Chapter I

Role of gonadotropin regulatory gene(s) polymorphisms in the pathogenesis of polycystic ovary syndrome
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1. Introduction

Polycystic Ovary Syndrome (PCOS) is a heterogeneous endocrine disorder of women. It was initially described as an association of polycystic ovaries (PCO) with amenorrhea, hirsutism, and obesity in women presented with infertility [1]. Of late, morphological and histological findings of the ovaries with a wide spectrum of biochemical and clinical changes, such as hyperandrogenism and menstrual irregularities, it is renamed as PCOS [2]. Apart from major phenotypes such as hyperandrogenism and ovarian dysfunction, PCOS is being associated with broad spectrum of metabolic abnormalities such as insulin resistance and hyperinsulinemia [3], abdominal obesity, and dyslipidemia [4] which in latter stages of life progress to type 2 diabetes mellitus (T2DM), endometrial hyperplasia and cardiovascular diseases.

Albeit, due to the broad spectrum of symptoms with overlapping biochemical parameters with other disorders, the diagnosis of PCOS, go undetected until puberty and lack of systematic guidelines for the diagnosis and management in the pediatric population [5].

Globally, 2-15% of women in their reproductive ages are found to have PCOS [6]. Variation in the prevalence of PCOS mainly depends on diagnostic criteria, ethnicity, lifestyle, geographic location and diet. Though the direct cause of PCOS remains unknown, both environmental and genetic factors have been implicated. Reproductive and metabolic phenotypes are found to be heritable and run as cluster in families suggesting a strong genetic susceptibility for the pathogenesis [7]. Abnormal secretion and regulation of gonadotropins, namely the luteinizing hormone (LH), follicle stimulating hormone (FSH), and associated excess secretion and action of ovarian
steroid hormones leading to sub-fertility in PCOS women. Further, they have an elevated LH/FSH ratio, as well as an increased frequency and amplitude of LH pulsations due to rapid hypothalamic gonadotropin releasing hormone (GnRH) secretion, a defect at the level of the hypothalamus. Elevated LH/FSH ratio is proposed as biochemical diagnostic assay for PCOS [8]. Comprehensive evidence suggests that several mutations or polymorphisms in candidate genes involved in steroid, gonadotropin and insulin pathways closely interact to set the stage for possible development of PCOS [9]. Expression of clinical and biochemical phenotypes vary among individuals and influenced by interactions between gene-gene and gene-environmental factors and largely depends on ethnicity and lifestyle. Inheritance of PCOS is also suggested to be seen in the first degree relatives. Sisters and brothers of PCOS women are found to have several of PCOS phenotypes [10]. While, the male relatives of PCOS women have increased serum levels of dehydroepiandrosterone sulfate (DHEAS) [11] and high prevalence of metabolic syndrome [12] in females were reported to have high levels of adrenal and ovarian androgens during puberty or adulthood [13].

1.1. Physiology of female reproductive system

The female reproductive system is an integrated and dynamic system comprised of paired ovaries and oviducts/fallopian tubes, uterus and vagina as principal organs. These structures function in coordination to perform physiological process like ovulation, fertilization of ovum by sperm and propagation of species.

1.1.1. Ovaries
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The ovaries are small paired intra-abdominal organ, amygdaloidal in shape, with white or yellowish in color. Each ovary is located on either side of upper pelvic cavity, against the back of the pelvic wall and near the uterus. The ovaries undergo structural changes continuously under the influence of hormones of hypothalamus and pituitary origin. The mature ovary measures about 2.5 to 5 cm in length and 1.5 to 3 cm wide. Ovaries are connected to the uterus and pelvic wall by supportive ligaments and are highly innervated by autonomic nerves and receive rich blood supply. Ovaries perform two principal functions such as progressive development and release of a mature oocyte to prepare for fertilization and synthesize steroid hormone required for follicle release, menstrual cyclicity and maintenance of reproductive tract [14]

1.1.2. Oviducts

The oviducts or fallopian tubes are paired tubes each measuring about 10cm in length extending from each ovary to the top of the uterus. Through its length, oviduct can be divided into several regions such as the oviductal infundibulum (the opening into the infundibulum, into which the ovulated ovum enters), oviductal ampulla, oviductal isthmus, and finally the part of the oviduct that is embedded within the uterine wall, the intramural oviduct.

