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Introduction

Nutritional, demographic, epidemiological, and socioeconomic transitions are occurring in many developing countries. Continuing undernutrition and escalating over nutrition has created double jeopardy of communicable and noncommunicable diseases (NCDs). This double burden poses health and economic challenges in resource-constrained populations, as observed in India. Amongst the various challenges, the increasing burden of metabolic syndrome in developing countries has created an urgent need to strategize health policies and mass intervention programs. Clearly these efforts require a thorough etiological identification of factors influencing and driving towards development of metabolic syndrome.

1.1. Metabolic syndrome

Metabolic syndrome represents a cluster of metabolic abnormalities that include hypertension, central obesity, insulin resistance, and atherogenic dyslipidemia, and is strongly associated with an increased risk for developing diabetes, atherosclerotic and nonatherosclerotic cardiovascular disease and infertility (Rochlani et al., 2017). Data from literature suggests a high prevalence of metabolic syndrome in most of the countries, i.e., 20% to 30% of the adult population (Grundy, 2008). The pathogenesis of metabolic syndrome is not completely elucidated; nonetheless, there are several factors that have been implicated, including dietary habits and sedentary lifestyles, increasing age and body mass index, central obesity, hyper triglyceridemia, low levels of high-density lipoprotein (HDL) cholesterol, hyperglycaemia, and hypertension (Shalini et al., 2013; Hadaegh et al., 2013). These factors directly contribute to pro-inflammatory as well as pro-thrombic state, an outcome that predisposes an individual to the development of Type 2 diabetes mellites and atherosclerotic cardiovascular diseases (Roa et al., 2008). The primary cause of such a metabolic predisposition is insulin resistance and hyperinsulinemia (Chaudhary and Chaudhary, 2010). Insulin resistance leads to elevated circulating insulin levels and androgen production as well as lipid metabolism (Akter et al., 2013). Having excess release of androgens could result in unfavourable metabolic problems resulting in central distribution of fat and manifesting dyslipidaemia Apart from metabolic

alterations, various reports have clearly shown the association of metabolic syndrome with reproductive dysfunction (Al Awlaqi et. al., 2016).

1.2. Association of Metabolic Syndrome with Female Infertility

In the past decades, several reports have depicted the clear association of metabolic disorders with female infertility. Female fertility is governed by the interplay of metabolic modulators with reproductive biomolecules. In this context, data show that elevated blood glucose (hyperglycaemia) triggers peripheral insulin resistance (Chia et al., 2018). Reports show that diabetic women experience sexual dysfunction, particularly dyspareunia, decreased desire, as well as changes in the arousal phase, which have been associated with depressive symptoms and marital problems (Salonia et al., 2006). Apart from glucose homeostatic molecules, there are compelling evidence to believe that energy stores and its metabolism could determine the onset of puberty and fertility, although the exact mechanism by which these occur, remains unclear (Hill et al., 2008; Roa et al., 2011). Women who are obese and diabetic have been shown to have an elevated risk of complications associated with pregnancy (Vahratian et al., 2009), along with increased risk of morbidity and mortality in the offspring (Downs et. al., 2010). Existing insulin resistance and hyperinsulinemia have been shown to cause Polycystic Ovary Syndrome (PCOS), which is a complex metabolic, endocrine and reproductive disorder (Soto et al., 2009). In addition to this, hyperglycaemia is also thought to affect ovarian function via the presence of advanced glycation products and its receptors, which are believed to express on theca and granulosa cells of healthy women (Diamanti-Kandarakis et al., 2007). Also, few studies have investigated ovulatory function in diabetic women (Wellons et al., 2017). Wherein, several reports have implicated that there exist delays in ovulation of women who wish to conceive, implying that these women exhibit a longer follicular phase (Lombardo et al., 2009; Picardi et al, 2008; Pralong, 2010).

The significance of insulin action on human reproductive function is underscored by insulin receptor expression in several tissues, including ovaries, uterus, hypothalamus, and pituitary (Codner and Escobar-Morreale, 2007). Insulin promotes ovarian steroidogenesis and follicular development through insulin receptors in granulosa cells. Insulin has also been shown to augment FSH-stimulated steroid secretion (Sirotkin, 2011). Additionally, its gonadotropic effects on folliculogenesis promote recruitment and pre-ovary follicular growth. These numerous actions of insulin form the basis for the potential impact of disturbed insulin

secretion on the development and functions of the ovaries (Codner et al., 2010). Further, hyperglycemia and insulin resistance have been shown to induce oxidative stress resulting in an imbalance between free oxygen radical activity and antioxidant defence mechanisms at the cellular level (Wener-Ozegowska et. al., 2011). Reports have suggested that some of the reproductive deficits associated with diabetes in women could result from ovarian alterations (Harvey et al., 2011; Cust et. al., 2007). Follicular growth and survival abnormalities, including increased granulosa and follicular apoptosis, as well as impaired oocyte-granulosa communication, oocyte maturation, and development of ovarian follicles have been observed in diabetic animal models. Ovarian steroidogenesis perturbation and ovulation have also been observed in female diabetic mice (Kurdoglu et. al., 2012).

In addition to this, there are several modifiers both intra and extracrine factors which causes an impact on women's fertility. Obesity, which is a component of metabolic syndrome, has been shown to have a significant impact on female fertility. Reports show that there is a high prevalence of obese women in the infertile population (Catalano, 2010). Obese women mostly have decreased insulin sensitivity putting them at an increased risk of several adverse outcomes in pregnancy. Higher incidence of follicular synchrony and higher failure rates has also been reported (Maheshwari et. al., 2007). Moreover, overweight and obesity have also been associated with menstrual irregularities leading to reduced rate of conception. Also, it increases the risk of miscarriage and contributes to perinatal and maternal complications (Elbers et. al., 2010). Obese women have been shown to have a 10%- 15% increased risk of preeclampsia, which has been associated with poor pregnancy outcomes (Barton and Sibai, 2008). Thus, metabolic disorders, including diabetes, obesity, and hyperlipidemia can affect women's fertility directly or indirectly by interfering with the pituitary-hypothalamic function or ovarian function. ***The most prevalent female pathology, wherein, endocrine-metabolic dysfunctions is predominant is Polycystic ovary syndrome (PCOS).***

1.3. Polycystic Ovary Syndrome

Polycystic Ovary Syndrome (PCOS) is an exceedingly prevalent metabolic disorder and possibly constitutes the most frequently encountered endocrinopathy to affect women. PCOS is a frequent medical condition characterized by both metabolic and reproductive disorders, affecting 4-26% of women at their reproductive age (Chatterjee and Bandyopadhyay, 2020). The clinical manifestations of this highly heterogeneous disease include amenorrhea, hirsutism,

obesity, hyperinsulinemia, hyperandrogenism, polycystic ovaries via ultrasound, and it attributes three fourth of the ovulatory infertility (Kini, 2012). Apart from the above manifestations, women with PCOS have incidences of diabetes, endometrial cancer, ovarian cancer, hypertension, and heart disease (Dumesic and Lobo, 2013). Newer studies in both serum and follicular fluid of PCOS patients demonstrated that the above-mentioned manifestations could be consequence of abnormal energy metabolism, lipid and amino acid metabolism that are interlinked or independent (Xu et al., 2020; Zhao et al., 2012). It is to be noted, manifestation and expression of PCOS symptoms such as polycystic ovaries, high levels of androgen hormones and irregular periods are variable from person to person. Also, the controversy concerning a PCOS diagnosis and treatment contributes to the overall current complexities of the syndrome.

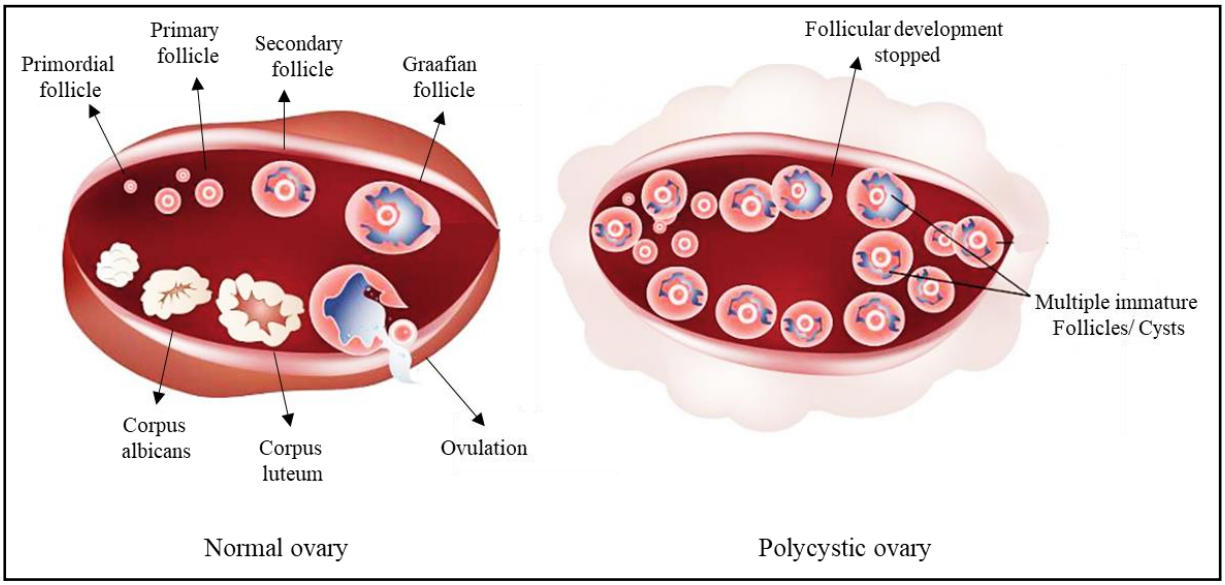

Figure 1.1: Cross-section of a normal and a polycystic ovary

1.3.1. Guidelines for diagnosis of PCOS

Diagnosis and treatment of PCOS remain controversial with challenges defining individual components within the diagnostic criteria, significant clinical heterogeneity generating a range of phenotypes with or without obesity, ethnic differences and variation in clinical features across different age groups. Reproductive symptoms are more common in younger women. Metabolic characteristics are more common as women get older, but they can also show up in younger women who are overweight. (Boyle and Teede, 2012). This complexity has made it very difficult for clinicians to find a clear-cut way to diagnosis a patient with PCOS and to

account for exact prevalence rates. This led to formulation of several guidelines, also known as global consensuses for the diagnosis of PCOS.

1. According to the National Institutes of Health (NIH) Criteria, which were established in 1990- the major criteria for PCOS should include the presence of clinical and/or biochemical hyperandrogenism, as well as oligo/amenorrhea anovulation (Zawadski and Dunaif, 1992).

