CLINICAL AND GENETIC STUDIES OF POLYCYSTIC OVARIAN SYNDROME

By

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In the name of Allah

The most merciful and

The most Compassionate

'O' Allah open our eyes to see what is beautiful

Our minds to know what is true

Our hearts to love what is good

DEDICATED TO

My Beloved Daughter Fatima Asif and husband for his love and moral support at all times

CERTIFICATE

This thesis, submitted by Dr. Fouzia Nazir is accepted in its present form by the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of Doctor of Philosophy in Reproductive Physiology.

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LIST OF ABBREVIATIONS

BMI	Body mass index
FSH	Follicle Stimulating Hormone
LH	Luteinising Hormone
PCOS	Polycystic Ovarian syndrome
TVS	Transvaginal Scan
TAS	Transabdominal scan
USG	Ultrasonography
Yrs	Years
S E	Standard error
P	Probability
%	Percentage
mIU/Ml	Milli international units per milliliter
<	Lesser than
>	Greater than
≥	Greater than or equal to
mg	Milligram
ng/ml	Nanogram per milliliter
mm	Millimetre
kg/m2	Kilogram per metre square
WHO	World Health Organisation
TSH	Thyroid Stimulating Hormone
χ^2	Chi square
vs	versus
RBC	red blood cells
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
NaOH	sodium hydroxide
NaCl	sodium chloride
TDT	transmission disequilibrium test
PCR	polymerase chain reaction
STRPs	short tandem repeat polymorphisms
SNP	single nucleotide polymorphism
INSR	insulin receptor
MPB	male pattern baldness
FST	follistatin

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"Aquire knowledge. It enableth its possessor to distinguish right from wrong; it lighteth the way to Heaven; it is our friend in the desert, our society in solitude; our companion when friendless; it guideth us to happiness; it sustaineth us in misery; it is an ornament amongst friends, and an amour against enemies." (Mohammad P. B. U. H)

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ABSTRACT

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Polycystic Ovarian syndrome (PCOS) is the most common endocrine disorder of reproductive age group and its familial presence has been proved. The present study was carried out in two parts. First part comprises clinical, sonographic, endocrine, biochemical characteristics and second part includes epidemiological aspect and association of known loci with microsatellite markers of PCOS. At the end, relationship among clinical, endocrine and association of microsatellite markers with PCOS loci was done. This study was carried out in 6 Pakistani families for which data were obtained through interview of the subjects from Gynecology advisory Centre, Islamabad. PCOS and non-PCOS women were diagnosed applying diagnostic criteria for PCOS of Rotterdam 2003 consensus. This study was carried out from Nov 2001 to December 2005. Main clinical features recorded for PCOS and non-PCOS women were menstrual problems, obesity, sub-fertility and hirsutism. It was found that 38.24 % of PCOS women were obese (BMI 30-35 Kg/m^2) in these families. Menstrual cycle problems (secondary amenorrhea and oligomenorrhea) of PCOS women were seen in 32.40 % and 52.90 % respectively. Sub-fertility was found in 50 % of PCOS women. Moderate and severe hirsutism in PCOS women was present in 47 % and 5.8 % respectively. Acanthosis nigrans was present in 52.9 % of PCOS women. Sonographic findings like follicular number with small diameter (2-8 mm), stromal thickness in mm, ovarian volume were significantly more in PCOS women than in non-PCOS women. Mean serum LH levels in pre-menopausal PCOS women were significantly more compared to non-PCOS pre-menopausal women. Raised serum LH: FSH ratio in PCOS women was seen in 32.4 % which was significantly high compared to non-PCOS women. Serum fasting insulin levels were more than 10 uIU/l in both PCOS and non-PCOS women which was a manifestation of hyperinsulinemia. Triglyceride levels and fasting blood sugar levels in non-PCOS were more compare to PCOS women. Diabetes mellitus type 2 was present in 17.8 % of PCOS and non-PCOS women.

