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Potential of utilizing specific miRNAs as biomarkers for polycystic ovarian syndrome (PCOS)

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POTENTIAL OF UTILIZING SPECIFIC MIRNAS AS BIOMARKERS FOR POLYCYSTIC OVARIAN SYNDROME (PCOS)

by

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DEDICATION

I would like to dedicate this work to my wonderful parents, my fiancé Ramana, and my dog Tommy.

ACKNOWLEDGMENTS

I would sincerely like to thank my advisors: Dr. Simon Levy & Dr. Amy Adkins for taking the time to read and review my thesis. Millie Agosto for helping me with graduation prep. My study buddies who turned out to be my best friends in the MAMS program: Mina Gardezi and Isabella Leon Calle.

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ABSTRACT

Polycystic Ovarian Syndrome is the one of the leading causes of infertility among women who are of child-bearing age. The syndrome's vast range of phenotypes has made it challenging for researchers to not only consistently diagnose but also discover a cure. Currently, there are several proposed treatments being looked into, however, much of the research focuses on employing promising biomarkers, micro ribonucleic acids (miRNAs), that can potentially aid in diagnosis. The four prominent locations of research for these biomarkers include: ovarian tissues specifically looking into granulosa cells (GC), adipose tissue, follicular fluid, and the serum. My goal is to determine which of these areas holds the most promise to diagnose this syndrome in the years to come.

This study reviewed a large collection of the current polycystic ovarian syndrome literature evaluating both reported miRNAs and how viable those would be as potential biomarkers to use for the future. The data showed that a majority of these promising biomarkers were found in granulosa cells, adipose tissue, and follicular fluid. Although there were miRNAs that were deemed promising in the serum, research is still far from conclusive in using these miRNAs as biomarkers for diagnosis of polycystic ovarian syndrome.

By comparing the miRNAs selected from each type of location, I was able to conclude that miR-21, miR-93, miR-223, and miR-let-7b hold the most promise for the

potential to become biomarkers for polycystic ovarian syndrome in the near future. Currently, there is a lot of research particularly surrounding these miRNAs and how they were shown to have been expressed in statistically significant levels among women with the syndrome. However, because of their complexity, miRNAs do not regulate one single pathway, it is hard to describe a mechanism that explains the pathophysiology of the syndrome. I believe we are still far away from successfully zooming in on one biomarker. By determining the most potential biomarker(s), we can focus resources and efforts towards finding a better diagnostic tool for this syndrome.

TABLE OF CONTENTS

TITLE……...i

COPYRIGHT PAGE……...ii

READER APPROVAL PAGE……..iii

DEDICATION iv

ACKNOWLEDGMENTS v

ABSTRACT vi

TABLE OF CONTENTS..... viii

LIST OF TABLES ix

LIST OF FIGURES x

LIST OF ABBREVIATIONS xi

INTRODUCTION 1

PUBLISHED STUDIES 21

DISCUSSION 46

REFERENCES 52

CURRICULUM VITAE 65

LIST OF TABLES

Table	Title	Page
1	Treatment of Clinical Symptoms in Patients with PCOS	3
2	Summary of miRNAs discussed with expression levels, where they are located, and possible target genes.	45

LIST OF FIGURES

Figure	Title	Page
1	Diagram representing some of the symptoms that can manifest in a patient with PCOS.	4
2	PCOS is a complex disease that can present with clinical hyperandrogenism, polycystic ovaries, and anovulation.	5
3	Schematic of Major Steroid Biosynthetic Pathways in the small antral follicle of the ovary.	7
4	A schematic of miRNA synthesis and function in animals.	9
5	Theca cells produce the androgens, whereas the granulosa cells convert the androgens into estrogens.	14
6	A schematic of an adipocyte cell when there is high levels of glucose detected in the blood stream.	16
7	A schematic representation of an oocyte with information of what can be found in the follicular fluid.	18
8	p53 is inactive when it is bound to mdm2.	29
9	This is a diagram showing the IRS1 signaling pathway.	39

LIST OF ABBREVIATIONS

AKT ..... Protein Kinase B

cAMP ..... Cyclic Adenosine Monophosphate

CASP3 ..... Caspase 3

CDKN1A ..... Cyclin-Dependent Kinase Inhibitor

CYP17..... Steroid 17α-monooxygenase

DHEA .....Dehydroepiandrosterone

ERK ..... Extracellular signal-regulated kinase

ERα.....Estrogen Receptor Alpha

ERβ..... Estrogen Receptor Beta

ET-1 .....Endothelin 1

FAI.....Free Androgen Index

FSH..... Follicle Stimulating Hormone

FSI .....Fasting Serum Insulin

FOXO1..... Forkhead Box O1

GATA6 .....GATA Binding Protein 6

GLUT-4 ..... Glucose Transporter Type 4

GC..... Granulosa Cell

GLUT-4 ..... Glucose Transporter Type 4

hASCs..... Human-Derived Adipose-Derived Stem Cells

HMGB1 ..... High Mobility Group Box 1

HOMA-IR ..... Homeostatic Model Assessment of Insulin Resistance

IGFBP-1 ..... Insulin-Like Growth Factor Binding Protein

IGF-1 ..... Insulin-Like Growth Factor 1

IGF-2 ..... Insulin-Like Growth Factor 2

IR ..... Insulin Resistance

IRS-1 ..... Insulin Receptor Substrate 1

KGN ..... Steroidogenic Human Granulosa-Like Tumor Cell Line

LH ..... Luteinizing Hormone

LTA ..... Lymphotoxin Alpha

MAPK ..... Mitogen-Activated Protein Kinase

MCM7 ..... Minichromosome Maintenance Complex Component 7

mRNA ..... Messenger Ribonucleic Acid

miRNA ..... Micro Ribonucleic Acid

PCOS ..... Polycystic Ovarian Syndrome

PDCD4 ..... Programmed Cell Death Protein 4

PI3K ..... Phosphoinositide 3-kinase

PTEN ..... Phosphate and Tensin Homolog

P450cc ..... Cholesterol side-chain cleavage enzyme

RBP ..... Ribonucleic Acid Binding Protein

RISC ..... Ribonucleic Acid- Induced Silencing Complex

RNA ..... Ribonucleic Acid

RTK ..... Receptor Tyrosine Kinase

SARA.....SMAD Anchor for Receptor Activation

SMAD3 .....Mothers Against Decapentaplegic Homolog 3

S6K1 .....Ribosomal Protein S6 Kinase Beta-1

TGF-β .....Transforming Growth Factor-Beta

TGFBR2..... Transforming Growth Factor-Beta Receptor Type 2

TNF-β .....Tumor Necrosis Factor-Beta

INTRODUCTION

Polycystic Ovarian Syndrome, also known as PCOS, is an endocrine and metabolic disorder that affects women who are of reproductive-age with a prevalence estimated somewhere between 5-10% (Asuncion et al., 2000). There have been studies suggesting that it is a multifactorial polygenic disease where the phenotypes very significantly different from woman to woman. As illustrated in Figure 1 some common signs and symptoms include: hyperandrogenism, ovulatory dysfunction, and polycystic ovaries to name a few (B. Chen et al., 2019). As a result of some these symptoms many women experience irregular menstrual cycles, infertility, hirsutism, and acne (De Leo et al., 2016).

PCOS has been traditionally viewed as an endocrinlogical issue. However, research within the past decade illustrated PCOS has essentially two components: endocrine and metabolic. Hyperandrogenemia has been identified as a very useful diagnostic for patient populations that are not within the adolescent range (Z. Chen et al., 2019). Lab tests are performed to measure levels of Luteinizing Hormone (LH), testosterone, free androgen index (FAI), and dehydroepiandrosterone sulfate (DHEAS) (Stracquadanio & Ciotta, 2015). Typically, physicians measure LH and testosterone levels since they are easier to measure. Sometimes measured DHEAS levels are mildly elevated along with serum testosterone suggesting that the patient has PCOS. In addition, PCOS is also associated with increased risk of developing Type 2 Diabetes, insulin resistance, hypertension, oxidative stress, and cardiovascular disease (Y. Zhang et al.,

2017). Other lab tests are also ordered such as fasting glycemia and insulinemia, because more than half the women that are diagnosed with PCOS also develop hyperinsulinemic insulin resistance (De Leo et al., 2016). With that said there is no standard diagnostic test to properly pinpoint this disease. In addition to the clinical indicators of the syndrome there may also be possible pregnancy complications that may arise such as gestational diabetes, placental abruption, and preterm birth to name a few (X.-D. Zhang et al., 2013). It is important to keep in mind that there is not an effective way at the moment to measure the risk of these complications.

The etiology of PCOS remains widely unknown and the syndrome as a result is not well understood. As of now there are treatment options to alleviate the symptoms, however, these treatments do not significantly improve prognosis of PCOS. Characteristically, physicians diagnose PCOS as an endocrine disorder and patients are prescribed birth controls and progestin therapy (Stracquadanio & Ciotta, 2015). However, since research has established that PCOS has a strong metabolic component, physicians are also prescribing metformin and statins (Janci et al., 2012). Other medications are prescribed to treat the other effects of PCOS such as hirsutism and acne (Table 1). Unfortunately, medications alone are sometimes not effective and research has demonstrated that keeping track of one's diet and exercise to maintain a healthy lifestyle has shown improvement. In terms of diet, there is no standardized PCOS diet but general guidelines to follow are: reducing total calories consumed based on sex, age, and activity; favoring complex carbohydrates; increasing fiber, and eating low glycemic index foods. Exercise is an important aspect as well because of its effect on weight loss, specifically a

reduction in the amount of visceral fat and a reduction to body mass index (BMI) (Stracquadanio & Ciotta, 2015). The reasoning behind why visceral fat reduction is good is because it is more metabolically active and has been linked to insulin resistance (IR). Research studies have shown that women with PCOS who implemented these lifestyle changes demonstrated improvement in insulin sensitivity, regular menstrual cycles and lower risk of type 2 diabetes and cardiovascular disease (Rocha et al., 2019).