2. Later in 2003, the Rotterdam Criteria introduced polycystic ovarian morphology on ultrasound as a new criterion to complement the two NIH criteria. The Rotterdam consensus of the European Society of Human Reproduction and Embryology and the American Society for Reproductive Medicine (ESHRE/ASRM) developed and expanded the PCOS diagnosis. The Rotterdam consensus is the most widely accepted across Europe, Asia and Australia (Boyle and Teede, 2012). According to the Rotterdam criteria, a clinical diagnosis of PCOS requires that a patient present with two of the following symptoms:

i. Oligo-ovulation or anovulation.

ii. Hyperandrogenism, clinical (including signs such as hirsutism) or biological (including a raised free androgen index or free testosterone).

iii. Polycystic ovaries visible on ultrasound.

Other aetiologies must be excluded such as congenital adrenal hyperplasia, androgen secreting tumours, Cushing syndrome, thyroid dysfunction and hyperprolactinaemia (Eshre, 2004; Fauser et al. 2004).

Fulfilling two of three diagnostic criteria implies that PCOS can be diagnosed in the absence of androgen excess or menstrual irregularity—the very factors that were once considered absolute requisites for the syndrome (Lujan et al., 2008). Despite the fact that the Rotterdam criteria are controversial (Azziz, 2006; Franks, 2006), they are still the most widely adopted criteria and are used by a wide range of medical practitioners as well as researchers globally (Wang and Mol, 2017).

3.The third and the newest definition was proposed in 2006 by Androgen Excess Society (AES) that has considered hyperandrogenism along with ovarian dysfunction or polycystic ovaries (Azziz et al., 2006). As a result, the Androgen Excess Society (AES) determined that androgen

excess is a key event in the development and pathophysiology of polycystic ovarian syndrome, and that androgen excess must be present and accompanied by either oligomenorrhea or PCOM, or both (Spritzer, 2014). They do not, however, recognize a mild variant of the syndrome in which little is known about metabolic status or long-term health risks.

The Rotterdam consensus and the androgen excess-PCOS society both recommended using ultrasound to detect polycystic ovarian morphology as one of the diagnostic criteria for PCOS. However, due to differences in sonographic procedures, the morphological evaluation of ovaries by ultrasonography is highly diverse. In addition, in obese patients, abdominal ultrasonography makes it harder to see the ovaries (Sahmay et al., 2014). Anti-mullerian hormone (AMH) testing for PCOS diagnosis is becoming more common to overcome these restrictions. The granulosa cells of tiny antral and pre-antral follicles release AMH, which regulates early follicular development. Serum AMH levels are reported to be elevated in PCOS patients, suggesting that it could be a useful diagnostic for diagnosing the condition (Cassar et al., 2014; Lauritsen et al., 2014; Sahmay et al., 2014).

1.3.2. Prevalence of PCOS

PCOS is the most widespread disorder across the globe affecting 4- 26% of women in their reproductive age (Diamanti-Kandarakis et al., 1999; Asuncion et al., 2000; Azziz et al., 2004b; Joshi et al., 2014). The variation in the prevalence is mainly due to different diagnostic criteria. Studies based on Rotterdam criteria have demonstrated PCOS prevalence in China (2-7.5%) (Chen et al, 2008; Li et al, 2013) and in Sri Lanka (6.3%) (Kumarapeli et al., 2008) whereas using NIH criteria, PCOS was found to be prevalent in 5-8% of Caucasian women (Asuncion et al., 2000; Azziz et al., 2004b; Joshi et al., 2014). In an Australian study, PCOS prevalence was about 12% using Rotterdam criteria which increased to 18% when imputed data was included (March et al., 2009). The prevalence of PCOS is on the rise, i.e., 3.7-22.5% in developing nations like India, which are undergoing rapid nutritional transitions due to westernized diets and lifestyle. The prevalence of PCOS in adolescents and young girls was found to be 3.7% in Lucknow, Uttar Pradesh (Gill et al., 2012), 9.13% in Andhra Pradesh (Nidhi et al., 2011), 18% in Tamil Nadu (Balaji et al., 2015) and 22.5% in Mumbai, Maharashtra (Joshi et al., 2014). In order to comprehend the etiopathology of PCOS, it is utmost important to understand the ovarian structure- function under normal physiological condition.

1.4. Ovarian follicular structure and function

Ovarian structure- function is driven largely by five hormones: follicle-stimulating hormone (FSH), luteinizing hormone (LH), androgens, estrogens and progesterone. An ovarian follicle, consisting of an oocyte surrounded by granulosa and theca cells are the main components of follicles, and the functions and interactions among them play crucial role in steroidogenesis, follicular development, and atresia (Orisaka et al., 2009).

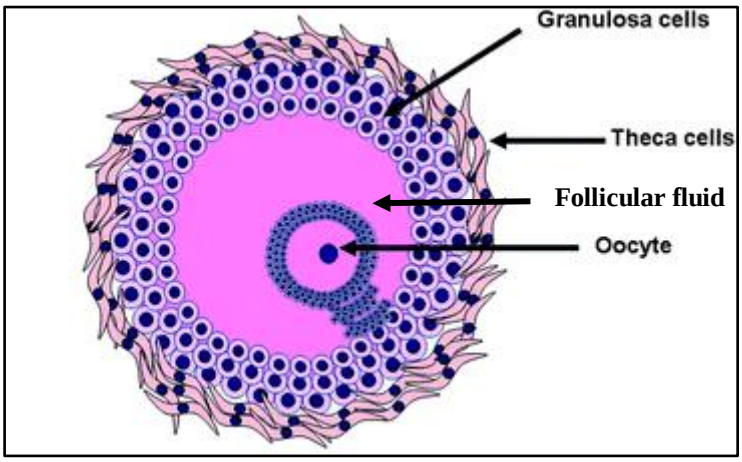

Figure 1.2: Structure of an ovarian follicle (Modified from Catalano et al., 2012)

1.5. Steroid hormones and steroid hormone receptors

As follicles mature, they secrete sex steroid hormones that control maturation in an autocrine/paracrine way and provide endocrine feedback that determines the ovarian functionality. Steroid hormones- progesterone, androgens and estrogens are synthesised by the ovary in sequential manner. The signalling of steroid hormones via nuclear receptors regulates various transcriptional events within ovary (Drummond et al.,2002). Figure.1.3 demonstrates the localisation steroid receptors in ovary. The role of steroid hormones and its receptor in maintenance of ovarian physiology is explained below.

1.5.1. Progesterone:

Progesterone via its receptors plays an important role in ovulation and maintenance of pregnancy (Garg et al.,2017). Progesterone receptors (PR) comprise two forms PR-A and PR-B (Jacobsen and Horwitz., 2012) and previously it has been reported that progesterone receptor

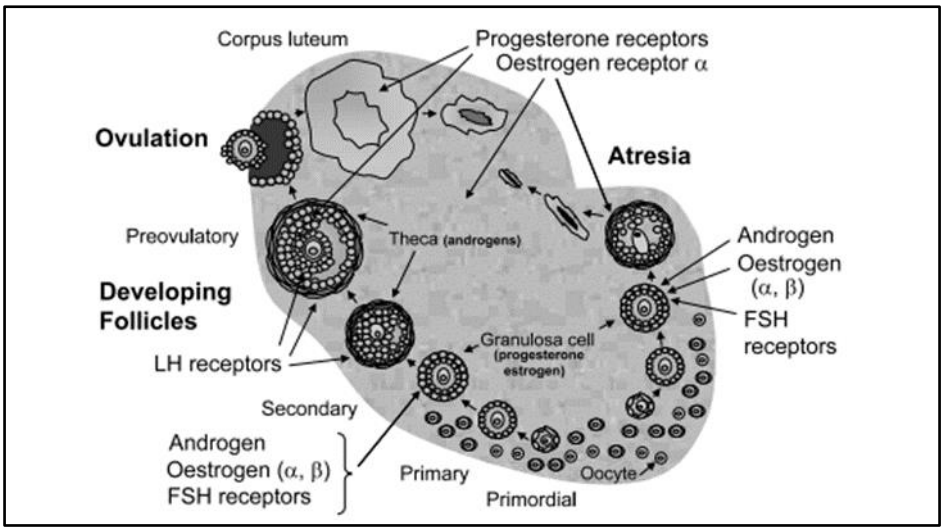

Figure 1.3. Schematic representation of the ovary indicating steroid hormone receptor sites and cellular sites of steroid hormone production (Adapted from Drummond et al., 2002)

knockout (PRKO), when expose to super ovulatory levels of gonadotrophins, PRKO mice fail to ovulate, along with ovarian histology revealed unruptured preovulatory follicles in the ovary and an absence of oocytes in the oviduct and uterine horns (Lydon et al., 1995). This indicate PR is required for ovulation. However, the differentiation of preovulatory granulosa cells follicles into luteal phenotype is still there and express the luteal marker, P450 side chain cleavage enzyme (Robker et al., 2000). Thus, PR is required specifically for LH-dependent follicular rupture leading to ovulation but not for differentiation of granulosa cells to form a corpus luteum (Conneely et al.,2001).

1.5.2. Androgen:

Androgens, mainly testosterone, are produced by theca cells in response to Luteinizing hormone (LH). Androgens target granulosa cell (GC) via their receptors (AR). AR mediated responses are important in the early stage of ovarian follicular development. AR antagonist (Bicalutamite) has shown to inhibit follicle growth and antral cavity in in vitro culture of pre-antral mouse follicles (Murray et al., 1998). Follicle development emerge not attributed to aromatisation, because AR antagonist (flutamide) suppressed the stimulatory effect on follicle growth and insertion of estrogen alone or with the presence of an aromatase inhibitor (fadrozole) did not show any effect on follicle growth (Wang et al.,2001). These results indicate

that effect was mediated via direct AR induced action and not because of aromatisation of testosterone to estrogen with subsequent ER-mediated effects (Yang and Fortune 2006).

1.5.3. Estrogen:

Estrogen actions are mediated via genomic pathways by ERα and Erβ (Tang et al.,2019). Local production of estrogen in ovaries known to regulate follicular maturation and induce proliferation of GC during dominant follicle growth. It has been reported that maturation of oocytes isolated from carp were significantly reduced when incubated with estrogen agonist (Majumder et al., 2015). Further to find out estrogen mediate action in the ovary, estrogen receptor knockout (ERKO) female mouse was acyclic, infertile and folliculogenesis is arrested at the antral stage with follicles becoming cystic and haemorrhagic (Lubahn et al.,1993; Couse et al.,1995).

Steroid hormones are produced primarily in the adrenal gland, gonads, and placenta through a process known as steroidogenesis, which is controlled by the hypothalamus–pituitary–steroidogenic gland (i.e., adrenal, gonads, and placenta) axis.