1.1.3. Uterus

The normal uterus is shaped like an upside-down pear, measuring about 7.5 X 5.0 X 2.5cm, lying superior to the urinary bladder and between the two ovaries in the pelvic cavity. The fundus is the upper portion above the entry of the fallopian tubes, and the body is the large central portion. The narrow, lower end of the uterus is the cervix, which opens into the vagina. The outermost layer of the uterus, the serosa or
epimetrion, is a fold of the peritoneum. The myometrium is the smooth muscle layer; during pregnancy these cells increase in size to accommodate the growing fetus and contract for labor and delivery at the end of pregnancy. The lining of the uterus is the endometrium, consists of two layers namely (i) basal layer, adjacent to the myometrium, is vascular but very thin and is a permanent layer, (ii) the functional layer is regenerated and lost during each menstrual cycle. Under the influence of estrogen and progesterone from the ovaries, the growth of blood vessels thickens the functional layer in preparation for a possible embryo. If fertilization does not occur, the functional layer sloughs off in menstruation. During pregnancy, the endometrium forms the maternal portion of the placenta. The uterus increases greatly in size, contains the placenta to nourish the embryo-fetus, and expels the baby at the end of gestation

1.2. Ovarian folliculogenesis

Ovarian folliculogenesis is a process of maturation of primordial follicles to preovulatory follicles by interacting with intra and extra ovarian factors. All the events related to follicular development are regulated by appropriate signals originating from the growing oocyte itself and from the somatic cells that surround it and also by complex interactions between gonadotropin hormones, sex steroids, and diverse growth factors. The development of ovarian follicles begins during fetal life with the transformation of primordial germ cells into oocytes enclosed in structures called follicles. Some of these follicles are recruited to start a long progress of growth and differentiation, during which the proteins required for oocyte maturation are progressively synthesized and accumulated [15]. Mechanism of ovarian
folliculogenesis unraveled by understanding the phenomena obtained from histologic, endocrine and ultrasonographic method such as, high-resolution trans-vaginal ultrasonography [16]. Folliculogenesis starts by recruitment of primordial follicles from a growing pool of follicles in order to enter the growth phase. Androgens play a key role on the regulation of early folliculogenesis via androgen receptors (ARs) or indirectly through aromatization to estrogen. ARs are highly expressed in the granulosa and theca cells of early stage follicles and slightly expressed in mature follicles. Primordial follicles are oocytes surrounded by single layer of granulosa cells. Upon puberty, the primordial follicles attain growth and ovulate. The early growing follicle undergoes developmental process of cellular proliferation and differentiation and most of them fail to complete the process of maturation and die in a process termed atresia [17]. The process of folliculogenesis can be categorized into three developmental phases: The first step in the process of folliculogenesis is preantral follicle growth (the primordial to primary follicle transition followed which formation and growth of secondary follicles); in the second stage, antrum formation and development of early antal follicles; and the last stage involves the terminal antral follicle (growth followed by development of antral to preovulatory follicles) [18]. The events of folliculogenesis is shown in the figure-1.1.
1.3. Ovarian steroidogenesis

The physiological process of conversion of cholesterol into biologically active forms of steroid hormones namely androstenedione, testosterone, dihydrotestosterone, progestogens, and estrogens under tightly regulated environment catalyzed by enzymes belongs to cytochrome P450 and hydroxysteroid dehydrogenases; each steroid serving as a substrate for the subsequent one in a cascade of events known as steroidogenesis under the influence of LH and FSH acting on ovarian theca and granulose cells respectively. Steroidogeneic acute regulatory protein transports cholesterol into mitochondria. The process of steroidogenesis in theca and granulosa cells and the enzymes involved in the synthesis of steroids are depicted in figure 1.2.
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Figure 1.2. Androgen synthesis by ovarian theca and granulosa cells. The androgens (androstanedione and testosterone) are produced in response to the luteinizing hormone (LH) stimulus in theca cells. Androgens diffuse into granulosa cells and are converted to estrogens (estradiol and estrone) by the enzyme aromatase under the influence of FSH. P450scc, enzyme responsible for the cleavage of the side chain of cholesterol; the 3β-, 3β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD,(Source: Gervasio et al. 2014 [20]).

1.4. Menstrual cycle

Menstrual cycle is a complex cyclic changes occurring at regular intervals in reproductively active women under the control of autocrine, paracrine, and endocrine factors. Menstrual cycle involves series of steps; (i) ovarian folliculogenesis, (ii) ovulation, (iv) luteinization, (v) growth and decline of endometrium in the uterus to receive the embryo followed by fertilization. Generally, a menstrual cycle spans for an average of 36 years starting from menarche to menopause [21]. Duration of menstrual cycle lasts for 25 to 34 days at an average of 28 days counting from first day of menstruation to next one. Based on ultrasound and hormonal studies, it was estimated that in fertile women, a follicular phase lasts for 14.6 days and luteal phase for 13.6 days [22,23], however variable length of menstrual cycle among fertile individuals was due to variability in follicular phase. Menstrual cycle initiated by hypothalamic
originated gonadotrophin releasing hormone (GnRH) pulse generator and surge center which releases LH and FSH from the pituitary to stimulate changes in steroid hormone secretion at ovaries. In parallel with hormonal changes, structural changes occur in the ovaries and the uterine endometrium. During these cycles, a woman’s reproductive system is in constant flux, and the hormonal changes affect non reproductive tissues as well. The menstrual cycle is, divided into three main phases namely (i) menstrual or “destructive phase, (ii) follicular or proliferative phase and (iii) luteal or secretory phase.

