Role of Oxidative stress and Detection of Bisphenol A in Women with Polycystic ovarian syndrome

KEERTHANA. M
(17PBC007)

A Thesis submitted to Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore – 641 043

In Partial Fulfillment of the Requirement for the Degree of

Master of Science in Biochemistry

April 2019

CERTIFICATE

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Signature of the Head of the department

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“GRATITUDE IS THE MEMORY OF HEART”

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1. INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most common endocrine and hormonal disorder of reproductive age affecting 5% to 10% of women worldwide. It is a heterogenous, multifactorial, complex genetic disorder (Spandana and Shetty, 2017). Women with PCOS are under an increased risk for infertility, preeclampsia and endometrial cancer. This heterogeneous endocrine disorder is strongly associated with a wide range of metabolic problems such as cardiovascular disease, carotid and coronary atherosclerosis, insulin resistance, lipid abnormalities, elevated triglycerides (TG) /highdensity lipoprotein (HDL) ratio, hypertension, endothelial dysfunction and dyslipidemia (Amini *et al.,* 2018).

Family history is one of the main risk factor of PCOS. The chance of getting PCOS is higher if other women in the family have the disease, irregular menstrual cycle or diabetes mellitus (Ibrahim *et al.*, 2018). Several lines of evidence suggest that women with PCOS may also be at an increased risk of having a personal history of ovarian cancer and breast cancer, particularly as these women are hyperandrogenic and infertile, which are risk factors for breast and ovarian cancers (Moini and Eslami, 2009).

Insulin resistance is a common feature of PCOS that has been observed both in obese and lean women. Hyper - insulinemia has been recognized as a contributory factor for ovarian disruption due to increased production of ovarian androgens. Since obesity is commonly seen with this disorder. (Kansra and Marquart, 2016). Insulin resistance is a major risk factor of women with PCOS. Women with PCOS have a higher risk of decreased high-density lipoprotein cholesterol (HDL-C) levels and increased total cholesterol, low-density lipoprotein cholesterol (LDL-C) and triglyceride levels. (Faris *et al.***,** 2017). The PCOS is associated with insulin resistance, evidence suggests that women with PCOS are at an increased risk for developing type-2 diabetes, dyslipidemia, hypertension, and heart disease (Gupta *et al.,* 2017).

Diabetes and obesity are among the leading causes of NAFLD. The presence of NAFLD in association with PCOS obese patients is raised and might grow up to 70 percent. NAFLD, free androgens and IR existence are found to be greater in obese patients having PCOS as compared to obese patients, BMI-matched controls, which suggest that PCOS and its related features are crucial

for the NAFLD instead of obesity. The metabolic abnormalities in addition to increase in androgen bioavailability might be implicated (Jaroudi *et al.*, 2017).

The imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system, which produces the oxidative damage is known as oxidative stress. Reactive oxygen species can affect a variety of physiological functions in the reproductive tract. When ROS increase to pathological levels such as in PCOS, they are capable of inflicting significant damage to cell structures. Moreover the body's defense mechanisms would play a role in the form of antioxidants and try to minimize these damages, thereby adapting itself to the above stressful situation. The antioxidants are compounds that dispose, scavenge and suppress the formation of free radicals or oppose their actions. Reproductive cells and tissues remain stable when ROS production and the scavenging antioxidants remain in balanced state. Levels of ROS are controlled and kept at physiological levels within the ovary by various antioxidant systems like vitamin C, which is known to have a protective effect within the follicle (Shirsath, 2015).

Insulin resistance causes hyperglycemia which triggers the release of reactive oxygen species from the mononuclear cells and, thus, induces oxidative stress. Oxidative stress leads to cellular damage and activates the transcription of pro-inflammatory cytokines such as tumor necrosis factor-alpha which is a known mediator of insulin resistance. This pro-inflammatory state may contribute to the development of insulin resistance and hyperandrogenism (Desai *et al.*, 2014).

Natural antioxidants are required to prevent and cure the disorders caused by free radicals. The free radicals are highly reactive chemical species produced in the body and have the potential to damage cells, DNA, organelles and other biomolecules, resulting in diseases such as cancer, cardiovascular disease and neurodegenerative disease (Ahmed *et al.*, 2015). PCOS is accompanied by oxidative stress in which increased production of free radicals is followed by decreased levels of serum total antioxidants. Possible contributors to oxidative stress in obesity include hyperglycemia, hyperleptinemia and augmented muscle activity to carry excessive weight, chronic inflammation and inadequate antioxidant defenses. Oxidative stress is increased in diabetes and also in obesity (Faris *et al.*, 2017).

Endogenous antioxidants are enzymes such as superoxide dismutase, catalase, glutathione peroxidase or nonenzymatic compounds, such as vitamin A, vitamin C and vitamin E. When endogenous factors cannot ensure a rigorous control and a complete protection of the organism against the reactive oxygen species (ROS), the need for exogenous antioxidants arises, as nutritional supplements and/or pharmaceutical products, which contain an antioxidant compound. Exogenous antioxidants can derive from natural sources like flavonoids, vitamins, anthocyanin, some mineral compounds, but can also be synthetic compounds, like butylhydroxyanisole, butylhydroxytoluene and gallates (Pisoschi and Negulescu, 2011).

The oxidative balance of the reproductive female tract depends on some types of free radicals and on different antioxidant mechanisms that neutralize them. There are two major groups of free radicals: reactive oxygen species and reactive nitrogen species (Barcelos and Navarro, 2012). A lot of investigations have revealed that Oxidative stress level is significantly increased in women with PCOS compared with the normal women, when oxidative status is evaluated by circulating markers, such as malondialdehyde (MDA) and tricyclic antidepressant (TAC). However, Oxidative stress level is also correlated with obesity, insulin resistance, hyperandrogenemia, cardiovascular disease and chronic inflammation. Though Oxidative stress is considered as a potential inducement of PCOS pathogenesis, it is still undetermined whether the abnormal Oxidative stress levels of women with PCOS derive from PCOS itself and/or if they are related to the potential complications (Zuo *et al.*, 2016).

There is some evidence that genetic susceptibility has been associated with PCOS and environmental factors such as environmental pollutants and geography have important role in the expression of those genetic traits. Endocrine disrupting chemicals are defined by the Environmental Protection Agency such as: "exogenous agents that interface with the synthesis, secretion, metabolism, transport, binding action, or elimination of natural blood-borne hormones that are present in the body and responsible for homeostasis, reproduction and development process". Among them bisphenol A (BPA) is one of the highest volume chemical produced worldwide and used by the manufacturers of plastics and epoxy resins which are pervasive in our environment and our daily lives (Rashidi *et al.*, 2017). Bisphenol A can easily move into the food samples from lacquer-coated cans and plastic products due to hydrolysis of the polymer during

thermal treatment. Consequently, it can also cause adverse health effects such as recurrent miscarriages, endometrial hyperplasia and polycystic ovarian syndrome. (Yildirim *et al.,* 2017).

Bisphenol A has the potential role in pathogenesis of PCOS. Hypothalamic BPA exposure activates GnRH pulse generator, which in turn leads to the increased LH and decreased FSH. It can stimulate androgen production in the ovarian theca. BPA can also interact with the receptors in adipose tissue and stimulate pancreatic beta cells to insulin production which both result in increased lipid accumulation in the adipose tissue. All of these effects impair ovarian folliculogenesis leading to anovulation (Rutkowska *et al.,* 2014).

Bisphenol A could stimulate prolactin release, alter thyroid hormone action, impair aromatase expression and act as an anti-androgen. Human exposure to BPA is nearly present and takes place through inhalation, ingestion, and dermal absorption. Females are born with a limited number of oocytes, it is significantly decreased in number from the second trimester of the fetal period until menopause through progressive apoptosis. Several cellular processes like epigenetic modifications, apoptosis and oxidative stress are all critical for oocyte maturation. Apoptosis is a programmed cell death which includes prenatal germ cell death, granulosa cell death during post-natal follicular atresia, plays a major role in the elimination of germ cells at all the stages of oogenesis and ovulation. Oxidative stress also inhibited oocyte maturation. Oocytes maturation inhibited by oxidative stress could be protected by melatonin (Wang *et al.,* 2016).

BPA has estrogenic activity and can bind to α- and β-estrogen receptors. It has been detected in almost all human body fluids, including follicular fluid, which indicates that oocytes are exposed to BPA during the folliculogenesis process. A large number of animal experiments suggest that BPA may act as a reproductive toxicant and affect fertilization rate, the onset of female puberty and estrous cycle. Moreover, the animal studies indicate that BPA has adverse effects on the maturing oocyte and meiotic cell division machinery. The environmental BPA exposure may adversely affect the oocyte quality and cause the decline of ovarian reserve and fertility in general population, though the magnitude of actual risk in human remains uncertain (Zhou *et al.,* 2016).

Bisphenol A also is a product of the condensation reaction between two moles of phenol and one mole of acetone in the presence of concentrated sulfuric acid. This chemical is soluble in

water upto 120-300 ppm. These chemicals are used extensively in epoxy resins, polycarbonates, dental fillings, food preserving containers, baby bottles, and mineral water containers. BPA have little stability in acid or alkaline conditions and they are unstable upon exposure to UV radiation. These chemicals have the ability to transfer from polymers to foods and they can contaminate foods at high or low temperatures. These chemicals have a variety of adverse effects on people, among which is the reduction of the fertility on men. Exposure of BPA can cause adverse effects, including decreases in the daily production of sperm and acceleration of growth and maturity. BPA used in food packaging materials increases the development of a specific type of prostate cancer with mutant androgen receptors. For management of most prostate cancers, testosterone is required to develop, and inhibition of the production of this hormone is one of the methods used in the treatment of prostate cancer (Kazemi *et al.*, 2016).

BPA is metabolized in the liver to its glucuronidated form (BPA glucuronide or BPAG) and eliminated mainly through urine. Because BPAG does not bind to the estrogen receptor, it is considered as a mechanism of detoxification in humans. However, BPAG can be deconjugated by β-glucuronidase, which is present at high concentrations in placenta, intestine, liver and kidney. The conversion of BPAG to BPA increases the potential for reactivation of BPA-induced effects. (Liao and kannan., 2012). With this background the present study entitled "**Role of Oxidative stress and Detection of Bisphenol a in women with polycystic ovarian syndrome"** was carried out with following objectives:

- To evaluate the clinical features and biochemical changes in PCOS and normal women
- To investigate the role of oxidative stress in women with PCOS
- To assess the level of bisphenol A in women with PCOS

2. REVIEW OF LITERATURE

Polycystic ovarian syndrome (PCOS), featuring problematic follicular development, is an endocrine system disorder that has life-long impact upon its patients. Polycystic ovarian syndrome is one of the common hormonal and metabolic disorders in women of reproductive age. Prevalence of PCOS among women at reproductive age was reported to be 5%–10%. The disease is characterized by oligomenorrhea or amenorrhea, unovulation, insulin resistance (IR), hyperandrogenemia and cysts on the ovaries and deemed as one of the main cause of anovulatory infertility (Shan *et al.,* 2015).

Women with PCOS often experience other conditions that can affect their short- and long-term physical and mental health. Decreased quality of life from mood disturbances, weight gain, acne vulgaris, and alopecia have all been documented. PCOS may represent one of the largest groups of women at high risk for the development of early onset coronary heart disease. Although it is important to treat the short term disturbances for women, research shows that it is important to think about the future of women with PCOS, as many of them will develop metabolic syndrome (Brady *et al.,* 2009).

The presence of polycystic ovarian syndrome is one of the common phenomena that can occur in the majority of PCOS women. About 95% of women with PCOS at their early follicular phase could have polycystic ovaries and reduced level of follicle stimulating hormone which may lead to increased luteinizing hormone level. In addition, PCOS is also associated with hyperandrogenism, menstrual dysfunction, oligo-ovulation. PCOS is considered as a complex androgen excess accompanied by different degrees of gonadotropic and metabolic dysregulation controlled by multiple gene interaction and environmental factors. Although, the genetic basis of abnormal follicular development, anovulation, metabolic disorder and other heterogenous clinical abnormalities of PCOS women seems to require detailed investigation, it is suggested that daughters from women exhibiting a characteristics of PCOS could have a higher chance of acquiring hyperandrogenism and other PCOS phenotypes (Salilew-Wondim *et al.,* 2015).

The review of literature related to the study **"Role of Oxidative stress and detection of Bisphenol - A level in polycystic ovary syndrome"** is discussed below

2.1 Polycystic ovary syndrome

2.2 Oligoovulation and / or anovulation

2.3 Hyperandrogenism

2.3.1 Hirsutism

2.3.2 Acne

2.3.3 Obesity

2.3.4 Androgenetic Alopecia

2.3.5 Acantosenigricans

2.4 Diagnosis of PCOS

2.5 Treatment of PCOS

2.5.1Weight Loss

2.5.2 Physical activity

2.5 Risk factors for PCOS

2.5.1 Insulin resistance

2.5.2 Dyslipidemia

2.5.3 Hypertension

2.5.4 Diabetes mellitus

2.5.5 Cardiovascular disease

2.5.6 Fertility

2.6 Oxidative stress and free radicals

2.6.1 Role of oxidative stress during PCOS

2.7 Role of antioxidant activity

2.7.1 Non enzymatic antioxidants

2.7.2 Enzymatic antioxidants

2.8 Role of liver marker

2.8.1 Alanine aminotransferase

2.8.2 Aspartate aminotransferase

2.9 Role Hormonal imbalance

2.10 Bisphenol A

2.1 Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is a complex condition characterized by menstrual irregularities, elevated androgen levels and/or small cysts on one or both ovaries. The disorder can be morphological (polycystic ovaries) and/or predominantly biochemical (hyperandrogenemia). Hyperandrogenism is a clinical hallmark of PCOS, can cause inhibition of follicular development, microcysts in the ovaries, anovulation, and menstrual changes (Ndefo *et al.,* 2013). Insulin resistance is a common feature of PCOS that has been observed both in obese and lean patients. This complex syndrome impacts across the lifespan and requires engagement in self-management and a multidisciplinary treatment approach Hyper insulinemia has been recognized as a contributory factor for ovarian disruption due to increased production of ovarian androgens (Kansra and Marquart., 2016).

. Multiple diagnostic criteria exist, giving rise to clinical phenotypes, heterogeneous and many women with PCOS undiagnosed. It is also a diagnosis of exclusion of thyroid dysfunction and hyperprolactinemia which should be screened in women with PCOS. Ovarian follicular arrest, metabolic features and infertility occur secondary to hormonal disturbances. Histologically, a cyst is an epithelial-lined, fluid filled sac usually greater than 2 cm. In PCOS, the ovaries are usually enlarged and contain multiple atretic follicles, typically less than 8 mm, that are not lined by epithelium. Although technology has improved, ultrasound still provides an indirect, nonfunctional ovarian assessment, and criteria for "polycystic ovary" remain highly controversial with poor sensitivity and specificity, prompting calls for a new name for the syndrome. The panel stated, "We believe the name 'PCOS' is a distraction and an impediment to progress. It causes confusion and is a barrier to effective education of clinicians and communication with the public and research funders". The name of a condition should reflect pathology, should promote both recognition and understanding by health professionals and consumers, and should not be inaccurate. Therefore, a revision of the name "polycystic ovary syndrome" is needed to reflect the condition's broader clinical features (Teede *et al.,* 2013).

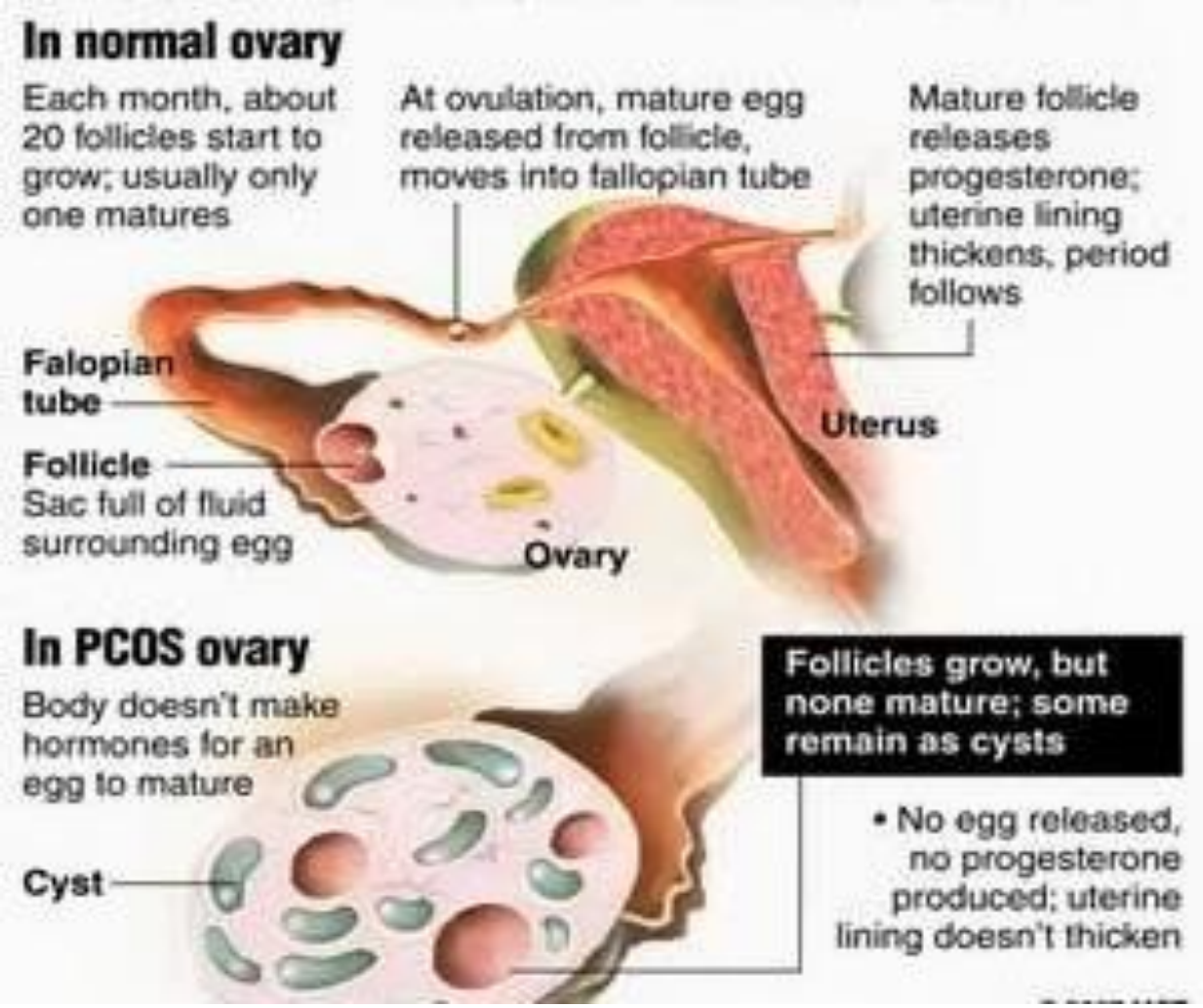


Figure 1 – PCOS and the menstrual cycle

2.2 OLIGOOVULATION AND / OR ANOVALATION

- Oligoovulation is infrequent or irregular ovulation (usually defined as cycles of ≥36 days or <8 cycles a year)
- Anovulation is absence of ovulation when it would be normally expected. Anovulation usually manifests itself as irregularity of menstrual periods, that is, unpredictable variability of intervals, duration, or bleeding. Anovulation can also cause cessation of periods (secondary amenorrhea) or excessive bleeding

Anovulation is the failure of the ovary to release ova over a period of time generally

exceeding 3 months. The normal functioning ovary releases one ovum every 25–28 days. This average time between ovulation events is variable, especially during puberty and the perimenopause period. For nonpregnant women aged 16–40 anovulation is considered abnormal and a cause of infertility in 30% of fertility patients. One of the cardinal signs of anovulation is irregular or absent of menstrual periods (Janneke *et al.*, 2010).