1.6. Steroidogenesis:

Steroidogenesis in ovary is bicompartmental, occurring in two major cells of ovary -granulosa cells and theca cells. A steroidogenic cell's range of enzymes corresponds to the cell's ability to biosynthesise particular steroids. Thus, steroidogenic capacity is primarily regulated by tissue and cell specific enzyme expression, rather than by selective activation or inhibition of a larger repertoire of enzymes. The levels of steroidogenic enzymes define the quantitative potential of steroidogenic cells for the production of particular steroids (Hanukoglu, 1992). According to Two Cell-Two Gonadotropin theory, FSH and LH regulates steroidogenesis in Granulosa cells and Theca cells. These cells respond differentially to FSH and LH, which is mainly due to following features: (1) granulosa cells have FSH receptors but not thecal cells; (2) thecal cells have LH receptors but not immature granulosa cells; (3) FSH stimulates aromatase activity but not androgen synthesis in granulosa cells; and (4) LH stimulates androgen synthesis but not aromatase activity in thecal cells (Hillier et al., 1994). It explains the endocrine regulation of follicular estrogen synthesis and implied paracrine signalling in the follicle wall.

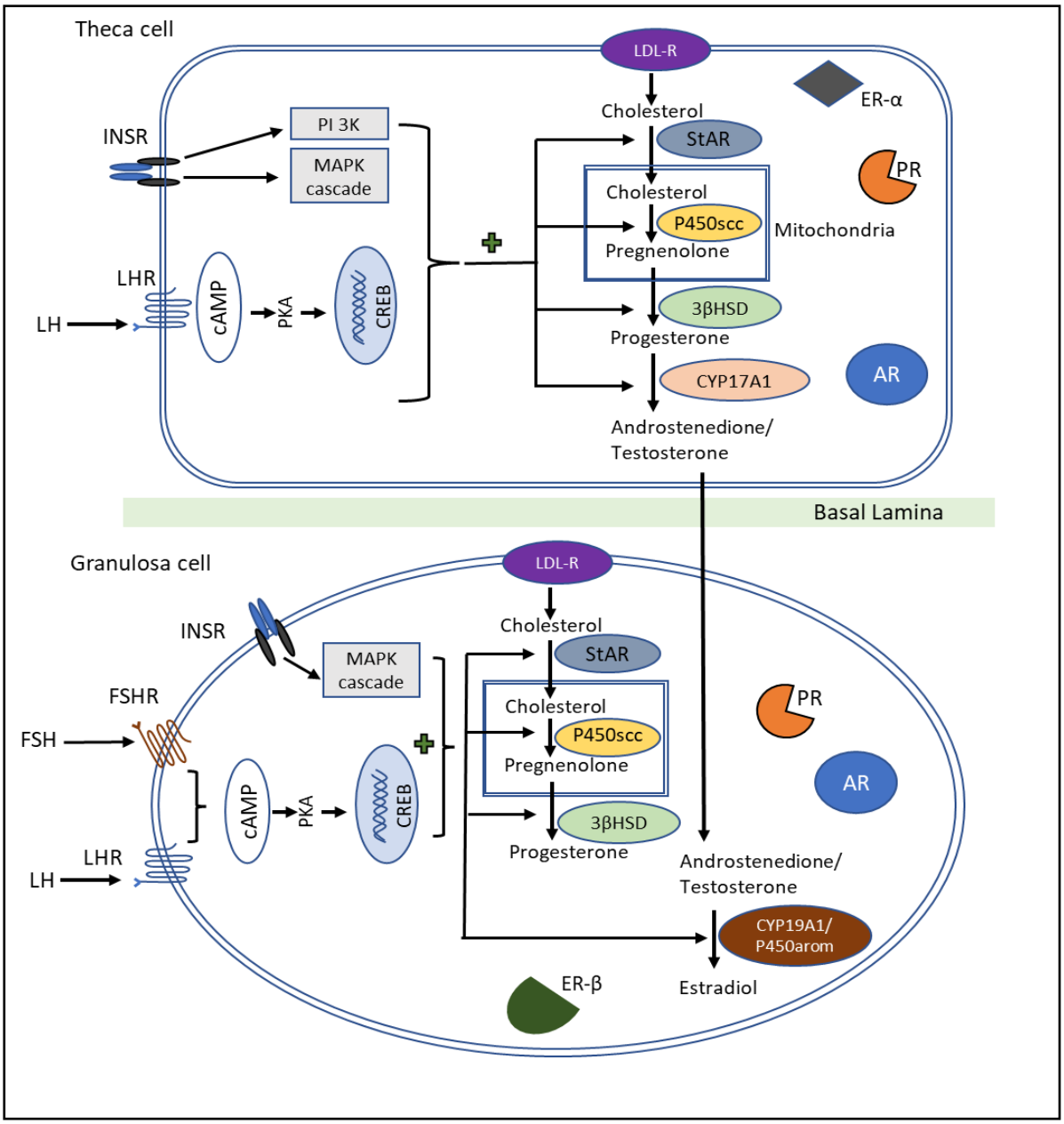

Figure 1.4: Intracellular pathways that affect the regulation of ovarian steroid synthesis. P450scc: cholesterol side-chain cleavage cytochrome P450; CYP17A1: 17- hydroxylase/C17–20 lyase cytochrome P450; 3-HSD: 3-hydroxysteroid dehydrogenase; P450arom: aromatase cytochrome P450; LDL-R: low-density lipoprotein receptor; StAR: steroidogenic acute regulatory protein; PI 3K: Phosphatidyl Inositol 3-kinase; MAPK: Mitogen-Activated Protein Kinase; PKA: Protein Kinase A; PR: Progesterone Receptor; AR: Androgen Receptor; ER-Estrogen Receptor; INSR: Insulin Receptor; LH: Luteinizing Hormone; LHR: Luteinizing Hormone Receptor; FSH: Follicle Stimulating Hormone; FSHR: Follicle Stimulating Hormone.

In the normal cycle, the dominant follicle maintains its growth and hormone output in the face of declining FSH concentrations by the acquisition of increased sensitivity to LH (Horcajadas et al.,2008). Through the follicular stage, FSH and LH both stimulate cyclic adenosine monophosphate (cAMP) production and elicit growth and oestrogen secretion through this same mechanism (Filicori et al,1999). Activation of the LH receptor in granulosa cells leads to the transcription and protein production of two key proteins of steroidogenesis: Cyp17(P450-17,20-Lyase & 17α-hydroxylase) and aromatase (P450arom). In a study by Yong et al., 1994, granulosa cells removed from follicles prior to the LH surge were differentially dependent upon follicular size, and both FSH and LH strongly stimulated P450arom mRNA expression and estradiol production in mature rat granulosa cells showed that P450scc mRNA expression and progesterone biosynthesis were maximal only in the presence of both FSH and LH.

The gonadotropins-LH and FSH regulate the expression of genes involved in steroidogenesis, including those that code for steroidogenic enzymes, cholesterol transporters, and electronic transporters. The biosynthesis of steroid hormones commences with cholesterol and is represented in Figure 1.4. Cholesterol is stored in lipid vesicles in an esterified state. Trophic hormones trigger a series of events that result in the hydrolysis of cholesterol esters into free cholesterol and the transport of cholesterol into mitochondria. The critical step in steroidogenesis is the initial conversion of cholesterol (a C-27 steroid) to pregnenolone (the primary C-21 product), from which all other steroids are generated. The cholesterol side-chain cleavage cytochrome P450 enzyme (P450scc), encoded by the *Cyp11a* gene, catalyses this step and is considered to be the rate-limiting step in steroidogenesis. (Miller et al.,2007; Wickenheisser et al.,2006). Pregnenolone is metabolised in one of three pathways: a) Hydroxylation of pregnenolone at the C-17a position yields 17-hydroxypregnenolone, and subsequent removal of the acetyl group forms the androgen precursor dehydroepiandrosterone (DHEA), b) Conversion of pregnenolone to progesterone by the action of 3β-HSD. The conversion is irreversible; progesterone can be converted to 17-hydroxyprogesterone by Cyp17 (the D4 pathway). This 17-hydroxyprogesterone has a possible role in modulating the biological activity of cortisol, thereby reducing the inflammatory-like reactions associated with ovulation (Andersen et al.,2002) & c) *Cyp17a1* is located exclusively in thecal/interstitial cells (i.e., the extra follicular compartment of the ovary), whereas *Cyp19a1*, encoding for aromatase is expressed only in the granulosa cells (i.e., the intrafollicular compartment). Thus, the theca cells provide the androgens required by the developing follicles for conversion into oestrogens

by the granulosa cells (Yong et al., 2010) & estradiol synthesis in the human ovary is via the D5 pathway only. Steroid hormones are hydrophobic molecules that can pass through biological membranes and enter into the bloodstream without being stored in intracellular vesicles when they are synthesised. Continuous steroid synthesis and secretion is required for a rise in steroid hormone levels in the blood.

1.6.1. Regulation of steroidogenesis:

The steroid hormone output is mainly regulated by mainly three factors. Firstly, the transcription, stability, and translation of the mRNAs encoding the steroidogenic enzymes determine their levels. Secondly, the activity of steroidogenic enzymes is regulated by the intracellular environment, cofactor availability, or post-translational modification of the enzymes. Finally, the availability of a substrate is usually determined by cholesterol mobilisation and transport to the mitochondrial P450scc, which catalyses the initial step in the steroid biosynthesis pathways (Hanukoglu 1992). Tropic hormones promote the transcription of genes that code for steroidogenic enzymes, resulting in a gradual regulation that determines the identity and capacity of steroidogenic cells. The ability to induce steroidogenesis 10–100-fold in minutes and quickly stop it is regulated at the level of cholesterol flow from the outer mitochondrial membrane to the inner mitochondrial membrane, which is mediated by the presence of StAR protein (Bose et al., 2002; Stocco and Clark, 1996).