1.4.1. Menstrual phase

In menstrual phase, functional layer of the endometrium is lost and terms as menstruation or the menses. Although this is actually the end of a menstrual cycle, the onset of menstruation is easily pinpointed and is, therefore, a useful starting point. Menstruation may last 2 to 8 days, with an average of 3 to 6 days. At this time, secretion of FSH is increasing, and several ovarian follicles begin to develop. Menstrual bleeding is the external symptom of cyclicity in women and occurs at the end of the luteal and the beginning of the follicular phase. In 80% of ovulatory women, a maximum bleeding is on second day with blood loss averages 33.2 ml (10–84 ml). An age-dependent decline of half a day is seen in women from the age of 35 years, and 50-year old women lose 6ml more than younger women [21]. Interestingly, regional, ethnic and even socioeconomic differences in follicular and luteal phase length, the duration of bleeding and amount of blood loss may exist [21].
1.4.2. Follicular phase

During follicular phase, FSH released from the pituitary gonadotrophs act on the ovarian follicles and stimulates its growth of ovarian follicles and secretion of estrogen by the follicle cells. Simultaneously, the secretion of LH is also increasing, but more slowly. While, FSH and estrogen promote the growth and maturation of the ovum, the estrogen stimulates the growth of blood vessels in the endometrium to regenerate the functional layer. This phase ends with ovulation, when a sharp increase in LH causes rupture of a mature ovarian follicle.

1.4.3. Luteal phase

Under the influence of LH, the ruptured follicle becomes the corpus luteum and begins to secrete progesterone as well as estrogen. Progesterone stimulates further growth of blood vessels in the functional layer of the endometrium and promotes the storage of nutrients such as glycogen. As progesterone secretion increases, LH secretion decreases by feedback regulation; if the ovum is not fertilized, the secretion of progesterone also begins to decrease. Without progesterone, the endometrium cannot be maintained and begins to slough off in menstruation. FSH secretion begins to increase (as estrogen and progesterone decrease), and the cycle begins again.

Thus, all those cellular and structural changes occurs in a menstrual cycle are tightly regulated by the respective hormonal changes. Altered secretions can results in normal reproductive physiology and may results in irregular cycle and associated fertility related conditions. PCOS is one such condition reported to be associated with abnormal ovarian functions.
1.5. Diagnosis of PCOS

Diagnosis of PCOS is critical for the treatment of affected individuals. Several diagnostic criteria’s and definitions are proposed; of which Stein and Leventhal [1] first described, such as presence of enlarged ovaries, obesity, hirsutism, and chronic anovulation as signs of PCOS. Of late, quantification of hormones, provided with broad incorporation of diagnostic tests for gonadotropin secretion and testosterone, availability of imaging techniques to examine ovarian morphology and metabolic disorders involved, resulted in new guidelines and criteria aids in accurate diagnosis.

Till date several criteria’s have been put forward to classify the PCOS as given below:

i. National Institute of Health Criteria (NIH-1990)

ii. European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine (Rotterdam 2003 criteria)

iii. Androgen Excess Society criteria (AES-2006)

iv. The Androgen Excess and PCOS Society criteria (AES-PCOS-2009)

The salient phenotypic features of diagnostic criteria of PCOS proposed by expert group are presented in table-1.1.
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Table-1.1. Proposed diagnostic criteria’s for PCOS

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Diagnostic criteria</th>
<th>Inclusion phenotypes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NIH, 1990</td>
<td>Simultaneous presence of i) Hyperandrogenism (Clinical or biochemical) and ii) Chronic an ovulation</td>
<td>[24]</td>
</tr>
<tr>
<td>2.</td>
<td>Rotterdam, 2003</td>
<td>Presence of any two of the following i) Hyperandrogenism (Clinical or biochemical) ii) Oligo and/or an ovulation and iii) Polycystic ovaries.</td>
<td>[25, 26]</td>
</tr>
<tr>
<td>3.</td>
<td>AES, 2006</td>
<td>i) Ovulatory dysfunction or PCO in the presence of hyperandrogenism (Clinical or biochemical)</td>
<td>[27]</td>
</tr>
<tr>
<td>4.</td>
<td>AES-PCOS, 2009</td>
<td>Simultaneous presence of i) Hyperandrogenism (Clinical or biochemical) and ii) Ovarian dysfunction</td>
<td>[28]</td>
</tr>
</tbody>
</table>

In addition to those characteristic phenotypes, exclusion of other disorders such as non-classical congenital adrenal hyperplasia, cushing syndrome, hyperprolactinemia, hypothyroidism, acromegaly, premature ovarian failure, a virilizing adrenal or ovarian neoplasm, or a drug-related condition ensures an accurate identification of affected individuals.