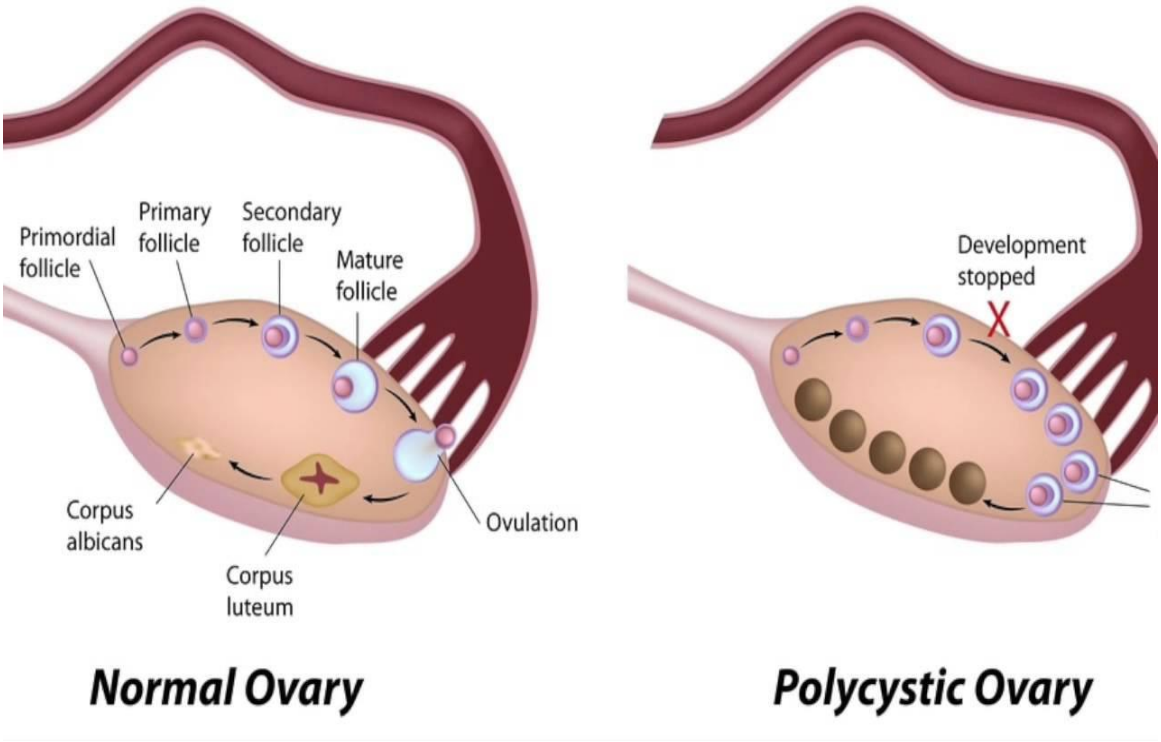


Figure 2 – Follicles with normal ovary and PCOS

Oligomenorrhea is defined as more than 36 days between menstrual cycles or fewer than eight cycles per year. In the absence of pregnancy, menstruation follows ovulation by approximately 14 days. Because menstruation is linked to ovulation, the clinical finding of oligomenorrhea correlates with oligoovulation. This predictable pattern of ovulation and menstruation is regulated by a cyclic change in hormones. Consequently, the diagnosis of ovulation dysfunction includes the assessment of the hormones and systems involved in ovulation and not just the symptom of amenorrhea.

Follicle stimulating hormone (FSH) is the primary gonadotropin responsible for this progression. As the follicles enlarge, FSH stimulates the production of more FSH receptors on the granulosa cells. This allows the follicle to become more sensitive to FSH and grow more rapidly. The larger follicles recruit more theca cells, which produce androstenedione. This androgen passes through the basement membrane and is converted to estradiol by FSH driven aromatization in the increasing number of granulosa cells. Follicular development is driven by FSH, but lutenizing hormone (LH) is responsible for ovulation. FSH acts on theca cells to induce LH receptor expression and render the cells sensitive to LH. LH also stimulates the theca cells to produce androstenedione, which is converted to estradiol by granulosa cells as described above. The estradiol produced further stimulates LH release from the pituitary. When a critical level of LH is reached, ovulation occurs and the follicle rapidly changes to a corpus luteum. Progesterone, produced by the corpus luteum, increases following ovulation and inhibits LH secretion by an effect on the hypothalamus. Without fertilization of the ova, the corpus luteum regresses, progesterone and estradiol levels drop, and FSH is again released to promote development of a new dominant follicle (Janneke *et al.,* 2010).

2.3 HYPERANDROGENISM

Hyperandrogenism is also known as androgen excess, is a medical condition characterized by excessive levels of androgens in the female body and the associated effects of the elevated androgen levels. The most common clinical manifestation of hyperandrogenism in women is hirsutism and excessive terminal hair growth in androgen-dependent areas of the body. Other clinical manifestations of hyperandrogenism include acne, weight gain and menstrual irregularities. PCOS is now considered as a disorder of androgen excess. In women with PCOS, there is increased gonadotropin-releasing hormone (GnRH) pulse frequency which favors increased LH secretion over that of follicle stimulating hormone (FSH). (Dadachanji *et al.,* 2018).

The chronology of symptom commencement and progression is important and can be indicative of specific disease processes.. Disruption of the timing of puberty can be associated with congenital adrenal hyperplasia and Cushing's syndrome (Meek *et al.,* 2013).

ANDROGEN ACTION

In the target tissues, androgens diffuse across cell membranes and bind to nuclear androgen receptors. The androgen receptor complex attaches to a specific DNA site and stimulates the production of messenger RNA which in turn stimulates the production of the enzymes and proteins necessary to affect androgen action. In the brain, the highest concentrations of androgen receptors are present in the preoptic area of the hypothalamus – in close proximity to estrogen receptors – and are thought to be involved in behaviour. In bone, androgens have an important role in bone mineralization both directly and through the aromatisation to estrogen. Lower androgen concentrations have been associated with bone loss in various age groups. Androgen receptors are also present in mammary epithelial cells in addition to estrogen and progesterone receptors (Chae *et al.,* 2008).

2.3.1 HIRSUTISM

Hirsutism is defined as an excessive hair growth in androgen dependent areas of women.it is one of the most widely used clinical criteria for the diagnosis of androgen excess and is observed in 50% - 80% of patients with hyperandrogenism. It is commonly graded according to the hormonal Ferriman-Gallwey system, which is quantitates the extent of hair growth in the most androgen-sensitive areas. Focal hirsutism (score < 8) is a common normal variant, whereas generalized hirsutism (score >8) is abnormal. (Barbosa *et al.,* 2015).

Hirsutism must be distinguished from hypertrichosis, the generalized excess growth of hair that sometimes occurs on a hereditary basis or in patients taking glucocorticoids, phenytoins, diazoxide, or cyclosporine. Hypertrichosis is distributed in a nonsexual pattern and is not caused by excess androgen, although it may be aggravated by excess androgen. The absence of hirsutism in approximately one third of hyperandrogenic adults appears to be because of relatively low sensitivity of their pilosebaceous unit to androgens. Conversely, hirsutism without elevated circulating levels of androgen—”idiopathic hirsutism”—accounts for approximately half of mild hirsutism and one sixth of moderate-severe hirsutism (Rosenfield *et al.,* 2014)

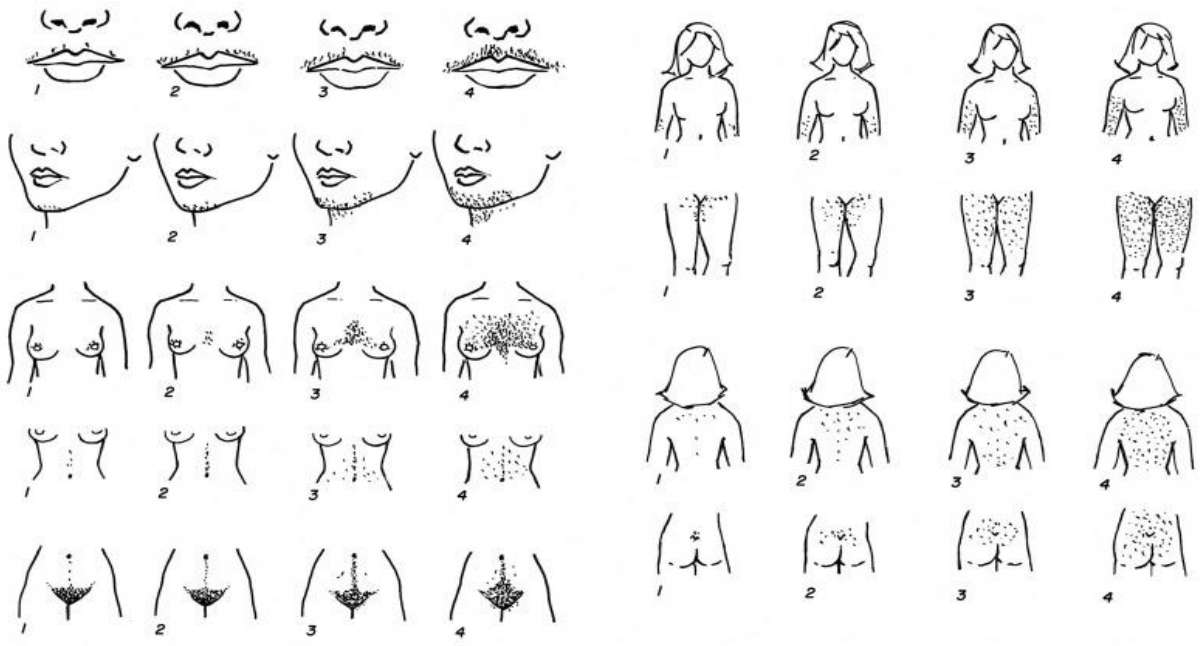


Figure 3 – Hirsutism scoring in each of nine body areas

2.3.2 ACNE

Acne is a disorder of the pilosebaceous unit, with scratch on the face, neck, back and chest area. As vulgaris acne, the androgen levels are usually normal. It is believed that the local conversion has been increased for a greater receptor sensibility for androgens in patients with acne in relations to normal population. Perhaps, it represents the most important cause in the disease activation. Numerous studies have tried to associate the clinical presentation of acne with hyperandroge-agency markers. (Barbosa *et al.*, 2015).

2.3.3 OBESITY

Reproductive disturbances are more common in obese women regardless of the diagnosis of PCOS. Obese women are more likely to have anvolatory infertility and menstrual irregularity than normal-weight women. In reproductive-age women, the relative risk of anovulatory infertility increases at a BMI of 25 kg/m2 and continues to rise with increasing BMI. (Susan Sam, 2007)

The sever hyperandrogenism seems to be amplified in obese women with PCOS. The obese women with PCOS have higher total and free testosterone levels as compared to non-obese PCOS. In fact, the increase of body weight and fat tissue, especially in the form of abdominal obesity, is

associated with abnormality of sex steroid hormone balance. This is mainly due to the reduction of sex hormone–binding globulin (SHBG) levels in circulation, that the result is increased fraction of free androgens in blood. Reduced SHBG synthesis in liver originates from hyperinsulinaemia that compensates for insulin resistance associated with obesity. Although hyperinsulinaemia is associated with PCOS, it is clear that obese women with PCOS exhibit a higher degree of insulin resistance and hyperinsulinaemia. Increased androgens in obese PCOS contribute to inhibition of SHBG secretion. (Rosenfield *et al.,* 2014).

2.3.4 ANDROGENETIC ALOPECIA

Androgen levels in women are much lower than in men so when the ovaries turn into cysts and then produce too much of androgen, that is known as hyperandrogenism. They can block the hair follicles thus can cause hair loss, it is called Androgenic alopecia or AGA. This means each affected hair becomes thinner in diameter, shorter in length and lighter in colour until it is finally not produced at all. Eventually, this hair loss can become severe near-baldness, especially as women pass menopause (Amiri, *et al.,* 2019))

In susceptible hair follicles, dihydrotestosterone binds to the androgen receptor, and the hormone-receptor complex activates the genes responsible for the gradual transformation of large terminal follicles to miniaturized follicles. Young women have much higher levels of cytochrome p-450 aromatase in frontal follicles than men who have minimal aromatase, and women have even higher aromatase levels in occipital follicles. The diagnosis of AGA in women is supported by early age of onset, the pattern of increased thinning over the frontal scalp with greater density over the occipital scalp, retention of the frontal hairline, and the presence of miniaturized hairs. Most women with AGA have normal menses and pregnancies. Extensive hormonal testing is usually not needed unless symptoms and signs of androgen excess are present such as hirsutism, severe unresponsive cystic acne and galactorrhea. (Vera, 2013)

2.3.5 ACANTOSIS NIGRICANS

The acantosis nigricans characterized by the presence of a brown and velvety plate with accentuation in the furrows of skin. The dermatopathology is most commonly observed in the neck

and intertriginous areas such as groin armpits and inflamammary region and it is reported in 5% of PCOS women. Although to be associated with obesity and diabetes, may be present in genetic diseases, drug reaction and malignancies. When severe, progressive and extensive, may be associated with malignancy, especially when the mucus is also involved (Barbosa *et al.,* 2016)

2.4 Diagnosis of PCOS

Having polycystic ovaries does not mean you have PCOS. Women with PCOS often have symptoms that come and go, particularly if their weight goes up and down. This can make it a difficult condition to diagnose, which means it may take a while to get a diagnosis.

A diagnosis is made when you have any two of the following:

- Irregular, infrequent periods or no periods at all
- An increase in facial or body hair and/or blood tests that show higher testosterone levels than normal
- An ultrasound scan that shows polycystic ovaries.

When a diagnosis is made, you may be referred to a gynaecologist (a doctor who specialises in caring for a woman's reproductive system) or an endocrinologist (a doctor who specialises in the hormonal system). (www.rcog.org.uk/ en/guidelines-research-services/guidelines/gtg33)

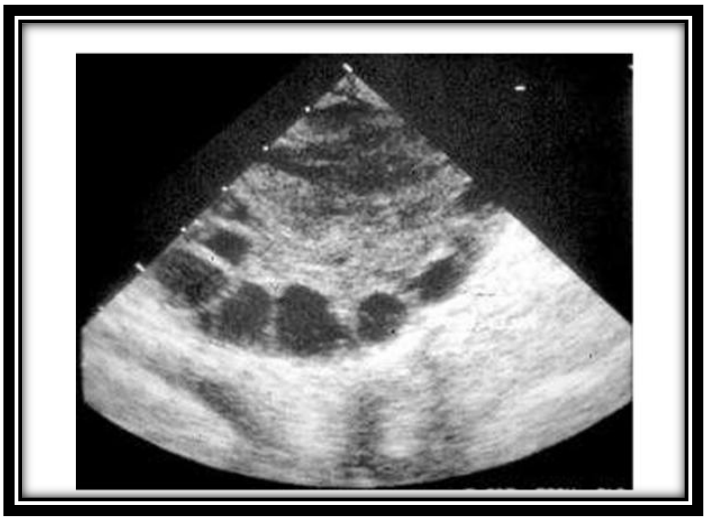


Plate 1 - Ultrasound picture of a polycystic ovary

2.5 Treatment of PCOS

2.5.1 Weight Loss

Obesity commonly is associated with PCOS. Fatty tissues produce excess estrogen, which in turn contributes to insufficient FSH secretion by the pituitary gland. Insufficient FSH prevents ovulation and may worsen PCOS. In addition, obesity is associated with the development or worsening of insulin resistance, which may further increase androgen production by the ovaries. Weight loss improves the hormonal condition of many PCOS patients.

2.5.2 Physical activity

Increasing physical activity is an important step in any weight reduction program. Start slowly with an aerobic activity such as walking or swimming. Increase speed and distance gradually. Regular activity improves state of mind as well as aiding in weight reduction. Recommendations include three to four exercise periods each week with at least 30 minutes of aerobic exercise. Extreme cases of obesity, unresponsive to medical management and behavioural modification, may be treated with bariatric surgery. Surgical risks have decreased over time and many procedures are performed in a minimally invasive way.

2.5 RISK FACTORS FOR PCOS

2.5.1 Insulin Resistance

Insulin is an atherogenic hormone and hyperinsulinemia may contribute to the development of diabetes, hypertension, and dyslipidemia that is often accompanied by increased total cholesterol and low-density lipoprotein (LDL), triglyceride (TG), and decreased high-density lipoprotein (HDL) levels in PCOS. Hyperinsulinemia accelerates ovarian androgen overproduction. Dyslipidemia and sex steroids also have important effects on cardiovascular disease. Women with PCOS have a relative risk for myocardial infarction. (Fenkci *et al.*, 2013).

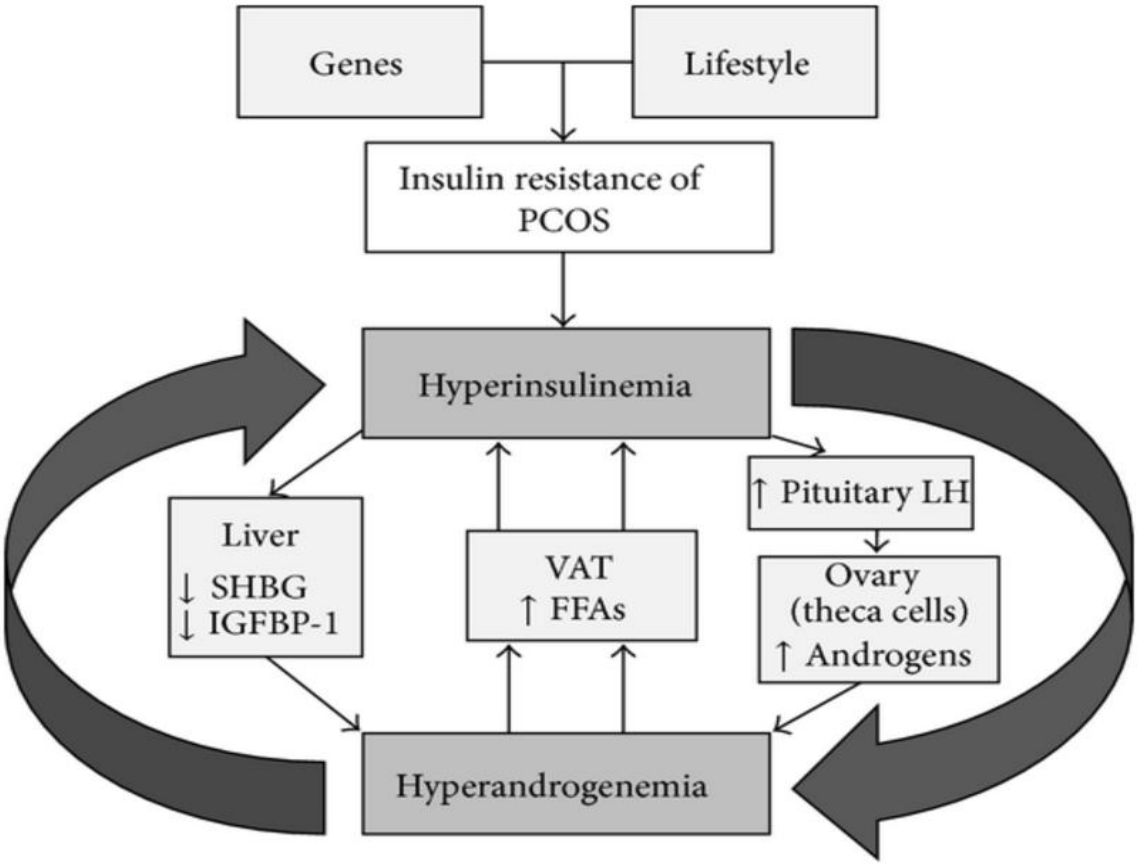


Figure 4 – Role of Insulin resistance in PCOS

Insulin acts to regulate glucose homeostasis by stimulating glucose uptake by insulin responsive target tissues, skeletal, adipocytes and cardiac muscle as well as by suppressing hepatic glucose production. Insulin also suppresses lipolysis, which decreases the circulating free fatty acid levels, which may mediate the action of insulin on hepatic glucose production. Insulin has other metabolic as well as mitogenic and reproductive actions (Kandarakis and Dunaif, 2012)

Most women with PCOS, particularly those who are overweight or obese, do in fact have insulin resistance and compensatory hyperinsulinaemia, partly attributable to an intrinsic insulin resistance mechanism. Using the homeostasis model assessment, 50% to 70% of women with PCOS demonstrate insulin resistance. (Yau *et al.,* 2017)

2.5.2 Dyslipidemia

The dyslipidemia in PCOS is similar to that seen in metabolic syndrome, characterized by low levels of HDL, small particle size of low-density lipoprotein cholesterol (LDL), and high triglyceride cholesterol levels. This pattern is more often seen in obese than in lean PCOS, likely secondary to the presence of greater insulin resistance in obesity. The level of LDL cholesterol is also increased in women with PCOS and is less dependent on obesity than HDL and triglyceride levels. There is also evidence for heritability of dyslipidemia, so these lipid patterns can be seen not only in women with PCOS but also in their family members (Lewis *et al.,* 2011).

