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Biomarker Development for Early Diagnosis of PCOS

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ABSTRACT

Polycystic Ovary Syndrome, most commonly known as PCOS, is a condition that affects at least 6 million women of reproductive age in the United States alone. Approximately 10% of women worldwide are affected by PCOS as it is the number one cause of female infertility. Diagnosis of PCOS is not a process that has been fully coagulated as many of the symptoms overlap with many other conditions. Biomarker development to diagnose PCOS during the earlier stages is critical to making advancements in the field as earlier detection means more time to treat the symptoms and possibly find a treatment.

Introduction

Polycystic Ovary Syndrome (PCOS) is a hormonal and reproductive condition in which ovaries become enlarged due to an excessive amount of androgen production, which leads to the formation of cysts that induces swelling in the organ. The excessive presence of androgen was coined hyperandrogenism and has become one of the key signs for diagnosis. There is no clear, set way to diagnose PCOS so the Rotterdam diagnostic criteria, which was established in 2018, was put into use, which was truly a paradigm shift for diagnosis. The criteria (which is known as polycystic ovary morphology or PCOM) states that in order to be diagnosed with PCOS, the patient must exhibit at least two of the following comorbidities: clinical/ biochemical androgen excess, oligo-ovulation or anovulation, and polycystic ovarian morphology (PCOM.). Some other symptoms of PCOS include abnormal and irregular menstruation and ovulation, fertility issues, hirsutism (excessive hair growth,) weight gain, severe acne, and androgenic alopecia. Some complications include endometrial cancer, Type 2 or gestational diabetes, increased risk of miscarriages and cardiovascular, sleep apnea and metabolic syndrome. Not only does this condition affect the women who have it, but it also sets up an unfavorable environment in the womb if the woman manages to get pregnant. After birth, the child will also have a greater risk of possessing the same health risks as the mother, especially if the child is female. Approximately 70% of women whose mothers had PCOS also end up getting diagnosed with the condition. PCOS is a struggle to diagnose because a single diagnostic test isn't available to use due to the fact that the multifaceted nature and overlapping symptoms could lead to misdiagnosis. Not much is known about the molecular setup and pathways underlying the disease as well as genes that directly affect this. The gene hereditary pattern isn't very clear but studies have found that in mice, PCOS and its symptoms can be passed down to three generations.The pathophysiology of this disease has multiple layers as it is known as a "polygenic and multifactorial syndromic disorder," which means that there are different traits from a multitude of genes.

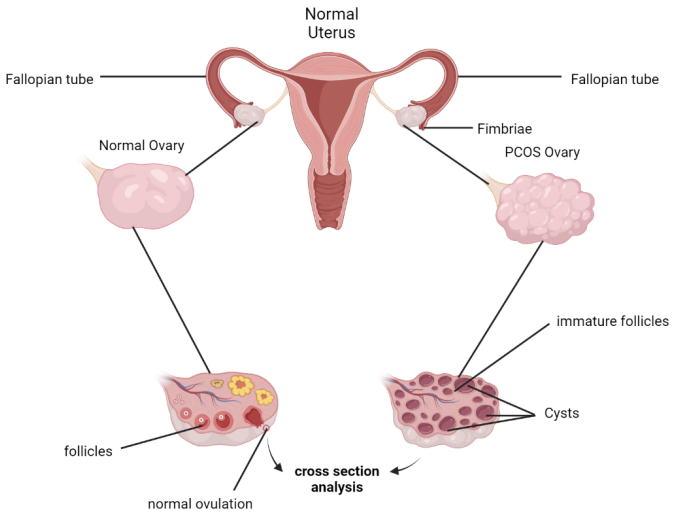


Figure 1. Diagram of Uterus with cross section analysis of both a normal human ovary and an ovary displaying the effects of polycystic ovary disorder. All figures created by Joshitha Senthil on Biorender.com

Biomarkers are a measurable sign that indicate that the body is experiencing abnormalities. Utilized by doctors, epidemiologists, and scientists alike, It is an indicator that can be objectively analyzed to determine whether the body's biological, pathological and pharmacological processes are running unerringly. Biomarkers are classified as diagnostic, monitoring, pharmacodynamic/response, predictive, prognostic, safety, and susceptibility/risk biomarkers. For PCOS specifically, diagnostic biomarkers are utilized to discover and determine the presence of PCOS in the body. Biomarkers can range from results from clinical examinations conducted by medical professionals to molecules such as proteins, hormones or DNA. To detect a biomarker and properly analyze it, the proteins must be extracted and separated, identified and verified.