1.6. Prevalence

Globally, the prevalence of PCOS has been reported as 2-15% distributed non-randomly in various populations. Further, a wide variation in the prevalence of PCOS among different world populations, is attributed mainly due to lack of consistency in diagnostic criteria, ethnicity, lifestyle, geographic location and diet [29]. The prevalence of PCOS reported from varied ethnic backgrounds are presented in table 1.2.
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Table-1.2. Prevalence of PCOS in different populations/countries

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Country / Population</th>
<th>Diagnostic Criteria</th>
<th>Prevalence (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.</td>
<td>United Kingdom</td>
<td>NIH- 1990</td>
<td>8.0</td>
<td>[31]</td>
</tr>
<tr>
<td>4.</td>
<td>Mexican American</td>
<td>Rotterdam 2003</td>
<td>12.8</td>
<td>[33]</td>
</tr>
<tr>
<td>5.</td>
<td>Thailand</td>
<td>Rotterdam 2003</td>
<td>5.7</td>
<td>[34]</td>
</tr>
<tr>
<td>7.</td>
<td>Chinese</td>
<td>Rotterdam 2003</td>
<td>2.2</td>
<td>[36]</td>
</tr>
<tr>
<td>8.</td>
<td>Mexican</td>
<td>Rotterdam 2003</td>
<td>6.0</td>
<td>[37]</td>
</tr>
<tr>
<td>10.</td>
<td>Indian</td>
<td>Rotterdam 2003</td>
<td>9.3</td>
<td>[38]</td>
</tr>
<tr>
<td>11.</td>
<td>Brazil</td>
<td>Rotterdam 2003</td>
<td>8.5</td>
<td>[39]</td>
</tr>
<tr>
<td>12.</td>
<td>Palestine</td>
<td>NIH – 1990</td>
<td>7.3</td>
<td>[40]</td>
</tr>
</tbody>
</table>

1.7. Pathogenesis

Observed phenotypes of PCOS like hyperandrogenism, ovulatory dysfunction, metabolic abnormality, and infertility implicate a strong involvement of endocrine system on the pathogenesis. Mechanism of PCOS pathogenesis mainly derives from abnormal co-ordination of hypothalamic-pituitary-gonadal axis. Synthesis and actions of hormones in neuroendocrine and ovarian steroid pathways are affected in PCOS. There exists strong regulation on the synthesis and secretion of tropic hormones by the hormones released from hypothalamus. In normal ovulatory women, faster GnRH pulses, favors LH secretion and slower pulses favors FSH secretion at pituitary. Thus, a change in pulse frequencies of GnRH stimulates the differential secretion of LH and
FSH from pituitary gonadotropes, is one of the key mechanisms during ovulatory cycles. In turn, the GnRH pulse frequencies are regulated by feedback mechanisms from ovarian steroids especially under the influence of estradiol [41]. In comparison to normal ovulatory women, 30-90% of PCOS women, have rapid GnRH pulses, which favors high LH secretion over FSH, thereby results in abnormal LH:FSH ratio (>1.0) [8]. Dysregulation of GnRH pulses are mainly due to lack of negative feedback inhibition from ovarian sex steroids, the progesterone and estradiol [41, 42]. High concentrations of LH levels aggravates androgen production in ovarian theca cells [43], where as low or relative FSH levels are insensitive to stimulate conversion of androgens to estrogens by aromatase enzyme in ovarian granulosa cells results in excess androgen to estrogens ratio, thereby leading to hyperandrogenism; defective follicular development and maturation thus leading to ovulatory dysfunction and subfertility [44].

Studies based on oral contraceptive pills and/or progesterone administration in hyperandrogenic conditions, the sensitivity of the GnRH pulse generator to suppression by progesterone is impaired [45, 46]. Recording directly from rodent-derived fluorescent labeled GnRH neurons in intact hypothalamic slices, progesterone (with estradiol) inhibits the firing of GnRH neurons. Dihydrotestosterone impairs the ability of progesterone to slow GnRH neuronal firing rates. In PCOS women, blockade with flutamide restored GnRH pulse generator sensitivity to progesterone [44], Further, GnRH antagonists showed reduction of testosterone and LH levels [47]. Thus, LH is the proximate physiological stimulus for androgen synthesis by ovarian theca cells and plays an important role in maintaining hyperandrogenism. Androgen
synthesis is augmented in PCOS women and ovarian theca cells are the principal
source of androgens excess [48], however few studies reported the contribution of
adrenal [28] and adipose tissues to some extent [49]. In vitro studies have
demonstrated that theca cells from polycystic ovaries produce high concentrations of
androstenedione both in basal conditions and after gonadotropin stimulation. Ovarian
theca cells isolated from PCOS follicles and maintained in long-term culture produced
elevated levels of progestins and androgens compared to normal theca cells is
associated with high expression of steroidogenic enzymes, such as cytochrome P450
cholesterol side-chain cleavage and 17alpha-hydroxylase/17,20-lyase. High adrenal
androgen excess, detectable primarily by elevated dehydroepiandrosterone sulfate
(DHEAS) levels in PCOS women. In adipose tissues, several of the key enzymes in
steroid synthesis are found to be expressed in high concentrations such as 17-
hydroxysteroid dehydrogenase 5 (17-HSD5) (responsible for the conversion of
androstenedione to testosterone) and 5-reductase (responsible for the conversion of
testosterone to dihydrotestosterone (DHT) that are mainly attributed to reduction in
expression of adiponectin, a key protein in regulating androstenedione synthesis [49].