2.5.3 Hypertension

High blood pressure is a common condition in which the long-term force of the blood against artery walls is high enough that it may eventually cause health problems, such as heart disease. PCOS is known to be associated with hypertension. BP in women with PCOS compared to body mass index (BMI)-matched controls, with differences in BP persisting after adjustment for adiposity and IR. Obesity is well known to be associated with hypertension. (Joham *et al.,* 2015).

PCOS women demonstrated a higher prevalence of hypertension among premenopausal women with PCOS compared to women without PCOS; however, the PCOS population was significantly more obese and the obesity could be responsible for the greater prevalence of hypertension. Hypertension was examined in menopausal women with PCOS who had undergone ovarian wedge resection. Menopausal women post-ovarian wedge resection had a three-fold increased likelihood of being hypertensive compared to non-PCOS women. These women with PCOS were also more obese than normal women and this comparison was not adjusted for BMI. Pregnant women with PCOS have a greater risk of perinatal morbidity from pregnancy induced hypertension (PIH) and preeclampsia (PE) than non-PCOS pregnancies as demonstrated in a meta-analysis of pregnancy outcomes in women with PCOS compared to controls (Lewis *et al.,* 2011)

2.5.4 Diabetes Mellitus

Polycystic ovary syndrome as an independent risk factor for diabetes and can be explained by several mechanisms. A unique post binding defect in insulin signal transduction in skeletal

muscle of women with PCOS, resulting in increased serine phosphorylation of the insulin receptor and insulin receptor substrate.

2.5.5 Cardiovascular Disease

There is increasing evidence that patients with polycystic ovary syndrome have increased cardiovascular risk compared with age matched controls. It has been estimated that myocardial infarction is seven times more likely in patients with PCOS and cardiac catheterisation studies have shown more extensive coronary artery disease in these patients than in women with normal ovaries. Furthermore, significant subclinical carotid atherosclerosis has been demonstrated on carotid artery ultrasound in women with PCOS. This increased cardiovascular risk is probably the result in part of the metabolic disturbance associated with PCOS. Dyslipidaemia, diabetes and obesity are all potent cardiovascular risk factors that tend to cluster in women with PCOS. However, it is not known whether the increased cardiovascular risk seen in PCOS is mediated through obesity or is independent of body mass index (BMI) and the result of other metabolic factors. In recent years, interest has grown in novel biochemical and biophysical markers of cardiovascular risk. C reactive protein (CRP) has been shown to be a good predictor of vascular events. In addition to being a marker of inflammation, there is evidence that CRP may have a direct role in atherogenesis via adhesion molecule expression, complement activation and mediation of low density lipoprotein (LDL) uptake by macrophages. Sialic acid has been proposed as a predictor of cardiovascular mortality, although the reason for this association remains unclear. Similarly, raised fibrinogen and homocysteine concentrations have been associated with an increased risk of ischaemic heart disease and atherosclerosis. Fibrinogen may promote cardiovascular disease by a variety of mechanisms including increased blood viscosity, thrombus formation or platelet aggregation. Homocysteine is postulated to damage the vascular endothelium directly. Finally, microalbuminuria is a well-established predictor of cardiovascular morbidity in diabetics and possibly in non-diabetic population also endothelial dysfunction is thought to occur at a very early stage of atherosclerotic plaque development and is an early marker for atherosclerosis (Bickerton *et al.,* 2004).

2.5.6 Infertility:

PCOS is characterized by anovulation due to a developmental defect of follicles beyond 10 mm in size. The clinical manifestations including infertility are related to the hypersecretion of LH (70%) present in women with hyperandrogenism anovulatory women (the ratio of LH/FSH ratio and high increase in ovarian androgen production). Most of the cycles are anovulatory making it essential to induce ovulation. The agency defined infertility as the absence of pregnancy after two years of regular intercourse, without using any contraception method. Regarding ovulation inducing drugs, all are associated with the increase in multiple pregnancies, obstetric and neonatal risks. Among the most commonly found female's causes of infertility, it is possible to observe structural changes, ovulatory changes, immune disorders and endometriosis. Infertility patterns may be influenced by many factors, such as the woman's age, frequency in sexual activity, woman's weight and smoking, among others. This way, different techniques should be used to reach an accurate diagnosis. About 50% of infertile women have also obesity. There is a clear association between obesity and menstrual irregularities, since the adipose tissue is the largest peripheral area for the aromatization of androgens to estrogens, contributing to estrogen production. Women with PCOS have a greater risk of anovulation and infertility. The progesterone dosage may be useful as an additional screening test. It is also recommended to exclude other infertility causes besides anovulation, in couples in which the woman has PCOS. The diagnosis of PCOS is very important, because it identifies the metabolic risks, the potential cardiovascular risk and mainly because such a diagnosis interferes directly with the fertility status of these patients (Barbosa *et al.,* 2016).

2.6 Oxidative stress and free radicals

Oxidative stress is an imbalance between the productions of free radicals that contain unpaired electrons, which increase the chemical reactivity. It causes changes to biological molecules, and these changes accumulate over time in the biological structures, which may cause molecular damage to cellular and tissue structures. Oxidative stress is implicated in the pathogenesis of several disease states including chronic diseases such as atherosclerosis, diabetes mellitus and cardiovascular disease. Chemical modification of amino acids in proteins leads to introduction of carbonyl groups, which is a consequence of the modification of protein by reactive

oxygen species (ROS) and implicated in the cause and progression of a number of physiological and pathological disorders. (Moti *et al.*, 2015)

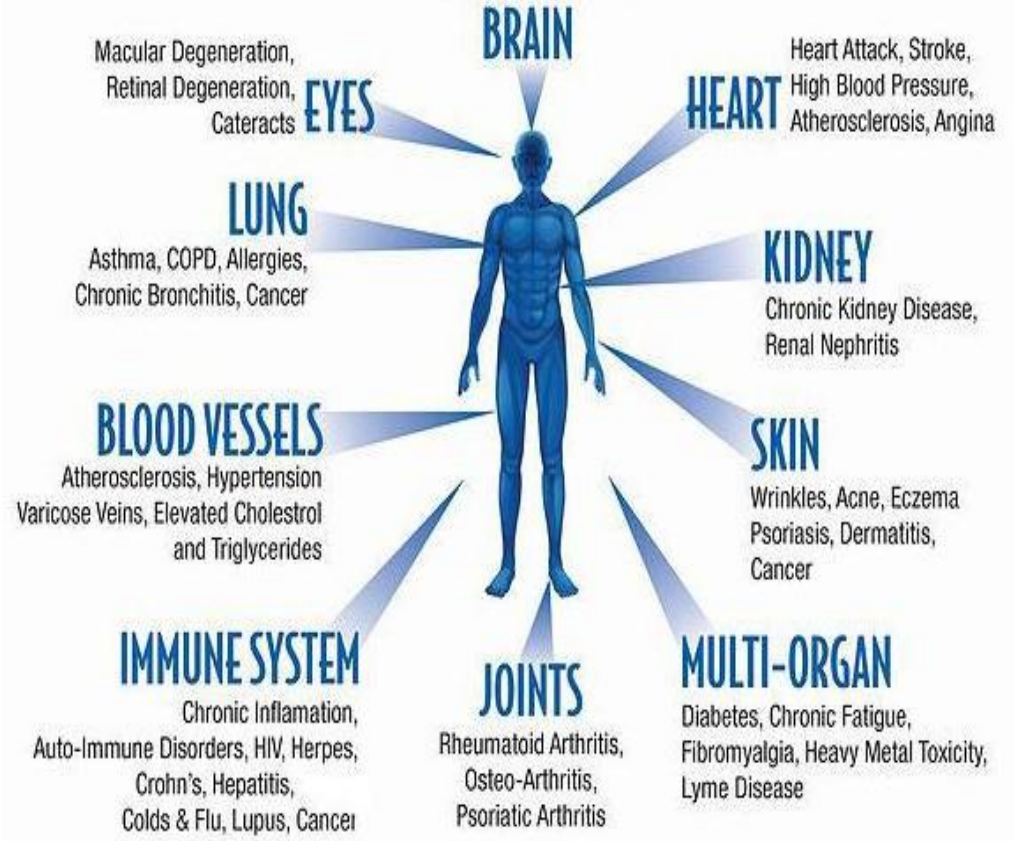


Figure 5 - Oxidative stress and free radicals

The increase in ROS generation or decreased antioxidant availability can result in a net increase in intracellular ROS. Normally, intracellular molecules including mitochondrial antioxidants prevent cellular damage produced by endogenous ROS. In living systems, free radicals and ROS are constantly generated and cause extensive damage to tissues and in various disease conditions, particularly degenerative diseases, and also lead to extensive lysis. Therefore, the mechanism of action of many synthetic drugs involves free radical scavenging, which protects against oxidative damage but has adverse side effects. (Saha *et al.*, 2016).

The free radicals are highly reactive chemical species produced in the body and have the potential to damage cells, organelles, DNA and other biomolecules, resulting in diseases such as cancer, cardiovascular disease and neurodegenerative disease. (Ahmed *et al.*, 2015)7. PCOS is

accompanied by oxidative stress in which increased production of free radicals is followed by decreased levels of serum total antioxidant. Possible contributors to oxidative stress in obesity include hyperglycemia, hyperleptinemia and augmented muscle activity to carry excessive weight, chronic inflammation and inadequate antioxidant defenses. Oxidative stress is increased in diabetes and also in obesity (Faris *et al.*, 2017).

2.6.1 Role of oxidative stress during PCOS

PCOS has been regarded as a chronic systemic disease instead of the simple local disease, and it is frequently associated with insulin resistance (IR), hyperandrogenemia, chronic inflammation and oxidative stress (OS). A lot of investigations have revealed that oxidative stress level is significantly increased in patients with PCOS compared with the normal, when oxidative status is evaluated by circulating markers such as malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx). However, oxidative stress level is also observed to be significantly correlated with obesity, insulin resistance, hyperandrogenemia and chronic inflammation. Though Oxidative stress is considered as a potential inducement of PCOS pathogenesis, it is still undetermined whether the abnormal oxidative stress levels of women with PCOS (Zuo *et al.*, 2016).

Lipid peroxidation mediated by free radicals is considered to be the major mechanism of cell membrane destruction and cell damage. Malonyldialdehyde (MDA) is a marker of lipid peroxidation and it increases in oxidative stress states while vitamin C and vitamin E are nonenzymatic antioxidants and helps in scavenging these free radical. PCOS is characterized by insulin resistance. Because of this insulin resistance glucose utilization by body tissue is decreased. It leads to hyperglycemia and further increasing insulin production. Hyperglycemia in turn leads to increased generation of ROS from mononuclear cells. These increased reactive oxygen species causes damage to all body cells including mononuclear cells leading to increased production of inflammatory markers like TNF –α and NF-Kappa B from the damaged cells. The mononuclear cells of women with PCOD are increased in this inflammatory state. Persistent hyperglycemia in PCOD acts on these mononuclear cells to generate furthermore ROS and the vicious cycle continues (Shirsath *et al.*, 2015).

2.7 Role of antioxidant activity

Antioxidants are compounds capable to either delay or inhibit the oxidation processes which occur under the influence of reactive oxygen species. Antioxidants are involved in the defense mechanism of the organism against the pathologies associated to the attack of free radicals. Endogenous antioxidants are enzymes like superoxide dismutase, catalase, glutathione peroxidase or nonenzymatic compounds such as uric acid, bilirubin, albumin and metallothioneins. Amongst the most important exogenous antioxidants, vitamin E, vitamin C, β-carotene, vitamin E, flavonoids, minerals are well Exogenous antioxidants can derive from natural sources (vitamins, flavonoids, anthocyanins, some mineral compounds), but can also be synthetic compounds, like butylhydroxyanisole, butylhydroxytoluene and gallates. There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, as well as the deterioration of fats and other constituents of foodstuffs (Pisoschi and Negulescu, 2011)

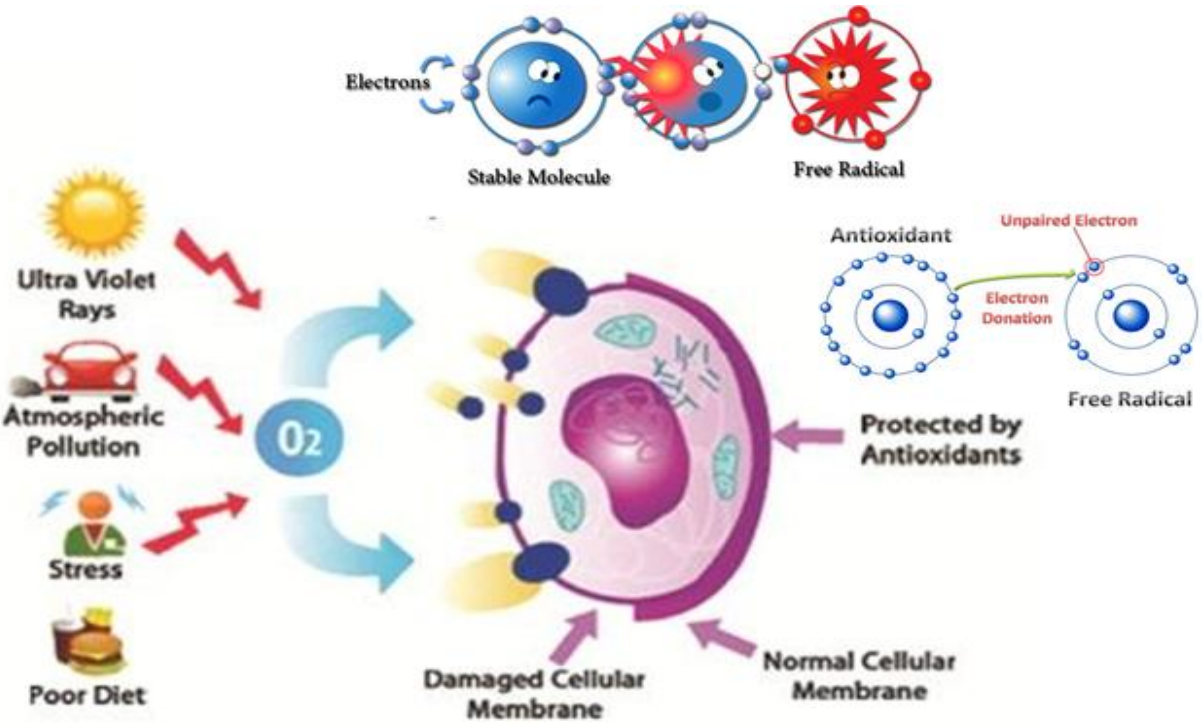


Figure 6 – Role of antioxidants

An antioxidant may be defined as ‘any substance that when present at low concentrations, compared with those of the oxidizable substrate, significantly delays or inhibits oxidation of that substrate. For convenience, antioxidants have been traditionally divided into two classes, primary or chainbreaking antioxidants and secondary or preventative antioxidants. Secondary or preventative antioxidants are compounds that retard the rate of oxidation. Primary antioxidants, when present in trace amounts, may either delay or inhibit the initiation step by reacting with a lipid radical. Chain-breaking antioxidants may occur naturally or they may be produced synthetically as in the case of BHT, TBHQ, BHA and the gallates. The synthetic antioxidants are widely used in food industry and are included in the human diet (Antolovich *et al.*, 2001).

Under normal conditions, all organisms have enzymatic and non-enzymatic mechanisms capable of neutralizing pro-oxidants species and/or repair damages caused by reactive species, converting them to H2O, to prevent overproduction. Many antioxidants of low molecular weight such as vitamins and polyphenols are usually found in nutrients (Barcelos and Navarro, 2012). Some characteristics of PCOS such as obesity and, androgen excess, insulin resistance and abdominal adiposity can develop oxidative stress in these patients. Antioxidant supplementation improve insulin sensitivity and other health threating conditions in women with PCOS. (Amini *et al.*, 2015). Antioxidant enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPX) may inactivate ROS and help to protect oocyte and embryo. A deficiency of antioxidants could be due to decrease in antioxidant intake, synthesis of antioxidant enzymes or increase antioxidant utilization (Panti *et al.*, 2018).

2.8 ROLE OF LIVER MARKER

2.8.1 Alanine aminotransferase

Alanine aminotransferase (ALT) is an enzyme found primarily in the liver and kidney. It was originally referred to as serum glutamic pyruvic transaminase (SGPT). Normally, a low level of ALT exists in the serum. ALT is increased with liver damage and is used to monitor liver disease. Alanine aminotransferase is usually measured concurrently with AST as part of a liver function to determine the source of organ damage. An alanine aminotransferase test measures the amount of this enzyme in the blood. ALT is found mainly in the liver, but also in smaller amounts

in the kidneys, heart, muscles and pancreas. High levels of these enzymes can be a sign that the liver is injured or irritated and the enzymes are leaking out of the liver cells (Hasan *et al*., 2017).

2.8.2 Aspartate aminotransferase

Aminotransferase (AST) is an enzyme that's present in various tissues of our body. An enzyme is a protein that helps to trigger chemical reactions that our body needs to function. AST is found in the highest concentrations in our liver, muscles, heart, kidney, brain and red blood cells. A small amount of AST is typically in our bloodstream.

2.9 Role hormonal imbalance

In PCOS, the ovaries produce upto 60% of androgens, whereas adrenal gland contributes the remaining 40%. That androgens from both the adrenal gland and the ovary are the underlying source of hyperandrogenism in PCOS women. Women with polycystic ovarian syndrome have abnormalities in the metabolism of estrogen and androgen. PCOS can result from abnormal function of the hypothalamic–pituitary–ovarian axis. In mammalian ovaries, LH induces androgen biosynthesis by theca interna cells. However, FSH stimulates aromatase activity by granulosa cells. Co-ordinated action of these two cells and pituitary hormones forms the basis of the two-cell, two-gonadotropin hypothesis for biosynthesis of estrogen. LH and FSH secreted by the pituitary gland in the brain are the hormones that encourage ovulation. LH and FSH usually range between 5 and 20 IU/mL. Most women have equal amount of LH and FSH during the early part of their life. However, there is a LH surge in which the amount of LH increases to about 25–40 IU/mL 24 h before ovulation. Once the egg is released from the ovary, LH level goes back down, although women with PCOS still have LH and FSH between 5 and 20 IU/mL. Their LH level is often two to three times that of FSH level. It is typical for women with PCOS to have an LH level of about 18 IU/mL and FSH of 6 IU/mL. This situation is called an elevated LH-to-FSH ratio. This change in LH-to-FSH ratio is enough to disrupt ovulation. It has been recently shown to elevate androgen production in PCOD women (Amini *et al.,* 2018).

2.10 BISPHENOL A

Bisphenol A (BPA) is one of the most abundant chemical produced worldwide and is used as a plasticizer in daily life. It can interact with estrogen receptors, androgen receptors and peroxisome proliferator-activated receptors gamma. BPA is the potential role of pathogenesis of PCOS. Hypothalamic BPA exposure activates GnRH pulse generator, which in turn leads to the increased LH and decreased FSH. It can stimulate androgen production in the ovarian theca. BPA can also interact with the receptors in adipose tissue and stimulate pancreatic beta cells to insulin production which both result in increased lipid accumulation in the adipose tissue. All of these effects impair ovarian folliculogenesis leading to anovulation (Rutkowska *et al.,* 2014).

BPA has estrogenic activity and can bind to α- and β-estrogen receptors (ER). It has been detected in almost all human body fluids, including follicular fluid, which indicates that oocytes are exposed to BPA during the folliculogenesis process. A large number of animal experiments suggest that BPA may act as a reproductive toxicant and affect fertilization rate, the onset of female puberty and estrous cycle. Moreover, the animal studies have been indicate that BPA has adverse effects on the maturing oocyte and meiotic cell division machinery. The environmental BPA exposure may adversely affect the oocyte quality and cause the decline of ovarian reserve and fertility in the general population, though the magnitude of actual risk in human remains uncertain (Zhou *et al.,* 2016).

Direct actions of BPA in the ovary

BPA accumulates in reproductive organs and disrupts the endocrine system. In the general population, BPA has been detected in follicular fluid at concentrations of ∼1–2 ng/ml [6]. Several epidemiological studies identified correlations between BPA and various abnormalities in the ovary of foetuses and adults. Moreover, the effects of BPA in the ovary, which goes through different stages such as folliculogenesis, ovulation and luteinisation, depend on the time of exposure (Ptak *et al.,* 2017).