1.6.1.1. Steroidogenic Acute Regulatory Protein (StAR)

StAR is expressed in the steroid producing cells of fetal and adult testes, adrenal cortex, ovarian theca, granulosa, and ovarian corpora luteal cells and fetal mouse giant trophoblast cells (Kiriakidou et al., 1996). StAR is directed to the mitochondria via its N-terminus and presumably utilizing C-terminal sequences, produces alterations in the outer mitochondrial membrane that result in the transfer of cholesterol from the outer to the inner membrane. This transfer of cholesterol is specific and up-regulated by LH, FSH, insulin, human chorionic gonadotropin (hCG), intracellular cAMP-inducing agents, IGF-I, growth hormone etc. It facilitates the initiation of steroidogenesis (Mishra et al., 2015). In the human ovary, the expression of StAR is regulated throughout the luteal phase and plays a key role in controlling luteal progesterone production during the development and demise of the corpus luteum (Devoto et al., 2002; Kohen et al., 2003; Sierralta et al., 2005). Several lines of evidence

demonstrate the involvement of multiple transcription factors and signalling pathways in StAR expression and steroidogenesis (Sugawara et al., 1995; Manna et al., 2003b, 2006a, 2009a, b; Stocco et al., 2005; Martin et al., 2008). Rapid expression of StAR in response to trophic hormone is required to initiate and maintain ongoing steroid hormone biosynthesis, and this expression is regulated via the cAMP second messenger signalling pathway (Manna et al., 2002). In many cases transcriptional induction by cAMP is mediated through the interaction of a cAMP response-element binding protein (CREB) family member with a consensus cAMP response element (CRE; 5'-TGACGTCA-3') found in the promoter of target genes (Manna et al., 2009). Transient expression of wild- type CREB in MA-10 mouse Leydig tumor cells further increased the levels of (Bu)2cAMP-induced progesterone synthesis, StAR promoter activity, StAR mRNA, and StAR protein (Manna et al., 2002). Another protein that plays an important role in the regulation of steroidogenesis is CYP17A1.

1.6.1.2. CYP17A1

CYP17A1 is a member of the cytochrome P450 superfamily of enzymes localized in the endoplasmic reticulum. CYP17A1 has both 17α-hydroxylase activity and 17,20-lyase activity. The 17α-hydroxylase activity of CYP17A1 is required for the generation of glucocorticoids such as cortisol, but both the hydroxylase and 17,20-lyase activities of CYP17A1 are required for the production of androgenic and oestrogenic sex steroids by converting 17α-hydroxypregnenolone to dehydroepiandrosterone (DHEA). In human adrenal steroidogenesis, in the zona glomerulosa CYP17A1 is absent, which means that the final product formed is mineralocorticoid aldosterone. In the zona fasciculata, where CYP17A1 hydroxylase activity is present but lyase is absent, the product is glucocorticoid cortisol. Both hydroxylase and lyase activity are present in the zona reticularis, the androgen precursor DHEA is produced in large amounts. (Miller et al.,1997). The hydroxylase and lyase activity of CYP17 are located at the same active site on the enzyme, but the discrimination between the two is regulated post-translationally. LH stimulates androgen production in theca cells via CYP17 proteins. Insulin and insulin-like growth factor (IGF-1) and (IGF-2) stimulates androstenedione production by human theca cells *in-vitro.* The effect of inhibin represents as an FSH-driven paracrine signal from the granulosa to the theca cells, affects the expression or the activity of CYP17, and thereby increasing androgen production. (Mishra et al.,2015). Also, this effect is enhanced by the addition of the action of LH on thecal cells (Nahum et al.,1995). Epidermal growth factor

(EGF), fibroblast growth factor (FGF), TGF-b, growth differentiation factor-9 (GDF-9) and activin inhibits the activity of CYP17.

1.6.1.3. 3β -Hydroxysteroid Dehydrogenase/Isomerase

3β-hydroxysteroid dehydrogenase, an enzyme localized in the endoplasmic reticulum is responsible for catalysing the conversion of pregnenolone, 17-OH pregnenolone, dehydroepiandrosterone (DHEA) and androst-5-ene-3β,17β-diol into progesterone, 17-OH progesterone, 4-ene-androstenedione and testosterone, respectively. This enzyme is in fact required for the formation of all classes of steroid hormones, namely, progesterone, mineralocorticoids, glucocorticoids, as well as androgens and estrogens (Simard et al., 2005). 3β-HSD is detected in a variety of peripheral tissues, including the skin, adipose tissue, breast, lung, endometrial, prostate, liver, kidney, epididymis, and brain, in addition to the traditional steroidogenic tissues of the placenta, adrenal cortex, ovary, and testis (Martel et al., 1994). Because of the extensive expression of 3β-HSD, this enzyme is believed to play a key role in the intracrine synthesis of sex hormones in peripheral target tissues. 3β-HSD mRNA has been localized to the theca interna of preantral, antral and atretic follicles as well as the corpus luteum, suggesting that it plays a crucial role in the folliculogenesis (Suzuki et al., 1994). The granulosa cell layer of most species does not contain 3β-HSD protein until late follicle development. Expression levels of 3β-HSD could reflect the steroidogenic capacity and physiological status of follicles. Very little is known about the regulation of 3β-HSD in ovaries. Previous studies have implicated that gonadotropin and steroid hormones regulate the expression of 3β-HSD in all mammals (Chedrese et al., 1990; Voss and Fortune, 1993; Keeney and Mason, 1992; Mcgee et al., 1995). Under culture conditions, LH, hCG, and cAMP-signalling agonists increase mRNA and protein levels of 3β-HSD in theca, granulosa, and leydig cells (Sun et al., 2011). 3β-HSD mRNA and protein levels are also stimulated by FSH in rat and human cultured granulosa cells (Eimerl and Orly, 2002). In addition to this, 3β-HSD mRNA expression is influenced or altered by a range of growth stimuli. 3β-HSD expression in granulosa cells can be increased by insulin and IGF-I. Insulin increases FSH stimulation of 3β-HSD mRNA in human granulosa cells in a more than additive manner (Belani et al., 2018).

1.6.1.4. P_{450} Aromatase/ CYP19A1

Aromatase enzyme encoded by the *Cyp19a1* gene, which contains an unusually large regulatory region. Its expression is under the control of two distinct promoters a gonad- and a brain-specific promoter & therefore, the coordinated and cell-specific expression of the aromatase (*Cyp19a1*) gene in the ovary plays a key role in the normal progress of the menstrual/estrus cycle. This enzyme is widely expressed in many tissues for example: breast, adipocytes, CNS, skin and placenta (Bulun et al., 2005). Its expression is limited to the follicle and the corpus luteum, wherein, cAMP is the main intracellular messenger mediating FSH stimulation of aromatase expression (Weniger and Zeis, 1988). Increase in intracellular cAMP levels induced by FSH leads to the activation of the cAMP-dependent protein kinase A (PKA) which catalyses the conversion of testosterone into the 17β-estradiol. Luteal cell function is greatly affected by locally produced estradiol, which stimulates both progesterone biosynthesis and luteal cell hypertrophy (Gibori et al., 2013).

There appears a complex network of signals that regulate steroidogenesis under different physiological conditions. Any disturbance in the signalling cascade can lead to disrupted steroidogenesis, as observed in case of Polycystic Ovary Syndrome (PCOS). PCOS is a complex pathological condition characterised by steroidogenesis abnormalities, in the form of excess circulating androstenedione and testosterone. Women with polycystic ovaries have a wide spectrum of presentation from anovulation and amenorrhoea to apparently regular, ovulatory menstrual cycles, suggesting that their ovarian structure-function is modulated. The exact pathophysiology of PCOS is not clear; however, disturbance in hypothalamic-pituitary-ovarian axis and abnormal steroidogenesis along with genetic and environmental factors act as main contributors to the multi organ reproductive endocrinopathy.

1.7. Pathophysiology of PCOS

The pathophysiology and intrinsic mechanisms underlying PCOS are complex because of varying etiologies and the different features are considerably intertwined. The interplay between these mechanisms results in and perpetuates the clinical features of PCOS, including hyperandrogenism, polycystic ovaries and ovulatory dysfunction, in addition to the associated mood disturbances, psychosexual dysfunction and long-term morbidities (Figure 1.5). In addition, the development of PCOS has a strong genetic component. In PCOS, there is a direct

link between hyperandrogenic and hyperinsulinemic conditions which leads to ovulatory dysfunction. Following sections will explain various etiopathologies associated with PCOS phenotype:

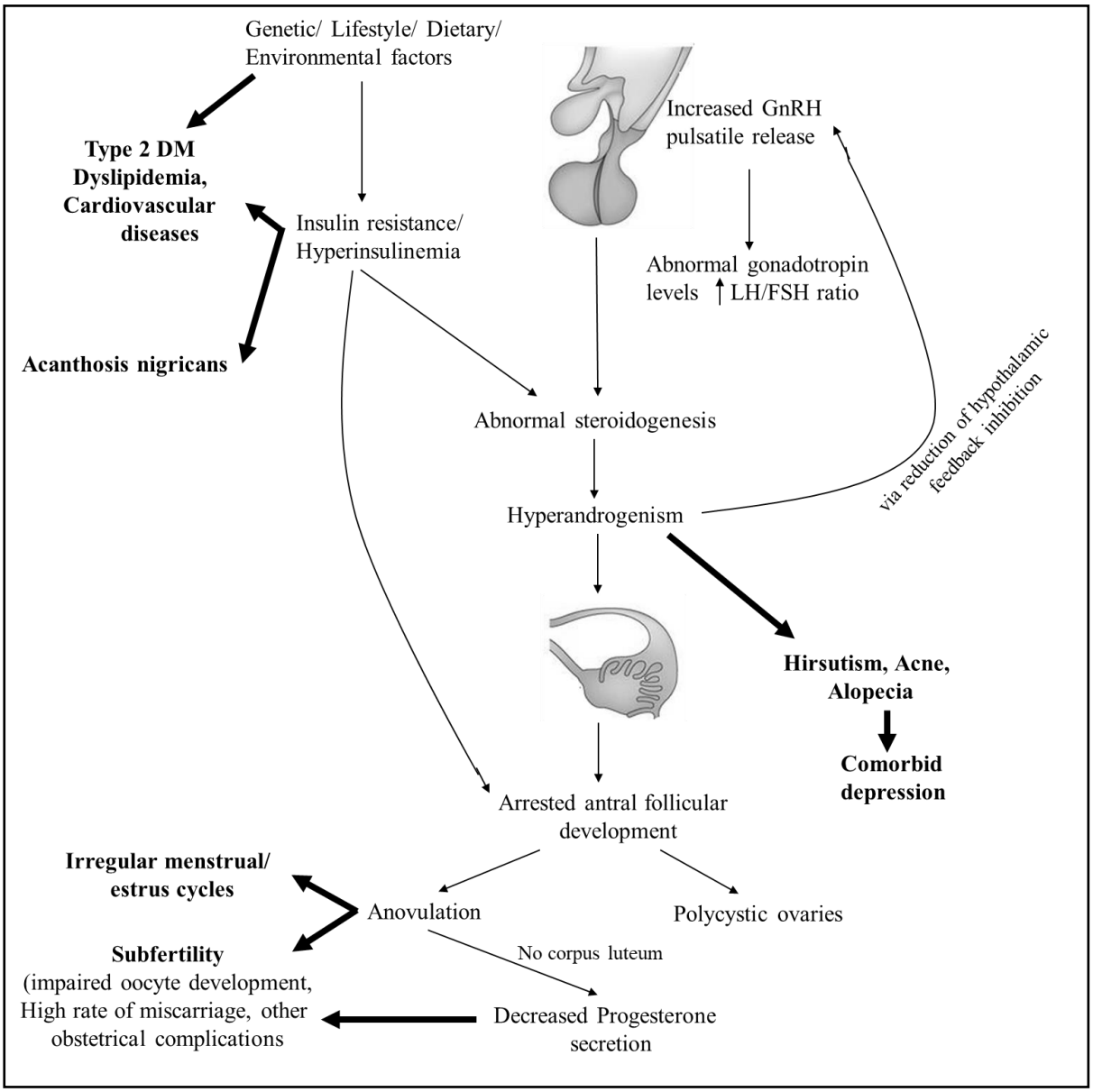

Figure 1.5: Pathophysiology of polycystic ovarian syndrome (PCOS)