Table 1. Treatment of Clinical Symptoms in Patients with PCOS

Symptom	Infertility	Hirsutism	Acne	Insulin Resistance	Obesity
First-Line	Clomiphene	Hormonal contraception, Spironolactone	Hormonal contraception, Topical Creams	Lifestyle changes	Lifestyle changes
Second-Line	Metformin, Gonadotropins	Spironolactone	Spironolactone	Metformin, Thiazolidinediones (TZDs)	Orlistat, Metformin

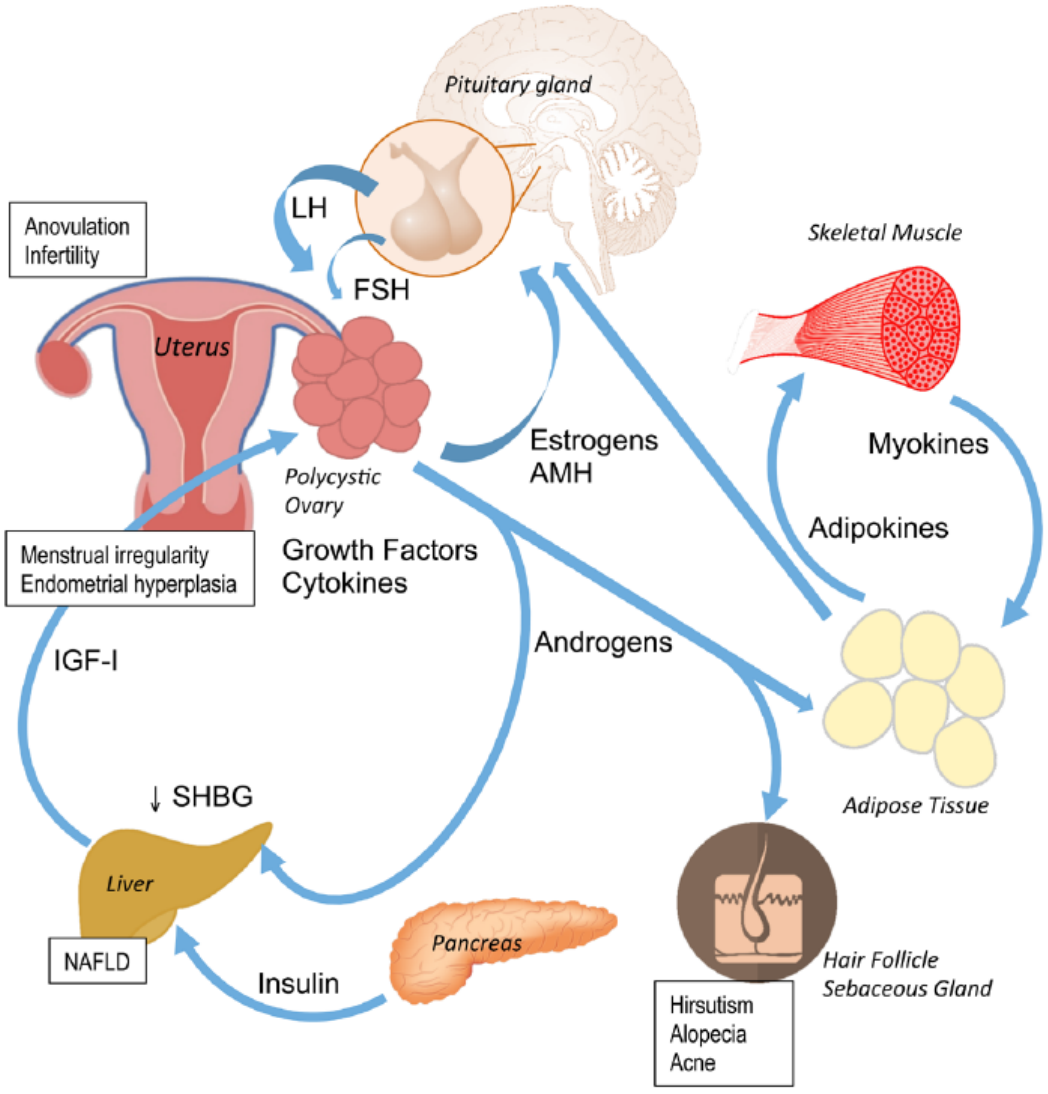

Figure 1. Diagram representing some of the symptoms that can manifest in a patient with PCOS. Androgens give rise to hirsutism and acne problems, whereas the estrogen produced by the ovaries and the adipose tissue gives rise to infertility and anovulation. AMH = Anti-Mullerian Hormone; FSH = Follicle Stimulating Hormone; IGF-1 = Insulin-Like Growth Factor 1; LH = Luteinizing Hormone; NAFLD = Non-Alcoholic Fatty Liver Disease; SHBG = Sex-Hormone Binding Globulin (Rocha et al, 2019)

PCOS is a complex disorder which has three different definitions established to aid proper diagnosis (Figure 2). The common denominator between all three criteria is that of androgen excess. According to the National Institute of Health (NIH) their definition includes hyperandrogenism /or hyperandrogenemia and oligo-anovulation, whereas Rotterdam encompasses as having the presence of two out of the following three features: clinical and/or hyperandrogenism, oligo-anovulation, and Polycystic Ovarian, PCO, on ultrasound. The Androgen Excess Society (AES) placed an emphasis on hyperandrogenism, and it can be defined principally by clinical and/or biochemical hyperandrogenism with either oligo-anovulation or polycystic ovaries.

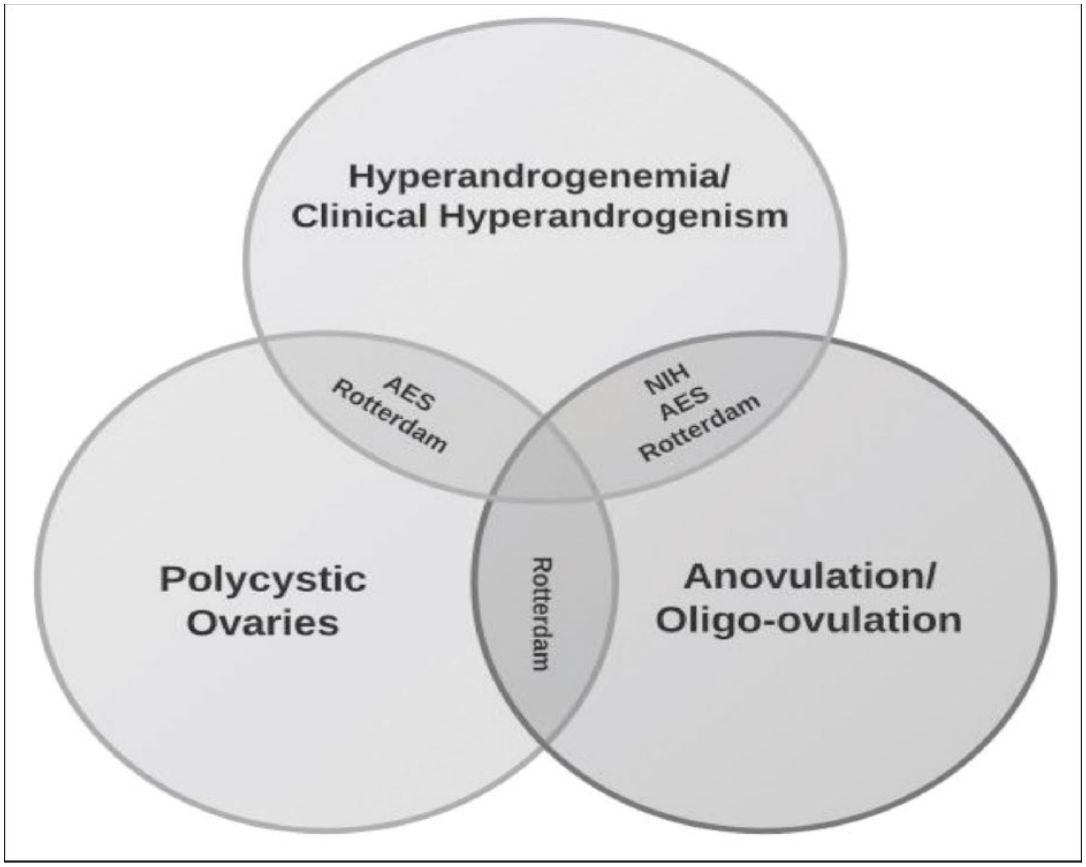

Figure 2. PCOS is a complex disease that can present with clinical hyperandrogenism, polycystic ovaries, and anovulation. The definition of PCOS is based on three different criteria: National Institutes of Health (NIH), Rotterdam, and Androgen Excess Society (AES). These three different criteria exclude other disorders that may present with androgen excess. (Lanzo et al., 2015)

Ovaries and adrenal glands in women typically produce similar amounts of testosterone. Usually, half of the testosterone comes from direct secretion by ovaries and adrenal glands, whereas the other half is made in the periphery via conversion from androstenedione (Rosenfield & Ehrmann, 2016). What is unique is that unlike estradiol and cortisol secretion, androgen production is not under direct negative feedback in

women. This becomes an important factor especially in women who are diagnosed with PCOS.

Many PCOS animal models hypothesize that the upstream Gonadotropin Releasing Hormone (GnRH) is hyperactive. Women who have been diagnosed with PCOS typically have higher levels of (LH), which means they have a higher LH pulse frequency. The high frequency contributes to ovarian thecal cell hyperplasia, which results in increased androgen production and secretion (Rosenfield & Ehrmann, 2016). It is important to note that not only is there an overall androgen production increase, but also the steroidogenic enzymes needed to make these androgens are also increased in PCOS (Xue et al., 2018).

Another hypothesis points to insulin binding to multiple sites to increase androgen levels. As shown in Figure 3, insulin can bind to Insulin-like Growth Factor -1 (IGF-1) receptors which can have a positive effect and lead to an increase in theca cells producing androgen. With higher insulin levels there is inhibition in the liver to produce Insulin-like Growth Factor Binding Protein -1 (IGFBP-1) leading to increased levels of IGF-1 and IGF-2, which play an important role in ovarian follicular growth and maturation as well as steroidogenesis (Rosenfield & Ehrmann, 2016). Although the exact relationship is still unclear, researchers have confirmed that there is interaction amongst insulin receptors and steroidogenesis causing hyperandrogenism.

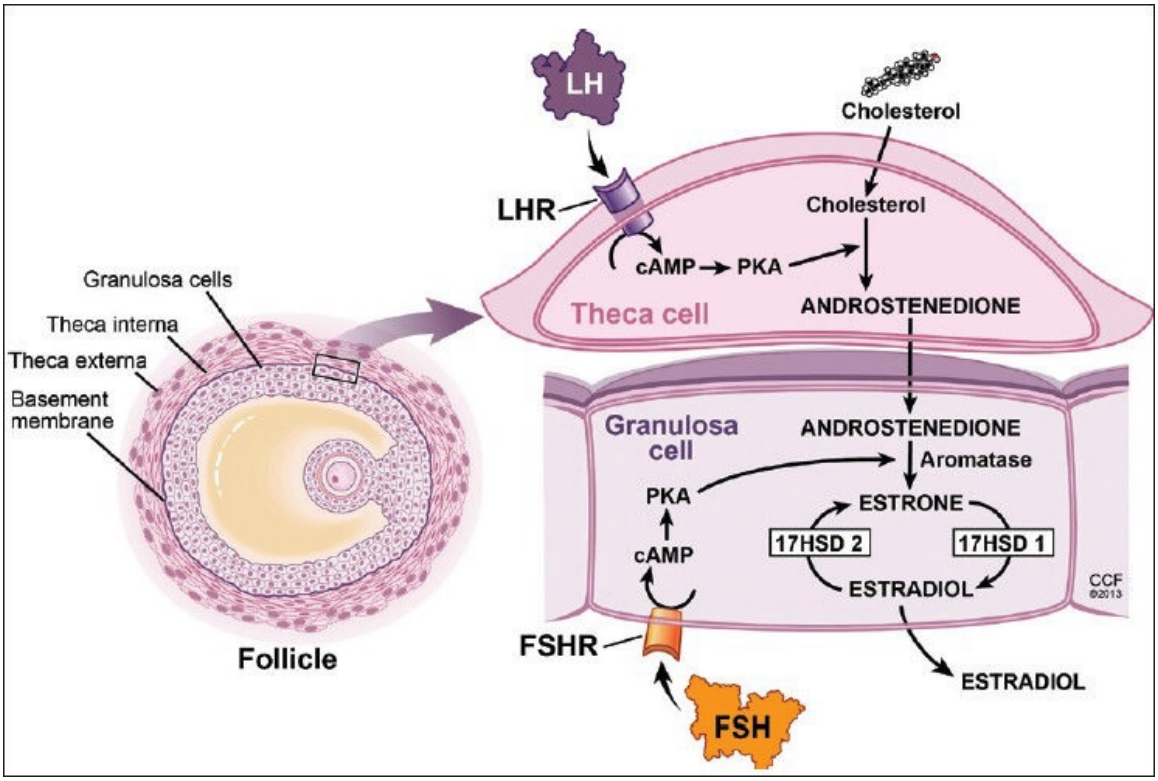

Figure 3. Schematic of Major Steroid Biosynthetic Pathways between the theca cell and granulosa cell. Luteinizing hormone (LH) promotes the creation of androgen synthesis in the theca cell via cholesterol, whereas the Follicle Stimulating hormone (FSH) promotes the creation of estradiol in the granulosa cell via the enzyme aromatase. cAMP = Cyclic Adenosine Monophosphate; FSHR = Follicle Stimulating Hormone Receptor; LHR = Luteinizing Hormone Receptor; PKA = Protein Kinase A; 17HSD1 = 17-Beta Hydroxysteroid Type 1; 17HSD2 = 17-Beta Hydroxysteroid Type 2. (Doshi & Agarwal, 2013)

MicroRNA (miRNA's) Formation and Function:

Like many other diseases, researchers within the field of PCOS have shifted gears by looking at microRNAs (miRNA) to see if there is a more reliable and effective way to diagnose PCOS. MiRNA's are small non-coding sequences that are transcribed from the genome via RNA polymerase II or III, forming a stem-loop hairpin structure (Catalanotto et al., 2016). Then, a nuclear protein complex that is made up of the Drosha enzyme

along with other proteins can cleave the pri-miRNA which results in a pre-miRNA that can leave the nucleus and enter the cytoplasm. Afterward, the pre-miRNA encounters Dicer, an enzyme that further processes and gives rise to a double-stranded miRNA: mature guide and passenger strand (Figure 4). The mature miRNA associates with Ribonucleic Acid- Induced Silencing Complex (RISC) and targets the 3' untranslated region of the target mRNA. This results in negatively regulating gene expression at the post-transcriptional level either via degrading the target mRNA or inhibition of translation. This is critical because many miRNAs' have important regulatory functions in many processes ranging from cell proliferation to apoptosis (Deswal & Dang, 2020).