Indirect actions of BPA in the ovary through adipokines

Leptin, apelin, chemerin and adiponectin are adipokines that are mainly produced by adipose tissues, but also by other tissues. Adipokines and their receptors are expressed by cells of both the normal and cancerous ovary in humans and other mammals. They play important roles in metabolic processes, such as in the regulation of insulin sensitivity, food intake, adipogenesis and inflammation. Adipokines also regulate ovarian function, including steroidogenesis and oocyte maturation. They also affect ovarian cancer cell proliferation, apoptosis, tumour invasion and angiogenesis (Ptak *et al.*, 2017).

3. METHODOLOGY

Polycystic ovary syndrome is one of the most frequent endocrine and metabolic disorders in premenopausal women. (Murri *et al.,* 2013). It is characterized by high levels of androgens in the body. Many women with this condition have elevated levels of insulin in their bodies (Latha *et al.,* 2015). Metabolic syndrome is a cluster of endocrine-metabolic dysfunction including Insulin resistance, dyslipidemia, hypertension, overweight/central obesity and high risk of cardiovascular disease. The Metabolic syndrome is more common among PCOS women due to the higher prevalence of Insulin resistance and hyperadiposity (visceral) in these women (Spinedi and Cardinali, 2018).

Oxidative stress has been associated with PCOS. The oxidative stress, which is involved in PCOS by causing altered steroidogenesis in the ovaries, which subsequently contributes to increasing androgen levels, disturbing follicular development, and infertility. (Sulaiman *et al.,* 2018). The potential role of Bisphenol A in the pathogenesis of PCOS. Hypothalamic Bisphenol A exposure activates GnRH, which in turn leads to increased LH and decreased FSH. It can stimulate androgen production in the ovarian theca. Bisphenol A can also interact with the receptors in adipose tissue and stimulate pancreatic beta cells to insulin production which both result in increased lipid accumulation in the adipose tissue. All of these effects impair ovarian folliculogenesis leading to anovulation (Rutkowska *et al.,* 2014)

3.1 PHASE I

EVALUATION OF CLINICAL FEATURES AND PREVALENCE OF PCOS WOMEN OF SELECTED AGE GROUP

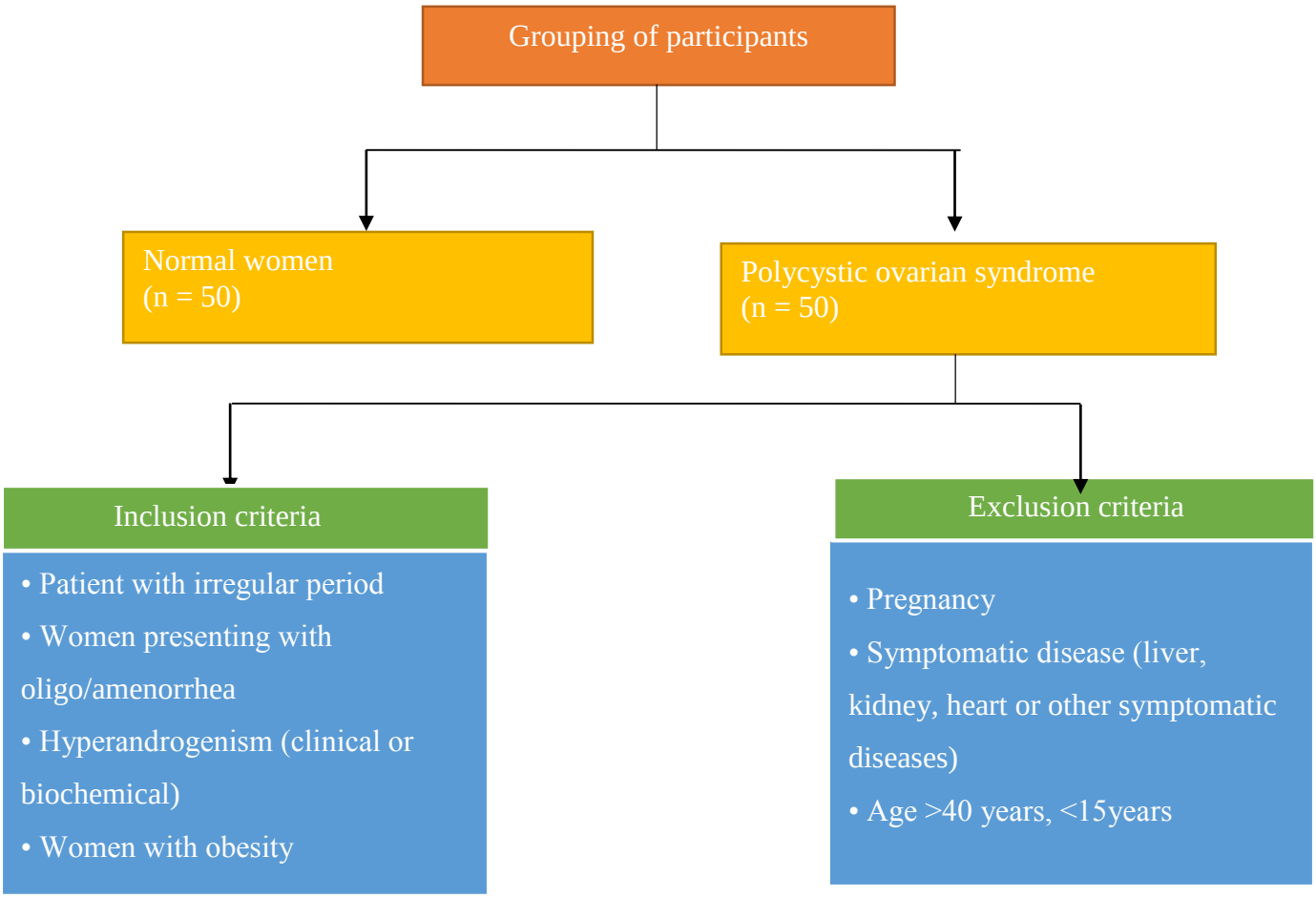
PCOS is a major public health concern in terms of a frustrating experience for women and a challenging complex syndrome for clinicians. Globally, prevalence estimates of PCOS are highly variable, ranging from 2.2% to as high as 26% of this age group depending on how it is defined. PCOS is associated with a wide spectrum of presenting features, including anovulation obesity and abnormal facial and skin hair growth (Guptha *et al.,* 2018)

PCOS is currently considered as a lifestyle disorder affecting 2.2-26% of young girls in their reproductive age in India. The signs of PCOS include excessive hair growth on the face and abdomen, acne, irregular or absent menstrual periods, failure of ovulation, and reduced fertility. PCOS usually begins at or soon after puberty and is a lifelong condition. Most clinical data suggests a prevalence of 6–7% of the population. The present Rotterdam criteria are current best practice but it is recognized that PCOS encompasses a wide spectrum of disorder, overlapping with normality. (Nivetha and suganya, 2016)

The experimental procedure followed for the study entitled, "**Role of Oxidative stress and Detection of Bisphenol A levels in women with Polycystic ovarian syndrome"** was studied under two different phases

3.2 Study design

The study protocol was approved by Institutional Human Ethics Committee (IHEC). The approval number is **IHEC/16 – 17/BC – 02**. A written informed consent was obtained from each of the participantts.



3.3 Selection of the participants

50 Women with PCOS and 50 women with normal reproductive cycles were selected from Avinshilingam institute for home science and higher education for women in Coimbatore. All cases met the diagnostic criteria of PCOS, which defines a PCOS patient as one who must have symptoms of oligomenorrhea and amenorrhea on abnormal uterine bleeding as well as one of the two following symptoms: hyperandrogenism and polycystic ovaries. Patients with malignant tumor, cardiovascular diseases, sever chronic diseases and psychiatric issues were excluded from the sample.

3.4 Grouping of participants

PCOS patients were selected based on the Rotterdam's 2003 criteria. After fulfilling the selection criteria, all women were counselled about the study and informed written consent was obtained. A detailed history was obtained from the participants for intake of any hormonal drugs, including OCP as well as medication for lowering blood pressure, blood lipids and glucose. Menstrual history in detail was taken. Secondary amenorrhea was defined as an absence of menstrual cycles more than 6 months. Oligomenorrhea was defined as a delay in menstruation for >35 days to 6 months. Family history was obtained regarding diabetes mellitus and hypertension in the first and second degree relatives, menstrual disorders, hirsutism and early baldness in male relatives. Patients were screened for clinical signs of hyperandrogenism (acne, oily skin and hirsutism). Clinical hyperandrogenism was defined using a modified Ferriman-Gallawey (FG) score for evaluating and quantifying hirsutism in women using nine body areas (upper lip, chin, chest, upper and lower abdomen, thighs, upper and lower back and upper arm). Hair growth was rated from 0 (no growth of terminal hair) to 4 (extensive hair growth) in each of the nine locations. A score >/= 8 was indicative of androgen excess. Height in cm, weight in kg, waist circumference in cm, hip circumference in cm was measured. Additionally, blood pressure is measured in patients in sitting position,>/= 140/85 was considered as hypertension. Obesity was assessed according to WHO criteria as a body mass index (BMI). Calculated as BMI=weight/height^2, kilogram per meter^2. Classified as <18.5 Underweight, 18.5-24.9 Normal, 25-29.9 Overweight, 30-34.9 Obese, >=35 morbid obese. Body fat distribution was assessed by measurements of the waist to hip girth ratio (WHR). A WHR <0.85 was considered normal. Fasting lipid profile (FLP) was done in PCOS

patients to diagnose dyslipidemia and metabolic syndrome, normal values taken were TC<200, HDL >50, LDL <130, TG <150 and VLDL <50. Clinical features, associated diseases, family history, hormone levels and ultrasonography results were all analysed.

3.5 Data collection

Self-designed questionnaire was given to both experimental and control groups. Data collected include age, address, BMI, age of menarche, marital status, menstrual disorder, mood, family history of infertility and levels of physical exercise.

3.6 Collection of blood sample

Blood sample for the experimental procedure was collected from selected age women. Biochemistry tests for free radical scavenging and antioxidant activity assay, total cholesterol, ALT, AST and bisphenol A were carried out using auto analyzer and HPLC.

3.7 Phase II – measurement of oxidative status

3.7.1 FRAP ASSAY

The FRAP assay was estimated by the method of Maruthamuthu and Kandasamy, (2016) as described in APPENDIX – I.

3.7.2 TBARS ASSAY

TBARS was estimated by the method of Bishayee and Balasubramaniam, (1971) as given in APPENDIX – II

3.7.3 Estimation of vitamin A

Vitamin A was estimated by the method of Bayfield and cole, (1994) as detailed in APPENDIX - III

3.7.4 Estimation of vitamin C

The ascorbic acid (vitamin C) was estimated by the method of Roe and Kuther, (1953) as described in APPENDIX – IV

3.7.5 Estimation of vitamin E

The α- tocopherol (Vitamin E) was determined by the method of Rosenberg, (1992) as given in APPENDIX – V

3.8 Estimation of total cholesterol

The level of total cholesterol in serum was determined by Allain *et al.*, 1974, method as depicted in APPENDIX – VI

3.9 Estimation of aspartate transaminase (AST/SGOT)

AST/SGOT was determined by the method of Reitman's and Frankel's, (1957) as given in APPENDIX – VII

3.10 Estimation of alanine transaminase (ALT/SGPT)

ALT/SGPT was determined by the method of Reitman's and Frankel's, (1957) as detailed in APPENDIX – VIII

3.11 MEASUREMENT OF BISPHENOL A LEVEL

Bisphenol A is an endocrine disruptor that can mimic estrogen and it has been causes negative health effects in animals and human. Bisphenol A closely mimics the structure and function of the hormone estradiol with the ability to bind and activate the same estrogen receptor as the nature hormone. Bisphenol A level was measured by the HPLC method.

3.12 Statistical analysis*:*

The categorical variables were described by percentages and the continuous as mean ± standard deviation. We used the independent Student's t-test to compare the values of the means between cases and controls. Differences in categorical characteristics between cases

and controls were assessed using $\chi 2$-test. Result values were expressed as mean ± SD, number of patient or percentage.

4. RESULTS AND DISCUSSION

Polycystic ovarian syndrome (PCOS) is the commonest endocrine system disorder among females in their reproductive age presented by menstrual dysfunction. Insulin resistance, hyperandrogenism and polycystic ovaries can lead to depression, social and marital crises and sexual dysfunction. Majority of patients with PCOS have overweight or obesity (Hanif *et al.,* 2015). This state corrects on its own after few years resulting into regular ovulatory cycles as the H-P-O axis matures. In some young girls however anovulation persists and continues as chronic anovulation. The resulting hyperestrogenism, leads to hyperandrogenism, manifesting clinically as irregular menses, amenorrhea, menorrhagia, hirsutism, acne, oily skin, alopecia, obesity or a propensity for weight gain and multiple unruptured follicles in ovaries, collectively known as polycystic ovarian syndrome (Choudhary *et al*., 2017). The imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system, which produces the oxidative damage is known as oxidative stress. Reactive oxygen species can affect a variety of physiological functions in the reproductive tract. When ROS increase to pathological levels such as in PCOS, they are capable of inflicting significant damage to cell structures. Moreover the body's defense mechanisms would play a role in the form of antioxidants and try to minimize these damages, thereby adapting itself to the above stressful situation. Reproductive cells and tissues remain stable when ROS production and the scavenging antioxidants remain in balanced state. Levels of ROS are controlled and kept at physiological levels within the ovary by various antioxidant systems vitamin C, which is known to have a protective effect within the follicle (Shirsath, 2015).

Natural antioxidants are required to prevent and cure disorders caused by free radicals. The free radicals are highly reactive chemical species produced in the body and have the potential to damage cells, DNA, organelles and other biomolecules, resulting in diseases such as cancer, cardiovascular disease and neurodegenerative disease. (Ahmed *et al*., 2015). PCOS is accompanied by oxidative stress in which increased production of free radicals is followed by decreased levels of serum total antioxidants. Possible contributors to oxidative stress in obesity include hyperglycemia, hyperleptinemia and augmented muscle activity to carry excessive weight, chronic inflammation and inadequate antioxidant defenses. Oxidative stress is increased in diabetes and also in obesity (Faris *et al*., 2017).

4.1 PHASE – I: Assessment of clinical features among normal and PCOS women

4.1.1 Anthropometric characteristics of the participants of the study

4.1.2 Age wise distribution of normal and PCOS subjects

4.1.3 Distribution of subjects according to BMI

4.1.4 Prevalence of menstrual cycle abnormalities in PCOS women

4.1.4.1 Distribution of irregular periods in PCOS women based on age

4.1.4.2 Phenotypic variables in PCOS and controls

4.1.5 Prevalence of skin disorder among normal and PCOS women

4.1.6 Prevalence of depression in women with and without PCOS of selected age group

4.1.7 Prevalence of hypertension in women with and without PCOS of selected age group

4.1.8 Family history of PCOS women

4.1.1 Anthropometric characteristics of the participants of the study

The phenotypic and genotypic data from PCOS (50) cases and controls (50) were obtained using the questionnaire. The mean values for age, height, weight and BMI among PCOS and controls are presented in table 1. The average age is 25 in PCOS women and 23 in normal subject. The average (Mean±SD) weight, height and BMI among the PCOS are 57.57±15, 156.45±6 and 24.05±6 respectively. The mean values for controls are 50.25±9, 157.60±6 and 20.17±3. BMI of the women with PCOS is found to be 24.05 ± 6, significantly higher BMI than the control group.

Table-1 Anthropometric profile of the participant of the study

Parameter	**PCOS group (n=50)**	**Control group (n=50)**
Age (years)	26 ±6	23±5
Weight (Kg)	57.57± 15	50.25± 9
Height (cm)	156.45± 6	157.60± 6
BMI (Kg/m2)	24.05± 6	20.17± 3

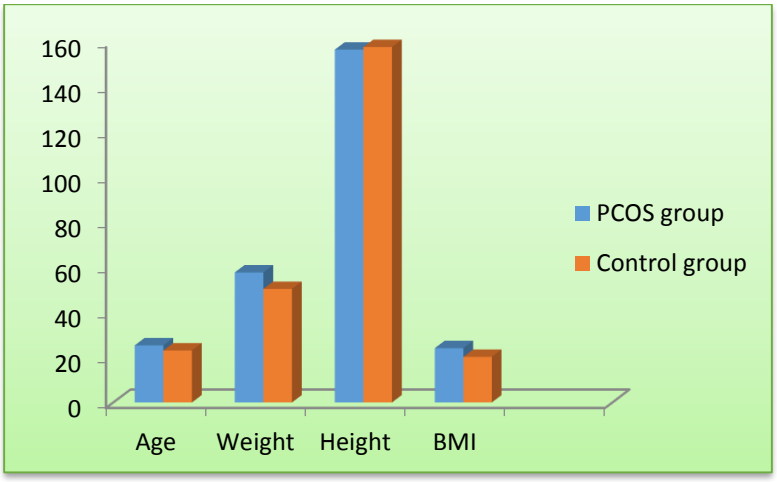


Figure 7. Anthropometric details of participants and control subjects

The present study showed that both groups are properly matched as far as age is concerned and there is no difference for both groups. Similar type of results were reported by panda *et al.* (2016).

The mean BMI of the PCOS group was higher than that of the control group, and the prevalence of overweight and obesity were significantly higher in women with PCOS, showing a similar relationship between PCOS incidence and BMI (Chitme *et al.*, 2016). Similar report was given by Smitha *et al.* (2018) regarding BMI. There was no significant difference between the mean body mass index of the two groups, but the mean of the waist circumference was significantly higher in the PCOS group, compared to the control group (Ahmadi *et al* ., 2013)

The mean waist circumference value for PCOS women is 37.67± 9 and 31.10± 4 in control group. The average hip circumference for PCOS women is 38.53± 8 and 35.20± 4 in control. The waist hip ratio in PCOS women is 0.96 ±0.16 and the control group is 0.852±0.12. The mean of the waist circumference, hip circumference and WHR (waist-hip-ratio) was significantly higher in the PCOS group, compared to the control group.

Table-1a Anthropometric characteristics of the participants

Variable	PCOS group **(n=50)**	Control group **(n=50)**
Hip circumference (cm)	38.53± 8	35.20± 3
Waist circumference (cm)	37.67± 9	31.10± 4
WHR	0.966±0.2	0.852±0.1

Central obesity characterized by increased waist circumference is another add-on risk factor for the development of metabolic diseases. It mainly happens due to excessive consumption of fast food, irregular eating habits leading to large fluctuations in blood glucose levels thereby hormonal imbalance posing higher risk for the development of PCOS.

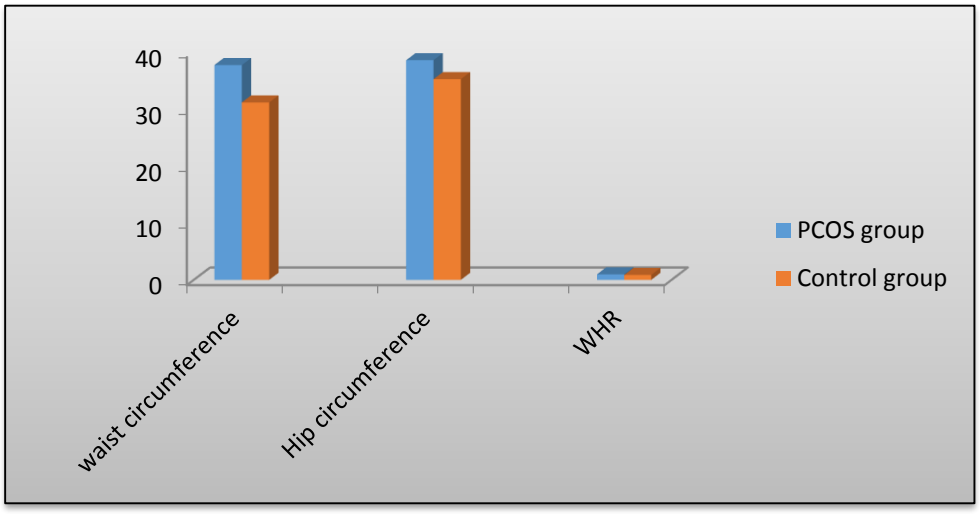


Figure 8- Anthropometric characteristics of the participants

Waist circumference measurement is a better predictor of central obesity than BMI. Several studies have described endocrine and metabolic differences between lean and obese women with

PCOS. In addition to alteration in insulin sensitivity that was independent of obesity, these studies have demonstrated more marked hyperandroge**ne**mia, insulin resistance and relative hyperglycemia and lower sex hormone binding globulin (SHBG) in obese compared with lean women with PCOS (Jefout *et al.,* 2017).