In the second part of the study, recessive mode of inheritance was ascertained in all of the six families. Association of steroid marker D15S519 was seen in family 5. Likewise, marker D5S822 was strongly linked to family 2. Weak association with insulin markers were found in family 1 and 6. Relationship of clinical features and

endocrine parameters with known loci was also an interesting feature of the study. In family 5, mean serum testosterone levels were maximum and association with steroid marker D15S519 was seen here. In family 2, mean serum LH levels were highest and association with marker for follistatin gene D5S822 was found.

In conclusion, we predict from this study that as there are more clinical manifestations in PCOS women of these families but due to its genetic etiology, endocrine and metabolic problems are more or less equalent in these families. So, early prevention by life style modification is the most understood method to save from long term complications.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is the most common but least understood endocrine disorder of reproductive age group (Legro and Strauss, 2002). It affects 5-10 % of the pre-menopausal women and is characterized by hyperandrogenism and chronic anovulation (Sattar et al., 1998). It is diagnosed after exclusion of specific diseases of pituitary, adrenal and thyroid glands (Urbanek et al., 1999; Joan et al., 2006). The field of reproductive endocrinology has not witnessed an issue as controversial as polycystic ovarian syndrome (PCOS). Despite improved diagnostic facilities and advances in in-vitro studies, the primary causes of polycystic ovary (PCO) have not been yet resolved.

This condition is characterized by a peculiar ovarian morphology and clinically by symptoms like oligo-amenorrhea, dysfunctional uterine bleeding, fertility deprivation, hirsutism, acne, obesity and perhaps recurrent miscarriages. The histological features of the PCO are thick smooth fibrotic pearly white capsule with small peripherally placed follicles and theca cell hyperplasia (Young and Goldzieher, 1988; Crum et al., 2003).

The major diagnostic reliance for PCO is on pelvic sonography which is a non-invasive, safe and quick technique for visulization of ovaries (Franks et al., 1997). It has a high concordance with surgical and biochemical diagnosis (Takahashi et al., 1994). Adams et al.,(1985) defined PCO as ovaries in which there are 10 or more fluid filled follicles of 2-8 mm in diameter, arranged around a dense stroma, giving the pearl neckless appearance or scattered throughout an increased amount of stroma. The ovarian volume being 9 cm^3 or more .

Biochemically, hyperandrogenism and raised serum luteinizing hormone (LH) levels are also found in these women (Ardaens et al., 1991). Hyperinsulinemia has also been documented to be the cause of hyperandrogenism in these women (Barbieri et al., 1988; Barnes, 1998). Many studies were based on biochemical phenotyping which offers several advantages over ovarian sonography. First, it can be accomplished with the single blood test assayed. Second, the background rate of abnormal values in the control population can be standardized. Third, biochemical criteria are objective and not subject to operator interpretation (Urbanek et al., 1999).

Sclerocystic changes in the ovary were described by Chereau in 1845(Young and Goldzieher, 1988), but it was in 1935 that Stein and Leventhal recognized an association between polycystic ovaries and complex of signs consisting of amenorrhea, hirsutism and obesity. They described the dramatic effects of ovarian wedge resection in 7 women with enlarged polycystic ovaries who complained of oligomenorrhae or amenorrhea. After wedge resection, menstruation occurred regularly in all cases (Stein and Leventhal, 1935; Battaglia, 2002).

Since the 1990 NIH-sponsored conference on polycystic ovary syndrome (PCOS), it has become appreciated that the syndrome encompasses a broader spectrum of signs and symptoms of ovarian dysfunction than those defined by original diagnostic criteria. The Rotterdam consensus workshop concluded that PCOS is a syndrome of ovarian dysfunction along with the cardinal features of hyperandrogenism and polycystic ovary (PCO) morphology. PCOS remains a syndrome and, as such, no single diagnostic criterion (such as hyperandrogenism or PCO) is sufficient for clinical diagnosis. Its clinical manifestations may include: menstrual irregularities, signs of androgen excess, and obesity. Insulin resistance and elevated serum LH levels are also common features in PCOS. PCOS is associated with an increased risk of type II diabetes mellitus and cardiovascular events (Fauser, 2004).