The next logical question to ask is how do miRNAs regulate gene expression? One proposed mechanism is that the nuclear complex RISC can mediate post-transcriptional gene silencing with miRNA in the nucleus as well as the cytoplasm. Another approach is that miRNAs can interact with the promoter region in association with the Ago protein and this can either lead to activation or suppression depending on the location of the target region and the epigenetic status (Hou et al., 2019). Finally, activation can be interrupted via miRNA's seed region by either being deleted, mutated, or when the enhancer locus is deleted. Enhancers are short regions of DNA that can bind to proteins to increase the probability that the gene will be transcribed.

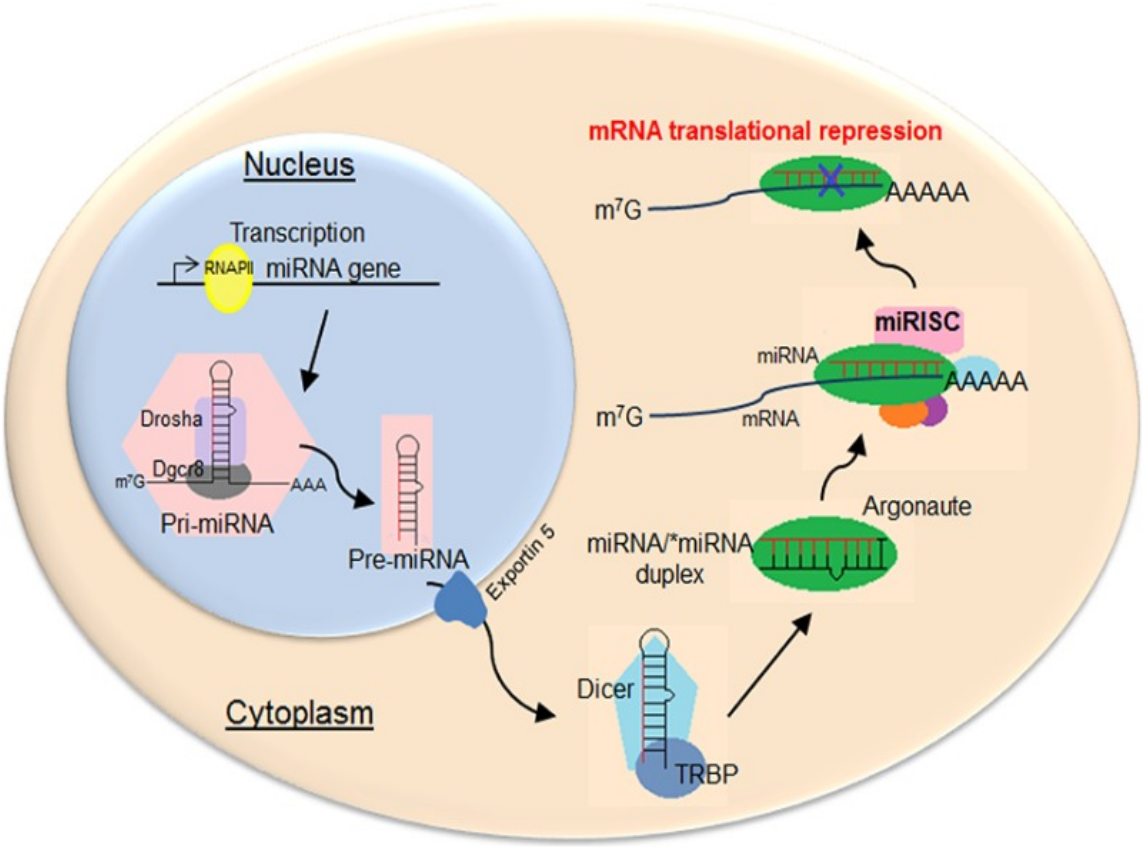

Figure 4. Schematic of miRNA synthesis and function in animals. The pri-miRNA is transcribed by RNA Pol II, then cleaved by Drosha-DGCR8 to produce pre-miRNA. Once the pre-miRNA is out in the cytoplasm it is further processed via Dicer upon which a mature miRNA is produced. Then, the mature miRNA combines with miRISC that typically results in silencing of the gene of interest. Dgcr8 = Digeorge Syndrome Chromosomal Region 8; miRISC = micro RNA Induced Silencing Complex; TRBP = Transactivation-Responsive RNA Binding Protein. (Hajarnis et al., 2015)

Subsection One:

Preliminary research in this field discovered that women diagnosed with PCOS have altered levels of microRNAs (miRNA) that can be found within many places throughout the body such as granulosa cells, theca cells, adipose tissue, follicular fluid,

and serum (B. Chen et al., 2019). In addition, miRNAs have been shown to play a role in the activation of primordial follicles, follicle development, oocyte maturation, and ovulation (Das et al., 2008). These miRNAs were shown to be found either encapsulated in microvesicles or free-floating. The hope is that by identifying some of these miRNA's we gain a better understanding of PCOS and they can potentially act as noninvasive biomarkers and become effective therapeutic targets in trying to better treat and cure PCOS (Atiomo et al., 2009).

As mentioned previously, there are many ways miRNA can affect us, and trying to understand which specific miRNAs are involved in the pathogenesis of PCOS can provide insight into discovering new treatment therapies (Liu et al., 2018). Hence, it would be ideal to discuss the physiology of PCOS and discuss different levels of expression of these miRNAs that can disrupt normal processes and lead to PCOS symptoms.

The following few paragraphs will provide a brief summary of how researchers are trying to identify where viable biomarkers for PCOS can be found. Numerous studies concurred about how hyperandrogenism and insulin resistance are amongst the common symptoms that many women have when diagnosed with PCOS. Rationally, it made sense to look at cells/tissues that are involved in these important processes and observe miRNAs that are significantly different in women diagnosed with PCOS *versus* those who are not.

Granulosa Cells:

Granulosa cells (GC) are critical to oocyte development because they provide nutrients and regulate the growth of oocytes. It is important to highlight that beyond helping oocytes grow, one of their major functions is the production of sex steroids as well as various growth factors. The production of sex steroids is triggered by Follicular Stimulating Hormone (FSH) from the anterior pituitary gland which stimulates the granulosa cells to convert androgens into estradiol via the enzyme aromatase (Figure 5). Also, granulosa cells are responsible for producing FSH receptors.

Artimani et. al (2015) concluded that abnormal folliculogenesis observed in PCOS occurs when there is a reduction in the number of genes related to estrogen and progesterone nuclear receptors expressed in GCs. As mentioned previously, the interaction between oocytes and GCs is important to the normal development of follicles. Specifically, the study was able to illustrate that the Estrogen Receptor β (ERβ) is predominantly expressed within GCs, which supports the hypothesis that these receptors are important for control of ovarian follicles. However, other studies suggest that there was a higher expression of ERα in GC cells (Jakimiuk et al., 2002). Researchers further probed and found that women diagnosed with PCOS have lower levels of ERα which can potentially lead to abnormal folliculogenesis (Jakimiuk et al., 2002).

Since these receptors are expressed within GC cells, researchers further questioned how to identify the miRNAs involved (Das et al., 2008). One of the conclusions that they arrived at is depending on the stage of oocyte development, there

are different levels of miRNA that were expressed. Many assays were performed and there was one miRNA that was deemed significant: miR-145 (Cai et al., 2017).

Theca Cells:

There is strong evidence that the granulosa and theca cells have to communicate with each other to produce estrogen that plays an important role in fertility (Magoffin, 2005). The theca cells are located in the ovary and are highly differentiated. The main function of these cells is to produce steroid hormones such as androstenedione (Figure 5). For theca cells to produce the right amount of hormones they are under the control of LH through stimulation of the cAMP signaling pathway. The reason why this important is because one of the key clinical manifestations in PCOS is hyperandrogenism.

According to Wood et. al (2004) experiments demonstrated there was an increased amount of mRNA related to the activity of the P450scc enzyme, which plays a role in converting cholesterol to pregnenolone and this is the first step in the process of steroidogenesis. Additionally, this first step is the rate-limiting step in steroid production. Also, there were increased levels of CYP17 promoter (Rosenfield & Ehrmann, 2016). The function of CYP17 is to hydroxylate 17α of pregnenolone to yield 17α-OH pregnenolone and cleavage of the c17, 20 bonds to yield the androgens DHEA or androstenedione.

Researchers further postulated that since these enzymes are important for steroid production, there might be miRNAs that can affect their production. Li et al. (2016) have identified possible miRNAs that may impact the functions of these enzymes. Their

research focused on *GATA6* and it is an important androgen-producing gene that stimulates CYP17 promoter. Results showed that miR-92a targets GATA6 and women with PCOS were shown to have lower levels of miR-92a. This ends up causing an increase in GATA6 expression and thus an increase in the activity of the CYP17 promoter.