1.7.1. Gonadotropic derangements:

In normal circumstances, immature oocytes mature under the influence of several hormones, most notably FSH, and ovulation as well as final maturation occur upon luteinizing hormone (LH) stimulation. A neuroendocrine abnormality in PCOS may include increased

gonadotropin-releasing hormone (GnRH) pulse frequency, which increases the frequency and pulse amplitude of LH over FSH production. In normal females, LH/FSH ratio is 1:1 whereas in PCOS females it is 3:1. This condition of increased LH over FSH is seen early during puberty in girls with hyperandrogenism indicates that abnormalities in the pulsatile release of GnRH might underlie the development of PCOS, at least in some patients (Solorzano et al., 2010). The increased LH/FSH ratio, and the resistance to FSH in the ovaries, further enhances hypersecretion of androgens in theca cells in ovarian follicles, which impairs follicular development and reduces the inhibition of GnRH pulse frequency by progesterone, further promoting the development of the PCOS phenotype.

1.7.2. Hyperinsulinemia and Insulin resistance:

The role of insulin resistance and hyperinsulinemia in the development of PCOS has been thoroughly explored, and it is generally accepted to play an important role in the molecular mechanisms implicated in the androgenic hypersecretion typical of this pathology (Diamanti-Kandarakis and Dunaif, 2012). Insulin may play a part in the development of the typical increased amplitude and frequency of GnRH and LH pulse secretion seen in PCOS. Indeed, elevation of LH and GnRH secretion in response to insulin infusion has been observed *"in vitro"*, both in dose-dependent and time-dependent fashions (Sekar et al., 2000; Morley et al., 1989). This effect may be mediated by insulin in GnRH-secreting cells of the hypothalamus, by potentiating GnRH gene transcription through the MAPK pathway. As a result, increased GnRH synthesis and secretion lead to a subsequent elevation of LH levels. This continuous stimulation would translate into augmented synthesis of ovarian steroid hormones, particularly androgens (Kim et al., 2005). Elevated insulin concentrations have been associated with lower levels of SHBG, leading to enhanced bioavailability of androgens (Wallace et al., 2013). Although insulin and the insulin-like growth factor 1 (IGF-1) have been demonstrated unable to directly repress SHBG gene (Selva et al., 2007), they may be key indirect mediators, as they have been associated with decreased total protein secretion in human hepatic cells (Crave et al., 1995). Insulin has been shown to repress insulin-like growth factor-1 binding protein (IGFBP1) synthesis in a direct, rapid and complete way in both the liver and the ovaries, allowing for greater IGF-1 availability, which in turn boosts insulin activity not only in the liver- further contributing to lower SHBG levels—but also in the ovaries, reinforcing PCOS pathophysiology (Mounier et al., 2006). The presence of Insulin receptor and IGF-1 receptors

in theca cells, granulosa cells and stromal cells of ovarian tissue unequivocally identifies this organ as a target of insulin activity (Dunaif et. al., 2001), confirmed by observations of decreased steroidogenesis in theca cells and granulosa cell from both healthy and polycystic ovaries, following in vitro administration of both anti-IGF1R and anti-Insulin receptor antibodies (Mukherjee and Maitra, 2010). One of the key players in this activity is the Steroidogenic Acute Regulatory protein (StAR), a molecule implicated in transportation of cholesterol to the internal mitochondrial membrane, where the cholesterol side-chain cleavage enzyme (CYP11A1) is anchored, the rate-limiting enzyme in steroid hormone synthesis (Zhang et al., 2000). Insulin appears to augment not only StAR expression, but also CYP11A1, 17-α-hydroxylase/17,20- lyase (CYP17A1), 3β-hydroxysteroid dehydrogenase (3β-HSD), and aromatase (CYP19A1) expression, contributing to an excess in the production of progesterone, 17-α-hydroxyprogesterone, and testosterone in polycystic ovaries in comparison to healthy ovaries (Jamnongjit and Hammes, 2006). Insulin appears to act in synergy with LH to elevate intracellular concentration of cAMP, which activates StAR, potentiating steroidogenic activity. Although this effect may be direct through the PI3K pathway, the requirement of cAMP for this activity suggests divergence from the usual insulin cascade; yet the differential molecular interactions exist and unexplained (Mendez et. al., 2005). Similarly, insulin and LH may also act in synergy to increase transcription of LDL-C receptors in GC through the PKA, PI3K, and MAPK pathways. On the other hand, insulin may also augment steroid synthesis through aromatase upregulation in GC, which would serve as substrates for theca cells for further conversion into androgens (Rice et al., 2005).

1.7.3. Hyperandrogenism:

Hyperandrogenism is the defining feature of women with PCOS. It is caused by the disruption of normal ovarian or adrenal function resulting in the production of excess androgens. The first impact of androgen excess in PCOS is impaired folliculogenesis. Increased androgens in the early gonadotropin-independent stage stimulate the formation of primordial follicles and increase the number of small antral follicles (Nisenblat and Norman, 2009). Normally, the gonadotropin-releasing hormone is secreted in a pulsatile manner by the hypothalamus that stimulates the pituitary gland to release gonadotrophins, i.e., LH and FSH. Luteinizing hormone acts primarily on the ovarian theca cells carrying LH receptors and induces the production of androgens. Concomitantly, FSH acts on the ovarian granulosa cells and converts

the androgens formed in theca cells into estrogens, principally estradiol, which is responsible for the development of follicles. However, in women with PCOS, it has been hypothesized that dysregulation in the neuroendocrine system leads to an imbalance in the hypothalamic–pituitary–ovarian axis, leading to the overproduction of gonadotrophins. An increased hypothalamic GnRH favors the production of the β-subunit of LH over the β-subunit of FSH that in turn favors the production of LH over FSH (Fauser et al., 1991; Van Santbrink et al., 1997), hence resulting in the classical hormonal hallmark of elevated LH/FSH ratio in PCOS. Owing to the increased LH stimulation, numerous follicles in the theca cells of ovaries get arrested mostly in the preantral and antral stages, causing hyperplasia of theca cells and subsequent accumulation of follicular fluid forming cyst-like structures along the periphery of the ovary giving it a string of pearls-like appearance (Abbott et al., 2005). Increased number of follicles and increased expression of key enzymes involved in the androgen synthesis thus produce an excessive amount of androgens. Furthermore, the hyperandrogenic state in PCOS also seems to be linked with the action of insulin. The increased insulin secretion possibly mimics the tropic action of luteinizing hormone on ovarian theca cells (Wu et al., 2014), which further causes an increase in androgens. This is further validated by the fact that the improvement of insulin resistance in PCOS women decreases the level of hyperandrogenism (Baillargeon et al., 2004). Insulin also promotes the Serine phosphorylation of multi-functional enzyme -C17 -20 Lyase/ 17 hydroxylase and thus increasing flux towards excess androgen production (Tonetta and Hernandez, 1989). Thus, suggesting relation of insulin with androgen.

The androgen excess is regarded as the major driving force in the development of signs and symptoms of this disorder. Excessive androgen production by ovaries as well as from adrenals contributes to hyperandrogenism. Genetic and clinical heterogeneity associated with hyperandrogenic condition indicates the possible involvement of abnormalities in the steroid synthesis pathway (Reddy et al., 2014). Recent studies indicate that hyperandrogenemic phenotype in PCOS is familial, suggesting maternal inheritance and hence the involvement of genetic factors particularly genes governing steroid hormone biosynthesis (Prapas et al., 2009). Furthermore, the altered expression of genes involved in the synthesis of androgens in PCOS mothers is known to alter the extent of androgen exposure in utero (Xita and Tsatsoulis, 2006). It has been hypothesized that exposure of the fetus to androgen excess in utero results in hypersecretion of luteinizing hormone, alteration in the differentiation process of thecal cells, and male-type fat distribution in female offspring (Xita et al., 2010). In addition, maternal

nutrition and epigenetic changes have also been found to influence fetal programming. Contrary to this, other studies have shown that normal aromatization in the placenta, if maintained, does not induce PCOS in the female fetus when subjected to increased levels of androgens from mother (Dumesic et. al., 2014). Legro et al.,1998 suggested a genetic basis of hyperandrogenism in PCOS. Genes involved in steroid synthesis are considered as candidate genes in the pathophysiology of PCOS.

The increased concentration of Total Testosterone or Free- Testosterone levels is a key diagnostic feature of biochemical hyperandrogenism. Other androgens like dehydroepiandrosterone and androstenedione may also be helpful in diagnosing biochemical hyperandrogenism. Androstenedione, DHEA, and dehydroepiandrosterone sulfate are all bound to albumin with low affinity (Moll and Rosenfield, 1979). DHEA-Sulphate is found abundantly in circulation, and due to the presence of its sulfate group, it is easily detected by commercial assays. Elevated levels of DHEA are seen in approximately 25% of PCOS patients (Huang et al., 2006). High androstenedione levels are found in 18% of PCOS women (Azziz et al., 2004). Hyperandrogenism in women with PCOS clinically presents as hirsutism, acne, and androgenic alopecia. Other manifestations like weight gain, menstrual irregularities, acanthosis nigricans, and insulin resistance are also manifested by increased androgen excess (Ashraf et al., 2019).