Table 1. Graphic displaying the criteria set by the NIH, Rotterdam criteria, and PCOS society guidelines that medical professionals must adhere to in order to diagnose PCOS.

	NIH	Rotterdam	PCOS Society
Clinical/ biochemical androgen excess	X	X	X
Oligo-ovulation or anovulation	X	X	X
Polycystic ovarian morphology (PCOM)		X	X
		must have 2 out of 3	must have red and either green or blue

What are Biomarkers?

The concept of biomarkers was established in the 1950s and was classified into three different groups: exposure, effect and susceptibility markers. Exposure biomarkers are correlated to the degree of exposure that the specimen has experienced with a certain chemical or foreign substance. Biomarkers of exposure are usually the products of metabolism which means they can be measured in the urine, blood, hair or saliva content of a specimen. The amount and level of a specific biomarker is an indication of the extent to which a person was exposed to a chemical. Conversely, biomarkers of effect connote the physiological, chemical or biological changes displayed in the organism that could potentially serve as indicators for a particular condition or disease. A susceptibility biomarker displays an individual's sensitivity to developing a certain condition without chemical exposure. It shows how an individual is likely to contract a certain disease even when it isn't present in any way in their body currently. The chief objective of biomarker development for any disease is utilizing all three types to understand and detect maladies to effectively combat them as early as possible.

Biomarkers are also commonly classified based on their physical characteristics. Molecular biomarkers are divided into subcategories— chemical, protein, DNA and karyotypic— based on their biophysical makeup which includes peptides, proteins, lipids, metabolites and nucleic acid. The molecular specimens are found in bodily fluids and excretion such as cerebrospinal fluid, plasma, bronchoalveolar lavage fluid, etc. Protein biomarkers convey the risk of disease and early treatment by offering a reliable prognosis, as well as the different biological stages of the condition progressing in the body. Proteins are known to be easier to measure than other substances due to it only needing non-invasive methods to collect and study. DNA biomarkers identify genetic predispositions and inbuilt signs in the genetic code that could point towards developing a certain abnormality. The ability to detect polymorphisms in the system could prove to be advantageous to find the root of the problem, which makes it easier to find treatment. Karyotypic biomarkers assess chromosome irregularities to detect genetic disorders and to specifically detect signs of cancer.

To detect biomarkers, there are different methods that are used such as western blotting, enzyme linked immunosorbent assay (ELISA,) immunodiffusion, polymerase chain reaction (PCR), flow cytometry, etc. Western blotting is a laboratory technique that is used to separate and identify proteins. This is executed by separating a protein mixture based on molecular weight through gel electrophoresis (a process where DNA and RNA strands are separated based on molecular weight by being put through a gel by an electrical field.) The results from this are then transferred to a membrane that produces a band for each protein which leads to the membrane being incubated with labels specific to the protein of interest. Enzyme linked immunosorbent assay, most commonly known as ELISA, is an assay (testing a specimen to find its quality) in an immunological nature and is used to gauge the quality of substances such as antibodies, antigens, proteins, glycoproteins, and hormones. A polymerase chain reaction (PCR) is a technique that is used to amplify miniscule sections of DNA. To do this, the sample is heated so the DNA denatures (separates) into two pieces of single-stranded DNA. The next step is an enzyme called "Taq polymerase" synthesizes two new strands of DNA, by using the original strands as templates. Immunodiffusion is a test where a given antigen or antibody moves through a diffusing medium to produce results. Flow cytometry is a lab test that is used to measure the characteristics of cells and particles. This is executed when a sample of cell or particle specimen is suspended in fluid and injected into a flow cytometer machine. It has been proven that 10,000 cells can be analyzed and processed by a computer in less than one minute with this process.