Incidence:

Clinical spectrum exists between typical Stein Leventhal picture PCOS on one hand and symptom less PCOS on the other hand. High resolution ultrasound scanning has made an accurate estimate of the prevalence of polycystic ovaries possible. Pelvic sonography has led to the reappraisal of the definition, diagnosis and prevalence of this condition in women. The prevalence of this shows a variable range as reported by various researchers. Hart et al., (2004) have documented prevalence of polycystic ovary syndrome in 10 % of Australian population and polycystic ovary morphology on ultrasound in 22 % of women. Diagnostic criteria used by them was presence of any of the following 3 criteria: (i) polycystic ovary, (ii) oligo/anovulation and/ or (iii) clinical or biochemical evidence of hyperandrogenism. Almost 60 % of the women referred because of clinical hyperandrogenism had the classical (anovulatory) form of PCOS, and the new Rotterdam consensus on diagnostic criteria added about another 15 % of ovulatory patients (Carmina et al., 2006).

Lane (2006) has given 3.4-6.8 % prevalence of polycystic ovary syndrome in women of California USA. The Kaiser Permanente Northern California PCOS study is also

currently the largest contemporary sample of women diagnosed for PCOS and one of the most ethically diverse PCOS cohorts, with 18.4 % Hispanic, 15.4 % Asian/Pacific Islander and 7.7 % Black among those in whom information was available. Women with PCOS who were Asians or Hispanic were more likely to have diabetes mellitus, independent of age and body mass index (BMI) (Fauser, 2004; Joan et al., 2006). Zargar et al., (2002) have studied prevalence of PCOS in 37.3 % of women presenting with hirsutism in Kashmiri women in Indian subcontinent. Mao et al., (2001) have studied that 37.4 % of mothers and 33.1 % of sisters of women with PCOS have irregular menstrual cycle in Chinese population. In Portugal population, polycystic ovary syndrome affects 6-10 % of women of childbearing age and in approximately 43 % have developed metabolic syndrome (Silva et al., 2006). In Pakistani women of reproductive age group, PCOS was found in 20.7 %, but the age of onset is not known (Nazir et al., 1999).

Bridge et al., (1993) performed 428 ovarian scans in girls aged between 3-18 year and found polycystic ovaries in 101 girls (24 %), in 6 % of the 6 years old girls, 18 % in 10 years old, and 26 % in 15 years of age girls. The onset of the symptoms range between 15-25 years. Onset of PCOS is gradual in young women who first become aware of a few excessive thick hairs which may be present on chin, upper lips, neck, periareolar areas, lower abdomen, or upper thighs or arms. Hirsutism may progress slowly and usually does not come to medical attention for at least one year after it was first noted. Some women with polycystic ovaries do not manifest hirsutism and may represent a degree of androgen resistance (Balen, 2000).

The earliest recognized PCOS phenotype to date is premature pubarche characterized by excessively elevated levels of dehydroepiandosterone sulfate and hyperinsulinemia. Such girls are at high risk to develop the PCOS phenotype, including ovarian hyperandrogenism and chronic anovulation. After menarche they become oligomenorrhic (Ibanez et al., 1999; Avvad et al., 2001).

Hirsutism and acne may have disturbing effect on the psychosocial make up of a teenager. Absent or infrequent menstruation is also a cause for worry. Screening can be performed relatively easily, by employing a fasting glucose: insulin ratio of < 7 as a useful index of insulin resistance. (Legro et al., 1998a; Kent and Legro, 2002).

Although the pathogenesis of this heterogeneous syndrome is still incompletely determined, the symptomatic management in adult women has been investigated intensively and is now fairly well determined, documented and relatively successful.

For the adolescent who is overweight or frankly obese, the first line of treatment should be a serious attempt to loose weight. Central obesity exacerbates insulin resistance and, if a more normal body weight can be maintained by correct diet and exercise instructions, it is equally as successful as metformin in correcting the stigmata of hyperinsulinism. A reduction of 5-10 % in the body weight can, for example, improve hirsutism in 40-55 % within 6 months of weight reduction (Stafford and Gordon, 2002).

Although the clinical manifestations of PCOS appears to be limited to women, genetic variants associated with hyperandrogenism and insulin resistance, are not limited to women. Thus, although the natural history of the premature pubarche in boys appears to be relatively benign (Potau et al., 1999).