1.7.4. Ovarian follicular arrest:

Ovarian folliculogenesis is regulated by a delicate balance between extra and intra-ovarian factors. Disruption of this balance may muddle follicular development and formation of mature oocytes, leading to infertility (De Leo et al. 2016). In healthy women, oocytes mature under the influence of various hormones, mainly FSH and LH stimulates ovulation as well as final oocyte maturation (Azziz et al. 2016). In women with PCOS, there may be early luteinization due to high levels of LH. These women tend to form several antral follicles whose development is interrupted prematurely. The combination of these factors induces most follicles to stop at a small antral stage, acquiring up to 2–5 mm in diameter, which is two to three times larger than that observed in normal ovaries (Kurobe et al., 2012). Amongst the various factors that regulate folliculogenesis, AMH (anti-Mullerian hormone) plays a very vital role in this process. It inhibits the process of recruitment of primordial follicles and modifies the growth of preantral and antral follicles by diminishing the sensitivity of follicles for FSH stimulation (Xu et al.,

2016). The level of this hormone decreases with age and in postmenopausal period is undetectable in blood. Therefore, AMH could be a useful marker of ovarian reserve. Multiple investigations have revealed that women with PCOS have very high levels of AMH in serum and follicular fluid (Chang et al., 2013). This hormone is produced by granulosa cells and, in normal women, acts on the primordial follicle, inhibiting the recruitment of many follicles and attenuates the effects of FSH on growing follicles. In women with PCOS, high levels of AMH lead to follicular resistance to FSH, which also impairs the follicle growth (Kurobe et al. 2012), selection of a dominant follicle and recruitment of more primordial follicles. Furthermore, considering that FSH is also important to stimulate granulosa cells to convert androgens to estrogens (Chang et al. 2013), the antagonism of AMH to FSH contributes to hyperandrogenism in PCOS. Increased serum AMH is a common feature of PCOS and thus a potential biomarker of this syndrome (Dumont et al. 2015; Quinn et al. 2017). Serum AMH levels correlate with the number of ovarian follicles and cysts and therefore, AMH can be used as an alternative to transvaginal ultrasonography to detect polycystic ovarian morphology, which is one of the criteria for diagnosing PCOS (Singh and Singh, 2015). Its measurement is also useful in the management of infertility in women with PCOS and as a marker of treatment efficiency in relieving PCOS symptoms (Dumont et al. 2015).

1.7.5. Alteration in steroidogenesis in PCOS:

The changes at the molecular level in granulosa and theca cells of polycystic ovaries in women with PCOS generally have elevated GnRH pulsatile activity, high levels of LH, hyperactivity of theca-stromal cell and altered activity of granulosa cell that causes reduced production of estradiol (E2) and progesterone (P4) by pre-ovulatory follicle. Various studies have inspected the activity of the enzymes involved in the biosynthesis of steroid hormones in PCOS (Meidoiross et al.,2013). There is an increased expression of certain alleles that are responsible for the expression of steroidogenic enzymes. The genetic basis of receptors in granulosa and theca cells of polycystic ovaries and reported higher mRNA expression levels of LH receptor (LHR), StAR, CYP11A1, and CYP17A1. Catteau-Jonard et al., 2008 showed dysregulation of granulosa cells in addition to intrinsic dysfunction leading to hyperandrogenism and increased expression of FSH receptor (FSHR) and androgen receptor (AR) in stimulated ovaries of women with PCOS. The ovarian programming induced by prenatal androgens in animals influences hypersecretion of androgens by altering LH sensitivity, resulting in up-

regulation of steroidogenic genes in theca cells to produce excessive amounts of androgens. One study also showed that in granulosa cells, LHR and CYP11A, but not StAR, mRNA expression was higher in PCOS than in control follicles indicating that granulosa cells in PCOS have increased LH responsiveness that may contribute to arrested follicle development. The intrinsic defect in the steroid biosynthesis in theca cells at genetic level is not due to overall differences in the regulation of cAMP or adenylate cyclase (activated by forskolin) but due to selective alterations in steroidogenic enzyme expression. Also, expression of CYP17A1 is elevated in the ovaries of women suffering from PCOS and it is partly responsible for the altered steroidogenesis (Comim et al.,2013) at both transcriptional and post-transcriptional level & demonstrates 4-folds greater CYP17A1 promoter activity in theca interna cells of human polycystic ovaries. (Wickenheisser et al.,2005). PCOS theca cells have cAMP-dependent CYP17A1 gene expression and there was slower degradation of CYP17A1 mRNA. It suggests that dysregulation of the processes involved in CYP17A1 transcription in PCOS theca cells may, in part, account for the increased ovarian androgen production. In addition to this, Aromatase enzyme, that catalyzes the formation of estrogens (estrone and E2) from androgens (androstenedione and T) during steroidogenesis shows downregulation and was found to be partially responsible for the altered steroidogenesis in PCOS. (Kirilovas et al.,2006). Multiple studies have demonstrated that there is dysregulation of P450arom in women with PCOS. Estradiol/Testosterone (E2/T) ratio, in females with PCOS is considered to be direct marker for Aromatase activity. In PCOS condition, reduced production of E2/T, FSH/LH with increase in LH, Testosterone. Follicles are resistant to FSH, but the follicle size remains small.

As evident from the above discussion, the pathophysiology of PCOS is not fully understood. This can be addressed by using appropriate experimental models to study PCOS.

1.8. Models to study PCOS

Studies on humans have limitations due to ethical issues, hence animal models are used to understand the different aspects of PCOS. Animal models of PCOS have been established to aid in the research of many aspects ranging from the origin to the management of PCOS. Given that developing an animal model that mimics all characteristics of this condition is difficult, the animal model's biological, anatomical and/or molecular similarities to human PCOS symptoms may boost its applicability. Many of the reproductive and metabolic phenotypes

associated with PCOS can be seen in prenatally androgenized rhesus monkeys and sheep (Abbott et al., 2009; Padmanabhan and Veiga-Lopez, 2013), but ethical and economic considerations, as well as the inability to genetically and transgenically manipulate these larger species to probe underlying mechanisms, limit the utility of nonhuman primate and large domestic animal models. On the contrary, the ovarian physiology of rodents is not very similar to that of human. However, because of the reduced costs, high sample size in similar animals, simplicity, and short duration of induction procedures, this model has been the model of choice for many researchers (Tamadon et al., 2018). The major aspects of PCOS that should be displayed in animal models, according to Rotterdam's criteria for PCOS diagnosis, are hyperandrogenism, too many antral follicles, and an irregular estrous cycle (amenorrhea or oligomenorrhea in case of non-human primates). Naturally occurring PCOS-like animals would be the best animal PCOS model. Laboratory rodents, unlike humans, do not naturally develop PCOS-like characteristics, although natural housed rats have been shown to have persistent ovarian follicles with a protracted estrous cycle (Evans and Long, 2021). Natural PCOS-like traits in rodents have yet to be discovered. As a result, several artificial methods have been utilized to research PCOS in rodents at different stages of the development of rodents like prenatal, postnatal, prepubertal and adulthood (Ryu et al., 2019).

Figure 1.6 provides the details of different experimental models used to study PCOS along with the salient features exhibited by them. Hyperandrogenism is the hallmark of PCOS (Ndefo et al.,2013). Hyperandrogenic condition is one of the culprits behind all the consequences of PCOS condition. Amongst, several approaches to develop rodent models of PCOS, hormone-regulated models are more popular, as they replicate several key PCOS features. Polycystic ovaries can be induced by androgen exposure including not only exogenous androgens but also as a result of secondary endogenous androgen excess (Walters et al., 2012). The latter includes a PCOS rodent model generated by letrozole, a nonsteroidal aromatase inhibitor that prevents androgens from being converted to estrogen (Kafali et al., 2004). Hence, administration of letrozole blocks the conversion of testosterone to estradiol and maintains the hyperandrogenic condition (a key feature for the establishment of PCOS model). Oral administration of letrozole (1 mg/kg, once daily, for 21–28 days) in pre-pubertal (Gong et al., 2015), post-pubertal (Rezvanfar et al.,2014) and adult (Kafali et al., 2014; Manneras et al., 2007) female rats can induce PCOS-like features, by causing hormonal imbalance, circulating hyperandrogenism and intra ovarian androgen excess leading to appearance of polycystic ovary. Follicular atresia and

abnormal follicular development are observed due to induced elevation of androgen levels inside the ovary (Choi et al., 2005). Letrozole induction was reported to cause hyperglycemic condition which may contribute to insulin resistance, hyperlipidemia leading to metabolic syndrome (Sasikala et al., 2009; Desai et al., 2012; Maharjan et al., 2010). This model has drawbacks due to the reversibility of reproductive function after letrozole discontinuation. While consistent and similar features reported in multiple studies to human PCOS have expanded the use of this model, the number of published studies on the letrozole model has been growing in recent years.

Although scientists use various animal models for understanding the molecular pathways that play a role in the creation of this syndrome, utilizing alternative strategies like testing on isolated organs and cell cultures, explants, cell lines, and even subcellular fractions, allows for rapid, inexpensive, repeatable, controllable tests. Recent research has emphasized on the usage and development of *"in-vitro"* techniques for better understanding of the mechanism of PCOS using primary culture of granulosa cells (Belani et al., 2016) and skeletal muscles (Allemand et al., 2005; 2009). There are few limitations in primary culture such as low primary cell yield and proliferation capability (Hashemian et al., 2020), therefore, immortalization of primary cells is an approach to overcome these limitations. In this regard, immortalized steroidogenic granulosa cell lines such as KGN cell-line can be used as an alternative model for mimicking human PCOS condition. These models can be used to understand the endocrine abnormalities, mechanism of actions of various drugs or natural herbal extracts or associated disturbances. To conclude, though these models being postulated and studies are being carried out, more light needs to be shed, and these models are yet to be validated for the use as an alternate for human PCOS model.