1.8. Symptoms

PCOS is multi-factorial origin with unclear etiology. Inspite of it, it was un-doubtly
demonstrated that the condition stems from reproductive (hyperandrogenism,
anovulation, menstrual dysfunction and polycystic ovaries) and metabolic
abnormalities (insulin resistance, obesity and dyslipidemia). The expression of clinical
and biochemical phenotypes vary among PCOS individuals likely due to interaction of
gene-gene and gene-environment factors, and lifestyle.
1.8.1. Hyperandrogenism

Hyperandrogenism (or androgen excess), is a clinical condition characterized by excessive levels of androgens synthesized in the body. Hyperandrogenism is a dominant phenotype observed in 50-80% of women with PCOS [50]. It is found to be associated with wide range of disorders such as reproductive disorders (amenorrhea, anovulation, and infertility), metabolic abnormalities (IR, T2DM, obesity, and metabolic syndrome) and breast and endometrial cancers [51]. Animal models showed an association of hyperandrogenism with ovarian volume and follicular number, follicular fluid and augment serum LH levels, which in-turn direct impaired follicle maturation and dominant follicle selection [52]. In PCOS women, hyperandrogenism can be ascertained either clinically by dermatological signs of androgen excess such as hirsutism, acne, androgenic alopecia, excess body hair or by biochemical indices such as high levels of free testosterone and DHEA in serum [28].

**1.8.1.1 Hirsutism**

Hirsutism is androgen dependent terminal development of hair on face, upper lips, chest, abdomen, and back in females and most common complaint of women in their reproductive ages [53]. It is one of the most common dermatological manifestations of hyperandrogenism in PCOS women. About 70-80% of women with hirsutism are found to be PCOS [54]. Degree of hirsutism varies by ethnicity [55] and most prevalent in American and European population by about 40-90% and very less in Asian populations like Chinese, Japanese and Koreans [56].
1.8.1.2 Acne vulgaris

Acne vulgaris (acne) is a disorder of the pilosebaceous unit commonly seen on skin in both male and females above 20 years of age. Acne is mostly observed on the face, neck, upper back, and in pectoral regions. Androgens play an important role in the development of acne. In the sebaceous gland, the enzyme 5α-reductase converts testosterone to a more potent androgen, DHT, which in turn increase sebum production from sebaceous glands and causing abnormal desquamation in follicular epithelial cells. Inflammation, genetic, environmental factors and bacterial infections like Propinibacterium acne are involved in the etiology of acne. In, PCOS women, 9.8–53% were reportedly to be with signs of acne vulgaris [57].

1.8.1.3 Male pattern baldness

Male pattern baldness or androgenic alopecia is a progressive form of losing terminal hair on scalp that is common with baldness in men and much less common in women. Generally, progression of alopecia associated with a familial predisposition to baldness and an associated increase in circulating androgens. The prevalence of PCOS in patients with alopecia may range from 67 to 77.8% [57].

1.8.1.4 Biochemical signs

Women with PCOS have evidence of biochemical hyperandrogenemia, and high levels of testosterone in circulation serves as a diagnostic marker for androgen excess. Testosterone is circulated as free and bound form with sex hormone binding globulin (SHBG) and albumin. The value of measuring testosterone is primarily to help to exclude other causes of androgen excess. The measurement of free testosterone or the
free androgen index (FAI) may also be used for assessing for hyperandrogenemia. Elevated levels of DHEAS and androstenedione are reported in PCOS [58].

### 1.8.2 Anovulation

Anovulation is a condition where arrest of multiple small follicles growth, characteristic of the PCOS [52]. In PCOS women, ovarian folliculogenesis is affected mainly due to dysregulation of intra and extra ovarian factors in the ovarian microenvironment. Generally, PCOS women are found to have high number of preantral and antral follicles compared to normal women. This might be explained by effects of androgens present in high levels may play a key role in the recruitment of more number of primordial follicles from growing follicles into the process of folliculogenesis [59]. Apart from high levels of androgen, several other factors necessary for folliculogenesis such as growth differentiation factor-9 and bone morphogeneic protein -15 are expressed in low amounts in PCOS compared to normal oocytes [60]. Thus, defects in growth in PCOS may begin at the earliest stages of folliculogenesis, well before arrest of the antral follicle. Similarly, the granulosa cells of preantral and antral follicles produce anti mullerian hormone (AMH), which antagonizes the actions of FSH in ovary, is found in high concentrations in PCOS women [61]. In-spite of accumulation of large pool of follicles, arrest of follicular development during mid-early developmental stages and failed in dominant follicle selection owing to low threshold of FSH. In parallel, LH concentrations tend to be higher in anovulatory cycles, due to failure of the negative feedback signal that is normally exerted by cyclical changes in estradiol and, in particular, progesterone. Thus the arrest of follicle growth and failure of dominant follicle selection for ovulation in
1.8.1 Early follicular phase