Cresswell et al., (1997) have studied the environmental risk factors for polycystic ovary syndrome (PCOS) which are present in prenatal and post natal life. In his retrospective study, he found 2 groups of patients with PCOS with following prenatal factors: (i) those who had above average birth weight and (ii) those born to overweight mothers. The second group comprised women of normal weight who had high plasma LH, but normal testosterone, concentrations. These women were born after term (40 weeks gestation). On the basis of these findings, the authors suggest that the two forms of PCOS have different origins in intrauterine life. Obese, hirsute women with polycystic ovaries have higher than normal ovarian secretion of androgens, associated with high birth weight and maternal obesity. Thin women with polycystic ovaries have altered hypothalamic control of LH release resulting from prolonged gestation.

Among postnatal risk factors, mainly chronic anovulation (Shoham et al., 1992) and obesity were the main problems (Kiddy et al., 1992; Britzow et al., 1996; Crosignani and Nicolosi, 2001).

Pathology:

Macroscopic appearance: ovaries are enlarged 3-5 times and are round or ovoid in shape. On cut section the superficial cortex is thickened, white and fibrotic. Situated below the cortex are multiple cysts, usually of equal size, which average less than 1 cm in diameter (Hernadez 1996). Despite overall smoothness of the outer cortex

which has a moderately irregular surface because of cysts these ovaries are called oyster ovaries (Lewis and Chamberlain, 1990).

Microscopic appearance: Hernandez (1996) have described the outer cortex of ovary as hypocellular and increased collagen fibres form appearance of true capsule. Nonluteinized granulosa cells are surrounded by an outer layer of hyperplastic, luteinized theca interna cells. Corpora lutea and corpora albicantia are generally absent. There is an increase number of thick walled blood vessels (Crum et al., 2003).

Etiological pathways of PCOS- leading to long term systemic complications.

Three major theories have been proposed to explain the causes of PCOS.

First, the luteinizing hormone-theca interstitial cell (LH-TIC) theory suggests that abnormally elevated levels of LH underlie the phenomenon of PCOS. It leads to the growth of theca interstitial cell (TIC) in developing follicles, which leads to androgen overproduction and follicular atresia (Dunaif et al., 1992).

The second theory, the follicle stimulating hormone-granulosa cell (FSH-GC) theory suggests that FSH leads to subnormal induction of cytochrome P450 aromatase in the granulose cell, leading to elevated androgen levels. This may be due to insufficient bioactive FSH in follicular microenvironment to induce P450 aromatase gene expression, dysfunctional FSH receptors signal transduction mechanism, or presence of inhibitors (such as epidermal growth factor and insulin like growth factor-binding protein-3) that prevent the normal expression of P450 aromatase activity (Rosenbaum et al., 1993).

The third theory relates to the growth factor-autocrine paracrine system. In PCOS, there is evidence of an altered IGF/insulin system, and these acts as mediators of biologic responses of selectogenic and atretogenic follicular hormones (Marseden et al., 1994; Odunsi and Kidd, 1999).

Several pathways have been implicated in the etiology of PCOS. These include the metabolic or regulatory pathways of steroid hormone synthesis, regulatory pathways of gonadotropin action, the insulin-signaling pathway, and pathways regulating body weight. Several genes from these pathways have been tested as candidate genes for PCOS (Urbenak et al., 1999).

weight. Several genes from these pathways have been tested as candidate genes for PCOS (Urbenak et al., 1999).

Abnormality in steroid hormone synthesis:

Barnes (1998) suggested that ovarian biosynthetic abnormality may be due to:

1-an enzymatic defect preventing the aromatization of androgen to estrogen, thus causing excessive secretion of androstenedione.

2- a defect in 3 ß-hydroxysteroid dehydrogenase enzyme causing increased secretion of dehydroepiandrosterone.

3- 17- ketosteroid reductase deficiency preventing the conversion of androstenedione to testosterone and estrone to estradiol, so there are elevated levels of androstenedione and estrone and the ratio of estrone to estradiol is elevated.

4- an increased testosterone production, resulting in its increase concentration in plasma and urine.

5- altered function of cytochrome P-450c 17 ά in ovary.