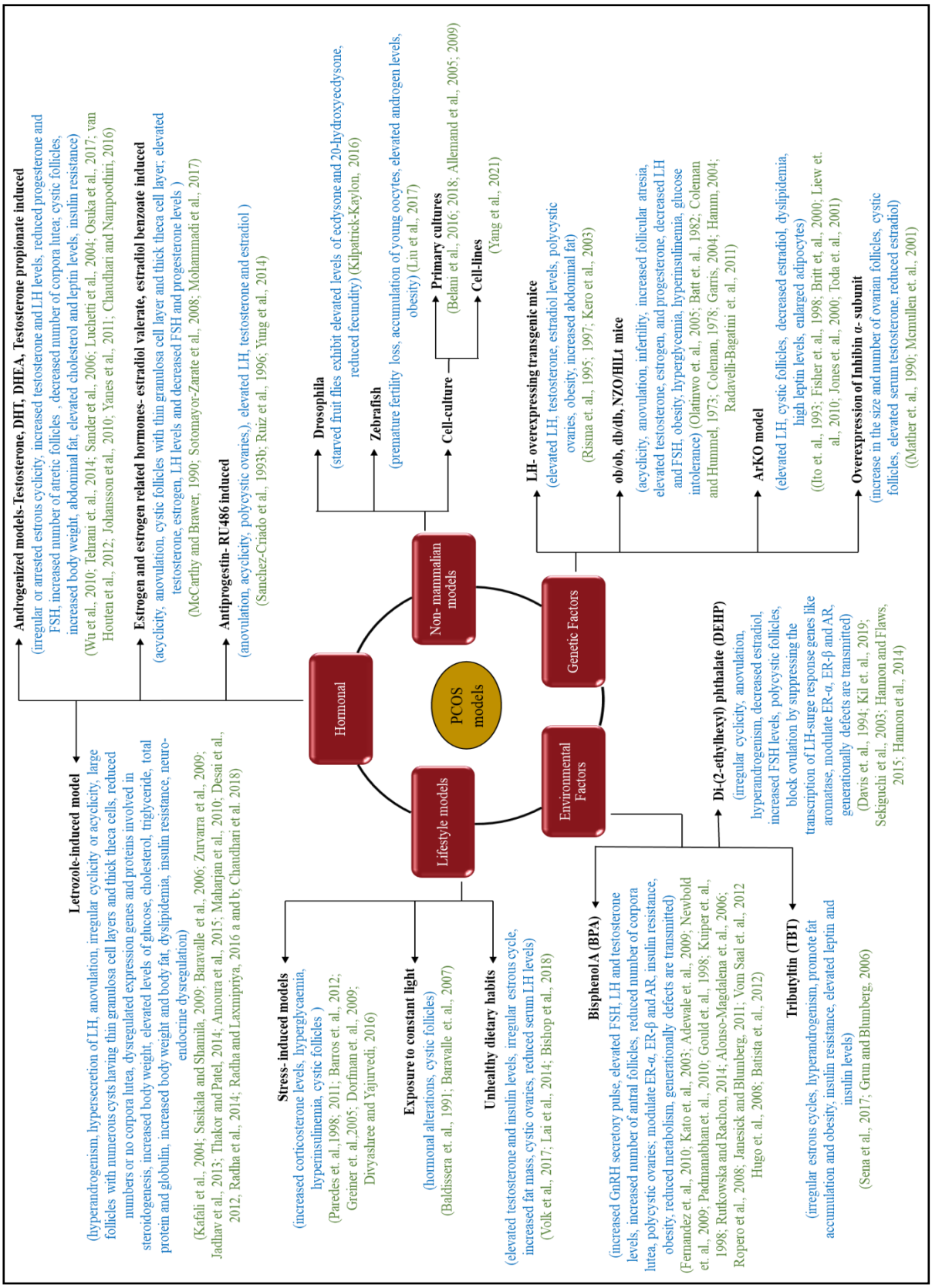

Figure 1.6: Salient clinical features demonstrated by different experimental models of polycystic ovary syndrome (Modified from Divyashree et al., 2019)

Based upon the previous discussion on the etiopathology of PCOS, the changes in steroidogenesis such as excess androgen production with hypersecretion of insulin leads to ovarian dysfunction. The above-mentioned conditions are very severe and can lead to various other symptoms such as obesity, hirsutism, alopecia, irregular menses, infertility, oligo or amenorrhea, etc. As discussed above, PCOS is a multi-etiological pathology for which several therapies have been attempted to manage the symptoms. The following sections will throw light on therapy and management of PCOS.

1.9. PCOS and therapeutic interventions

Different pharmaceutical treatments have been proposed for PCOS. Mostly, the available medication is to treat the symptoms of PCOS and to reduce the severity of the disorder and its maintenance. Till today no cure for this disorder is found, but there are several ways to maintain this condition without any further increase in severity.

1.9.1. Diet, Exercise:

These are lifestyle modifications. Various types of epidemiological surveys and based on their result shows that maintaining a proper health regime including diet and daily exercises can reduce the life-threatening condition of PCOS. This can effectively help in maintaining the symptoms to a certain extent, but has its own shortcomings. A proper treatment is required only then these modifications will help in the maintenance.

1.9.2. Drugs:

Various Drugs are available for the management of PCOS with different mode of actions.

1.9.2.1. Metformin:

It is the most widely used drug for treatment of PCOS. The active component of this drug is Metformin hydrochloride and it is anti-hyperglycemic in nature. (Lashen, 2010; Maruthur et al., 2016). According to FDA, it improves glucose tolerance, and is an insulin sensitizing drug. The mode of action of this drug is it decreases hepatic glucose production, decreases intestinal absorption of glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization. Based upon the pharmacokinetic studies, it was found that the single loading dose of metformin is 1000mg, Tmax is 7-8 hrs, Area under curve (AUC) for 0-36 hrs is 6705

± 1918 ng.hr/ml and Cmax is 868 ± 223. The volume of distribution (Vd) in the body is 654 ± 358 L. It is very rarely found in bound form with plasma proteins. The clinical doses and dose regime of metformin, steady state concentrations of plasma (Cp) for metformin are reached within 24-48 hours and are generally more than 1 µg/mL and less than 5 µg/mL. In PCOS, rectifying insulin uptake and hyperinsulinemic condition effectively reduces excess androgen production, controls SHBG and LH regulation etc. hence improving the present state of the woman with PCOS. (Palomba et al., 2009)

1.9.2.2. Clomiphene Citrate:

It is used in infertility treatment; it rectifies anovulation or oligo-ovulation and corpus luteum insufficiency in woman with PCOS. According to FDA, it has anti-estrogenic properties and competes with estrogen for binding sites at the hypothalamic level. It increases LH and FSH gonadotropins, which results in ovarian follicle maturation, followed by the pre-ovulatory LH surge, ovulation, and the subsequent development of the corpus luteum (Sawant and Bhide, 2019).

1.9.2.3. Rosiglitazone:

It is an insulin sensitizing drug of thiazolidinedione class, improves glucose tolerance, also, has shown to improve ovulation patterning in females of child bearing age with PCOS. (Batista et al.,2012). It reduces excess androgens in blood by increasing SHBG levels, it mainly controls regulation via glucose transporters. (Moran et al.,2010). Mode of action of this drug, according to FDA is by activation of the intracellular receptor class of the peroxisome proliferator-activated receptors (PPARγ) which regulates the transcription of insulin-responsive genes involved in the control of glucose production, transport, and utilization. It is a selective ligand of PPARγ, and has no PPARα-binding.

1.9.2.4. Side effects of drugs available for PCOS management

Various studies are done with respect to combination of Metformin and Rosiglitazone, where in they have said that these drugs give better effect at lower concentration but leads to hypoglycemic shock at higher doses or due to prolong use. (Arslanian et al.,2013; Zinman et al.,2010; Liao et al.,2011). These drugs on prolong abuse shows adverse effects:

a. Hepatic function impairment, Hepatotoxicity (Clomid,1998);

b. Scotoma (blurred vision in the periphery of the normal lens)

c. Prolonged usage of Rosiglitazone can cause skin flushing, alopecia, heart burn, liver failure, chest pain, edema and hypoglycemic shock leading to coma (Sepilian et al.,2005)

d. Increased risk of vascular thromboembolism, digestive complications such as nausea, diarrhea and dizziness, and Vitamin B12 deficiency upon prolonged usage of Metformin (Mokaberinejad et al., 2019; Saha et al., 2012; Lashen et al.,2010)

e. Prolonged usage of clomiphene can lead to ovarian cysts, increase in ovarian size. Hence, this drug is now banned and should not be used due to its long list of side effects.

These adverse effects via consumption of drugs, combination of drugs leading to drug-drug interaction, and complications gave rise to optional therapies that has less or no side effects even after prolong use of it. To present, there is no perfect cure for PCOS. Current medical treatments do not provide a comprehensive therapy strategy capable of completely reversing the underlying hormonal imbalances. Aside from the financial burden, the wide range of phenotypic presentation of PCOS has posed a difficulty in diagnosis and management of the pathophysiology. Complementary medicine use by women has increased during the past ten years with rates of use ranging between 26% and 91% (Chauhan et al., 2015). According to Sasikala et al.,2010, herbs have to be used as ayurvedic medicines since ancestral period. One such useful and rapidly upcoming therapy is Nutraceuticals (Herbal remedies). Herbal medicines are known to contain pharmacologically active constituents with physiological effects on female endocrinology and have been positively associated with reduced incidences of breast cancer, osteoporosis and cardiovascular diseases (Abu Bakar et al., 2021; Leung, and Siu, 2013; Shaito et al., 2020; Kwon et al., 2020). Thereby, currently herbal medicines are been studied scientifically for its use in multi-etiology metabolic endocrinopathy like PCOS.

1.9.3. PCOS and herbal therapy

A variety of medicinal plants have been studied in context of PCOS. There is evidence suggesting that the bioactive compounds found in plants which include flavonoids, polyphenols, phytoestrogen, polyunsaturated fatty acids and phytosterols show positive effects in attenuation of the syndrome (Rao, 2021, Shi et al., 2019; Tabrizi et al., 2020; Mihanfar et

al., 2021; Najafi et al., 2018; Wang et al., 2020; Salek et al., 2019; Radha and Laxmipriya, 2016a; Dey et al., 2017b; Arpi and Laxmipriya, 2019; Pachiappan et al., 2020). Evidence suggests that phytocompounds increase ovulation, fertility as well as cause a reduction in insulin resistance and hyperandrogenism by improving the hormonal milieu and ovarian structure-function (Iervolino et al., 2021).

Some of the potential medicinal plants mentioned used in Ayurveda for treatment of PCOS are highlighted below: -

1. ***Withania somnifera:*** (Ashwagandha) also known as "Indian Ginseng"belongs to the family Solanaceae. It is used for millennia in Ayurveda as a Rasayana. It is known to promote reproductive health and maintain balance. Stress is one of the key variables that affects female reproductive health. Menstrual irregularities, amenorrhea, and anovulation may occur as a result of this (Allsworth et al., 2007). Withania somnifera's anti-stress properties have been demonstrated in numerous preclinical and clinical investigations (Ashok et al., 2014). Active compounds of the herb include alkaloids like anahygrine, cuseohygrine, isopelletierine etc. and saponins. The root of this herb is known to support overall functions of the endocrine system and is used in the treatment of female infertility (Amrin et al., 2016). The postulated mechanism connects antioxidant properties to a beneficial effect on testosterone, LH, and FSH hormone abnormalities. In addition to this, the gamma-aminobutyric acid (GABA) mimetic property of Withania somnifera extract plays a key role in stimulating gonadotropin-releasing hormone secretion and restoring hormonal balance (Nasimi Doost Azgomi et al., 2018). Treatment with *W. somnifera* root hydroalcoholic extract elongates the estrus phase and shortens the diestrus phase. In letrozole-induced PCOS rats, it also raises FSH while decreasing LH, testosterone, and estradiol (Saiyed et al., 2016). In addition to this, *W. somnifera* demonstrated hypoglycemic potency by increasing the insulin secretion and improving the insulin sensitivity in muscles, as well as hypolipidemic effect by increasing the bile acid synthesis for the removal of body cholesterol in PCOS animals (Gorelick et al., 2015; Visavadiya and Narasimhacharya, 2007).