In women with PCOS. These observations support the findings in several studies, which have shown According to Chitme *et al.* (2016) the prevalence of overweight, obesity and central obesity were significantly higher that increased BMI directly and significantly increases the incidence of PCOS. Higher percentage of PCOS patients were in the category of large and extra-large circumference than reported previously in women only having PCOS.

4.1.2 Age wise distribution of normal and PCOS subjects

Among the 50 people with PCOS group, 15 (30%) participants were aged up to 18 years, 20 (40 %) participants aged between 19 to 25 years, 15 (30 %) participants aged above 30 years. Among the 50 people without PCOS group, 18 (36 %) participants aged up to 18 years, 20 (40%) participants aged between 19 to 25 years, 12 (24 %) participants were aged above 30 years. The difference in the proportion of PCOS group and age group was statistically not significant.

The women diagnosed with PCOS are between 16 and 35 years old. Mean age in PCOS group is 25 and 26 in the control group.

Table 2: Comparison of age among PCOS and control groups

Age in total years	**PCOS (n=50**)		**Control (n=50**)	
	n	percentage	n	Percentage
< 18	15	30	18	36
18- 25	20	40	20	40
>25	15	30	12	24
Total	50	100	50	100
Mean age(years)	25		26	

Table 2 indicates the mean age of PCOS women which was found to be 25 and in control group it was found to be 26 which was more are less same. It's clear that both groups are properly matched as far as age is concerned. So there is no significant difference for age in both the groups.

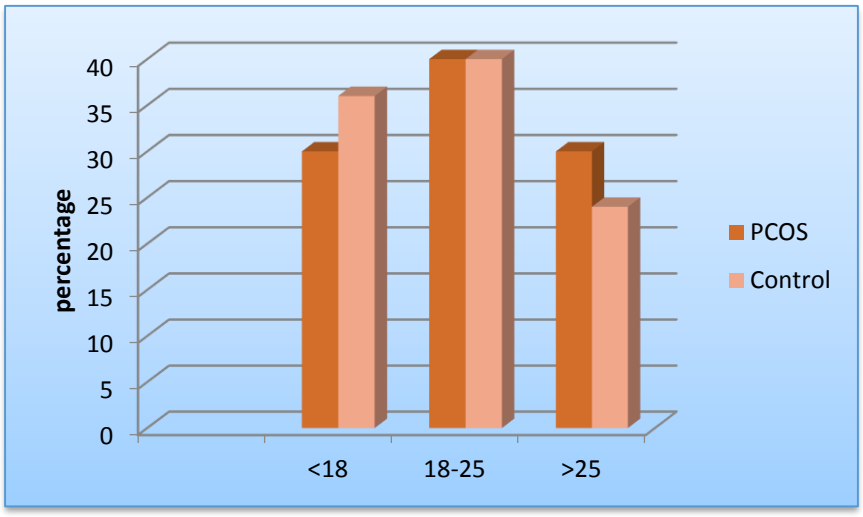


Figure 9- Comparison of age among PCOS and control groups

Diagnosis is recommended by NIH L (1999) criteria or Rotterdam criteria, with polycystic ovaries, androgen excess and an ovulation (any two criteria) in our observation from above study there is a rising incidence of PCOS in young women. Bronstein *et al.* (2011) found that not only there is a rising incidence but PCOS may be occurring at a younger age than previously thought. Changing lifestyle, eating habits, lack of physical exercise increased incidence of adolescent obesity and rising stress levels may be responsible. Adolescent girls with PCOS have a higher prevalence of metabolic syndrome than normal adolescent girls with 3.8 times increased risk. Hyperandrogenamia in these girls is responsible for metabolic syndrome independent of obesity and insulin resistance. In recent years India has witnessed a 30% rise in the incidence of PCOS in young adolescent girls due to changes in lifestyle (Coviello, *et al.*, 2006).

4.1.3 Distribution of participants according to BMI

Obesity is reported to be associated with various diseases such as cardiovascular, metabolic syndrome and Insulin resistance. In particular, it was also demonstrated that the obesity have adverse effects on reproductive potential in females leading to impaired ovulation, irregular

menstrual cycle, high rate of miscarriage, lower implantation and pregnancy rates.

Table 3: Distributions of subjects according to BMI

BMI	**PCOS case (n=50)**		**Control (n=50)**	
	n	percentage	n	Percentage
Normal body weight (BMI 18-25)	14	28	20	40
Over weight (BMI 25-30)	17	34	14	28
Obese (BMI >30)	9	18	6	12
Total	50	100	50	100
Mean BMI	24.05±6.08		20.17±3.18	

From the table 3 it is evident that prevalence of PCOS is more in over weight (34%) and obese (18%) than the normal women.

Obesity is common in women who have PCOS. Presence of obesity is a risk factor to amplify the consequences of PCOS. It increases the risk for metabolic dysfunction. Insulin resistance is worsened by the presence of obesity. The prevalence of obesity in these women varies, many patients of PCOS have a normal BMI. Obesity is not essential to make the diagnosis of PCOS. The results of present study show that PCOS was present in both obese and nonobese subjects.

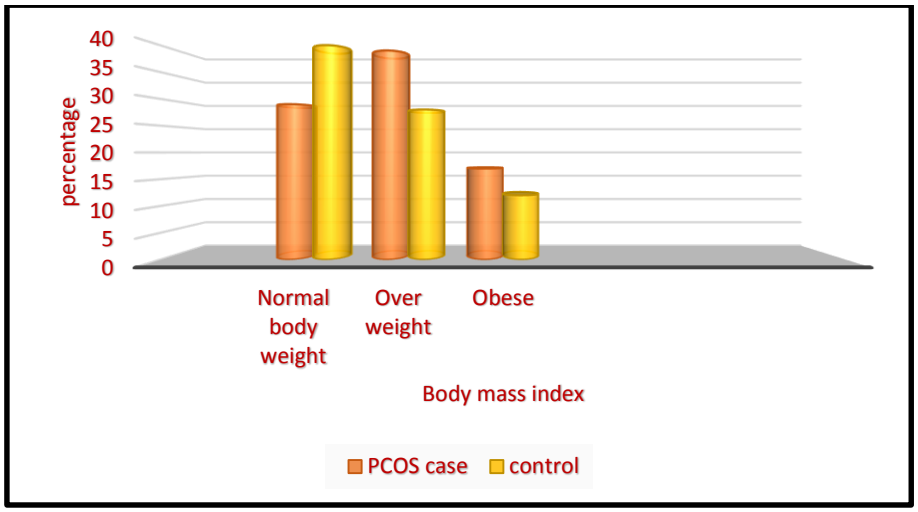


Figure 10- Distributions of subjects according to BMI

The prevalence of obesity in women with PCOS is highly variable depending on age, ethnicity and geographic regions in the general population. The cut-off BMI with body fat as standard consensus statement for Indian population was considered, as follows i.e., normal BMI: 18.0 – 22.9 kg/m2, over weight: 23.0 – 24.9 kg/m2, obesity: >25 kg/m2. Considering the significance, the anthropometric data was compared among the study subjects. The mean B MI in PCOS did not deviate from controls. Through BMI positively affect the development of PCOS, it is not observed in all the prospective studies (Misra *et al.*, 2012).We observed that obese participants are at 1.74 times more risk for development of PCOS compared to participants with normal BMI. This is probably because of aggregation of factors that is lack of physical exercise and unhealthy dietary habits.

4.1.4 Prevalence of menstrual cycle abnormalities in PCOS women

The precise triggering factor(s) and the chronology of events which lead to PCOS remain less wellknown. During pubertal development, adolescents typically have relative androgenemia, insulin resistance, cystic ovaries and anovulatory cycles, which transits to an estrogenic state later in puberty. Failure to make this transition may result in regular menstrual cycles (28 to 35 days cycle). Anovulation is the pathognomic feature of PCOS and results in irregular menstrual cycles. Therefore, persistent menstrual irregularities (resulting from anovulation) seem to be better predictors compared to biochemical parameters. Oligomenorrhea is one of the diagnostic criteria

of PCOS. Documentation of anovulation is usually not necessary in view of menstrual irregularities with periods of amenorrhea (Ramanand *et al.*, 2016).

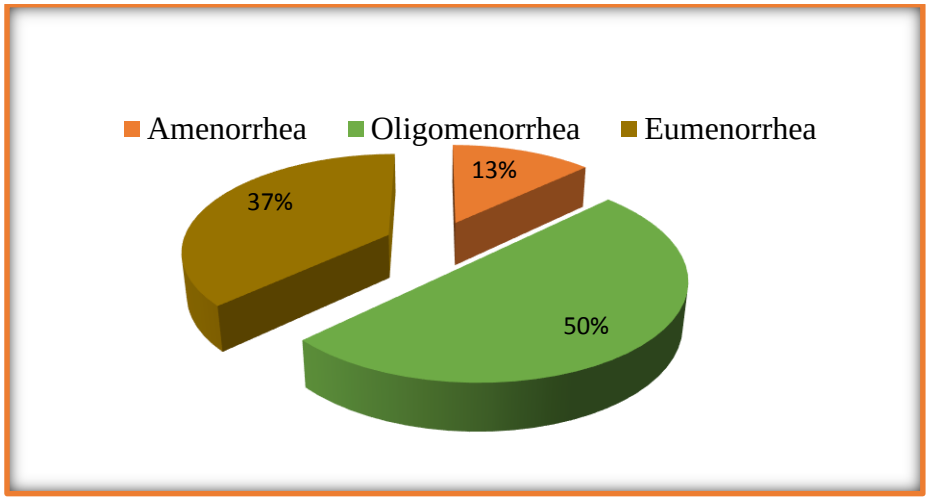


Figure 11 - Distribution of women diagnosed with PCOS according to menstrual cycle

This study also found that from a total number of 75 women included, the higher percentage of PCOS patients are with oligomenorrhea 37(50 %), with amenorrhea 10(13 %) and cases with regular menstrual cycle are 28 (37%). Though more number of obese patients had oligomenorrheoa, the difference between obese and non-obese w not significant. Oligomenorrheoa is considered as a highly predictive surrogate marker of PCOS. Approximately 85-90% of women with oligomenorrhea have PCOS, usually defined as cycle length greater than 35 days.

According to Fouzia Hanif et al. (2015) PCOS patients have the additional ratio of oligmenorrea as 59 %, amenorrhea as 5% and eumenorria as 5%. About 27.3% of the women in the study group had menstrual disturbance, oligomenorrhea being the most common (Viswanathan *et al.*, 2016).

4.1.4.1 Distributions of irregular periods in PCOS women based on age

Menstrual irregularity is a common gynaecological condition affecting especially women in early reproductive life. Women with menstrual irregularity at 16 years had a significantly higher prevalence of menstrual irregularity suggestive of anovulatory cycles, PCOS than women with

regular menstrual cycle. Less than 18 PCOS group showed an increased percentage of irregular period. Many factors can cause a hormonal imbalance, from polycystic ovarian syndrome to extreme weight loss and excessive exercise.

Table 4 indicates the distribution of irregular periods in PCOS women based on age according to the methods of contraception. There may be an imbalance of the reproductive hormones estrogen and progesterone. A wide range of factors influence menstrual cycle characteristics including body size, smoking, alcohol intake and physical activity as well as pathologic conditions including polycystic ovary syndrome (Harris *et al.,* 2017).

Table 4: Distribution of irregular periods in PCOS women based on age

Age	No. of. Subjects studied	No. of subjects having irregular periods	Percentage of women with irregular periods
< 18	20	18	90
18-25	40	34	85
>25	15	11	73

Menstrual irregularity in adolescence has been shown to be a good marker of hyperandrogenaemia and it has been proposed to lead to the development of PCOS in adulthood (Lewy *et al.*, 2001). In addition, adolescent girls with irregular menstrual cycles have higher androgen levels than girls with regular menstrual cycle (Pinola *et al.*, 2012).

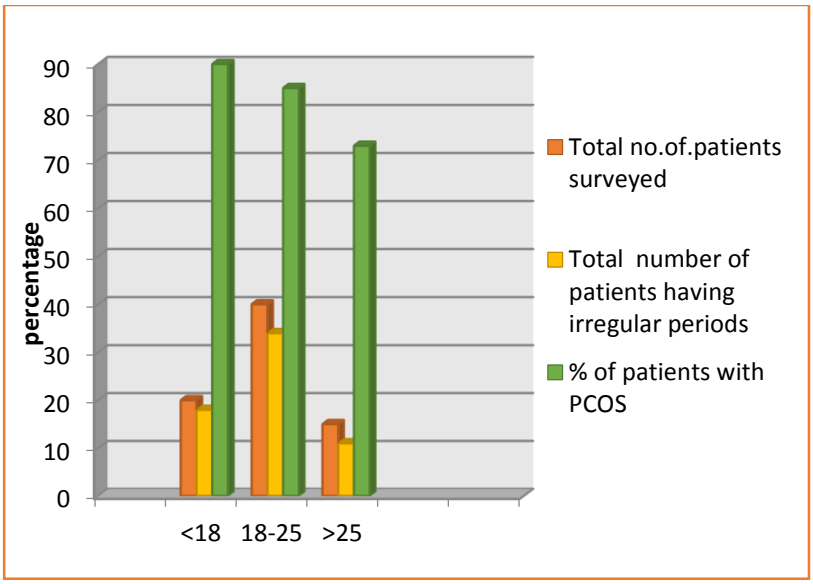


Figure 12- Distribution of irregular periods in PCOS subgroups based on age

Longer menstrual cycle length and irregular cycles have been associated with higher androgen and lower sex hormone binding globulin levels (SHBG) and this altered hormonal environment may increase the risk of specific histologic subtypes of ovarian cancer. Cirillo *et al*. (2016) recently reported that among parous women, those with irregular menstrual cycles had a two-fold increase in ovarian cancer risk.

In a study by Nidhi *et al.* (2013) prevalence of PCOS in Indian adolescents was found to be 9.13%. Majority of women present with irregular periods but women with regular menses may have anovulation and PCOS. Irregular period is the characteristic feature of PCOS women (Begum *et al.*, 2012 and Edison *et al.*, 2016).

4.1.4.2 Phenotypic variables in PCOS and controls

Age at menarche, duration of menstrual cycles were collected between PCOS and control group. Menarche attained by PCOS group were 30% in 10-12 years, 30% between 13-15 years at the same age group, it was 11% and 39% in the control subjects. Age at menarche showed a less significant difference between PCOS and controls (p-value<0.05). While, control group shows a regular menstrual cycle occurring in normal intervals (25 to 34 days at an average of 28 days counting from first day of menstruation to next one), among the PCOS group 28% experienced

25-37 days and 72% are >37 days. Days between me nstrual are highly significantly differed between PCOS and controls (p-value<0.05).

Table 5- Phenotypic variables in PCOS and controls

S.NO	Phenotype	PCOS (n=50)	Control (n=50)
1	Menarche		
	10-12 (yrs)	15 (30%)	11 (22%)
	13-15(yrs)	35 (70%)	39 (78%)
	>16	0	
2	Duration of menstrual cycles		
	25-37 days	14 (28%)	48 (96%)
	>37 days	36 (72%)	2 (4%)

4.1.5 Prevalence of skin disorder among normal and PCOS women

PCOS is one of the most common endocrine disorders in women of reproductive age and can be associated with multiple long-term health risks and substantial psychological impact. The dermatologic manifestations of PCOS play a significant role in diagnosis and constitute a substantial portion of the symptoms experienced by women with this syndrome. Patients must be counseled regarding the long duration of treatment that in cludes lifestyle modifications along with systemic treatment (Keen and Sheikh, 2017).

Acne is one of the common skin disorders. It has a possible correlation between PCOS and androgenism in females. Acne develops earlier in females than in males, it often reflects early puberty. Acne is an inflammatory disease of pilosebacious glands. Severity of disease changes from one individual to other. The pathogenesis of acne is a complex one (Tom *et al.,* 2008). The main factors involved in the aetiology of acne are seborrhoea, sex hormones, hyperandrogenism, propionibacterium and its colonisation (Simpson *et al.,* 2004). Acne was graded according to WHO criteria in grades. The prevalence of PCOS among the women with acne was found to be 40%. According to an association between acne and clinical markers of androgenecity was revealed. It was found that patients aged above 20years are more likely to get acne.Hirsutism is

defined as the presence of terminal hair with male distribution in women, and polycystic ovary syndrome (PCOS) is the most common etiology of hirsutism.

Table -6 indicates the hirsutism, acne and skin pigmentation of normal and PCOS women. Acne, hirsutism and skin pigmentation was found to be high (84%, 74% and 72%) in PCOS subjects and showed a significant difference between PCOS and controls (p-value<0.05).

Table 6- Prevalence of skin disorder among normal and PCOS women

S.NO	Skin disorder	PCOS (N=50)	Control (N=50)
1	Acne		
	Yes	37 (74%)	14 (28%)
	no	13 (26%)	36 (22%)
2	Hirsutism		
	Yes	42 (84%)	0 (0)
	No	8(16%)	50 (100%)
3	Skin pigmentation		
	yes	36 (72%)	9 (12%)
	no	14 (28%)	41 (82%)

Young adolescent girls experience full range of symptoms from irregular menses, amenorrhea, menorrhagia, hirsutism, acne, skin pigmentation, alopecia and ovarian cysts. Other symptoms like anxiety, depression, thyroid problems and galactorrhea, may exist. Obesity or propensity to weight gain is a common feature, though it is not uncommon in non-obese women. Characterized by chronic anovulation, hyperandrogenism, hormonal imbalance and metabolic disorder, it has an unclear origin.

Hyper androgenism is a main feature of PCOS. It is responsible for the male type obesity, hirsutism, oily skin, acne and alopecia. We found that 64% women had varying degrees of hirsutism, 54% with acne and 24% had alopecia. Andrea Hsu Roe quoted that majority of women (80%) show signs of androgen excess and androgen excess plays a major role in the pathophysiology of the condition in PCOS.

The prevalence of hirsutism in our study group of PCOS women was found to be 84%. Some studies reported a prevalence of hirsutism in women with PCOS in the range of 50–76%. Saxena *et al.* (2010) in their study reported that the prevalence of hirsutism was 89% and 80% in obese and lean PCOS, respectively.

The next common clinical manifestation of hyperandrogenism noted in our study was acne, which was seen in 74% of the women and hirsutism was seen in 84% of women. According to Wijeyaratne et al. (2014) 74.6% of the PCOS patients had significant hirsutism and acne was present in 39.2% patients.

The difference in frequency of oligmenorrea, hirsutism and acne in different studies as compared to our study may be due to difference in diet, atmospheric conditions, socioeconomic status and level of exertion of our patients. It has proved that age ≥35 years, BMI ≥25 kg/m2 and acne are as significant predictors of metabolic disorder in PCOS women (Usmani *et al.,* 2014).

4.1.6 Prevalence of depression in women with and without PCOS of selected age group

Women who are diagnosed with PCOS are at high risk of developing a variety of symptoms. Many women with PCOS struggle with mood disorders including depression. Others have lower mood issues which manifest as irritability, lethargy and short temper. The fact that PCOS can make a women feel physically uncomfortable, emotionally insecure or unfeminine.

Table 7: Prevalence of depression in normal and PCOS women

Subjects	Depression	
	n	Percentage
Women with PCOS	47	63
Women without PCOS	9	36

Table 7 indicates the prevalence of depression in women with and without PCOS subjects. The depression was found to be higher (63%) in PCOS subjects and showed significant difference between the PCOS and control.

4.1.9 Prevalence of hypertension in women with and without PCOS of selected age group

Women who are diagnosed with PCOS are at high risk of developing a variety of symptoms, some of which are more devastating such as heart disease which may arise due to high blood pressure (hypertension). Insulin resistance (IR) and hyperandrogenism are the key hormonal abnormalities in PCOS. IR occurs in the majority of women with PCOS and has been demonstrated in 75% of lean and 95% of overweight PCOS women. IR in PCOS occurs independently of obesity but is also exacerbated by obesity. In this setting, the majority of women exhibit clustering of metabolic features including visceral obesity, IR, dysglycemia, dyslipidemia and potentially hypertension.

The association between PCOS and hypertension remains unclear. It is postulated that IR may be a potential mechanism for hypertension in PCOS, yet studies of hypertension in PCOS are inconsistent. Some show no difference in 24-hour ambulatory blood pressure (BP) between women with PCOS and age- and sex-matched controls.