The elevated androgen levels in patients with PCOS are of combined adrenal and ovarian origin (Erel et al., 1998). Although, secretion of androgens by the adrenal gland is increased in PCOS but main source is ovary in this syndrome (Frank et al., 1989).

In PCOS women with anovulation levels of androgens are raised. Oestrone levels are raised mostly because of extra-ovarian conversion of androstenedione, which largely takes place in adipose tissues. It is well known that there is a primary abnormality in the theca cells of PCOS patients leading to excessive production of progesterone and androgen (Gilling-Smith et al., 1994; Gharani et al., 1997). The androgens are reversibly bound to the carrier protein, sex hormone binding globulin (SHBG). In the case of circulating testosterone, 98 % is bound. It is however the tiny free fraction which is responsible for the biological activity at the target organs (Handelsman, 2006). Excessive estrogen levels in PCOS is a risk factor for endometrial carcinoma (Kousta et al., 2005).

Abnormality in gonadotropin action on ovarian activity:

Normal ovulation will only occur if there is coordination between hypothalamo-pituitary-ovarian axis, the feedback signals and local responses within the ovary. The loss of ovulation can be due to any factors operating at any of the levels. The end

resulting in disturbed steroid synthesis (Ehrmann et al., 1989; Willis et al., 1996; Gambineri et al., 2002).

FSH (follicle stimulating hormone) stimulation of ovarian function is believed to be important for proliferation of granulosa cells, production of follicular fluid, induction of aromatase enzyme activity starting at mid follicular phase, and induction of LH receptors in the late follicular phase. LH (luteinizing hormone) stimulates theca cells production of androstenedione, which is the substrate for subsequent estrogen formation. In the late follicular phase, LH may also play an important role in further growth of the follicle (Zeleznik and Porl, 2006).

Various studies have shown that in PCOS women, LH pulse is usually higher in frequency and amplitude due to high GnRH (gonadotropin releasing hormone) pulstile release by the hypothalamus (Waldstreicher et al., 1988; Abbott et al., 2002). LH pulse amplitude is higher in PCOS than in hirsute women with normal menstrual cycle or in healthy women. The LH pulse frequency was increased only in PCOS compared with healthy women and not in hirsute women with normal menstrual cycle (Minanni et al., 1999). The cause of excess LH is not known. Hypersecretion of LH occur in 40 % of the women with PCOS. Several suggestions have been made to explain this hypersecretion of LH (Balen et al., 1993). A primary CNS (central nervous system) abnormality has also been suggested by the abnormal diurnal pattern of LH secretion in both postmenarcheal teenagers and adults with PCOS (Zumoff et al., 1983; Porcu et al., 1987). Burger et al., (1985) suggested that there is an increase pulse frequency of gonadotropin releasing hormone (GnRH) which increases the pituitary sensitivity leading to a prompter response to GnRH stimulus. This increase frequency and amplitude of GnRH alters the LH and FSH ratio greater than 2 or 3 times, a characteristic feature of PCOS (Fauser and De Jong, 1993). Also high amplitude of GnRH often results in desensitization of the pituitary and a selective suppression of FSH. The circulating levels of LH remain ineffective suggesting an incomplete pituitary desensitization by GnRH. Consequently, the cyclicity of gonadotropins is disturbed acquiring a steady state resulting in chronic anovulation (Minanni et al., 1999).

The aromatase system in peripheral adipose tissues are independent of FSH control and readily able to convert excess circulating androgens to estrogens i.e. oestrone. This peripheral conversion is increased in obese women (Edman and Macdonald, 1978). The circulating levels of oestrone and androgens appear to exert both a positive

and negative feedback controls on the hypothalamic-pituitary axis. The result is increased LH output to negative feedback effects (Devane et al., 1975). It is .hus evident that feedback mechanisms remain intact in women with PCOS. Similiarly, increased levels of oestradiol have a positive feedback on LH release in response to GnRH. Supportive data showed that the circulating levels of sex hormone binding globulin (SHBG) were significantly lower in PCOS, therefore, unbound estradiol concentration are enhanced, resulting into an increase in LH (Stirrat et al., 1987).