2. ***Trigonella foenumgraceum L*:** (Fenugreek), belongs to family Leguminosae, its seed extract is successfully used in lowering blood glucose levels. In a study, PCOS women were given *T. foenumgraceum* seed capsule along with metformin for eight weeks and the results showed improved insulin sensitivity, improvement in menstrual cycle as well as improved ultrasound

report (Bashtian et al., 2013). A recent study has demonstrated the ameliorative effect of *T. foenumgraceum* on sex hormonal levels in Letrozole induced female rats (Alkalby and Hamzah, 2017).

3. ***Tribulus terrestris*:** (Puncture Vine), belongs to family Zygophyllaceae, is known to have beneficial effects in PCOS. The treatment of estradiol valerate induced PCOS rats with hydroalcoholic extract of *T. terrestris* normalises menstrual irregularity, hormonal abnormalities, and effectively eliminates ovarian cysts and restores ovarian normal function. It could be owing to its luteinizing impact, which is linked to its gonadotropin-like action or follicular cyst luteinization (Dehghan et al., 2012; Esfandiari et al., 2011). *T. terrestris'* antiestrogenic activity is thought to be the major mechanism for regulating hormone levels and inducing ovulation in PCOS. The bioactive phytoestrogens- diosgenin, gitogenin, chlorogenin, ruscogenin, and essential oils present in *T. terrestris*, bind to estrogen receptors ER-α and ER-β, acting as a pure estrogen antagonist by stimulating the secretion of gonadotropin-releasing hormone (Saiyed et al., 2016). The ethanolic extract of the herb showed increased FSH levels in healthy women (Milanov et al., 1981). Another study found that herb and Clomiphene were equally effective at inducing ovulation in females with oligomenorrhea and infertility caused by anovulation (Tabakova et al., 1984). T. terrestris' aerial component and fruit have long been known to induce ovulation and minimise ovarian cysts (Amrin et al., 2016). Additionally, in a randomized control study, women with diabetes mellitus type 2 were given 1000 mg/day of hydroalcoholic *T. terrestris* extract, which had a promising hypoglycemic effect and significantly reduced total cholesterol and low-density lipoprotein (Samani et al., 2016).

4. ***Cinnamomum zeylanicum:*** (Cinnamon), of family Lauraceae, is proven to improve both the reproductive and metabolic aspects of PCOS. Oral administration of Cinnamon extract reduces testosterone levels in dehydroepiandrosterone-induced PCOS mice by downregulation of insulin and insulin-like growth factor-1 (IGF-1). The increasing levels of insulin-like growth factor-binding protein -1 (IGFBP-1) in plasma and the ovary is linked to the downregulation of IGF-1 (Bergh et al., 1993). According to a clinical trial, Cinnamon supplementation (1500 mg/day for 6 months) was found to be beneficial for menstruation dysfunction in women with PCOS by enhancing insulin sensitivity (Kort and Lobo, 2014). Cinnamon has been reported in both *"in-vitro"* and *"in-vivo"* studies to reduce insulin resistance. Insulin resistance is reduced by increasing glucose consumption and increase phosphatidylinositol 3-kinase (PI-3 kinase)

activity. It aids intercellular glucose transport and increases glycogen synthesis, resulting in the translocation of the glucose transporter type-4 (GLUT-4) receptor and enhanced glucose utilisation in rats (Qin et al., 2003) and PCOS women (Wang et al., 2007), and thereby, improved the insulin sensitivity. In addition to this, oral cinnamon supplementation (1500 mg per day for 8 weeks) in women with PCOS, dramatically reduced total cholesterol and low-density lipoprotein cholesterol levels while increasing high-density lipoprotein cholesterol levels (Borzoei et al., 2018). Cinnamon's hypolipidemic impact may be related to its polyphenol and cinnamaldehyde content, which restrict intestinal cholesterol absorption while increasing lipolysis in adipose cells (Khare et al., 2016).

5. ***Ocimum tenuflorum L:*** (Holy Basil), commonly known as Tulsi in India belongs to family Lamiaceae. It is known to be an excellent antioxidant (Shantaram et al., 2019), has anti-androgenic properties and hence is effective in the management of PCOS. It also finds its use in treating multiple co-morbidities associated with obesity (Pachiappan et al., 2017; Satapathy et al., 2017).

6. ***Cimicifuga racemose:*** known as Black Cohosh Root, belongs to family Ranunculaceae, is known to be very effective for PMS related symptoms like excessive menstrual cramps and hormone related symptoms (Dehghan et al., 2012). Black Cohosh has the ability to induce ovulation in women with PCOS (Bency et al., 2016). The herb is known to suppresses the LH secretion in cell cultures from ovariectomised rats (Düker et al., 1991) and PCOS women (Kamel, 2013). Reduction in LH levels increase the FSH sensitivity, thereby improving folliculogenesis. Kamel et al.,2013 demonstrated that phytoestrogens are responsible for increasing the endometrial thicknesses, which improves the implantation rate and pregnancy outcome in PCOS women. The proposed mechanism of action is the selective binding of the phytoestrogens on the estrogen receptors present on the pituitary and hypothalamus (Seidlova-Wuttke et al., 2003) and hence lead to inhibition of estrogen.

7. ***Taraxacum officinale:*** (Dandelion Root), belong to family Asteraceae, has hepatoprotective properties, stimulates bile flow and detoxifies liver. It is used to cleanse the liver and get rid of any build-up of hormones. This clean up can stimulate the production of SHBG which reduce the free testosterone in the blood (Cai et al., 2017). Extracts of *T. officinale* is used in PCOS treatment, because menstrual irregularities are often affected by the liver which is being backed

up with excessive hormones. It also helps in removal of toxin from the body, thus helping the women who are experiencing fertility problems and menstrual issues (Wang et al., 2018).

8. ***Asparagus Racemosus:*** (Shatavari), belongs to Asparagaceae, is known to contain phytoestrogens, which helps revitalize reproductive system, promote normal development of female ovarian follicles and regulate the menstrual cycle (Kumar et al., 2008). It also helps in combating the hyperinsulinemia (Pachiappan et al., 2017).

9. ***Tinospora cordifolia:*** (Guduchi), belongs to family Menispermaceae, is a well-known medicinal plant for its hypoglycemic effects and has anti- inflammatory property. Chronic inflammation in tissues is the root cause for insulin imbalance and ovarian cysts. *T. cordifolia* helps in lowering insulin resistance, revitalizes and boosts body's metabolism naturally (Chandrasekaran et al., 2012).

10. ***Aloe barbadensis Miller:*** commonly known as *Aloe vera* and belongs to family Liliaceae. Traditional knowledge of Ayurveda and Siddha has several evidences which substantiate the effectiveness of *Aloe vera*, also called as kattrali, kani or kumari towards management of female reproductive system and its associated disorders like PCOS (Nadkarni, 1976; Risvan et al., 2017; Sahu et al., 2013). PCOS, being a metabolic syndrome, is characterized by glucose intolerance, insulin resistance and dyslipidaemia. There are several evidences that have proved *Aloe vera* gel is an efficient modulator of metabolic status by exhibiting hypoglycemic, anti-dyslipidemic, antioxidant and anti-inflammatory properties (Desai et al., 2012; Tanaka et al., 2006; Misawa et al., 2008; Misawa et al., 2012). The varied pharmacological properties of *Aloe vera* gel is due to its abundant phytochemicals such as polysaccharides, glycosides, flavonoids, carbohydrates, coumarins, tannins, chromones, alkaloids, anthraquinones, organic compounds, pyrones, phytosterols, anthrones, fatty acids, sterols, terpenoids, hormones, vitamins, proteins, and mineral constituents (Nalimu et al., 2021; Kar and Bera, 2018; Radha and Laxmipriya, 2015). Though its ethnopharmacological use has been documented in traditional medicine system, its thorough scientific evidence is lacking.

In this context, data from our lab demonstrated that *Aloe vera* gel (10mg dry weight daily for 60 days) could restore ovarian structure-function and decrease co-morbidities like hyperglycemia and dyslipidemia in PCOS rat model (Maharjan et.al., 2010; Radha et al., 2014; Desai et al., 2012; Radha and Laxmipriya, 2015). Further, it is interesting to note that PCOS

rats treated with *Aloe vera* gel (AVG) before conception could increase implantation rate, leading to healthier pups with few or no resorptions, suggesting that AVG is a good pre-conceptive agent and help in management of complications associated with women (Radha and Laxmipriya, 2016b). Further, solvent based extraction of AVG demonstrated that oral administration of non-polar petroleum ether extract (NPE) (25 µg/kg body weight for 60 days) in Letrozole induce PCOS rat model could affectively improve the reproductive and metabolic complications associated with PCOS. The observed efficacy was attributed to the presence of fatty acids, phytosterols and terpenoids in the NPE, which acted at various molecular targets leading to improve the ovarian structure- function along with metabolic modulation (Radha and Laxmipriya, 2016a).

Since plant extracts are typically a mixture of different types of bioactive compounds or phytochemicals with different polarities, separating them remains a significant challenge in the identification and characterization of bioactive compounds. The varied phytochemicals present in the extract may potentiate undesirable side-effects. Moreover, certain plant-derived compounds are effective in combination with others, while others are active as single entities. The current advancement in science has made it possible for the isolation of phytochemicals and studying their therapeutic potential individually or in combination in order to understand their key molecular targets. Thereby, isolation of the non-polar phytocomponents present in *Aloe vera* gel by chromatographic techniques help us to identify their molecular targets by using *"in-silico", "in-vitro"* and *"in-vivo"* approaches. ***Hence, it can be hypothesized that non-polar phytocomponents present in Aloe vera gel may act as potential therapeutic alternative for reproductive and metabolic disturbances in PCOS.***

Aim of the study

In light of the above literature, the aim of the present study was to isolate the bioactive non-polar phytocomponent/s of *Aloe barbadensis* Mill, gel and elucidate their potential molecular targets using *"in-silico", "in-vitro"* and *"in-vivo"* approaches; with defining therapeutically possible molecular mechanism that be directed towards the better management of PCOS and its associated metabolic co-morbidities.

Specific Objectives:

Major objectives of the present study are –

I. To isolate and characterize the non-polar phytocomponents of *Aloe vera* gel and evaluate their pharmacokinetics, distribution and pharmacodynamics in rats.

II. To identify the targets of the isolated phytocomponents of non-polar fraction of *Aloe vera* gel Extract using "in-silico" approach and its validation.

III. To evaluate the role of isolated phytocomponents of non-polar fraction of *Aloe vera* gel Extract in PCO-like ovarian cellular models.

IV. To evaluate the role of isolated phytocomponent/s of non-polar fraction of *Aloe vera* gel Extract in Letrozole induced PCOS mouse model.