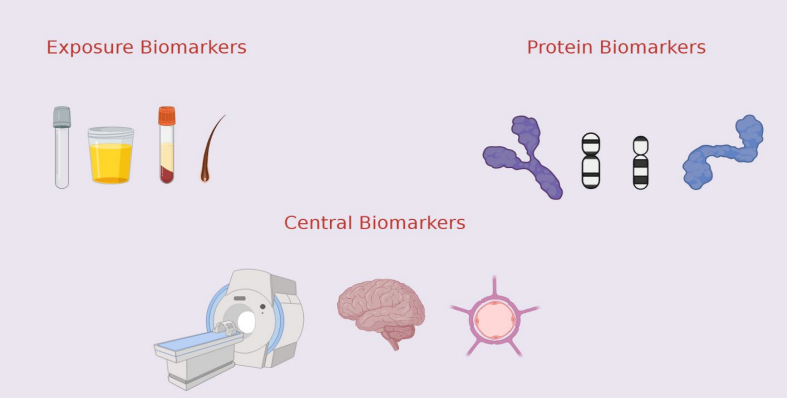


Figure 2. Visual displays of many types of biomarkers used for finding and classifying abnormalities in the body.

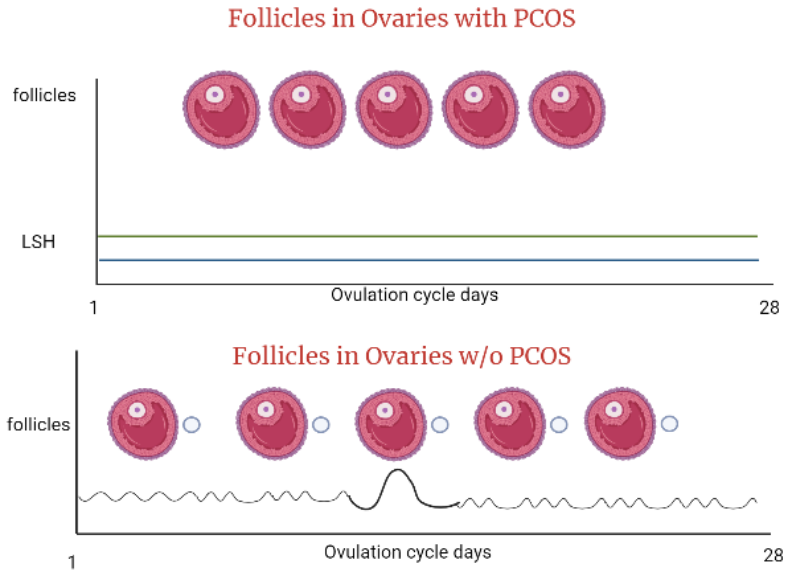
Hormonal Makeup and Effects of PCOS

At its core, PCOS is a hormonal disorder. The excessive androgen production from the ovaries interferes with ovulation and as a result of that, menstrual cycles. The shaken cycles entail that eggs don't develop on a regular cycle and aren't released from the follicles that they grow and develop in. This affects all the other hormones and sets off a domino effect. The hormones that specifically play a part in PCOS include: gonadotropin-releasing hormone (GnRH) (regulator of reproductive axis which mediates transition to reproductive age starting from the pituitary gland,) insulin, the luteinizing/follicle-stimulating hormone (menstrual cycle and eggs,) androgens (hair growth and puberty,) estrogens (female characteristics,) growth hormones (GH), cortisol, parathyroid hormone (PTH) and calcitonin (regulates calcium levels.) These hormones play a big part with theca cells, a group of ovarian cells that surround the follicle and manage folliculogenesis (the production of follicles) by synthesizing all necessary hormones, regulating crosstalk, and providing structural support as the growing follicle moves through all of the developmental stages.

To understand why the damage PCOS has on these systems is so adverse, it is important to know what the normal biological balance of the hormone physiology is. Without PCOS, the adrenal glands and ovaries play an equal part in testosterone production. Half of the testosterone supply comes from the ovaries and adrenal gland directly secreting testosterone that's from the gonads, whereas the other half is produced by conversion of circulating a steroidal hormone called androstenedione, which also can be identified with its equivalent ovarian and adrenal secretion. Due to the abnormal androgen secretion from PCOS, the body's systemic metabolism is affected. Normally, normal amounts of leptin and insulin, sex hormones, and growth hormones all affect our metabolism through appetite and receptivity to food intake, so with normal amounts, the body's metabolic rates can be deemed normal. However, with PCOS, general adiposity increases in tandem with the insulin sensitivity greatly decreasing and this leads to a significantly slower metabolism.