Table 8- Prevalence of hypertension in normal and PCOS women

Subjects	**Hypertension**	
	n	percentage
Women with PCOS	47	63
Women without PCOS	9	36

Relationship between hypertension, BMI and PCOS Of women reporting hypertension, mean BMI tended to be higher in women reporting PCOS compared to women not reporting PCOS, although not significantly (BMI: 29.7 vs. 28.9 kg/m2, P = 0.72). In the normal BMI category, the proportion of reporting hypertension was higher in women with PCOS than women without PCOS (5.1% of women with PCOS vs. 1.0% of women without PCOS, P < 0.001). In the overweight and obese BMI categories, the proportion of reporting hypertension was similar between women with and without PCOS (4.0% of overweight women with PCOS vs. 2.0% of overweight women without PCOS, P = 0.19 and 6.9% of obese women with PCOS vs. 4.9% of obese women without PCOS, P = 0.31).

The prevalence of hypertension was higher in women reporting PCOS, compared to control women (5.5% vs. 2.0%, P < 0.001). However, the trend towards an association between PCOS and hypertension and a clear association between BMI and hypertension. Women reporting PCOS had higher BMI, yet hypertension was only associated with BMI in women not reporting PCOS (Joham *et al.*, 2015).

The higher mean ambulatory BP and higher daytime systolic BP in women with PCOS compared to BMI-matched controls, with BP differences persisting after adjustment for adiposity and IR was found by Holte *et al.* (1996).

Hypertension in PCOS despite significant IR. It is hypothesized that hyperandrogenism seen in women with PCOS may be associated with hypertension. Androgen levels were significantly and positively correlated with both systolic and diastolic BP in women with PCOS.We show a higher prevalence of hypertension in women with PCOS, which may be related to the higher BMI in the PCOS group. This is consistent with epidemiological studies that have revealed a strong relationship between obesity and hypertension.There is an evidence of hypertension noted in 15% of women with PCOS (Chen *et al.*, 2007).

4.1.8 Prevalence of family history of PCOS among the selected patients

The main risk factor for polycystic ovarian syndrome (PCOS) is family history of it. The chance of having it is higher if other women in the family have PCOS or the irregular periods or diabetes. Cases of PCOS cluster in family's revealed hereditability of both hyperandrogenaemia and hyperinsulinaemia in affected siblings.

Table 9: Family history of PCOS women

Age	Women of PCOS with obese		Women of PCOS without obese	
	n	percentage	N	Percentage
< 18	4	36	3	33
18-25	7	32	5	27
>25	2	22	2	33

The presence of a genetic component to PCOS and familial clustering of reproductive and metabolic abnormalities results in increased risk of PCOS among first-degree relatives of PCOS patients. This is evident from our study results where participants with a positive family history of PCOS carried little higher risk of the development of PCOS.

Some evidence suggests that women with PCOS may also be at an increased risk of having a personal history of ovarian and breast cancer and high levels of hormones, particularly estrogens are casually associated with increased breast cancer risk. However, in our study, there was not any case of ovarian or breast cancer either in PCOS patients or in total infertile population. This contradiction may be due to several factors. First, it is probable that estrogens act as promoters rather than being directly causal. Even as promoters, lifetime exposure to estrogens is not necessary. The cause is most probably a lifestyle factor, changes wherein can rapidly alter risk. It has been suggested that a high body mass index (BMI) can be associated with irregular or long menstrual cycles or with PCOS. It has been suggested that anovulation, which is associated with such characteristics and with decreased estradiol and progesterone levels, may explain the lower risk of breast cancer in these women.

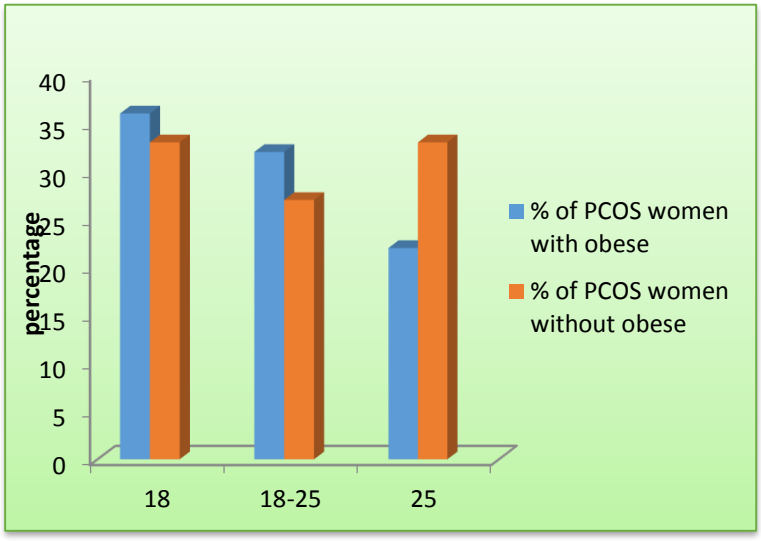


Figure 13- Family history of PCOS women with and without obese

The frequency of diabetes was significantly higher in women with PCOS and their relatives. Collina *et al.* (2001) noted that there was a heritable component of B-cell dysfunction in

the families of women with PCOS. The rate of diabetes in maternal l family was higher than in paternal family. Whether maternal or paternal inheritance is more significant in diabetes has been a matter of controversy and differs in various populations and races.

PHASE II

4.2 ASSESMENT OF OXIDATIVE STRESS BIOMARKERS IN NORMAL AND PCOS SUBJECTS

4.2.1 Thiobarbituric acid reactive substances (TBARS) assay

4.2.2 Ferric reducing antioxidant power (FRAP) assay

4.2.3 Levels of non – enzymatic antioxidants in normal and PCOS women

4.2.4 Assessment of total cholesterol level in PCOS and normal subjects

4.2.5 Assessment of Liver marker enzymes in PCOS subjects and control

4.2 Assessment of oxidative stress biomarkers in normal and PCOS subjects

Oxidative stress has been implicated as a causal factor for hyperandrogenism in these women. Leucocyte ROS generation, p47phox gene expression, and plasma TBARS to promote oxidative stress in the presence of hyperglycemia. Thus in PCOS, hy perandrogenism may be the progenitor of diet-induced oxidative stress independent of obesity or excess abdominal adiposity (Gonzalez *et al.,* 2012)

Oxidative stress was linked to obesity commonly seen in these PCOS women. Oxidative stress has been implicated in a number of diseases such as cardiovascular disease, neurological disease, malignancies, renal disease, diabetes, inflammatory problems, skin diseases, aging, respiratory diseases, liver diseases and different types of viral infections. (Sundharan *et al.*, 2016). Two mitochondrial ATP synthesis enzymes, NADPH oxidase and xanthine oxidoreductase, are associated with the generation of ROS, mainly the superoxide radical. Oxygen is needed to generate energy for folliculogenesis and oocyte maturation, and ROS production is inherent in these processes (Agarwal *et al.*, 2005).

4.2.1 Thiobarbituric acid reactive substances (TBARS) assay

Malondialdehyde is one of the final products of lipid peroxidation, and it is a stable product, it can be used as a cumulative measurement of this process (Petean *et al.,* 2008). Androgen excess in PCOS women increases leukocytic ROS generation, p47phox gene expression, and plasma TBARS to promote oxidative stress in the presence of hyperglycemia in healthy reproductive-age women. Thus in PCOS, hyperandrogenism may be the progenitor of diet-induced oxidative stress independent of obesity or excess abdominal adiposity. This could explain the presence of oxidative stress in PCOS women (Desai *et al.,* 2014)

In the presence of pelvic endometriosis, there would be macrophage activation in the peritoneal cavity that might promote oxidative stress. Peroxidized lipids when undergoing decomposition may generate products such as malondialdehyde (MDA) and may be recognized as foreign bodies, triggering an antigenic response with the consequent production of antibodies. This process may result in oxidative damage to red blood cells and to endometrial and peritoneal cells, which in turn may stimulate the recruitment and activation of more mononuclear phagocytes, perpetuating the oxidative damage to the pelvic cavity (Petean *et al.*, 2008).

MDA levels, which were very harmful effects on the cells, MDA levels are higher in men compared to women. Hyperandrogenemia in PCOS may be the reason for these higher MDA levels (Karabulut *et al.*, 2012)

Table 10 indicates the mean serum MDA level in norma l and control group. MDA level were measured by Thiobarbituric acid reactive substances. The serum MDA level is increased in PCOS women (5.42 ± 0.58) compared to normal women (3.35 ± 0.58).

Table 10 – Level of Thiobarbituric acid reactive substances in normal and PCOS subjects

Groups	**TBARS**
PCOS	5.42 ± 0.58
Control	3.35 ± 0.58

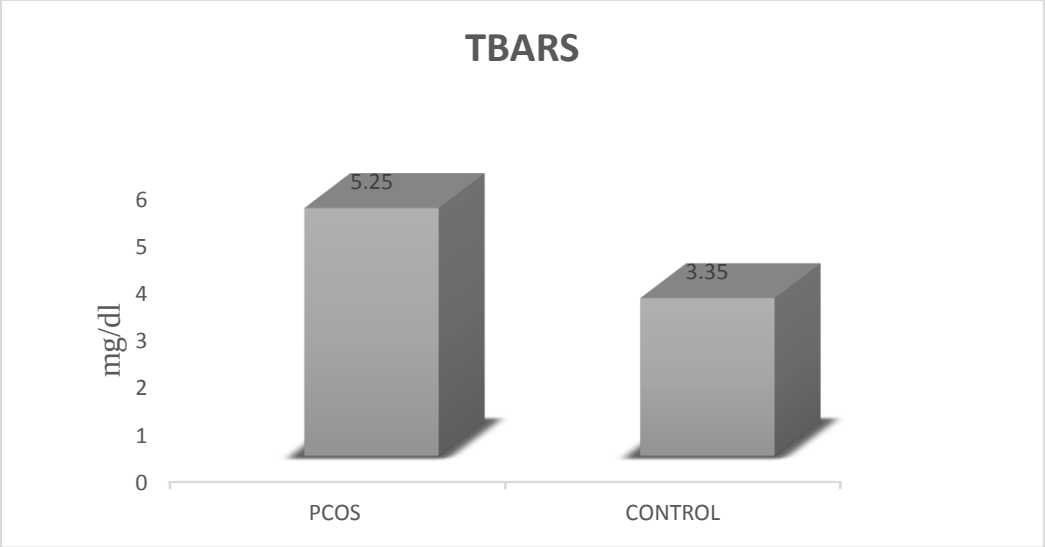


Figure 14– levels of Thiobarbituric acid reactive substances in normal and PCOS women

From the above results it is evident that the PCOS group women showed a significant ($p<0.05$) increase in the levels of MDA compared to control group. Serum MDA levels were significantly higher in women with PCOS compared with healthy women ($P < 0.001$). Total antioxidant status levels were significantly lower in women with PCOS compared with healthy women ($P < 0.005$). Total antioxidant levels ($P < 0.005$) increased and MDA levels ($P < 0.001$) declined after the treatment, but the parameters did not change after the treatment ($P > 0.05$) (Yilmaz *et al.,* 2005)

MDA level was found to be significantly increased in the study group when compared to the levels of various oxidant and antioxidant parameters like FRAP and Uric acid. (Desai et al., 2014). According to Shirsath *et al.* (2015) serum levels of MDA was higher in PCOD group (6.96±1.29), when compared with control group (3.56±1.00)

The results presented suggest that an increased oxidative stress could be associated with pathogenesis of PCOS.

4.2.2 Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power(FRAP) is a measure of total antioxidant capacity which represents the effect of the reducing power of plasma constituents, contributed by low molecular weight antioxidants of a hydrophilic and hydrophobic character especially vitamin C, vitamin E, bilirubin and uric acid and thus gives more biologically and clinically relevant information on antioxidant capacity. Mostly TAOS was found to be decreased in PCOS women compared to normal women (Desai *et al.,* 2014).

Lower levels of total antioxidant capacity (TAC) in the follicular fluid are predictive of decreased fertilization potential. Ferric reducing antioxidant power assay to estimate TAC in follicular fluid. Lower levels of TAC were associated with increased viability of the embryos till the time of transfer. The fertilization potential decreased as TAC levels decreases (Agarwal *et al.,* 2005)

Table - 11 indicates the mean serum TCA level in normal and control group. TCA level were measured by FRAP. The FRAP level decreased in PCOS women (27.53 ± 21.69) compared to normal women (38.44 ±12.65).

Table 11 – Ferric reducing antioxidant power of normal and PCOS

Groups	**FRAP**
PCOS	27.53 ± 21.69
Control	38.44 ±12.65

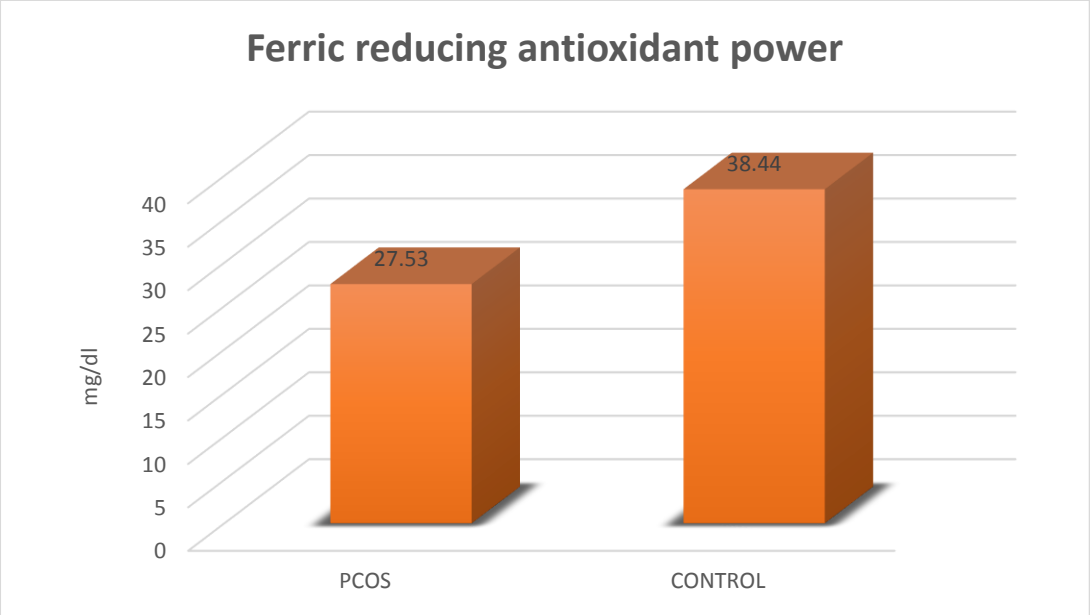


Figure 15 - Ferric reducing antioxidant power in normal and PCOS women

From the above result it is noticed that there is significantly decreased FRAP level in PCOS group when compared with the control group ($P < 0.001$)

Desai *et al.,* (2014) showed that the levels of various oxidant and antioxidant parameters such as MDA, FRAP and Uric acid in the study group and control group. FRAP was found to be significantly decreased in the study group compared to control group ($p = 0.09$).

Liu *et al.,* (2012) reported that oxidative stress in PCOS patients was linked to obesity. However, a few other studies were able to detect increased oxidative stress even in lean PCOS patients. Oxidative stress is increased in PCOS patients. The women with PCOS were found to have significantly higher advanced oxidation protein products and significantly lower total antioxidant status when compared to those of the age- and body mass index-matched healthy controls (Moti *et al.,* 2015). Najafi *et al.,* (2017) reported that the difference between the mean Total Antioxidant Status in PCOS group and control group was insignificant (1.35±.60 and 1.33±.78, respectively, P=0.583.

The finding of the present study indicate the presence of oxidative stress as evidenced by an increase in MDA levels and decrease in FRAP levels.

4.2.2 Levels of non enzymatic antioxidants in normal and PCOS women

The fact that antioxidant supplementation in subjects with PCOS may improve their chances of achieving pregnancy or correct the endocrinopathies associated with PCOS (Panti *et al.,* 2018). Systemic supplementation with antioxidants may help to overcome oxidative stress in female infertility. Systemic supplementation with vitamin C (ascorbic acid) has been used in patients who are infertile, in those with luteal phase defects and in those who have experienced recurrent abortions. Vitamin C may play a role in fertilization. In patients undergoing in vitro fertilization–embryo transfer, vitamin C supplementation was given during the period of hormonal stimulation, which resulted in higher follicular fluid concentrations of vitamin C. Antioxidants help protect the embryo from damage caused by pro-oxidants (Agarwal *et al.*, 2005). Vitamin C is a known redox catalyst that can reduce and neutralize ROS. Its reduced form is maintained through reactions with GSH and can be catalyzed by protein disulfide isomerase and glutaredoxins (Agarwal *et al.,* 2012).

Vitamin A is essential for the health of our muscle, connective tissue and eyes. When there is too much estrogen in the body. Vitamin A converts excess levels of estrogen into inactive estrogen. It is essential for the growth and development of the embryo specifically for the d evelopment eyes and nerves.

Vitamin E is not a hormone, but it acts like a hormone. Specifically progesterone. It will reduce the negative effects of too much of androgens. Vitamin E deficiency are associated with anovulation and it is also associated with luteal phase defect and frequent miscarriage. Vitamin E is directly correlated with the amount of progesterone and it will cause the ovaries to produce more, small and weak corpus luteum (Izadi *et al.,* 2018)

Table 12 – indicates the levels of vitamin A, vitamin C and vitamin E mean values. The mean vitamin A is decreased in women with PCOS (3.82 ± 0.60) compared to control group (6.13 ± 1.05) as well as the mean vitamin C in PCOS (3.01 ± 1.39) is also decreased compared to normal

women (14.05 ± 9.85). The mean value of vitamin E in PCOS group (0.29 ± 0.19) is decreased compared to normal group (0.41 ± 0.2).

Table 12 – Levels of non – enzymatic antioxidants in normal and PCOS women

Groups	Vitamin A	Vitamin C	Vitamin E
PCOS	3.82 ± 0.60	3.01 ± 1.39	0.29 ± 0.19
Control	6.13 ± 1.05	14.05 ± 9.85	0.41 ± 0.2

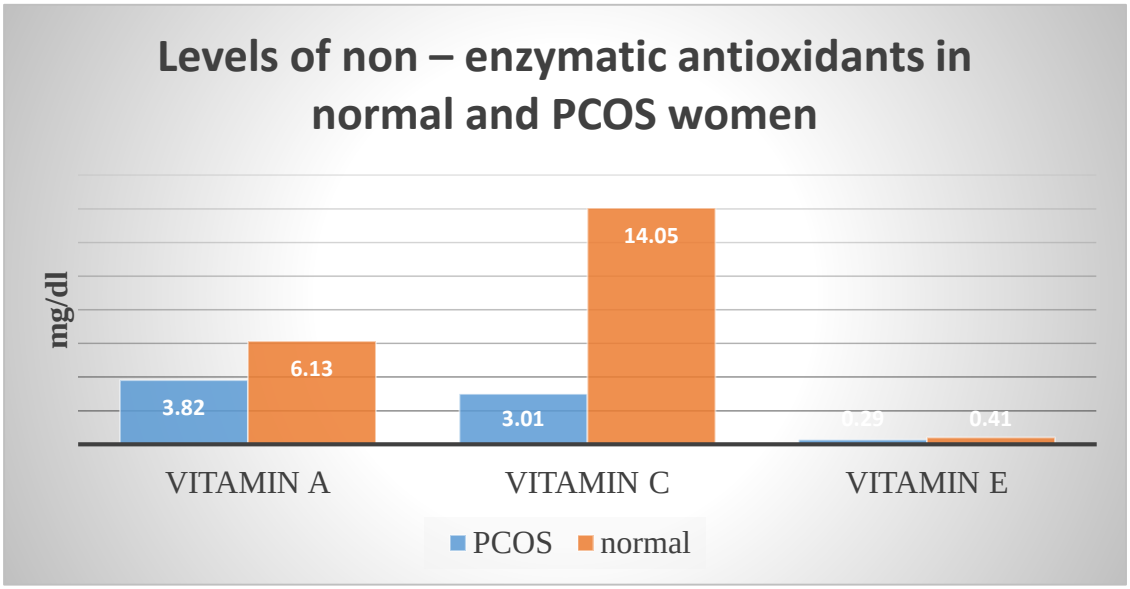


Figure 16 - Levels of non – enzymatic antioxidants in normal and PCOS women

It is noticed that there is a significant decrease in the levels of vitamin A and vitamin E in PCOS group when compared with the control group ($P < 0.05$). And also high significant levels of vitamin C in PCOS group when compared with the control group ($P < 0.001$). The Serum vitamin E levels were significantly lower in the group with endometriosis, compared with the case of the control group (Peaten *et al.*, 2008)

Kateen *et al.* (2010) reported that PCOS produce significant reduction in serum antioxidant Vitamins A, C and E when compared to control. Antioxidant supplementation has been shown to improve insulin sensitivity and other health threatening conditions in women with PCOS.