Early follicular phase to stimulate normal follicle maturation is attributed to inadequate secretion of FSH; further due to lack of negative feedback inhibition by estrogens to induce progesterone receptors at hypothalamus to regulate GnRH pulses. Restoration of normal ovulation and regular menstrual cycles by reducing number of small antral follicles using invasive techniques like wedge resection and ovarian drilling had been shown in PCOS women [62]. Furthermore, PCOS women treated with anti-androgen such as flutamide restores the sensitivity of GnRH pulse generator to inhibition by estradiol and progesterone [44], and treatment with insulin sensitizing agents, particularly, metformin reduces insulin resistance by decreasing insulin levels, and thereby the excess ovarian steroid synthesis [63,64].

1.8.3 Polycystic ovaries

Polycystic ovaries (PCO) are defined by the presence or accumulation of more than twelve follicles (2–9 mm in size) in each ovary, which are commonly seen on the periphery with increased central stroma, and/or increased ovarian volume (>10 ml) [25,26,65]. In comparison to normal ovaries, PCO can be characterized by increased follicle number and volume due to 2-3 fold increase in growing preantral and antral follicles [66]. Though the mechanism of increase in follicle formation in PCO is unclear, however possibly due to increased primordial follicle activation, slower preantral follicle development, high rate of follicle survival and/or decreased atresia are postulated as causes. Growth of follicles is arrested and show a peculiar degenerative change and accumulation of excess follicular fluid and expansion of antrum is observed; upon enlargement of follicle, granulosa cells undergo apoptosis and atretic and appears as thin walled cyst due lack of granulosa cell layer.
1.8.4 Menstrual dysfunction

Menstrual dysfunction appears to be an excellent surrogate marker for anovulation in PCOS [67]. In general, first encounter of irregular menstruation occurs in adolescents, upon which the menstruation will occur at regular time intervals in normal healthy young and reproductively active women. In contrast, PCOS women have an abnormal pattern of menstrual cycles frequently associated with insulin resistance and hyperandrogenemia; approximately 85–90% with oligoamenorrhea and up to 30–40% of those with amenorrhea [68]. Menstrual irregularities are often associated with abnormal endocrine and metabolic profile in PCOS women [69]. Thus vast majority of women with PCOS, regardless of how defined, demonstrate anovulation and frequently overt menstrual dysfunction [28].

1.8.5 Insulin resistance

The insulin resistance (IR) has been defined as an impaired metabolic response to either exogenous or endogenous insulin. Insulin exerts its actions by binding to their receptors which initiates a series of signaling cascades and initiate a wide range of pleiotropic actions via different signaling pathways at the target tissue such as cellular metabolism, growth and differentiation. IR and hyperinsulinemia are frequent findings in lean and obese PCOS patients. About 50-70% of women with PCOS demonstrate IR [70]. IR induces hyperandrogenism in PCOS women by various mechanisms such as by increasing synthesis and pulsatility of LH; stimulates increased theca cell sensitivity to LH, enhances adrenal sensitivity to ACTH, increased adrenal and ovarian 17alpha-hydroxylase/17,20-lyase activity, reduces insulin growth factor binding protein-1 (IGFBP-1) levels and upregulates ovarian IGF-I receptors, ovarian
enlargement and cyst development in synergy with LH and hCG and by inhibition of hepatic synthesis of sex hormone binding globulin (SHBG). Hyperinsulinemia, resulting from IR, plays a pathogenic role in PCOS by stimulating ovarian testosterone production, decreasing serum SHBG concentrations, and impending ovulation. Insulin decreases IGFBP-1 production, both in the liver and in the ovary and upregulates IGF-I, IGF-II. Further, it increases the LH receptor number at the ovary and sensitizes LH secreting pituitary cells to GnRH stimulation [71]. Thus the IR either in isolation or in association with LH aggravates androgen production in ovarian theca cells thus leading to hyperandrogenism, and reduces hepatic synthesis of SHBG

1.8.6 Obesity

Obesity is defined by abnormal or excessive lipid storage due to increase in number and volume of adipocytes. According to the World Health Organization (WHO) criteria, an individual with a body mass index (BMI) of 30 kg/m$^2$ or higher is considered obese. Obesity is associated with various diseases like congenital vascular defects, metabolic syndrome, T2DM, and has adverse effects on reproductive potential in females leading to impaired ovulation, irregular menstrual cycle, high rate of miscarriage, lower implantation, and pregnancy rates [72]. About 20-80% of women with PCOS are obese, displaying a central or abdominal pattern of obesity, which is characterized by an increased waist to hip ratio. Obesity results in increased androgen production and suppression of SHBG [73]. Despite the close association between PCOS and obesity, the prevalence of obesity in women with PCOS is highly variable depending on age, ethnicity and geographic regions in the general population [74].
combined effect of obesity, along with IR, worsens reproductive features of PCOS including hyperandrogenemia [75], anovulation [76] and dyslipidemia [77].