One such adverse effect of hormonal imbalance can be traced back to the follicle stimulating hormone. Inositol is a sugar that balances chemicals in the body and an intracellular second messenger for both the follicle and thyroid stimulating hormone. There are several different types of inositol but the two most relevant forms to PCOS are myo-inositol and chiro-inositol Myo-inositol is a growth factor of mammalian cells and an intracellular second messenger for both the follicle and thyroid stimulating hormone, as well as for insulin. Chiro-inositol is best known for inducing ovulation in women who have difficulty due to cysts in the ovaries.

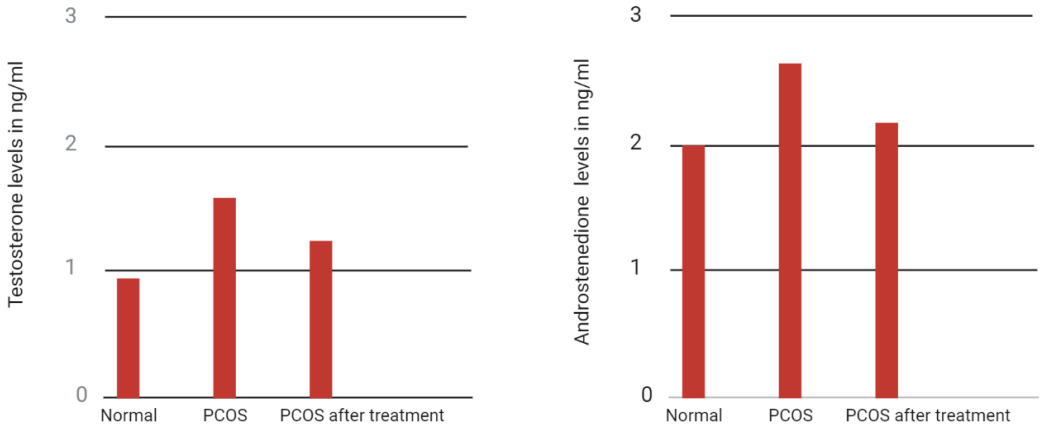
Another hormonal imbalance brings the pathophysiology of PCOS to light. The gonadotropin-releasing hormone (GnRH) is the hormone that signals the pituitary gland to secrete the follicle stimulating and luteinizing hormone. Its pulsatile secretion establishes the pattern of secretion of the hormones, which regulates both the endocrine organs and gamete maturation in the gonads. A lack of a stable process contributes to the ovarian hyperandrogenemia and dysfunction in the body. The levels of GnRH pulse frequency increase by 40% for women with PCOS leading to excessive luteinizing hormone secretion and in turn increased ovarian androgen production. To elaborate, ovarian hyperandrogenism in PCOS is LH dependent; PCOS manifests during or right after the puberty induced increase in LH secretion, and GnRH agonists significantly reduce androgen production in women with PCOS. On the neurological side, GnRH neurons showcase the pathway for the center of reproductive function. High and low frequency GnRH pulses favor LH and FSH production respectively. Ergo, consistently high GnRH pulse frequencies prominently add to the gonadotropin abnormalities. The increase in GnRH pulsatility is stimulated by a lower sensitivity to negative feedback from sex steroids, particularly progesterone and estradiol, which is due to abnormally high androgen levels.



Figures 3 & 4. Graphs demonstrating follicular function and proper ovulation fluctuation in non PCOS bodies versus lack of function in bodies with PCOS.

Metabolic Processes of PCOS

A key element of female reproductive function is Steroidogenesis, which can be defined as the biosynthesis (formation of biological products) of hormones, specifically steroids in tandem with regulation of ovulation. To elucidate this, normally, androgen production is from adrenal and ovarian secretion as well as conversion from precursors that come from peripheral tissues (precursor cells in tissues are stem cells that have evolved to the stage where the process of transforming into a new cell with an advanced purpose.) On the contrary, when it comes to metabolic processes in women with PCOS, studies documented higher androgen production rates for both androstenedione and testosterone in women with PCOS, associated with a less pronounced increase of their metabolic clearance rate. The Follicle stimulating hormone also leads to the proliferation of ovarian cells, which leads to steroidogenesis and hyperandrogenism. Both of the rates were also shown to be varied as body type changed and fluctuated which could point to the fact that factors such as peripheral conversion and possible binding to the sex hormone binding globulin, which attaches itself to testosterone and dihydrotestosterone, could influence testosterone production as well.