Sekhon *et al.,* (2010) did observational study and found that up to 50-60% of recurrent pregnancy loss may be attributable to oxidative stress. Hence the antioxidant supplementation has been shown to improve insulin sensitivity and restore redox balance in patients with PCOD. In our study we found a significant negative correlation ($p<0.001$) between serum MDA and serum vitamin C levels.

Fenkci *et al.* (2003) reported a decreased levels of TAOS in women with PCOS, and increased levels of protein carbonyls, confirming free radical attacks on proteins. Shirsath *et al.*(2015) found that the serum vitamin C levels were lower in PCOD group (0.42±0.22) compared to control group (0.93±0.44) and difference was statistically significant ($p<0.001$).

4.2.4 Assessment of total cholesterol in PCOS and normal subjects

Obesity and dyslipidemia which may predispose patients to metabolic syndrome, are common in PCOS. Dyslipidemia is found in women with PCOS, independently of the excess weight (Halasawadekar *et al*., 2016). hyperandrogenism and obesity were independent predictors for the presence of a more atherogenic lipid profile in women with PCOS and found that free androgen index and BMI were independent predictors for serum ApoA-I levels in women with PCOS (Kim and Choi., 2013).

Dyslipidemia is common in PCOS characterized by higher triglycerides and lower high density lipoprotein cholesterol Dyslipidemia in PCOS has multifactorial causation. Insulin resistance plays a pivotal role by stimulation of lipolysis and altered expression of lipoprotein lipase and hepatic lipase (Richa *et al*., 2015).

Table 13 – indicates the level of total cholesterol in PCOS and control group. Total cholesterol mean value is increased in PCOS women (165.35 ± 20.38) compared to normal group (136.04 ± 24.23)

Table 13 - Assessment of total cholesterol level in PCOS and normal subjects

Groups	Total Cholesterol
PCOS	165.35 ± 20.38
Normal	136.04 ± 24.23

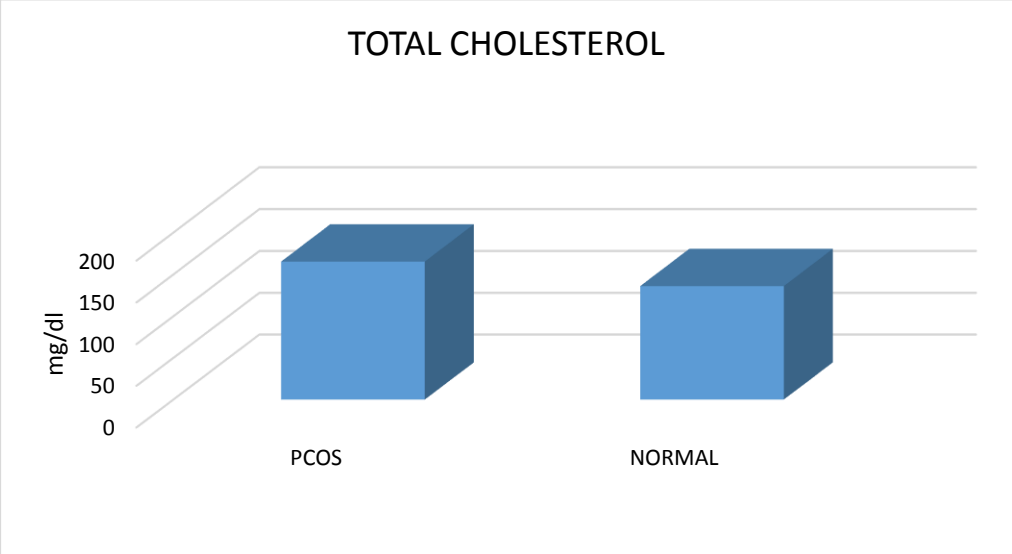


Figure 17 - Assessment of total cholesterol level in PCOS and normal subjects

There is a significant increase in the levels of total cholesterol compared with the control group ($P < 0.05$). The current study shows that a total of 43 out of 49 PCOS patients in the study group had high total cholesterol (TC). The fall in TC was significant ($p<0.001$) when compared to placebo group after 6 months of Metformin treatment (Singh *et al.*, 2017).

The levels of total cholesterol, TG and LDL-C were statistically higher and level of HDL-C was lower in PCOS patients, when compared with age-matched healthy females. (Shoaib *et al.*, 2018) Moreover, similar results found by some studies suggest that PCOS patients were hyperlipidemic with higher total cholesterol, LDL-C and TGs concentrations and lower HDL-C levels than control. PCOS is associated with a more pronounced atherogenic Lipid profile and it

seems to constitute e an additional risk factor for an atherogenic Lipid profile. Changes in serum lipid profile, which are possible risk factors for cardiovascular disorders play important roles in the development of cardiovascular disease in both obese and non-obese patients with PCOS. Increased serum concentration of LDL-C is atherogenic, whereas increased HDL-cholesterol (HDL-C) is considered cardio protective. Hence, the present of high LDL-C and low HDL-C in PCOS patients made them high risk for cardiovascular diseases.

High levels of the total and LDL cholesterol and low HDL-cholesterol were observed in women with PCOS when compared with healthy women. Dyslipidemia is common in PCOS compared to weight matched controls with higher triglycerides and lower high density lipoprotein cholesterol. The dyslipidaemia occurs independent of BMI. The causes of dyslipidaemia in PCOS are again multifactorial (Cristian-Ioan *et al.,* 2012)

Bickerton *et al.,* (2005) found that there were no significant differences in Lipid or lipoprotein concentrations between the women with PCOS group and controls. Yilmaz *et al.* (2005) found no difference in serum TC, LDL-C, TG, levels between PCOS and control groups, whereas HDL-C was lower.

4.2.5 Assessment of Liver marker enzymes in PCOS subjects and control

Hyperandrogenism may exert direct effects on the liver and indirect effects by modulating insulin sensitivity and favoring visceral adiposity. Insulin resistance/hyperinsulinemia contributes to hyperandrogenism by affecting the production, the clearance and bioavailability of ovarian androgens (Vassilatou E., 2014)

Nonalcoholic fatty liver disease (NAFLD) is one of the most important hepatic manifestations of metabolic disturbances with a spectrum from hepatic steatosis, inflammation, fibrosis to hepatocellular carcinoma. Women with PCOS were recently reported to have a higher level of alanine aminotransferase (ALT) and prevalence of NAFLD than women without PCOS. ALT is a sensitive indicator of liver cell injury, is a cytosolic enzyme and is thought to be a more specific indicator of liver damage than aspartate aminotransferase (AST) (Chen *et al.,* 2010).

Most common biochemical indexes in NAFLD patients are increased levels of alanine aminotransferase (ALT) and to a lesser extent aspartate aminotransferase (AST). ALT is a more sensitive biomarker than AST for impaired insulin signaling and NAFLD (Ramezani-Binabaj *et al.*, 2014). NAFLD frequently occurs in women with PCOS. A recent meta-analysis has reported that women with PCOS have an approximately four-fold increased risk of NAFLD. The prevalence of NAFLD in women with PCOS may vary worldwide because of their different genetic, ethnic, and lifestyle backgrounds. (Minato *et al.*, 2018)

Table 14 indicates the serum levels of ALT and AST. It was found to be 17.76±7.4 and 31.41±8.19 in PCOS and 14.98±7.4 and 25.14±3.78 in control.

Table 14 - levels of liver marker enzymes in PCOS and control group

Groups	ALT/ SGPT	AST/SG OT
PCOS	17.76 ± 15.22	31.41 ± 8. 19
Control	14.98 ± 7.40	25.14 ± 3.7 8

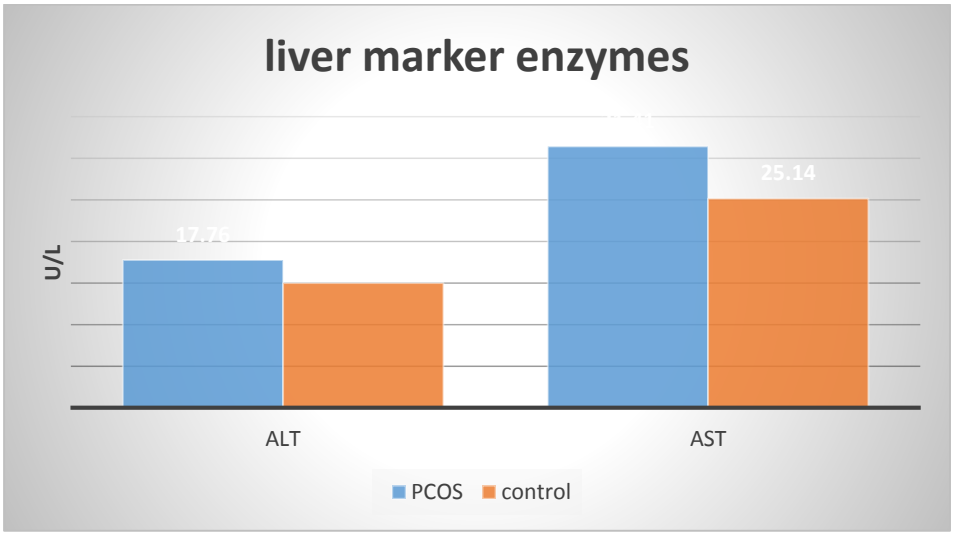


Figure 18- levels of liver marker enzymes in PCOS and control group

It is noted that there is a significant increased levels of ALT and AST in PCOS when compared to normal women. ($P < 0.05$)

Economou *et al.,* (2009) found that Lean PCOS patients did not exhibit evidence of NAFLD or elevated liver enzymes in respect to lean controls, whereas obese PCOS women were shown to have significantly elevated liver enzymes in comparison to controls.

Hasan *et al.,* (2017) shows that the mean of liver enzymes levels ALT and AST were significantly higher in patients (7 ± 21) and (6 ± 21) U/L comparing with control (6± 2) and (62 ±) U/L respectively p= (0.00001), (0.0008). ALT level found to be weakly correlated with BMI and LDL (0.22), (0.31) respectively, and negatively correlated with age, TG. F.B.G and HOMA-IR (-0.30), (-0.52) (-0.20), (-0.20) respectively. Also AST level found to be weakly correlated with HDL (0.27), (0.31) and negatively correlated with TG and F.B.G (-0.44), (-0.24) respectively.

The finding of our study shows that women with PCOS presented elevated levels of ALT and AST and it was correlated with insulin resistance, obesity and dyslipidemia

PHASE III – Detection of Bisphenol A in PCOS women

Bisphenol A is an Endocrine disrupting chemicals (EDCs) are natural or synthetic chemical compounds that can interfere with the endocrine system by antagonizing endogenous steroid hormones. Bisphenol A (BPA) is widely used in the manufacture of resins such as polycarbonate plastic products and epoxy resins (Zhou *et al.,* 2016).

BPA has weakly estrogenic properties. It binds to the classical nuclear estrogen receptors (ER-α and ER-β) as well as the non-classical membrane bound ER receptor. Estrogen production in premenopausal women is a prime target for BPA activity and indeed, BPA is commonly found in ovarian follicular fluid. Early exposure to BPA has also been linked to earlier pubertal timing. BPA can directly affect oocyte or indirectly affect the local, intra-ovarian and intrafollicular environment (i.e., by affecting the granulosa cells that are essential for ovarian follicle growth, steroidogenesis, oocyte survival and nourishment). (Barrett and Sobolewski, 2015).

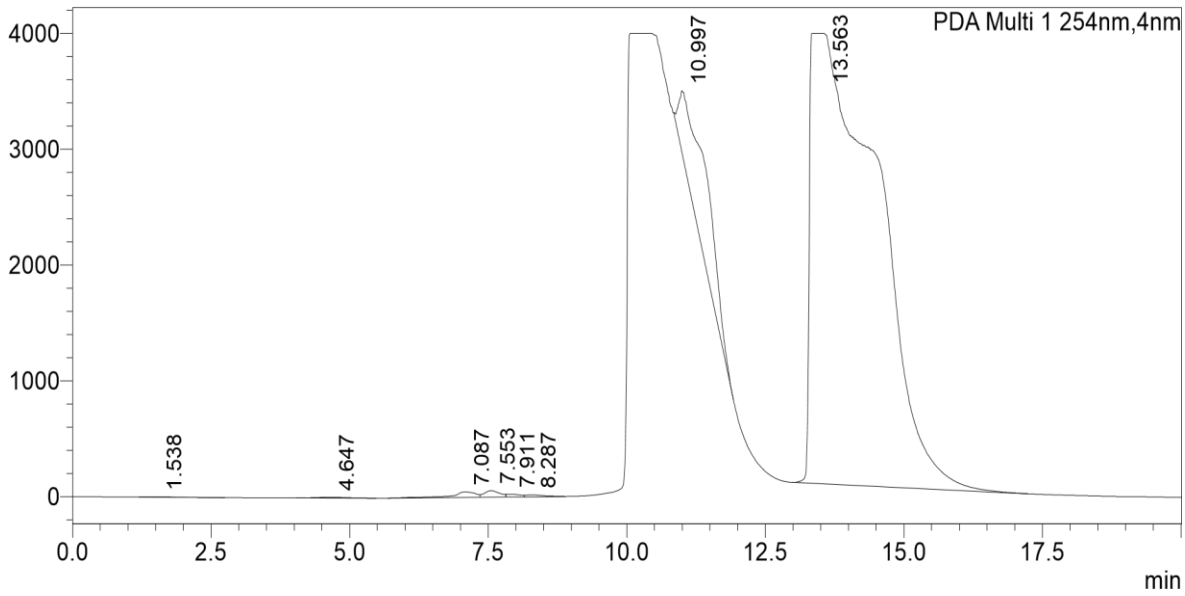
BPA accumulates in reproductive organs and disrupts the endocrine system. In the general population, BPA has been detected in follicular fluid at concentrations of 1–2 ng/ml. Several epidemiological studies identified correlations between BPA and various abnormalities in the ovary of foetuses and adults. Moreover, the effects of BPA in the ovary, which goes through different stages such as folliculogenesis, ovulation and luteinisation, depend on the time of exposure (Ptak *et al.*, 2017).

BPA can directly affect oocyte or indirectly affect the local, intra-ovarian and intrafollicular environment (i.e., by affecting the granulosa cells that are essential for ovarian follicle growth, steroidogenesis, oocyte survival and nourishment). For example, rodents exposed to BPA during the early postnatal period had a reduced ovarian follicular reserve, including a decline in the stock of primordial follicles, increase in antral atretic follicles, higher incidence of multiple oocyte follicles (MOFs) and lower ovarian weight. The increased incidence of MOFs may also contribute to the decline in the primordial follicle pool. (Ptak *et al.*, 2017).

The effects of BPA on DNA methylation of imprinted genes and its potential influence on oocyte development in mice. They demonstrated that BPA exposure during early postnatal period resulted in remarkably decreased methylation of imprinted gene IGF2R and PEG3 and suppressed expression of DNA methylation transferases (Dnmts) which were closely related to oocyte growth (Zhou *et al.*, 2016).

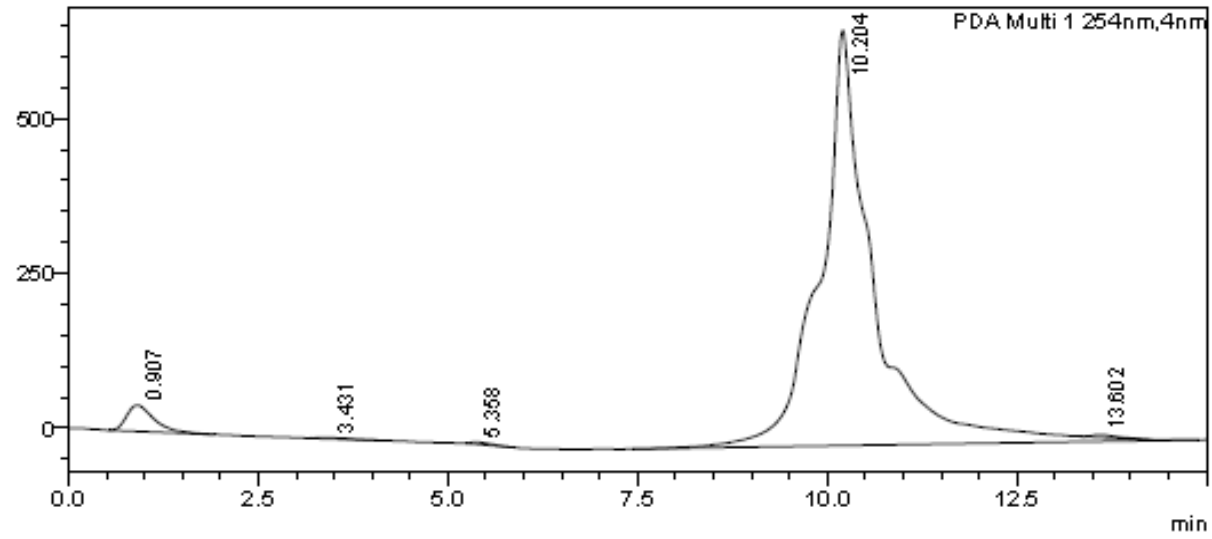
In our study the levels of Bisphenol A and in serum samples were assayed using HPLC equipped with fluorescence detector. The HPLC method was allowed to perform rapid analyses of BPA in the serum with good detectability and quantification. A larger sample zone obtained by separation with the precolumn could be effectively introduced into the separation column, which resulted the increase in the sensitivity for BPA detection. Bisphenol A was observed at 254nm.

Figure 19 : High Performance Liquid Chromatogram of standard Bisphenol A



From the standard the effect of the column-switching time was observed in 8 different retention time. The range of retention time is 1.538 – 13.563 min. The maximum peak height was observed at the r ange of 10.997 – 13.563 min. then the peak will decreased with expansion of the switching time is 13.563 mins.

Figure 20: High Performance Liquid Chromatogram of serum sample from PCOS Women



From our sample the effect of the column-switching time was observed in 5 different retention time. The range of retention time is 0.907 – 13.602 min. The maximum and constant peak height was observed at the range of 10.204 min. then the peak will decreased with expansion of the switching time is 13.602 mins.

The standard peak was compared to the peak serum sample of PCOS women. The standard peak is mat ched to the sample peak in the same retention time. Approximately the retention time at 10.0 min high concentration of bisphenol A was separated.

From this result Bisphenol A was identified in PCOS women. The possible presence of BPA in the serum was confirmed by comparison with the chromatograms of the same samples. It will proved that Bisphenol A is a one of the reason for PCOS. From the sample peak Bisphenol A shows high concentration, so it is also one of the strong reason for PCOS.

The result of the present study manifested BPA as endocrine disrupting chemicals may be are one of the new risk factors for the development of PCOS. The result of this study were consistent with other studies those who were evaluated in serum samples, they found serum BPA level is significantly higher in PCOS women compared to women without PCOS (Akin *et al.,* 2017).

Hu *et al. (*2017) reported the higher levels of BPA in PCOS patients. Their results indicated that BPA may play a major role in the PCOS pathogenesis.

The results conclude that serum BPA may be positively associated with women with PCOS and BPA might be involved in the insulin – resistance and hyper androgenism of PCOS.

5. SUMMARY AND CONCLUSION

Polycystic ovary syndrome (PCOS) is one of the most common endocrine and hormonal disorder of reproductive age affecting 5% to 10% of women worldwide. It is a heterogenous, multifactorial, complex genetic disorder. Women with PCOS often experience other conditions that can affect their short- and long-term physical and mental health. Decreased quality of life from mood disturbances, weight gain, acne vulgaris, and alopecia have all been documented. Polycystic ovarian syndrome (PCOS) is the commonest endocrine system disorder among females in their reproductive age presented by menstrual dysfunction, insulin resistance, hyperandrogenism and polycystic ovaries can lead to depression, social and marital crises and sexual dysfunction majority of patients with PCOS have overweight or obesity.

The present work entitled, " **Role of oxidative stress and Bisphenol A in women with polycystic ovarian syndrome"** was organized in three phases

In phase I, assessment of clinical features of normal and PCOS women was carried out. The result of the phase I study proved that the average age is 25 in PCOS women and 23 in normal subject. BMI of the women with PCOS is found to be significantly higher BMI than the control group. The mean of the waist circumference, hip circumference and WHR (waist-hip-ratio) was significantly higher in the PCOS group, compared to the control group.