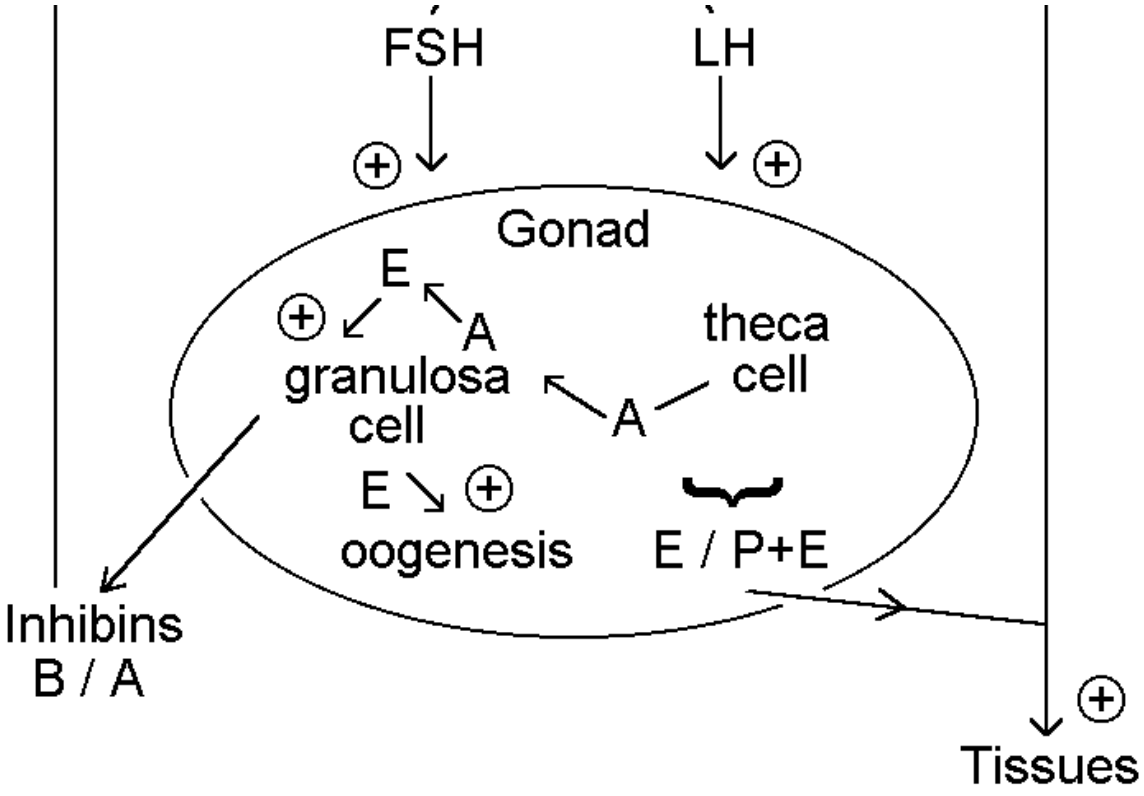

Figure 5. Theca cells produce the androgens, whereas the granulosa cells convert the androgens into estrogens. Estrogens then stimulate the growth of the granulosa cells and they acquire LH receptors as a result of this process. A = Androgens; E = Estrogens; FSH = Follicle Stimulating Hormone; LH = Luteinizing Hormone; P = Progesterone. (Mahalingaiah, 2019).

Adipose Tissue:

Adipose tissue is seen as a place for storing and releasing energy to make sure the body's metabolic needs are met (Figure 6). This is regulated via insulin binding to its receptor on the adipocyte which triggers an exocytosis reaction of GLUT-4 to the plasma membrane and allows glucose to flow inside the cell. In turn, glycolysis is activated and produces Adenosine Triphosphate (ATP). Also, there is increased lipid synthesis that allows the storage of fat droplets. Lastly, there is an increase in protein synthesis to make lipoprotein lipase (LPL) to breakdown the very low-density lipoprotein (VLDL) or chylomicron coming from the liver so they can be repackaged upon entering the adipocyte.

Women who have been diagnosed with PCOS, especially those women who are overweight or obese, are 50-70% more likely to also be diagnosed with IR (Cai et al., 2017). Researchers have concluded that there is a link between IR & PCOS but the cellular mechanisms have not been well identified and are an active area of research. IR is when the bloodstream has excess glucose and that reduces the function of cells to uptake and use that glucose for energy. As a result, the pancreas ends up producing more insulin to help glucose enter cells.

Chen et al. (2013) collected data where they compared women who had PCOS with and without IR. They concluded that miR-93 was found in adipocyte tissue irrespective of IR, however, miR-223 was found in women that had IR. This led the researchers to further hypothesize higher expression levels of both miR-93 & miR-223

decrease GLUT-4 protein in adipocyte tissues. As a result, providing a potential explanation of how PCOS can affect IR in adipocytes.

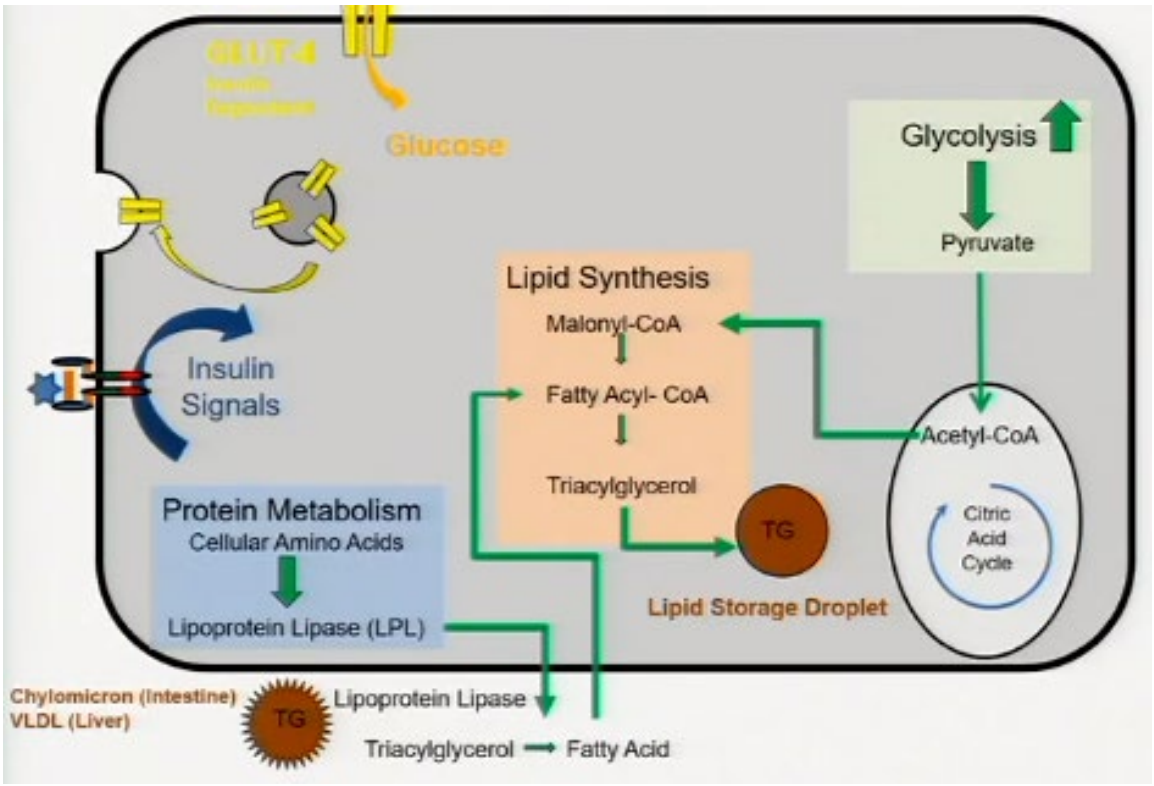

Figure 6. Schematic of an adipocyte when there is high levels of glucose detected in the blood stream. The adipocyte upon sensing this information performs many mechanisms such as: increasing the rate of glycolysis to produce Adenosine Triphosphate (ATP) to sustain lipid synthesis. Lipid synthesis is important as it packages lipid droplets so the body has stores of energy available for future use. Lastly, there is increased protein synthesis directed towards making the enzyme lipoprotein lipase (LPL). GLUT-4 = Glucose Transporter Type 4; TG = Triacylglycerol; VLDL = Very Low-Density Lipoprotein. (Atkinson, 2019)

<u>Follicular Fluid:</u>

Rosenfield and Ehrmann (2016) have briefly summarized how granulosa and theca cells are imperative to the proper growth and function of the ovary. An additional compartment to focus on finding probable miRNA biomarkers is follicular fluid. The follicular fluid is produced from granulosa and theca cells and it serves as an important microenvironment to allow proper growth and development of oocytes (Butler et al., 2019).

Follicular fluid is not however just made of only growth factors, hormones, and nutrients to allow the oocyte to mature, but according to Revelli et al. (2009), it also includes anti-apoptotic factors, proteins, and nucleotides (Figure 7). There is active research being performed to identify possible biomarkers within this fluid, however, there is little consensus on the research. For example, Roth et al. (2014) reported that miR-9, miR-32, & miR-135a were found at high levels in PCOS follicular fluid. Upon further examination, researchers found that these miRNA biomarkers were associated with carbohydrate metabolism: insulin receptor substrate 2 & interleukin 8 (Cirillo et al., 2019).

Another study illustrated that miR-132 and miR-320, which have been closely associated with estradiol secretion, were expressed at low levels in women with PCOS (Delitala et al., 2017). On the contrary, Ling et al. (2009) showed that there were high expressions of miR-320. Researchers from this study hypothesized miR-320 could suppress the production of estrogen which might explain the hyperandrogenemia in PCOS. Researchers have determined that a common theme among women diagnosed with PCOS is the miRNAs are involved in the regulation of steroid synthesis.

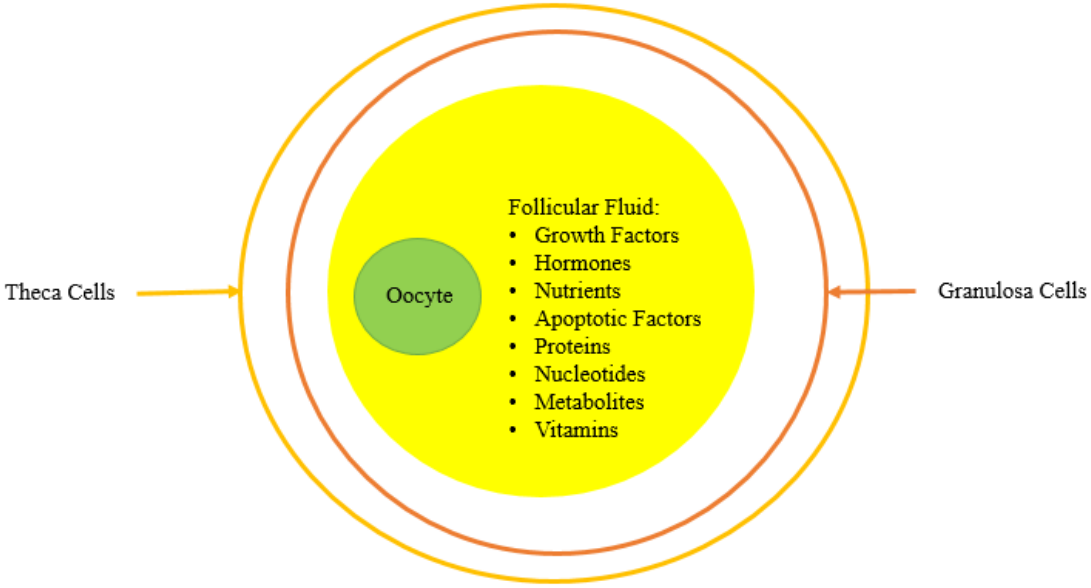

Figure 7. A schematic representation of an oocyte with information of what can be found in the follicular fluid. The follicular fluid is an amalgamation of growth factors, hormones, nutrients, apoptotic factors, proteins, nucleotides, metabolites, and vitamins.

Extracellular Fluid:

Imagine the scenario where researchers have discovered viable, accurate, and stable miRNAs as possible markers for PCOS, but having to get to a theca or granulosa cell seems invasive and difficult. Whereas if researchers were able to find just as comparable biomarkers in a more accessible compartment of the body to diagnose

whether the patient has PCOS or not. The extracellular compartments of the body is an important aspect to consider when thinking long term.

Surprisingly, miRNAs compared to the longer mRNAs were observed to be more stable due to encapsulation into microvesicles and association with protein complexes (Dufourd et al., 2019). So, why aren't researchers solely focusing on extracellular miRNAs? According to Kirschner et al. (2011) it has to do with collecting the blood and processing it. This poses a hemolytic threat and ends up degrading the miRNAs in the red blood cell. The shortcoming of this research is that only a few select miRNAs have been selected to study and we do not know whether this applies to every miRNA in the bloodstream. Despite shortcomings, numerous studies have shown that there are distinctive levels of miRNA expression present in the serum of PCOS patients (Arancio et al., 2018).