Figures 5 & 6. Bar graphs that display the amount of the given hormone in normal, PCOS and PCOS after treatment conditions respectively.

To foreshadow the role of steroidogenesis with PCOS biomarkers, it is necessary to look at the different steroidogenic pathways. As a whole, all the pathways are mainly for converting cholesterol to steroid hormones. In order to do this, a multitude of pathways are necessary to aid and catalyze the reaction to form specific steroids for specific purposes. The purposes focused on here are all reproductive and endocrine in nature so the pathways covered will pertain to that. The production is referred to as biosynthesis, which is producing more natural products through a biological substrate. The biosynthesis of steroid hormones specifically in the ovaries, testes, adrenals, brain, skin and even the placenta are regulated by signals and hormones that are tropic in nature, which mean that class of hormones are actually targeted towards affecting the function of other endocrine glands. When a woman has PCOS, there are disruptions in the synthesis of the cholesterol in the body and their overall metabolism, which is what leads to complications and issues in hormone production, regulation and functions of the body that are driven based on that.

Identification of Various PCOS Biomarkers

The impact of steroidogenesis is clearly displayed as 50% of women with polycystic ovarian morphology (PCOM) have clinical steroidogenic defects. These defects frequently pertain to functional ovarian hyperandrogenism due to dysregulation in the body. The theca cells from the ovaries of the patients account for this as the cells overexpress most steroidogenic enzymes, especially an enzyme called cytochrome P450c17. Cytochrome P450c17 manifests as the key enzyme that regulates androgen synthesis as it is the only enzyme known to have the ability to convert C-21 (C-21 is a steroid that can be converted into another cell or substance) precursors to androgen pre-hormones. In recent years, it has been discovered that the mutation of the normal theca cells reproduced the phenotype in vitro and in vivo. Other variants that have been mapped are allelic variants of the biomarker fibrillin-3 (FBN3) and variants of the luteinizing hormone receptor. Fibrillin is a glycoprotein rich in cysteine and its primary function is the synthesis of microfibril and elastic fibers of the body's connective tissues. These microfibrils are the providers of tensile strength for tissues that are non-elastic and initiate the assembly of tropoelastin, all contributors to PCOS.

FBN3 specifically has been found to encode an extracellular matrix protein, which is what regulates the growth hormone. The expression of this covers the range of early to mid-gestation for the ovary, which is when fetal development is impacted by the growth of this. When monitoring levels of expression, it has been found that the FBN3 marker's levels are consistently the highest in prenatal tissues but are also imperative in

developing microfibrils in skeletal, muscular, lung, skin and kidney formations. Unlike FBN1 and FBN2, FBN3 functions are placed primarily in the brain and alternatively spliced, which removes the exon encoding cbEGF2. Similar to FBN1, FBN3 contains three alternate exons (a DNA or RNA segment that contains the information coding for a protein or peptide sequence.) This also works with a protein called a transforming growth factor (TGF) that is responsible for cellular and physiological growth through cytokine signaling. When the TGFs are stimulated, this triggers fibroblast replication and collagen production, which aligns with behaviors known to be exhibited from the standard PCOS ovarian phenotype. The aforementioned behaviors are an increase in stromal collagen (arranged collagenous tissue) and overall collagen production. What happens in the ovary is key to understanding the different PCOS phenotypes because the many variants of the luteinizing hormone receptor can either increase or decrease stimulation of theca cells, follicle development and ovulation. The presence of FBN3 as well as other phenotypes could determine the chance of the individual getting PCOS in the future.