The prevalence of PCOS is more in over weight (34%) and obese (18%) than the normal women. The higher percentage of PCOS patients are with oligomenorrhea 37(50 %), with amenorrhea 10(13 %) and patients with regular menstrual cycle are 28 (37%). Oligomenorrheoa is considered as a highly predictive surrogate marker of PCOS. Approximately 85-90% of women with oligomenorrhea have PCOS

The percentage of irregular periods with PCOS women is greater in less than 18 years when compared to <25 and 18-25 years. Menarche attained by PCOS group was greater than control and duration of menstrual cycle in PCOS women was greater in >37 days while comparing normal women shows a regular menstrual cycle occurring in normal intervals (25 to 34). Acne, hirsutism and skin pigmentation was found to be high (84%, 74% and 72%) in PCOS subjects and showed significant difference between PCOS and controls.

Prevalence of depression was found to be higher (63%) in PCOS subjects and showed significant difference between the PCOS and control. The prevalence of hypertension was higher in women reporting PCOS, compared to control women. The women of PCOS with obese have a higher percentage of family history than the non-obese PCOS. This is evident from our study results where participants with a positive family history of PCOS carried little higher risk of the development of PCOS. Thus results of Phase I cleared that PCOS women of all the age group showed irregular periods, acne, hirsutism, skin pigmentation and family history of PCOS.

In second phase, assessment of oxidative stress biomarkers were analyzed. The serum MDA level were measured by TBARS. The TBARS level is increased in PCOS women compared to normal women. Total antioxidant status level was measured by FRAP. The FRAP level is decreased PCOS women compared to normal women. Vitamin A, E levels were significantly reduced in PCOS group than the normal women. The levels of total cholesterol was significantly increased in PCOS group. The liver marker enzymes such as ALT and AST ware increased in PCOS women when compare to normal women. This shows the oxidative stress play a major role in the pathogenesis of PCOS.

In phase III, The HPLC method was allowed to perform rapid analysis of Bisphenol A in the serum of PCOS women. The concentration of the standard peak was compared to sample peak. The concentration of the standard peak is matched to the sample peak in the same retention time. Approximately the retention time at 10.0 min high concentration of bisphenol A was separated. The possible presence of BPA in the serum was confirmed by comparison with the chromatograms of the same samples. A higher concentration of Bisphenol A in PCOS is suggested from the sample peak. Serum BPA may be positively associated with women with PCOS and BPA might be involved in the insulin – resistance and hyperandrogenism of PCOS.

To conclude that women with PCOS most frequently exhibit dyslipidemia, and increased oxidative stress levels. The results presented suggested that decreased antioxidative capacity could be associated with pathogens PCOS. The higher levels of Bisphenol A in PCOS women, which can exacerbate the androgen production. It seems that Bisphenol A may play role in PCOS pathogenesis.

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APPENDIX-I
PCOS Questionnaire

SELF TEST: do you have PCOS

PCOS is characterized by multiple small ovarian cysts, obesity, hypertensions, insulin resistance and hirsutism (elevated levels of male hormones). The ovarian cysts may not produce any definite symptoms, and may come and go, so they may not show on ultrasound at the time of the test is done. It may, therefore, be difficult to prove the presence of this disorder. in view of the combined cluster of problem you are experiencing (Weight gain , glucose intolerance and almost certain insulin resistance , hormone imbalance, high blood pressure) it may be reasonable to make treatment recommendations based on what would be appropriate for **PCOS**.

1. Age _____ yrs
2. Height _____cm
3. Weight _____kg
4. Body Mass Index (BMI) _____kg/m2 (calculated)
5. Waist circumference_____ (cm)
6. Hip circumference_____ (cm)
7. Age of first period_____
8. period comes once in
   a) 35 – 45 days b) 45- 60 days c) more than 60 days1
9. Pelvic pain during menstruation, YES [ ] / NO [ ]
10. Did you have continuous weight gain, YES [ ] / NO [ ]
11. periods are unpredictable, YES [ ] / NO [ ]
12. Is there any difficulty in losing weight YES [ ] / NO [ ]
13. Periods are very heavy and prolonged
    a) 3 – 5 days b) 5 – 7 days c) more than 7 days
14. Do you have Hirsutism (Unwanted hair growth) on face and body, YES [ ] / NO [ ]
15. Do you have Hair loss, YES [ ] / NO [ ]
16. Do you have acne, YES [ ] / NO [ ]
17. Do you notice any Skin colour change or pigmentation, YES [ ] / NO [ ]
18. Did you have menstrual period with clots, YES [ ] / NO [ ]
19. Is there abdominal bloating, YES [ ] / NO [ ]
20. Is there menstrual cramps, YES [ ] / NO [ ]
21. Did you have any premenstrual syndrome symptoms YES [ ]/ NO [ ]
22. Did you undergo depression or mood changes during the period, YES [ ]/ NO [ ]
23. Did you have any symptoms of hypoglycemia, YES [ ] / NO [ ]
24. Blood pressure during the period is
    a) Low b) normal c) high
25. Increased appetite, YES [ ] / NO [ ]
26. Did you have vitamin D deficiency YES [ ] / NO [ ]
27. Do you feel extremely hungry , irritable, sleepy or fatigued after eating sweets during the period, YES [ ]/ NO [ ]
28. Are you taking physical activities for PCOS, YES [ ] / NO [ ]
29. If so for how many days _____
30. Are you feel better by doing this physical activities YES [ ] / NO [ ]

31. Did you have the regular basis of eating fast foods YES [ ] / NO [ ]
32. Did you have the drinking habit of alcohol YES [ ] / NO [ ]
33. Do you have the habit of consuming caffeine (coffee), YES [ ] / NO [ ]
34. If so,
    a) Twice per day b) 3 or more times per day c) once per day d) none
35. Are you taking excess carbohydrate and sugar, YES [ ] / NO [ ]
36. Do you have sleep apnea, YES [ ] / NO [ ]
37. How many hours you sleep
    a) More than 8 hours per day b) 6 – 8 hours per day c) less than 5 hours per day
38. Other systemic disorders
    Diabetes_____, Hyperthyroidism_____
    High Cholesterol _____, Stress_____

MARITAL STATUS, *YES [ ]/ NO [ ]*

39. Fertility problems , YES [ ] / NO [ ]
40. Use of oral contraceptive Pills YES [ ]/ NO [ ]
41. Do you have difficulties in getting pregnant YES [ ] / NO [ ]

FAMILY HISTORY

42. Mother with PCOD, YES [ ] / NO [ ]
43. Siblings with PCOD, YES [ ] / NO [ ]
44. Is there anybody having Diabetes mellitus YES [ ] / NO [ ]
45. CVD YES [ ] / NO [ ]
46. Gestational diabetes YES [ ]/ NO [ ]
47. High blood pressure YES [ ] / NO [ ]

APPENDIX II

FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

(Vijayalakshmi and Ruckmani, 2016)

PRINCIPLE

The flavonoids and phenolic acids are present in the blood sample exhibit strong antioxidant activity which is depending on their potential to form the complex with metal atoms, particularly iron and copper. This method is based on the principle of increase in the absorbance of the reaction mixtures, the absorbance increases the antioxidant activity increases. The antioxidant compound present in the samples forms a colored complex with potassium ferricyanide, trichloroacetic acid and ferric chloride, which is measured at 700 nm by UV-Spectrophotometer.

REAGENTS

1. 0.2M phosphate buffer (pH 6.6)

   8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium hydrogen phosphate, 0.24 g of potassium dihydrogen phosphate was taken in a 1,000 mL standard flask and add 800 mL of distilled water and adjust the pH 6.6 using hydrochloric acid and adjust the volume with deionized water.
2. Potassium ferricyanide (1%)

   1 g of potassium ferricyanide was dissolved in 100 mL of deionized water.
3. Trichloroacetic acid (10%)

   10 g of trichloroacetic acid was dissolved in 100 mL of deionized water.
4. Ferric chloride (0.1%)

   100 mg of ferric chloride was dissolved in 100 mL of deionized water.
5. Ascorbic acid (0.1%)

   1 mg of ascorbic acid was dissolved in 1 mL of water.

PROCEDURE

Different concentrations of the methanolic extract of M. serratulum and its various fractions (10-50 µg/mL) was added to 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide [K3Fe(CN)6] solution. The reaction mixture was vortexed well and then incubated at 50°C for 20 min using vortex shaker. At the end of the incubation, 2.5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged at 3,000 rpm for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 0.1% ferric chloride. The colored solution was read at 700 nm against the blank with reference to standard using UVSpectrophotometer. Here, ascorbic acid was used as a reference standard, the reducing power of the samples were comparable with the reference standard.

APPENDIX III

ESTIMATION OF SERUM THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS) COLORIMETRIC METHOD

(Jentszch *et al*., 1996)

PRINCIPLE:

One molecule of malondialdehyde reacts with two molecules of thiobarbituric acid to produce a pink pigment with absorption at 535 nm. Amplification of peroxidation during the assay is prevented by the addition of the chain breaking antioxidant butylated hydroxy toluene (BHT).

REAGENTS

1. Standard malondialdehyde (MDA): 0.2 –4 µM 1,1,3,3 tetramethoxy propane
2. Ortho phosphoric acid: 0.2 M
3. Butylated Hydroxy Toluene (BHT): 2 mM
4. Thiobarbituric acid reagent: 0.11 M in 0.1M NaOH
5 .n butanol

PROCEDURE:

Added 400 µl serum/ tissue homogenate to sample tubes. Added 400 µl water to the blank tube. Added 400 µl of each dilutions of MDA to the standard tubes. Added 400 µl of orthophosphoric acid reagent, 50 µl BHT reagent and 50 µl TBA reagent to all the tubes. Mixed well and incubated at 90°C for 45 minutes in a water bath. Transferred the tubes to ice bath to stop the reaction. Added 1 ml of n butanol to all tubes. Vortexed for 20 seconds and transferred the butanol phase to fresh tubes. Read the absorbance of the butanol phase at 535 nm and 572 nm (for base line correction). MDA equivalents were calculated using the difference in absorption at two wavelengths. TBARS levels were cacalculated by constructing a linear graph of the standard concentrations.The values are reported in µmol/l in human serum, nanomoles of TBARS formed per mg protein in animal serum.

APPENDIX IV

ESTIMATION OF α-TOCOPHEROL

(Emmerie-Engel method, 1938 as described by Rosenberg, 1992)

PRINCIPLE

Tocopherol can be estimated using Emmerie – Engel reaction which based on the reduction of ferric to ferrous ions by tocopherols, which then forms a red colour with 2, 2'-dipyridyl. Tocopherol and carotenes are first extracted with xylene and the extraction read at 460nm to measure carotenes. A correlation is made for these after adding ferric chloride and reading at 520nm.

REAGENTS

1. Absolute alcohol
2. Xylene
3. 2, 2'- dipyridyl
4. Standard solution:

Dissolved 10mg/ 10ml of α-tocopherol in absolute alcohol 91mg of œ- tocopherol is equivalent to 100mg of tocopherol acetate.

PROCEDURE

Into 3 stoppered centrifuge tubes (test, standard and blank), pipetteed out 1.5ml of sample, 1.5ml of standard, 1.5ml of water respectively. To the test and blank added 1.5ml of ethanol and to the standard,added 1.5ml of water. Added 1.5ml xylene to all the test tubes, stoppered, mixed well and centrifuged. Transferred 1.0ml of xylene layer into another stoppered tube, taking care not to include any other ethanol or protein. Added 1.0ml of 2, 2'-dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5ml of the mixture into colorimeter cuvettes and read the extinction of the test and standard against the blank at 460nm. Then in turn beginning with the blank, added 0.33ml of ferric chloride solution.

The amount of vitamin E can be calculated using the formula,

$$\text{Amount of tocopherols in µg} = \frac{\text{Reading at 520nm – Reading at 460nm}}{\text{Reading of standard at 520nm} \times 0.24 \times 15}$$

APPENDIX V

ESTIMATION OF ASCORBIC ACID

(Roe and Kuether, 1953)

PRINCIPLE

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal and bromine. Dehydroascorbic acid then reacts with 2, 4- dinitrophenyl hydrazine to form osazones, which dissolves in sulpuric acid to give an orange coloured solution whose absorbance can be measured spectrophotomerically at 540nm.

REAGENTS

1. 4%TCA
2. 9N H_2SO_4
3. 2% 2, 4-dinitrophenyl hydrazine: Dissolved 2g of DNPH in 100ml of 9N H_2SO_4
4. 10% thiourea
5. 80% sulpuric acid
6. Stock standard solution: Dissolved 100mg of ascorbic acid in 100ml of 4%TCA
7. Working standard: Diluted 10ml of the stock solution to 100ml with 4%TCA

PROCEDURE

0.5 ml of sample were taken for the assay. The assay volumes were made up 2.0ml with 4%TCA. 0.2 to 1.0ml of the working standard solution containing 20-100 µg of ascorbate respectively were pipetted out into clean dry test tube, the volume of which were also made

up to 2.0ml with 4%TCA. Added 0.5ml of DNPH reagent to all the test tubes, followed by 2 drops of 10% thiourea solution. Incubated at 37°C for 3 hours.

The osazones formed were dissolved in 2.5ml of 85% sulpuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea were added after the addition of H_2SO_4. The tubes were incubated for 30 minutes at room temperature, and the absorbance was read spectrophotometrically at 540nm. Calculated the content of ascorbic acid in the sample using the standard graph.

APPENDIX VI

ESTIMATION OF VITAMIN A

PRINCPLE

The colour produced by vitamin A, its acetate or palmitate with TCA is proportional in intensity to its concentration, which property is used for the spectrophotometric estimation.

REAGENTS

All reagents were prepared fresh. Exposure of samples and reagents to light was avoided at all times.

1. TCA – saturated solution in chloroform

   15 g of clear TCA crystals were dissolved in 25 ml of alcohol-free chloroform, stored in the dark (stable for 5 hours)
2. Standard vitamin A: 15 MG OF retinol in 10 ml of chloroform

EXTRACTION OF THE VITAMIN FROM SERUM

To 0.2 ml of the serum, added equal volumes of 95% ethanol and 0.6 ml of petroleum ether (40° - 60°C). The tube was corked and the contents were agitated in a cyclomixer for 45 seconds to extract the vitamin into the petroleum ether. The tube was then centrifuged. The petroleum ether layer was separated into a test tube and evaporated to dryness at 60°C. The residue was dissolved in 1 ml of chloroform and used for the determination of vitamin A

PROCEDURE

Aliquots of the standard were pipetted out into a series of clean dry test tubes, in the concentration range of 0 – 7.5 µg. The volumes in all the test tubes were made up to 1 ml with chloroform. From a fast delivery pipette, added 2 ml of TCA reagent, rapidly mixing with the vitamin A solution. Measured the absorbance immediately at 620nm in a spectrophotometer. A standard curve was constructed in linear regression mode of a scientific calculator and the unknown concentration calculated from it.

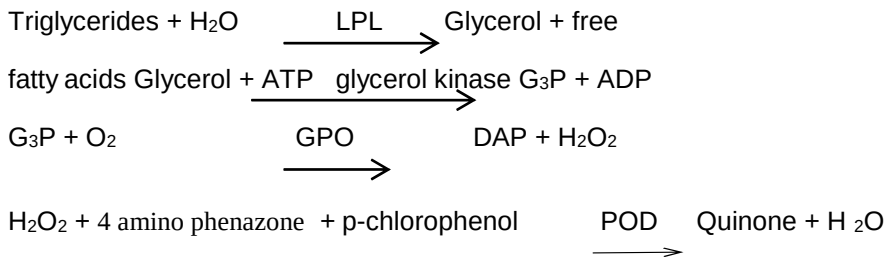
APPENDIX VII

ESTIMATION OF TOTAL CHOLESTEROL

(Allain et al ., 1974)

PRINCIPLE

Cholesterol esterase (CHE) hydrolyses cholesterol ester to free cholesterol is oxidized by the cholesterol oxidase (CHOD) to 4-cholestenone and hydrogen peroxide. Hydrogen peroxide formed reacts with 4-amino antipyrine and phenol in presence of peroxidase(POD) to produce pink coloured compound called quinonimine dye.



The intensity of the colour formed is proportional to cholesterol concentration in the sample.

REAGENT

1. Cholesterol standard: 200 mg/dl
2. Cholesterol Reagent: It is Constituted with Sodiumcholate11.6mmol/l, 4-minoantipyrine1.6mmol/l, Phenol 21.2mmol/l, peroxidase 10000U/l, cholesterol oxidase 250U/l, cholesterol phosphate bufferpH7.5 and esterase.

PROCEDURE

1 ml of cholesterol reagent and 20 µl of serum sample pipetted out into a clean dry test tube. Standards were prepared by adding 1ml of reagent and 20 µl of cholesterol standard. Blank contains 1 ml of reagent and 20 µl of distilled water. Mixed well and incubated at 37 ºC for 10 minutes. The absorbance of the samples and blank were measured against the blank at 505 nm.

Calculation

$$\text{Total Cholesterol (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}}$$

APPENDIX VIII

ESTIMATION OF ASPARTATE TRANSAMINASE (AST)

(Reitman and Frankel, 1957)

Principle

Serum glutamine oxaloacetate transaminase (SGOT) catalyses the reversible transfer of an amino group from aspartate to α-keto glutarate forming glutamate and oxaloacetate. SGOT catalyses the following reaction:

SGOT (pH 7.4)

L–Aspartate + α–Keto glutarate ⟶ Oxaloacetate + L–Glutamate Alkaline medium

Alkaline medium

Oxaloacetate + 2,4 DNPH ⟶ 2,4 dinitrophenyl hydrazine

Reagents

1. Tris buffer, pH 7.5-100mmol/l

2. L-aspartate-500mmol/l

3. 2-oxoglutarate-15mmol/l

4. 2, 4 dinitrophenyl hydrazine reagent 5. Working sodium hydroxide (4N)

Procedure

Five hundred µl of buffered substrate was incubated at 37°C for 3 minutes and 0.1ml of serum was added, mixed well and incubated at 37°C for 30 minutes. Then 0.5ml of 2, 4 dinitrophenyl hydrazine (DNPH) reagent was added, mixed well and kept at room temperature for 20 minutes and 0.5ml of 4N working sodium hydroxide was added and kept at room temperature for 10 minutes. Blank and standards were also processed in a similar way and the absorbance was measured spectrophotometrically at 505nm. Activity of AST was expressed as U/L.

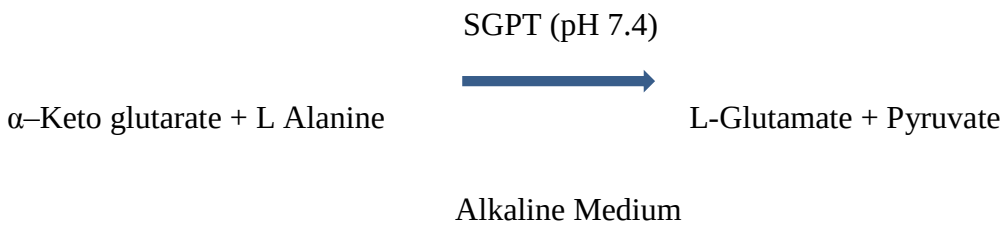
APPENDIX IX

ESTIMATION OF ALANINE TRANSAMINASE (ALT)

(King, 1965)

PRINCIPLE

SGPT catalyses the reversible transfer of amino group from L-alanine to alpha ketoglutarate with the formation of pyruvate and glutamate. The pyruvate so formed is allowed to react with 2-4 dinitrophenylhydrazine (DNPH) to produce 2, 4- dinitrophenyl hydrazone derivative, which is measured spectrophotometrically.



Pyruvate + 2,4 DNPH ⟶ 2,4 dinitrophenyl hydrazine (Brown Colored)

REAGENTS

1. Tris buffer, pH 7.5-100mmol/l

2. L-alanine-500mmol/l

3. 2-oxoglutarate-15mmol/l

4. 2, 4 dinitrophenyl hydrazinereagent

5. Working sodium hydroxide (4N)

PROCEDURE

Five hundred µl of buffered substrate was incubated at 37°C for 3 minutes and 0.1ml of serum was added, mixed well and incubated at 37°C for 60 minutes. Then 0.5ml of DNPH reagent was added, mixed well and kept at room temperature for 20 minutes and 0.5ml of 4N working sodium hydroxide was added and kept at room temperature for 10 minutes. Blank and standards were also processed in a similar way and the absorbance was measured spectrophotometrically at 505nm. Activity of ALT was expressed as U/L.