Serum:

As previously mentioned, hyperandrogenemia is a common feature of PCOS. Song and colleagues (2015) have determined that the reduced levels of miR-592 were associated with high levels of LH/Chorionic Gonadotropin Receptor (LHCGR). This is an important relationship because this receptor plays a role in how patients develop hyperandrogenemia. Other studies have shown that there were certain miRNAs that were negatively correlated with serum testosterone such as miR-146a, whereas miR-21, miR-27b, and miR-155 were positively correlated (Hou et al., 2019).

Women diagnosed with PCOS were also found to be more at risk of developing insulin resistance and diabetes later. Another study compared patients who have damaged

glucose breakdown *versus* those who did not, and found that there were three miRNAs' that stood out: miR-122, miR-193b, and miR-194 were found to have increased expression levels (Kong et al., 2011). Besides, the listed miRNA's, Long et al. (2014) found that miR-222 was found to be positively correlated with serum insulin.

PUBLISHED STUDIES

Previous Studies:

As a result of this brief introduction to some possible target sites a common theme was observed: many of these miRNAs that are discovered as possible biomarkers in some way affected carbohydrate metabolism and the steroidogenic synthesis pathway (Lerchbaum et al., 2014). These results correctly map onto clinical manifestations that so many women have when diagnosed with PCOS. Furthermore, it is interesting to see that the downstream targets of these miRNAs are associated with proteins that have important roles in metabolism and steroid synthesis (De Leo et al., 2016).

The next part of the thesis will focus on specific miRNAs which I will describe the mechanism they act by, their role, association with other proteins, and viability/accessibility as a biomarker. The latter is what defines a good biomarker from a bad biomarker (Holland, 2016). Ultimately, physicians want to be able to use a biomarker that is accurate and precise to diagnose whether a patient has PCOS.

miRNA 145:

Undoubtedly, granulosa cells are necessary for sustaining follicular growth and endocrine signaling to other tissues as well as synthesis of estrogen. Typically, GCs try to maintain a balance of follicular growth and death. When GCs are programmed for cell death, they stimulate and form atresia follicles (Cai et al., 2017). However, a multitude of studies have confirmed that GCs contribute to the abnormal follicle growth seen in

PCOS. According to Das et al. (2008), lower apoptotic rates and higher proliferation rates were seen in GCs from patients that have PCOS. Researchers realized that these cells might not only provide insight into the possible identification of biomarkers but also present as an avenue for the potential treatment of PCOS. miR-145 was isolated from GCs in women with and without PCOS, and many other studies focused on this target as well (Naji et al., 2018).

Cai et al. (2017) studied the effect of miR-145 on cell proliferation and its mechanism of action. In addition, identifying what proteins miR-145 might be associated with could lead to the effect it has on GCs. The researchers started by trying to establish how miR-145 suppresses cell proliferation in human GCs. Naturally, the next line of thought would be how miR-145 is promoting cell apoptosis. Utilizing an algorithm based on mircoRNA.org-Targets and Expression, Cai and colleagues (2017) were able to predict that *IRS1* might be a potential target.

IRS1, also known as Insulin Receptor Substrate-1, plays a role in providing signals from insulin and insulin-growth factors receptors in critical pathways such as PIK3/Akt and Erk/MAPK (Martini et al., 2014). Both pathways are involved in essential processes that affect glucose metabolism, apoptosis, and cell proliferation. To determine whether IRS1 plays a role in GCs a knockout experiment was performed (Cai et al., 2017). When researchers silenced IRS1 they found that GCs had a lower cell survival rate. This demonstrated that IRS1 could inhibit GC proliferation. What role does miR-145 have on IRS1? To find out a luciferase reporter assay, QRT-PCR, & Western blotting

was performed. The results showed that the IRS1 is a target of miR-145 and that it ends up binding to the 3'-UTR region and prevents expression of IRS1 (Cai et al., 2017).

As mentioned previously, Erk/MAPK signaling pathways are important for GCs. A plausible mechanism of how miR-145 causes suppression of cell growth might be: suppressing IRS1 which leads to the Erk/MAPK pathway not being activated (Naji et al., 2018). miR-145 could serve as a potential avenue of therapy with patients who have PCOS, however, miR-145 is located in GCs, so trying to obtain samples might be considered invasive for many patients, but this alone is not a reason to eliminate miR-145 and other biomarkers that are being found. miR-145 shows promise and other researchers like Naji and colleagues (2018) have also arrived at a similar conclusion that miR-145 levels are starkly reduced but also have managed to find another potential target, miR-182.

To deem a biomarker credible, it needs to have three characteristics: analytical validation, clinical validation, and clinical utility (Holland, 2016). While research with miR-145 is hopeful, researchers agree that a more thorough mechanism as to how this biomarker functions needs to established. Thus far, research has provided us with a basic outline of how miR-145 acts and what it does. Once, a proper mechanism has been established it is logical to assess the validity, accuracy, and precision of miR-145 to be a potential diagnostic tool for PCOS.

miR-200b & miR-200c:

Other miRNAs like miR-200b and miR-200c have received a lot of attention as potential biomarkers, but for a different disease (Belgardt et al., 2015). Numerous studies focused on how these miRNAs were involved in endometrial cancer and nasopharyngeal carcinomas, while it was relatively limited in PCOS (Sirotkin et al., 2009). However, He and colleagues (2019) realized that these miRNAs might play a role in the development of PCOS.

Like miR-145, miR-200b and miR-200c have been demonstrated to effect GC proliferation as well, but the mechanism is different (Dou et al., 2013). Researchers showed that miR-200b and miR-200c might also play an important role in the dysregulation of GCs within PCOS (He et al., 2018). He et. al (2019) found that there are two significant forms of miR-: miR-200b and miR-200c.

He and colleagues (2019) further explored the underlying mechanism of how miR-200b and miR-200c can effect GCs. These researchers employed a set of techniques similar to Cai et al. (2017) to try and identify the mechanism of miR-200b and miR-200c. First, they had to demonstrate that both miRNAs suppressed GCs proliferation. The team utilized KGN cells instead of GCs because these cells come from a steroidogenic human granulosa-like tumor cell line. Through the process of transfection, researchers inserted miR-200b and mR-200c mimics and, compared to the control, the results demonstrated that there was an overall inhibition of KGN cell proliferation. The next step was to

identify any possible gene targets by using TargetScan, researchers found PTEN to help further elucidate the mechanism by which miR-200b and miR-200c might work.

PTEN, also known as phosphatase and tensin homolog, is a gene that encodes instructions for making an enzyme that acts as a tumor suppressor, which is essential in regulating cell division by keeping cells from growing or dividing too rapidly Genetics Home Reference, 2015). The PTEN enzyme is part of the signaling pathway that signals cells to stop dividing and triggers cells apoptosis. Evidence suggests that this enzyme also helps control cell movement, the adhesion of cells to surrounding tissues, and the formation of new blood vessels (Genetics Home Reference, 2015). These functions help to avert uncontrolled cell growth that has the potential to form tumors.

According to the results, a miR-200b inhibitor led to increased levels of PTEN mRNA, whereas the miR-200b mimic led to a decrease in the levels of PTEN mRNA (He et al., 2019). The same results were also seen with the miR-200-c mimic as well as the inhibitor. He et al. (2019) then proposed that PTEN is a target of both, miR-200b as well as miR-200c. Other researchers have also observed that overexpression of miR-200b and miR-200c might play a role in contributing to insulin resistance, which is characteristic of PCOS (O'Reilly et al., 2014). This study, however, was not able to establish a thought-out mechanism. All we learned from this study was that PTEN is a potential target, however, PTEN is located in a lot of tissues throughout our body. In trying to figure out whether these miRNAs would be probable biomarkers, the first thing that comes to mind is how can we isolate the target located specifically GCs and not affect other cells along the way.

Akin to miR-145, miR-200b & miR-200c more research needs to be performed to see if these are valid biomarkers to utilize and diagnose patients with PCOS. There needs to be a statistical difference that shows that women with PCOS have increased expression of these miRNAs. Even prior to that, a more elucidated mechanism needs to be proposed to better understand how these miRNAs contribute to the abnormal folliculogenesis that is seen in patients with PCOS.

miR-93:

Based on a multitude of studies that have been demonstrated, miRNAs have been discovered that are not limited to target one mRNA (Liu et al., 2018). As a result, one can deduce that a miRNA does have the potential to cause multiple diseases. On this basis, Jiang et al. (2015) studied miR-93, which has been implicated in ovarian cancer, but also in steroidogenesis. Based on PCOS diagnostic criteria hyperandrogenism is a symptom, so it makes sense to see if miR-93 might be involved in contributing to ovarian abnormality and trying to determine the mechanism of increasing granulosa cells proliferation.

Jiang et al. (2015) have chosen to study multiple miRNAs using similar techniques described in previous experiments. Jiang and colleagues (2015) were able to see a statistically higher level of miR-93 expressed among women with PCOS compared to control. This was further compared to the target gene predicted by bioinformatics which yielded the same result. Jiang et al. (2015) utilized flow cytometry to identify what phase of the cell cycle miR-93 had a potent role on. The results demonstrated that there

was an increased proportion of cells that were in the S-phase. To further support this result, an miR-93 inhibitor was transfected into cells and this illustrated a decreased proportion of cell in the S-phase.

To better explain how miR-93 promotes growth, Jiang and colleagues (2015) utilized starBase2.0 and the most probable target gene was hypothesized to be *CDKN1A*, a cyclin-dependent kinase inhibitor. This gene encodes for a protein that inhibits the activity of cyclin-cyclin-dependent kinase2 or -cyclin-dependent kinase4 complexes, and functions as a regulator of cell cycle progression at G1 (CDKN1A Gene-NCBI, 2020). This gene is controlled by the tumor suppressor protein, p53 (Figure 8). p53 plays an important role in the initiation of the cell cycle arrest pathway to either repair damages or undergo apoptosis. In addition, this protein has a regulatory role in S phase DNA replication and DNA damage repair. p53 is reported to be cleaved by CASP3-like caspases, which leads to activation of cyclin-dependent kinase2, and is important to stimulate apoptosis following caspase activation (Kanapathipillai, 2018).

To observe the effect miR-93 would have on CDKN1A: KGN cells were transfected with miR-93 mimics and the results demonstrated a decrease in CDKN1A levels and therefore stimulated cell proliferation. Whereas, miR-93 inhibitors showed increased levels of CDKN1A and inhibited cell growth (Jiang et al., 2015). Logically, high levels of miR-93 were correlated with low levels of CDKN1A mRNA. This is strong evidence that CDKN1A is a direct target of miR-93. Even though previous research established that miR-93 had played a major role as a tumor promoter implicated

in certain cancers. This study was able to successfully demonstrate that it not only has a role as tumor promoter, but also the potential to be a granulosa cell promoter.

The hope of this research was to provide a new perspective and a possible explanation on how the dysregulation of granulosa cells can contribute to PCOS (Miles et al., 2012). Despite its strong candidacy, miR-93 has not moved forward as a potential biomarker. The reason is two-fold: 1) the sample size in this study was very small (N=24) and that is not enough evidence for reproducibility which is needed in a diagnostic test and 2) like most of the miRNAs that was seen thus far there has not been a well-established mechanism.