A8, the marker that is the variant that affects PCOS, is a metabolically distinct phenotype that specializes in insulin resistance. These are multifunctional cytokines because they regulate TGFs through apoptosis, cell proliferation, and differentiation. The problem begins when mutations manifest in A8 which disrupts the regulation of TGF which contributes to the damages to the body's reproductive and cardiometabolic functions. Women with higher A8 levels also had higher DHEA content. DHEA, which is also known as dehydroepiandrosterone, is a sex hormone that comes from the adrenal glands (some are known to come from the ovaries or the testes) involved in the production of testosterone and estrogen. It has been determined that approximately 30% of women with PCOS have demonstrated an excess of APA (adrenal precursor androgen) production, which is determined through the DHEA marker through DHEA synthesis. On the note of sex hormones of an androgenic nature, the body's adrenal cortices are known to produce three primary steroids that manage androgenic activity. These include the aforementioned DHEA or dehydroepiandrosterone, androstenedione (more commonly known as A4,) and testosterone. Looking at the steroidogenesis process will shed a light on how all of these steroids are interconnected. The pathway for androgenic steroidogenesis is through the Δ5 pathway, via pregnenolone and 17-hydroxypregnenolone. This along with the entrance of the C17 side chain is what forms DHEA. To convert this to A4, the DHEA is partially transmitted through the bloodstream and then converted to A4 through the 3β-hydroxysteroid dehydrogenase enzyme, which acts as a catalyst for the process of steroidogenesis.

Alternatively, A4 could also be produced through the Δ4 pathway instead by converting pregnenolone to progesterone with the enzyme 3β-HSD, a catalyst who's specific duty is to catalyze the biosynthesis between the two aforementioned steroids. Then, a conversion to 17α-hydroxyprogesterone takes place via P450c17. It is important to note that the latter process takes place mostly in animals and the former is most commonly seen in humans. To tie testosterone into this, A4 can be converted to testosterone in the liver. This steroidogenesis pathway is known to be incredibly important in women because this is what accounts for 60% of circulating testosterone in their bodies. Not only can these three primary steroids be produced through DHEA, but even more potent androgens can be formed in this way, with DHEA acting as a pre-hormone. This formation could serve to be incredibly important to women during menopause and after as steroid levels are disrupted during this time.This takes place through hepatic (liver) and peripheral conversion to A4. Hepatic conversion specifically has been demonstrated in vitro in hepatocytes in human samples.

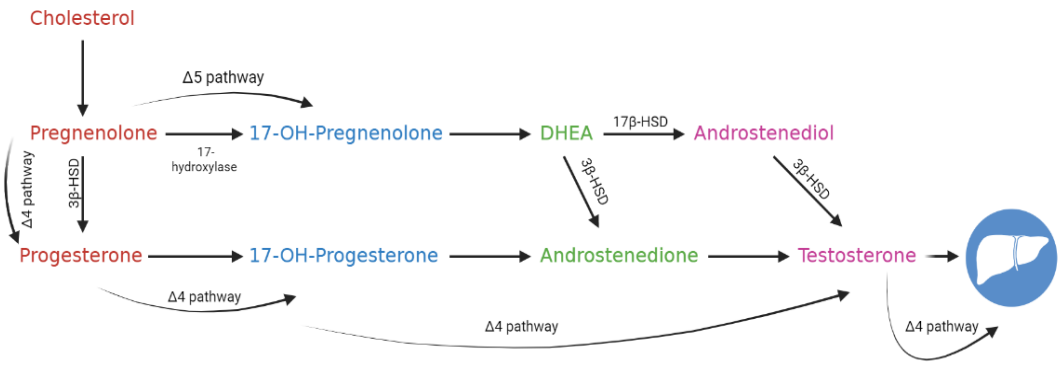


Figure 7. Diagram showcasing the steroidogenic pathways and how a multitude of hormones and steroids come into play to convert into DHEA and testosterone as well as alternate pathways to reach the same product.

