated with PCOS. The regulatory roles of lncRNAs in gene expression have proven to be important in the recent years. Thus, it is a possibility that abnormal gene expression levels regulated by lncRNAs may be involved in the development of PCOS.

Previously published studies have shown that *CYP11A1* gene is expressed significantly different in the oocytes of PCO patients (Al-Omar et al., 2020). *CYP11A1* has an important role in the steroidogenesis pathway, which provides the production of estrogen and androgens from cholesterol. While the association of hyperandrogenism with PCOS is evident, it is not surprising that in oocytes, *CYP11* is overexpressed in PCO patients, unlike healthy individuals. In this study, a hypothesis was developed on the regulatory effects of lncRNAs on gene expression (Sebastian-delaCruz et al., 2021) causing *CYP11A1* to be overexpressed. However, the results of this study showed that there was no significant difference in the expression patterns of these three lncRNAs in the PCO samples and the control group. Thus, it does not seem that these lncRNAs are involved in the regulation process of *CYP11A1* gene in human oocytes. Despite this, there are still numerous lncRNAs associated with *CYP11A1*, and as more studies are conducted and the number of samples increased, the statistical significance of the change may occur.

Two lncRNAs, *RP11-573D15.8* and *RP11-156E8.1*, which were examined in this study, have a strong interaction with *CYP11A1* and may affect mRNA stability. In addition, although lnc-CYP11A1-1 does not directly interact with CYP11A1, it can indirectly affect CYP11A1 gene expression by binding to ncRNAs that strongly target CYP11A1 such as RP11-573D15.8, MIR6820-001 and RP3-323A16.1-001 (Fukunaga et al., 2019).

Previously published studies showed an association between altered expression of *PVT1* and *microRNA-17-5p* in the development of PCOS (Liu et al., 2020). Increased expression of *NEAT1* lncRNA, which also interacts with *CYP11A1*, in mouse and human granulosa cells were associated with PCOS (Zhen et al., 2021). This is the first lncRNA that has been associated with *CYP11A1* and may be associated with increased *CYP11A1* in oocytes as well. Over-expression of

lnc-MAP3K13-7 was shown to be associated with the development of PCOS (Geng et al., 2021). Although there is no lncRNA study in oocytes with PCO/PCOS yet, two studies in this field showed that circular RNAs are important in the development of oocytes, which can indicate that expression changes or junction anomalies can cause PCOS development (Dang et al., 2016; Cheng et al., 2017). A number of lncRNAs were examined in human oocytes and neighboring cells, and a different lncRNA expression pattern between the oocytes and neighboring cells was observed (Bouckenheimer et al., 2018). In healthy oocytes, lncRNAs such as, *TUNAR, LINC01118, C3orf56, CASC8* and *BCAR4* appear to be higher expression levels than in cumulus cells. In addition, expression analysis of 943 lncRNAs in cumulus cells were shown to be associated with mRNA expression in the oocyte, suggesting that the varying expression levels may originate from neighboring cells in the ovary (Bouckenheimer et al., 2018). This situation seems to create difficulties in understanding the mechanism of PCOS development. On the other hand, it can be concluded that more ncRNA studies are required in this field.

There are a number of studies examining the relationship between *CYP11A1* and ncRNAs. In the first study, the expression relationship between various miRNAs and *CYP11A1* was examined in the corpus luteum (CL) obtained from cattle, and no correlation was found between the selected miRNAs and the target gene (Donadeu et al., 2020; Andreas et al., 2021). Another ncRNA study on granulosa cells in sheep showed that microRNA-150 has negative regulation on the *CYP11A1* gene. A study in Leydig cells of goats also showed that miR-1197-3p increases testosterone synthesis by promoting *CYP11A1* (An et al., 2019). MiR-628-5p obtained from the serum of pregnant women was associated with *CYP11A1* expression level and lncRNA-*miR-628-5p-CYP11A1* networks indicating that it may play a role in increasing the risk prenatal abnormalities and post-natal abnormalities in PCOS patients (Martinez-Fierro et al., 2019). Whether miR-628-5p and its associated lncRNAs play a role in PCOS development in oocytes remains to be clarified.

CHAPTER VI

Conclusion and Recommendations

The current study investigated the gene expression levels of the selected lncRNAs targeting the *CYP11A1* gene involved in the steroidogenesis pathway in oocytes donated by PCO patients and the control group, respectively.

6.1 Conclusion

In this study, no statistically significant results could be obtained between the lncRNAs with transcript IDs ENST00000627551, ENST00000607453, ENST00000607453, and upregulated *CYP11A1* gene expression in human oocytes obtained from PCO patients. However, studies show that ncRNAs have a strong relationship with PCOS and the regulation of related genes.

No ncRNA study has yet been performed in human oocytes obtained from PCOS/PCO patients. This study shows that the selected lncRNAs are highly expressed compared to the housekeeping gene. Although the functions of lncRNAs in oocytes are not well known, miRNAs are known to be important in oocyte development, and this study may show that they may have important roles with the high expression levels.

In conclution, this study shows that there is no regulatory relationship between selected ENST00000627551, ENST00000607453, ENST00000607453, and *CYP11A1*. It can be concluded that the three selected lncRNAs are not associated with PCOS, but it would not be wrong to conclude that they also show a high expression in human oocytes.

6.2 Recommendations

Altered expression levels of genes such as *CYP17*, *HSD17B1*, and *CYP11A1*, which have important roles in the steroidogenesis pathway, may be associated with hyperandrogenism. This can cause the arrest of follicular development. Future studies may examine ncRNAs and epigenetic mechanisms associated with genes involved in this pathway. In addition, by examining the changing ncRNA expression levels in the neighbouring tissues of oocytes, a

relationship can also be established. Understanding the developmental mechanism of PCOS seems promising to develop treatment strategies for PCOS and other reproductive disorders.

The major limitations for this study were the very few number of samples and the difficulty of primer design because lncRNAs are mostly composed of repeat regions. However, the difficulty of collecting human oocytes at the MII stage proves that studies with low number of oocytes were sufficient for the study. In addition, not performing hormonal checks from patients donating oocytes for this study was another limitation. In the future studies, it may be useful to use RNA-seq analysis for miRNAs where primer design is almost impossible and to identify high numbers of lncRNAs.

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Tez

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%12	%7	%10	%
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EXPERIENCES

July 2021– Jan 2022 **Full-time Internship** • Near East University • Nicosia, Cyprus. Covid 19-qPCR lab

Jan 2020– Mar 2020 **Research Intern•** Gebze Technical University • Kocaeli/Turkey. Project "Virus-induced gene silencing (VIGS) of possible microRNA genes regulating plant growth and development".

June 2019– Aug 2019 **Full-time Internship** • Girne Akcicek Hospital • Girne, Cyprus. Medical Microbiology and Biochemistry Diagnosis Laboratory

July 2018– Aug 2018**Full-time Internship** • Dr.Burhan Nalbantoglu State Hospital. Nicosia/Cyprus. Tumor testing, Carier state testing and post-symptomatic diagnostic testing based Molecular Genetics Laboratory

EDUCATION

MSc Medical Biology and Genetics, Near East University, Nicosia/Cyprus
High Honor Student
Thesis: "The expression of long noncoding RNAs in human oocytes".
2020-2022

BSc Molecular Biology and Genetics, Gebze Technical University, Kocaeli/Turkey
Thesis: "Investigation of the effects of plant growth promoting bacteria (PGPB) on maize plant growth"
2015-2020