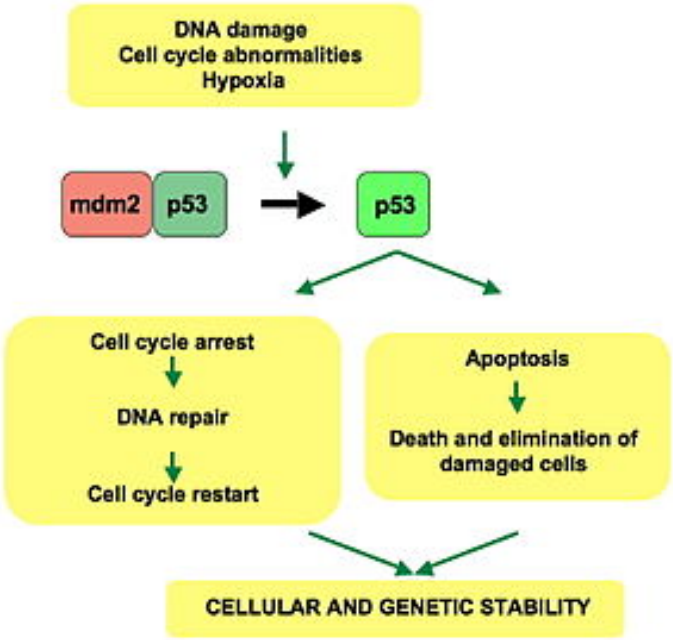

Figure 8. p53 is inactive when it is bound to mouse double minute 2 (mdm2). When there is DNA damage, stress, or shock, there is a pathway that is triggered that leads mdm2 to dissociate from the p53 protein. The p53 protein then becomes activated and will signal the cell cycle to arrest to either allow for repair of the damage or apoptosis of the cell. (Soussi, 2007).

miR-223:

Research validated that GCs are a source of finding plausible miRNAs to utilize for diagnosing PCOS. However, it provides a limited perspective of PCOS because its clinical manifestations are beyond the ovary. PCOS also impacts the body's metabolism. (Bagge et al., 2012). Peng et al. (2014) postulated women with PCOS that are either overweight or obese are at a more increased risk of developing diabetes, insulin resistance, hypertension, oxidative stress, and cardiovascular disease. Rationally, researchers explored the adipose tissue to discover another source of potential miRNAs that can be utilized (Ezeh et al., 2020).

Unlike other studies that chose to look at a few select miRNAs and find a possible mechanism, Qin and colleagues (2019) took on an additional task. They discovered a potential miRNA and hypothesized that Dicer may play a role in adipocytes and obesity. Dicer is an enzyme involved in cleaving the pre-miRNA into short double-stranded RNA fragments. Further research has discovered that Dicer and many miRNAs play a regulatory role in biological processes associated with obesity such as adipocyte differentiation & lipid metabolism. Researchers postulated how obesity has an effect on women with PCOS and that obesity may very well be involved in the etiology of PCOS (Murri et al., 2013). Thus, the reasoning behind to study Dicer as well as miR-223.

Qin et al. (2019) performed experiments utilizing similar techniques as other researchers in the field. The results demonstrated that the expression of Dicer is reduced in those with IR. The results further revealed that the adipocytes in women with PCOS and IR showed decreased levels of Dicer. miR-223 is important because Qin and

colleagues (2019) determined that it plays a vital role in the differentiation of adipocytes. This was illustrated when miR-223 resulted in low expression of genes related to differentiation in adipocytes.

Chuang et al. (2015) studied miR-223 because it has been implicated in many studies as being abnormally expressed especially in PCOS women who have IR (Dou et al., 2013). As of now, research is still being conducted to discover how abnormal expression of miR-223 plays a role in the dysregulation of metabolism among PCOS women and IR. Using techniques such as western blotting and a luciferase reporter assay several observations were noted (Chuang et al., 2015). The most significant observation was women who were diagnosed with IR had higher levels of miR-223 compared to their counterparts. Chuang and colleagues (2015) also discovered that high levels of miR-223 inhibited glucose uptake stimulated by insulin in adipocytes. Understanding how glucose can enter the adipocyte is important and this led researchers to postulate that GLUT4 protein is decreased (Lu et al., 2010). Chuang et al. (2015) were also able to verify this through experimentation. Even though protein levels of GLUT4 were decreased, the mRNA levels were not affected. This allows us to conjecture that miR-223 may regulate GLUT4 expression by binding to the 3'-UTR of the mRNA. Ultimately, this research pointed out that miR-223 may have a role in explaining IR, but it was irrespective of PCOS status.

Most of the current research agrees that miR-223 is implicated in IR, but the mechanism is still unknown (Y. Zhang et al., 2017). This may be because miRNAs have a multitude of targets they can act on. There needs to be strong evidence that miRNA-223

does play a role in the etiology of PCOS and this is done by establishing a mechanism and increasing the sample size of the studies. Hitherto, studies at most had 30 subjects and by increasing the sample size, the results that come out of the study are statistically more powerful. This leads us a step closer in trying to figure out the complex way the PCOS affects women and potentially not only discovering biomarkers but also a therapeutic agent.

miR-25 and MCM7:

As noted earlier, 60-70% of women with PCOS have been diagnosed with IR and puts them at risk for acquiring other diseases such as type 2 diabetes and cardiovascular disease (Ezeh et al., 2020). Research has not found a clear mechanism to explain how IR is related to PCOS. As a result, studies have been published that postulates adipose tissue is an area to look for potential dysregulation (Delitala et al., 2017). Scientists have pointed out that PCOS women have problems with glucose transport, GLUT4 production, and lipolysis (Lu et al., 2010).

Previously miR-93 was thought to affect granulosa cells proliferation. However, Wu et al. (2014) demonstrated that high levels of miR-93 directly inhibit GLUT4 expression. This study observed how miR-93 affects its target gene, *maintenance-deficient 7* (*MCM7*), and other miRNAs like miR-25 to understand how regulation works in adipocytes. Some observations that were deduced are intriguing and may provide some perspective. miR-25 was expressed at high levels in subjects with IR irrespective of

PCOS (Wu et al., 2014). Even though researchers found that miR-25 levels were higher in adipocytes in subjects with PCOS and IR it was not statistically significant.

Now we turn towards the other target: *MCM7* gene. The protein is synthesized via transcription and translation contributes to the initiation of DNA replication (Cao et al., 2015). Logically, if there are high levels of miR-93, then there would be low levels of MCM7. Wu et al. (2014) discovered a similar result: a negative correlation was shown between MCM7 and miR-93. MCM7 levels were lower in adipocytes in women with PCOS. This suggests that miR-25 and MCM7 are regulated through different regulatory pathways. This may be due to a couple of reasons: the stability of the transcript or further miRNA post-transcriptional regulation (Miles et al., 2012). Due to these inconsistencies, there is no strong enough evidence provided that would indicate miR-25 plays a regulation in the development of IR.

Unfortunately, miR-25 does not have much research published that suggests it would be a viable biomarker in PCOS. Further studies performed to examine the role of miR-25 and how it regulates IR in the etiology of PCOS. Wu et al. (2014) presented an interesting opportunity to study miR-25, even though their research indicated miR-25 did not reflect the same expression levels of MCM7. What if the attention was turned towards identifying plausible RNA binding proteins (RBP)?

RBPs are proteins that bind to double or single-stranded RNA and form highly intricate complexes (Hentze et al., 2018). RBPs play an important in post-transcriptional processing which can affect mRNA transcription, transport, translation, and degradation. In addition, RBPs aid in determining the function of miRNAs. Future studies should be

focused towards studying the targeted miRNAs along with trying to identify their associated RBPs to gain a better understanding of how miRNAs can regulate certain mRNAs (Hentze et al., 2018). The challenge I surmise is a large number of these RBPs have not been categorized and researchers might have to characterize these newly discovered RBPs.

miR-21:

Studies demonstrated that the metabolic component of PCOS plays an influential role in increasing the risk of developing diabetes, metabolic syndrome, and IR (Carletti et al., 2010). Researchers theorized a pathway that is continually being fed where excess levels of androgens favor visceral fat which stimulates ovarian and adrenal cells via autocrine, paracrine, or endocrine signaling to induce hyperinsulinemia (Lerchbaum et al., 2014). Evidence has strongly indicated that we start targeting obesity, visceral adiposity, and IR to reduce the signs and symptoms of PCOS (Peng et al., 2014). Therefore, developing a better understanding of the mechanism that lead to PCOS can aid in identifying novel diagnostic targets.

miR-21 has been associated with hormone metabolism, adipogenesis, and insulin signaling(Jiang et al., 2015). Murri et al. (2013) performed a series of experiments to determine the biological function and regulatory role that miR-21 might play in PCOS. One of the experiments performed looked at obesity and androgen excess to see which miRNAs were statistically significant. The results indicated there were four miRNAs:

miR-21, miR-27b, miR-103, and miR-155. Then, Murri and colleagues (2013) were able to identify possible target genes for miR-21 utilizing TargetScan, MicroCosm, and Pictar. Based on the target genes that were analyzed miR-21 affects various biological functions like carbohydrate metabolism, lipoprotein synthesis, energy reserve metabolic process, and insulin signaling pathway (Naji et al., 2017). Their results further illustrated that there was a negative correlation between obesity and expression levels of miR-21. Logically, future studies should look at obesity and how it plays a role in giving rise to metabolic dysfunction.

Kim et al. (2009) performed a series of experiments to determine the function of miR-21and arrived at a similar conclusion: obesity is a metabolic disorder that plays a definite role in the metabolic abnormalities observed in PCOS. Furthermore, cell communication is a regulated process and it has been reported to be altered in obesity and PCOS. Other studies concurred that the communication between inflammatory and metabolic cells contributes to metabolic dysfunction (Cirillo et al., 2019) (Dou et al., 2013). To better understand obesity, the researchers examined adipocyte differentiation with human-derived adipose-derived stem cells (hASCs) to see whether these cells are regulated by miR-21 and if they can give rise to metabolic diseases. After performing PCR analysis and a northern blot analysis with a miR-21 probe the results demonstrated increased levels of miR-21 expression. Employing TargetScan Kim et al. (2009) were able to identify on the target gene, TGFBR2.

These results were confirmed by Naji et al. (2017) and they performed a study to see whether TGFBR2 downregulated TGF-β and examined the overall effect it had in

adipocytes. The results demonstrated low levels of SMAD3 which led to increased adipocyte differentiation and is strong evidence that SMAD3 plays a role in adipocyte differentiation. This novel correlation of SMAD3 along with previous studies illustrating the role of TGFBR2 is strong evidence that the TGF-β signaling pathway modulates adipocyte differentiation (Qin et al., 2001). As a result, this provides a potential mechanism in understanding obesity and how these results can be extrapolated to pinpoint the etiology of PCOS.

Obesity is one aspect to better understand the metabolic component of PCOS. Since obesity is controlled by various genes this leads to a variety of symptoms in women with PCOS (Wang et al., 2020). This can lead to many etiological mechanisms to manifest and sometimes inconsistencies in the data. Prior to deeming miR-21 a credible biomarker more studies need to be performed to better elucidate what mechanisms in PCOS are affected. Thus far, research has focused mainly on the TGF-β pathway, but I believe to gain a better-understanding the MAPK signaling pathways and the insulin signaling pathways should be looked into as well.

miR-320:

PCOS is a complex syndrome that presents with abnormal follicular development and growth (Eisenberg et al., 2017). As a result, this ends up affecting the fertility of women, because their bodies fail to release eggs properly and on time. Scientists focused on looking at cells that directly impact these processes such as GCs and theca cells. This

led researchers to discover potential miRNAs as biomarkers but also paved the way to look outside the cells and see that the environment of the oocytes plays an equally important role. The follicular fluid is an important microenvironment because depending on the types of proteins, lipids, and metabolites that are present, it provides vital information about the state and quality of the oocyte (Martinez et al., 2018). Researchers have identified that some of these proteins/metabolites have been associated with reproductive disorders.