Another potential set of biochemical biomarkers that have been demonstrated to be clear indicators are syndecan 2 and syndecan 4, which are proteins that are encoded in the SDC2 gene in the body. Syndecan 2 and 4 are variants of the protein syndecan, which is a class of proteins that are coreceptors with growth factor receptors that aid extracellular signal transfer across the surface of a cell. Syndecan 4's role in identifying PCOS specifically can be found in what it affects. Syndecan 4 acts as a coreceptor for not only growth factors like TGF-β, but also for the fibroblast growth factor (FGF2) and vascular endothelial growth factor (VEGF.) When reduced, the syndecan can affect the regulation of the cell matrix interaction during the process of assembly and expansion of the COC matrix and affect overall growth factor signaling. A COC matrix stands for a Cumulus Cell-Oocyte matrix, which is a heavily extended coat of tightly packed granulosa cells (called cumulus oophorus) that forms around an oocyte (an ovarian cell that is going to undergo division through meiosis to produce an ovum) before the process of ovulation. This coat also remains surrounding the oocyte as it travels into the peritoneal cavity and down the fallopian tubes. The coat also ensures that no changes are being made to the oocyte and that it is receiving the proper nutrients needed. The overall stability of the formation and maintenance of the COC matrix is invaluable for the process of ovulation to be as smooth as possible as well and for proper sperm selection and reception. When these factors are affected, PCOS symptoms such as ovulation issues and infertility come into play.

Not only can biomarkers with complicated compositions serve as indicators, but aforementioned hormones that play a role in the body's regulation can also be considered as indicative markers. Some such hormones include leptin, adiponectin, DPP-IV, and RBP-4 especially in cases where the BMI was increased. Leptin and adiponectin especially have displayed the strongest levels of FAI (free androgen index) and women with hyperandrogenemia will have the biggest leptin, adiponectin, and DPP-IV level difference with women without hyperandrogenemia. This bolsters the fact that adipocytokines are found in women with metabolic complications, especially when they possess metabolic complications. To look at leptin's role in a physiological lens, the process starts off when leptin is secreted from white adipose tissues to regulate both food intake and expenditure of energy while collaborating with the central nervous system. As insulin activity and body weight increase, hyperandrogenism and infertility rates also steadily increase, which are key attributes of PCOS. This can also cause damage to ovarian function by decreasing the ovaries' sensitivity to gonadotrophins. If the gonadotropins don't stimulate the gonads and work with the ovaries to produce needed amounts of estrogen and progesterone, the delicate hormonal balance could be tipped and cause flare ups in PCOS symptoms. Adiponectin is also secreted by white adipose tissues which expresses anti-inflammatory, anti-inflammatory, and insulin-sensitizing effects. Concentrations of this are also shown to be inversely correlated to effects such as insulin

resistance, obesity and diabetes mellitus type 2. Women with PCOS have lower concentrations circulating around in their body in general.

Like previously mentioned, not only are the women with PCOS affected, but so are their offspring. Biomarkers that are found in offspring specifically are MMP-9 and S100A8. MMP-9 is a proteolytic enzyme which is responsible for extracellular matrix remodeling. Pertaining to the ovary specifically, the MMPs are involved in development of the follicles and the process of ovulation as a whole. Due to this, MMP-9 concentrations could fluctuate with the pathophysiology of PCOS because when the extracellular matrix has changed, there will be an increase in ovarian stroma tissue and follicular atresia. An increase in MMP leads to more diseases as well, such as cystic fibrosis, cardiovascular disease, atherosclerosis, ulcerative colitis, and asthma. As for S100A8, it is known as a damage associated molecular pattern (DAMP.) A DAMP is a molecule released due to cells that are exposed to stress or death. The S100A8 is chemotaxin (a substance simulating a chemotaxis which is the movement of a cell that correlates with a concentration of a particular substance) that is involved in processes such as cellular migration, energy metabolism and calcium homeostasis and within the ovary, it plays a part in primordial follicle formation.

Conclusion

Polycystic ovary syndrome is an all-enveloping condition that affects every aspect of a woman's body. Not only does it just affect the woman with the diagnosis, but it also affects their future offspring. It is critical that methods for earlier diagnosis can be found to further mitigate the painful symptoms and effects. The extra time could prove indispensable to even finding a cure by attacking the problem at the root to find out what factors (whether they are genetic, environmental, biological) are the cause. The inner workings of the body transcend all that we know so far but if utilized correctly, we can use the body itself to find the answers we are looking for. The research explored analyzing proteins in the human body such as FBN3, A8, syndecan, leptin and more to serve as indicators for abnormal behavior in the body as well as the utilization of these samples to explain the effect of PCOS on the body. If the fight against PCOS is specifically concentrated towards looking for a solution inwards, the progress made for women suffering from this condition from all backgrounds could be powerful.

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