1.8.7 Dyslipidemia

Dyslipidemia with insulin resistance is a common metabolic abnormality found in 70% of women with PCOS and seen in the very young adolescent or during reproductive ages [76]. Women with PCOS have higher LDL-cholesterol, decreased levels of high-density lipoprotein-cholesterol and apolipoprotein A-I, and increased levels of triglycerides, apolipo protein B and very low-density lipoprotein [77].

1.9 Etiology

Stimulation and injuries during embryonic stage cell differentiation have long-term effects in adult providing evidence for fetal origins of adult disease. High concentration of testosterone exposure in early stages of animals and humans exhibit clinical and biochemical phenotypes of PCOS in adult stages suggests its developmental origin. Epidemiological studies reported that early androgen excess in adolescent girls is at high risk for PCOS [78, 79]. The association is further supported by animal studies: animal fetuses exposed to high androgens develops PCOS like phenotypes in adult stages, provided insights into the fetal origins [64, 79, 80] and suggesting the developmental origins of PCOS even in early intrauterine life. Exposure of the pregnant ewe to large doses of testosterone resulted an enhanced LH secretion and abnormal ovarian cycles in female offspring [81, 82, 83]. Recent studies proved that in-utero androgen exposure in adult female rats could also develop the PCOS related metabolic syndrome [80, 84]. Reproductive and metabolic phenotype is
found to be heritable and runs as cluster in PCOS families suggesting a strong genetic susceptibility for PCOS [7].

1.9.1 Environmental factors

In complex traits like PCOS, it is often impossible to fully identify all the non-genetic contributors to the observed phenotype, and accurately quantify their effects. Lifestyle modifications, such as weight loss and moderate exercise may alter phenotypic expression of PCOS substantially [37]. Contributions of other factors, such as exposure to certain chemicals, are still under debate [85].

1.9.2 Genetics of PCOS

PCOS is a complex infertility disorder of unclear etiology associated with interplay of several gene-gene and gene-environmental factors. Studies showed an association of genes with different phenotypes observed in PCOS, however an inconsistency in replicating the results; it was attributed due to limitations such as lack of retrospective studies, lack of universally accepted standard diagnostic criteria, phenotypic heterogeneity in reproductive and metabolic symptoms etc. As factors responsible for PCOS phenotypes differ among individuals and the susceptible genes affecting these phenotypes may perhaps also be different. The approaches used to identify a genetic locus for PCOS genes are (i) candidate gene association studies where a predisposing allele is expected to be found more frequently in the affected population than the normal individuals and (ii) family studies where the probands inherits PCOS phenotypes in their families are screened to identify specific genomic loci are distributed independently or in linkage with the phenotype [86]. Although candidate gene approach is a very promising one to study the genetic disorders, several candidate
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Genes have proposed as susceptibility genes to PCOS but none gene has been successfully replicated and identified as truly causative across all studies.

1.9.2.1 Genetics of gonadotropin pathway

Genes involved in gonadotropin synthesis, action and regulation such as GnRH1, LHβ, LH receptor (LH/CGR) FSH and FSH receptor (FSHR) and follistatin are proposed as candidate genes for PCOS. Very few studies are available on GnRH1 gene polymorphisms rs6185 (C/G; Trp16Ser) in PCOS [87], and estrogen associated bone disorders [88], however did not found any significant association with PCOS [87]. LHβ gene SNPs rs1800447 (Trp8Arg); rs34349826 (Ile15Thr) and rs5030774 (Gly120Ser) are extensively studied in reproductive disorders such as premature ovarian disorder [89], PCOS [90], endometriosis [91], however did not found any significant association with PCOS. The presence of luteinizing hormone/choriogonadotropin receptor (LH/CGR) gene mutations has been examined in PCOS patients with normal serum LH levels and hyperandrogenemia. LH/CGR SNP may affect the actions of LH and luteinizing hormone/choriogonadotropin receptor (LHCGR) bioactivity [92]. The LH/CGR gene G935A polymorphism in Egyptian women [93] and S312N variants in Sardinian origin, Italy [94] are associated with several clinical and biochemical characteristics of PCOS. FSHR gene SNP rs6165 and rs6166 are strongly associated with the severity of clinical features such as levels of gonadotrophic hormones and the presence of hyperandrogenism in PCOS [87]. Follistatin, a monomeric glycoprotein encoded by a single gene, is linked functionally through its role as a high-affinity binding protein for activin and inhibits the FSH synthesis. Though follistatin is proposed as strong candidate gene, did not found any
association with PCOS in Indian and Asian Singapore populations [95]. Dasgupta et al. [95], screened for mutations in a large scale of samples comprising 549 women consisting of 250 PCOS cases and 299 controls from India did not find any mutations in Follistatin gene exons [95].