Other research has established that miRNAs have been found in areas of the ovarian tissues, Sang et al. (2013) hypothesized that relevant miRNAs might be present in follicular fluid and aid in explaining the pathophysiology of PCOS. Sang and colleagues (2013) used follicular fluid and performed techniques such as electron microscopy, RNA isolation, and miRNA array and profiling to grow and transfect cells to identify possible miRNAs. Sang et al. (2013) found many miRNAs but one stood out: miR-320. When they compared expressions of the various miRNAs between PCOS women and their controls, there was a statistical significance for both miR-132 and miR-320. Both miR-132 and miR-320 demonstrated to be lower in subjects with PCOS. By transfecting KGN cells, Sang et al. (2013) determined that miR-320 played a part in the regulation of hormonal secretion, specifically estradiol secretion.

Many studies were published to further aid in identifying a possible mechanism of how miR-320 affects PCOS. miR-320 has widespread biological effects as it regulates many molecules, but of particular interest is endothelin (ET-1) (Zhang et al., 2017). ET-1

is a protein produced by endothelial cells and is involved in cell mitosis (Vignon-Zellweger et al., 2012). Rashad et al. (2019) investigated the relationship present among PCOS women by comparing expression levels of miR-320 and ET-1. The results illustrated among PCOS subjects that also had IR, miR-320 was expressed at low levels, whereas ET-1 was expressed at high levels. In addition, PCOS subjects with IR also showed high levels of fasting serum insulin (FSI) and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) levels. These results were largely concordant with other studies published studying the expression levels of miR-320 (Sørensen et al., 2016). Emerging evidence postulates that miR-320 acts through its target ET-1 which inhibits IR in PCOS women via IRS-1, insulin receptor substrate 1. The protein, IRS-1 regulates the Erk 1/2 signaling pathway that ultimately plays a role in follicular growth and maturity (Figure 9).

In conclusion, there is strong evidence supporting that miR-320 has the potential to be a plausible biomarker for PCOS. There is still uncertainty with regards to the mechanism and I agree that there need to be more experiments directed towards how miR-320 plays a role in PCOS. However, these studies alone are not enough and further analysis is needed to deduce whether miR-320 is a credible biomarker. Future studies that focus on evaluating the prognostic, diagnostic, and therapeutic significance need to be performed in a larger sample size (Rupaimoole et al., 2011).

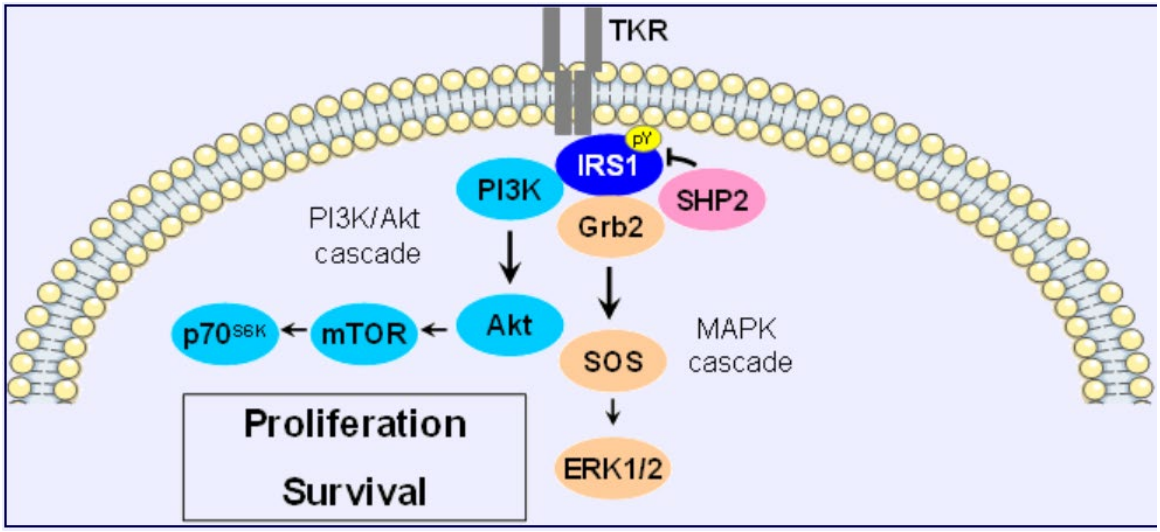

Figure 9. Diagram showing the IRS1 signaling pathway. When the tyrosine kinase receptors (TKR) are activated this attracts IRS1 to bind and there are other signaling molecules and effectors called to aid in important biological processes such as proliferation and cell survival. Akt = Protein Kinase B; Erk1/2 = Extracellular Signal-Regulated Kinase 1/2; Grb2 = Growth factor receptor-bound protein 2; MAPK = mitogen-activated protein kinase; mTOR = mammalian target of rapamycin; PI3K = Phosphoinositide 3-kinase; p70S6K = ribosomal protein S6 kinase; SHP2 = Src homology 2-containing phosphotyrosine phosphatase; SOS = Son of Sevenless. (Machado-Neto and Traina, 2013).

miR-let-7b:

As indicated earlier, experiments were performed comparing PCOS women and controls in order to identify good biomarkers. Results illustrated different expressions of a variety of miRNAs in follicular fluid among women with PCOS. Thus, representing a promising approach to identify new biomarkers. It is worth reiterating that the follicular fluid is not just an environment that provides support for the oocytes to grow and mature, but also serves as a predictive factor of the quality of the oocyte (Scalici et al., 2016).

However, just like the GCs, adipocytes, and other ovarian tissues the miRNAs that have been found and explored are complex. It is hard to pinpoint one mechanism and explain how they regulate pathways which ultimately lead to PCOS.

Another potential miRNA that researchers have come across is miR-let-7b. Scalici et al. (2016) compared the expression of miRNAs in follicular fluid with women with PCOS and controls. Three miRNAs were shown to be statistically significant: miR-140, miR-let-7b, & miR-30a. These results were further analyzed via a multivariate analysis. This study highlighted the expression of miR-140 and miR-let-7b were lower compared to miR-30a which was higher. The three miRNAs demonstrated high sensitivity and specificity which is an essential first step to be considered a potential biomarker.

Further studies were performed surrounding the expression and function of miR-let-7b that effects GCs and cumulus cells. As previously stated, Scalici et al. (2016) found lowered levels of miR-let-7b in PCOS women, and other researchers have postulated that this may lead to the abnormal folliculogenesis that is seen in PCOS. Cao et al. (2015) showed that miR-let-7b could play a role in follicular development via regulation of TNF-β, also known as tumor necrosis factor-beta. TNF-β is a protein that arises from the lymphotoxin alpha (LTA) gene and has a variety of important roles from providing immunity to cell survival, proliferation, and differentiation (Bauer et al., 2012).

According to a study performed by Zhang et al. (2017) miR-let-7b can potentially regulate TGF- β via Activin Receptor-1 and Smad 2/3. SMAD proteins act as modulators that are involved in many signaling pathways. TGF-β and its receptor recruit SMAD and

activate the SMAD anchor for receptor activation (SARA) protein. In response, this protein is phosphorylated by the TGF-βreceptors (Kim et al., 2009). The phosphorylation stimulates the dissociation of SMAD and SARA. Thereafter, exposing the nuclear import sequence which allows the SMAD to enter the nucleus and bind to a target gene along with other proteins (Qin et al., 2001). So far, it seems from the variety of published studies that miR-let-7b affects cell survival and proliferation, but there is no consensus as to which pathway.

Another study demonstrated by Miles et al. (2012) observed miR-let-7b and how it functions by suppressing Myc. Myc is a proto-oncogene that encodes an important protein involved in stimulating cell proliferation and can promote cell cycle progression (Genetics Home Reference, 2017). Studies have illustrated that miR-let-7b has many different targets and is involved in multiple pathways. This led researchers to conjecture that miR-let-7b contributes to the reproductive abnormalities that are seen among subjects with PCOS. Based on this, miR-let-7b shows promise as a potential biomarker, however, its role and regulation in the follicular fluid should be further investigated.

miR-29a:

Up to this point, the selected miRNAs have been focused on either the endocrine or metabolic aspect of PCOS (Villa & Pratley, 2011). This selection makes sense in light of the result of the heavy involvement of hormones like testosterone and estrogen which contribute to irregular follicle development as well as other symptoms like hirsutism (Xue et al., 2018). Also, IR is a common symptom of PCOS. Many studies were directed

towards adipocytes and their differentiation to explain what may lead to IR (Rocha et al., 2019). Logically, this intrigued researchers to look for possible miRNAs involved in these processes. However, thinking long-term, patients want a diagnosis without many invasive procedures involved. For example, a blood test would be ideal in trying to minimize invasiveness. So identifying miRNAs in the serum that might aid in explaining the pathophysiology of PCOS (Arancio et al., 2018).

Of interest to many researchers is the family of miR-29a. This is because it has been strongly associated with being in the serum and therefore easier to measure levels of this miRNA compared to the ovarian tissues and adipocytes (Bagge et al., 2012). Sorenson et al. (2016) designed a study with 49 women with PCOS: 19 expressed hyperandrogenism, whereas 30 had normal levels of androgens, and 21 women who did not have PCOS. Afterwards, miRNA levels were estimated using PCR. The results showed that among all PCOS women there was a statistically significant decrease in miR-29a. Other miRNAs found were: miR-24, miR-151, & miR-574. All of the listed miRNAs via TargetScan are involved in the regulation of cell proliferation and biosynthetic processes as well as negative transcription. With respect to miR-29a, a certain form, miR-29a-3p, effected androgen receptor transcript levels and protein kinase B (AKT2) levels.

AKT along with phosphatidylinositol 3-kinase (PI3K) is involved in a signaling pathway that promotes cell survival and growth (Martini et al., 2014). This pathway is highly regulated and can communicate with other signaling pathways. One of the many ways this pathway can be activated is via insulin or IRS-1. There is a negative feedback

loop where S6K1 phosphorylates IRS-1 and prevents it from binding to receptor tyrosine kinases (RTK). AKT is located in the cytosol and is inactive until phosphorylation occurs by multiple proteins such as: phosphoinositide-dependent kinase 2 and integrin-linked kinase among others (Martini et al., 2014). Problems with this pathway has been linked to many diseases of interest to the PCOS researchers like type II diabetes as well as IR (Nandi et al., 2014). These are the same results that Sorenson et al. (2016) came across. In addition, they saw an impaired glucose-stimulated insulin secretion of β cells. As mentioned previously, IR & type II diabetes are closely linked to the dysfunctional features of PCOS.

These results were further supported and confirmed by Arancio et al. (2018). Even though their results concluded that miR-155 might be a better biomarker than miR-29a, the target genes and pathways that miR-29a effects were similar. These researchers witnessed a negative correlation between androstenedione concentrations and miR-29a. Research demonstrated that women with high levels of androstenedione had an adverse metabolic phenotype (Lerchbaum et al., 2014). As discussed above, Sorenson et al. (2016) also saw similar results, but with androgen receptors, however if we take both results we can extrapolate that miR-29 can play a role in leading to hyperandrogenemia among women with PCOS.

The evidence gathered so far touches upon the metabolic component and illustrates that PCOS is not only endocrinological in nature but also metabolic as well. miR-29a is a strong contender for being a potential biomarker for PCOS. It has been associated with IR and type II diabetes that PCOS women are at a higher risk of

developing. It is worth reiterating that to deem a biomarker credible there needs to be more concrete evidence establishing a mechanism prior to moving onto a larger sample size. Further experimentation needs to be performed about which forms of miR-29a affect which part of the androgen and insulin pathway, because research has focused on one.

miRNA-155:

Altering levels of miRNAs among women with PCOS have been associated with diabetes, IR, and inflammation (Dou et al., 2013). The pathway related to IR in terms of PCOS is not fully known and studies are still being conducted to figure it out. As mentioned previously, miR-155 was also a contender as a potential biomarker (Sirotkin et al., 2010). This miRNA was chosen because studies have shown it has a role in regulating insulin and is involved in chronic inflammation. Cirillo et al. (2019) studied miR-155 among other selected miRNAs to see if there is a possible correlation between insulin, IL-6, and 17-beta estradiol.

Upon collection of samples, techniques were performed to filter out the selected miRNAs. The preliminary analysis measured levels of the selected miRNAs that were chosen to study, Cirillo and colleagues (2019) illustrated that miR-155 were significantly upregulated in both GCs and follicular fluid in subjects with PCOS. Also, miR-155 is correlated with HMGB1 and insulin concentrations. A target gene analysis was performed and some notable genes that miR-155 was associated with were: KRAS, PAK2, NFKB1, and FOXO1. *FOXO1* is of particular interest because previous studies have established that it is an important transcription factor that plays a role in regulating

gluconeogenesis and glycogenesis via insulin and promotes adipocyte differentiation (Nakae et al., 2003). This study was able to illustrate that insulin sensitivity within the ovary is a major factor when investigating PCOS.

Previous literature explored the possible roles of miR-155 in cancer cell proliferation, invasion, and metastasis (Liu et al., 2018). However, increasing studies are exploring the potential role that miR-155 plays in the pathophysiology of PCOS such as Xia et al. (2020). Xia and colleagues (2020) designed an experiment to study the effects and regulatory mechanisms by which miR-155 acts. Utilizing similar techniques to other researchers in the field, the results demonstrated that there was a significantly higher level of expression of miR-155 in PCOS women compared to control. Levels of cyclin D1, p53, and p21 were measured along with levels of miR-155 to see whether or not they were correlated. Results showed levels of cyclin D1 were increased, whereas p53 and p21 were reduced. As a result, this promotes cell cycle proliferation. This hypothesis is further supported when cells were transfected with an miR-155 inhibitor, Xia et a. (2020) saw apoptosis rates had increased. Once this was established, it made sense to study PI3K/AKT and JNK signaling pathway to see if *programmed cell death protein4*, *PDCD4*, had an effect on this pathway (PDCD4 Gene - NCBI, 2020). The findings resulted in the following conclusion: PDCD4 was a target of miR155 and down-regulated by miR-155 in the process of PCOS and this supports increased cell proliferation.

In conclusion, miR-155 is a promising biomarker that needs more research elucidating the cell proliferation and insulin sensitivity pathways. It is worth noting that

the experiments performed thus far have shown strong evidence, but future studies need to implement a larger sample size.

Table 2. Summary of miRNAs discussed with expression levels, where they are located, and possible target genes.

miRNA	Level of Expression	Location	Target Gene(s)
miR-145	Down-regulated	GC	IRS-1
mirR-200b & miR-200c	Up-regulated	GC	PTEN
miR-93	Up-regulated	GC, Follicular Fluid	CDKN1A
miR-223	Up-regulated	Adipocyte	GLUT4
miR-25	Up-regulated	Adipocyte	MCM7
miR-21	Up-regulated	Serum, Adipocyte	TGFBR2
miR-29a	Down-regulated	Serum	AKT
miR-155	Up-regulated	Serum, GC	FOXO1, PDCD4
miR-320	Down-regulated	Follicular Fluid	ET-1
miR-let-7b	Down-regulated	Follicular Fluid	TNF-β, Myc

DISCUSSION

There are several proposed biomarkers for polycystic ovarian syndrome, many of them require further elucidation of the mechanisms that might contribute to the pathophysiology of the disease (Ma et al., 2007). Throughout this paper, I have explored ten miRNAs as listed and summarized in Table 2. Through this review of current PCOS literature, I have been able to come to several conclusions regarding which miRNA offers the most promise based on the results of these studies. In this section, I will discuss the results of the research described previously and then hope to provide some novel conclusions.

Granulosa cells within the ovary have been the focus of much research as scientists works to understand pathophysiology of PCOS. Advancements in understanding what role the GCs play in the hormonal pathway have allowed for the successful identification of plausible miRNAs that might regulate this pathway and contribute to PCOS (Sirotkin et al., 2009). By determining where the miRNA is able to act upon scientists are one step closer in identifying a promising biomarker as well as a therapeutic agent. Human trials are currently in the process of discovering new miRNAs as well as demonstrating that these miRNAs are not just restricted to one pathway but can have an effect on multiple pathways (Wissing et al., 2014). So far, the reports have described multiple genes that a miRNA can regulate. Almost every miRNA discovered in GCs I have reviewed not only affects ERα and ERβ receptors, but also the metabolic aspect of PCOS (Jakimiuk et al., 2002). These results displayed establish the fact that

PCOS is most definitely an endocrine and metabolic disorder and discovering a biomarker that regulates both aspects would be a good start.

There are still improvements that must be made to the type of studies being conducted to demonstrate a plausible miRNA before it is considered a good biomarker. Rupaimoole et al. (2011) reported that one of the key aspects of identifying miRNAs is that they have a long half-life so the levels are accurately represented. The researchers provide several solutions like chemically modifying the miRNAs, but this falls short because this may lead to off-target effects and degradation can lead to toxic by-products being made. These are areas that need to be further perfected to optimize and produce viable biomarkers. Additionally, more research needs to be targeted at not only elucidating mechanisms by which miRNAs regulate pathways, but also looking at larger sample sizes. It is clear after viewing the results of these experiments that miRNAs such as miR-93 show more promise compared to miR-145, miR-200b or miR-200c. There also needs to be consistent results showing that levels of miR-93 are up-regulated among women with PCOS, because some research that has been published is contradictory and it becomes difficult to establish a baseline (Naji et al., 2017). Overall, GCs offer great promise in finding potential biomarkers for PCOS.

Adipocytes and its tissues, like GCs, produced and resulted in the discovery of more miRNAs that can function as future biomarkers for PCOS. There is still a great deal of research that needs to be completed before these experimental approaches become clinically applicable. As research has demonstrated PCOS patients are more at risk of developing type II diabetes and IR. However, a problem with this is that scientists are

unsure of the pathways that lead to impaired insulin signaling and insulin resistance (Wang et al., 2019). Many researchers like Samuel and Shulman (2012) know it is not one single pathway but a common pathway that acts diversely depending on the type of tissue. Another issue that needs to be examined is that much of the research has linked obesity as being a clinical manifestation of PCOS, however, there are also non-obese patients with PCOS, and when trying to identify miRNAs in adipocytes, the miRNAs selected still need to show a statistically significant result in both groups. A concern is that these miRNAs still need to be valid among patients who are non-obese as well. Research focused on IR and insulin signaling pathways as well as adipocyte differentiation among subjects with PCOS *versus* those who did not and realized that different expression of miRNAs was seen amongst both groups (Delitala et al., 2017). Scientists found miR-21 to regulate differentiation in adipocytes via manipulation of TGF-β pathway. A possible avenue to find a reliable biomarker might also be to observe the differentiation process to see if that can partially explain PCOS.

Still, even in light of all these problems, the current research is promising in that the miRNAs that were identified offer a great deal of promise for the future, in not only trying to diagnose PCOS, but for shedding light on IR and insulin signaling pathway as well. Many of the researchers used a TargetScan to try and associate the miRNAs of interest with potential genes that they affect. For example, miR-223 was associated with the gene GLUT4 which research has established is critical for allowing glucose to be taken in by adipocytes (Chuang et al., 2015). The results showed that the protein levels of GLUT4 were decreased because miR-223 was bound to the 3'-UTR of the GLUT4

mRNA and prevented GLUT4 from being translated among patients with PCOS (Chuang et al., 2015). However, as mentioned previously there needs to be more studies conducted to ensure that this is a reliable measurement across all patients diagnosed with this disease. The results are far from conclusive and there are still improvements that need to be made before miR-223 can be used to diagnose patients with PCOS.

Much research has been dedicated to follicular fluid. This intrigued me the most and I found quite informative that miRNAs were in the follicular fluid. Many researchers have dedicated a great deal of time and effort to explore the follicular fluid and inform the scientific community that it is more than just an area where metabolites and nucleic acids are stored, but also provides pertinent information like the quality and growth of the ovary (Butler et al., 2019). Although they have produced promising results, for the most part, just demonstrated the selected miRNAs are involved with major growth and development pathways such as MAPK, PI3K-AKT, and TGF-β. Many of the current researchers now hypothesize that cell proliferation and growth play an influential part in explaining the etiology of PCOS (Song et al., 2015). This is because research based on the selected miRNAs show that many of these target genes that were associated were with these pathways. Using this knowledge in combination with what we already know about PCOS might lead us in further trying to identify prospective miRNAs. Coincidentally, there were numerous studies that studied the GCs and cumulus cells proliferation and apoptosis rates by utilizing selected miRNAs. The most notable miRNA that researchers came across in the follicular fluid is miR-let-7b, because not only is it associated with TNF-β but also has been associated with a proto-oncogene Myc which

can also aid in informing researchers not only about PCOS but also lymphomas (Cao et al., 2015).

The serum is another a location where possible miRNAs were found, but more importantly it is worth noting that it is the most readily accessible area in term of human testing. Considering that many diagnostic and lab tests look out for patient's preferences to be minimally invasive, this would be a favorable area of research for PCOS. Unfortunately, with the research that is currently available there were few miRNAs that are considered promising (Arancio et al., 2018). Much of the research regarding PCOS and possible miRNAs have been studied in ovarian tissues and its surroundings as well as adipocyte tissues. One of the more prominent miRNAs that were discovered was miR-21. It has received attention due to the fact that it has been linked in a variety of cancers, but also has recently received attention because it has been implicated in PCOS. Various studies have supported that miR-21 has been associated with TGFBR2 and it is involved in once again cellular growth and survival (Carletti et al., 2010). Further studies need to be done in this area of PCOS and provide valuable information, however, there are small amount of miRNAs in a given blood volume and this is an important perspective to consider when moving forward.

After careful analysis of the current literature, it appears that GCs, adipocytes, and the follicular fluid hold the most promise in trying to identify miRNAs as biomarkers for PCOS in the years to come. Not only do these areas have a multitude of selected miRNAs that were studied, but they also tried to explain the underlying etiology of PCOS by looking into the endocrine and metabolic aspects. Aside from having multiple gene

targets for many of these miRNAs, all the gene targets that were found seem to be clustered under one of three groups: production/synthesis of hormones, IR and insulin signaling, and cell proliferation and survival. As for the serum, this compartment might be able to give us insight but until better qualitative and quantification methods are introduced it might be considered an alternative place to look. As much promise as the miRNAs hold for the future, it will take a while before human subjects are recruited to diagnose PCOS based on biomarkers (Y. Zhang et al., 2017). A common theme across studies performed to find miRNAs associated with PCOS is that they were preliminary studies: in trying to figure out a well explained mechanism and the sample size of the studies was not enough to move onto the next phase.

It is my personal hope, as well as thousands of people afflicted with this horrible syndrome that more progress is made in the years to come. Because science has seen the potential of miRNAs, especially in the field of oncology, it is possible that it will not be too long until miRNAs are found within the realm of PCOS. All research needs is one breakthrough which will allow an accurate diagnosis of PCOS and simultaneously will also provide a permanent treatment as well. By taking a hard look at the current literature and identifying the most promising miRNAs, we can allocate resources and efforts to finding a proper biomarker.

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