1.9.2.2 Genetics of steroidogenic pathway

Genes involved in steroidogenesis pathway are overly expressed in PCOS. Several genes involved in the steroidenic pathway have been proposed as candidate genes such as CYP11A (Cholesterol side chain cleavage enzyme (P450 SCC), CYP17 (encodes 17-alpha-hydroxylase/17,20 lyase) and CYP19 (encodes aromatase), androgen receptor (AR), SHBG. CYP11A1 gene promoter (TTTTA)n microsatellite polymorphism may alter the activity and expression level of these enzymes may induce insulin resistance, thereby affecting an individual’s susceptibility to PCOS. CYP11A gene promoter (TTTTA)n microsatellite polymorphism is found to be associated with PCOS in Caucasian women [96], Greek [97] and Indian women [98], however in PCOS women from Spanish [99] and United Kingdom [100] did not show any association. CYP17A1 encodes for an enzyme 17- alpha -hydroxylase /17–20 lyase that catalyzes pregnenolone to 17-hydroxy-pregnenolone and progesterone into 17-hydroxyprogesterone (17-OHP), which are rate-limiting steps in androgen biosynthesis. It also has a lyase activity, acting to convert 17-OHP and 17-hydroxypregnenolone to androstenedione and dehydroepiandrosterone (DHEA), respectively. CYP17 SNP rs743572 (−34 C/T), a promoter polymorphism, found to be associated with androgen levels [101], adiposity and insulin levels [102], PCOS [101] on the other hand, further studies on rs743572 found no association with PCOS [103]
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and serum androgen levels [104,105]. CYP19 encodes for aromatase, which is a key enzyme for androgen metabolism, catalyzing the conversion of androgens to estrogens [106]. CYP19 SNPs are associated with aromatase expression or bioactivity and lead to steroid hormone disorders, causing the occurrence of some steroid hormone-dependent diseases such as breast cancer and osteoporosis [107]. Several SNPs rs2470152 and rs2414096 had been found to be associated with serum levels of estradiol and estradiol:testosterone ratio in males [108,109] and PCOS [110]. CYP19, [TTTA]n tetra nucleotide repeat polymorphism is associated with breast cancer [111] and gynecomastia [112], and not associated with PCOS in Chinese population [113]. SHBG is a glycoprotein that is synthesized in the liver, and regulates bioavailability of androgens to target cells. Low levels of serum SHBG are characteristic feature of PCOS [114], IR and T2DM [115]. (TAAAA)n pentanucleotide repeat polymorphism in the promoter region of SHBG have been shown to affect transcription efficiency and extensively studied in PCOS, however several studies show inconsistency in results [114]. Several other SNPs such as rs6259, rs6258 are widely studied apart from (TAAAA)n repeat polymorphism, but did not find strong association with PCOS. Genetic studies on AR (CAG)n repeat polymorphism are extensively studied in PCOS and found influence on AR activity and sensitivity towards androgens [116], however, with respect to PCOS, studies on AR CAG polymorphism showed discrepancies [117,118].

In PCOS, steroid and gonadotropin pathways are primarily affected, however deregulation of peripheral mechanisms such insulin pathway, energy homeostasis, inflammatory etc., are found to be associated with several clinical and biochemical
phenotypes in PCOS. However studies on the genetic variations in the genes involved in those pathways such as insulin (INS), insulin receptor (INSR), insulin receptor substrate proteins (INSRP), tumor necrosis factor-α (TNFα) failed to show association with PCOS [119, 120, 121, 122].
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**Hypothesis**

Any change or defect in genes either results in abnormal gene expression, influences bioactivity or induces confirmations changes which may affect the binding capacity. Based on the review of literature, altered gonadotropin physiology is the principal causative mechanism for impaired reproductive physiology in PCOS. Any genetic variations such as mutation, single nucleotide polymorphism (SNP) and insertion/deletion in the genes involved in gonadotropin metabolism, action and regulatory pathway gonadotropin releasing hormone (*GnRH*), beta subunit of luteinizing hormone (*LHβ*), follicle stimulating hormone receptor (*FSHR*), androgen receptor (*AR*) and sex hormone binding globulin (*SHBG*) may affect the hypothalamic pituitary axis leading to cessation of ovarian folliculogenesis and abnormal steroidogenesis and on PCOS.

**Aim**

To evaluate candidate genes SNP involved in the gonadotropin action, regulatory pathway and its association for PCOS.

**Objectives**

I. To obtain systematic phenotypic data with respect to PCOS among patients and a sample of normal control population.

II. To study the SNP in the gonadotrophin releasing hormone (*GnRH*), β-subunit of leutinizing hormone, (*LHβ*), follicle stimulating hormone receptor (*FSHR*), sex hormone binding globulin (*SHBG*) and androgen receptor (*AR*) genes.

III. To investigate genotype –association with PCOS.
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