The ovary is not normally a major source of androgen production, but once the vicious circle of PCOS is established, it becomes the major source of androgens. So the increased levels of ovarian and adrenal androgen and low levels of (sex hormone binding globulin) SHBG along with increase in LH, is responsible for intense stimulation of the theca cells, thus causing thecal hyperplasia (Shoham et al., 1992). A comparable reduction of FSH due to various factors mentioned above leads to inhibition of aromatase activity thus causing accumulation of androgens. FSH inhibition also leads to impaired follicular development and therefore, granulose cell atresia (yen, 1987).

Among the disorders of ovulation, PCOS is probably unique in that it is associated with normal or elevated estrogen levels. This estrogen may arise from the ovary and also peripherally mainly from the fatty tissue. Thus, an abnormal estrogen environment could feedback on gonadotrophin secretion leading to a relative excess of LH secretion compared to that of FSH, which may indeed be suppressed. This disturbance may lead to a failure of ovulation as the developing Graafian follicle depends upon stimulation from FSH. FSH stimulates the conversion of androgens to estrogens by inducing the activity of ovarian aromatase which resides in granulose cell. The androgen for granulose cell is derived from an outer layer of theca cells in the graafian follicle. Androgen production is controlled by LH. Inappropriate release of LH may profoundly affect this process such that the released egg is either unable to be fertilized or if fertilized, miscarries (Filicori, 1999).

Chapman et al., (2002) have pointed out towards some local intra ovarian factors influenced by systemic mediators and they act on hypothalamo-pituitary ovarian axis. Some important factors are described as following:

Inhibin:

Inhibins are produced locally in many tissues including brain, pituitary, placenta and adrenal glands (Fujimura et al., 1999). The major production site is gonads (Schwall et al., 1990). Castration of male or female rats results in the loss of circulating inhibin and subsequent rise in pituitary FSH (Burger et al., 2001). It also acts as antagonist of Activin (Welt, 2002; Bernard et al., 2003). High levels of inhibin have been found in the PCOS and this provides an additional means by which FSH is reduced (Ehremann et al., 1992).

Follistatin:

Follistatin is a high affinity binding protein that modulates the bioactivity of activin. Activin, a member of the transforming growth factor-β superfamily, and follistatin is expressed in numerous tissues, including the ovary, pituitary, adrenal cortex and pancreas. Activin promotes ovarian follicular development, inhibits theca cell androgen production, increases pituitary follicular stimulating hormone s pancreatic β cells. It increases LH binding sites and progesterone production and may play a role in preventing premature luteinization of the ovarian follicle. Follistatin reverses the enhancing effect of activin on FSH-stimulated steroidogenesis and inhibin production and inhibits activin-induced FSH receptor number and basal inhihin production by granulosa cells. Thus, follistatin may modulate granulosa cell function in an autocrine fashion and its mechanism of action is through binding and neutralization of activin action, and it is likely to favor the process of follicular luteinization or atresia. Overexpression of follistatin will therefore be expected to lead to increased ovarian androgen production and reduction in circulating FSH levels, which are characteristics of PCOS (Urbanek et al., 1999). Follistatin is not expressed in primordial and primary follicles but is expressed in antral stage (Rajkovic et al., 2006).

Leptin:

Leptin is a polypeptide that is secreted by the fat cells in response to insulin and glucocorticoids. Leptin is a 167 amino acid peptide synthesized solely in adipose tissue. The name comes from the Greek for thin. Leptin is thought to be a "satiety" hormone, sending a message to the brain from fat tissue about the adequacy of fat

stores. Leptin concentrations in obese humans are increased in direct proportion to the fat mass. Per unit fat mass in obese persons produce exactly as much leptin as do lean people. Leptin deficiency appears to be a rare cause of obesity in humans. Leptin mutations have been described in infants and recently in adults (Zhang et al., 1994). The two infants described were from a highly consanguineous Pakistani family'' Both children were severely obese, with very low serum levels of leptin. The patients had a frame shift mutation that resulted in a string of incorrect amino acids. These mice displayed massive obesity. hyperphagia, lack of sexual development, and low levels of sex steroids The phenotypes of these adults suggested that leptin is also important in reproductive function and perhaps vital to the initiation of puberty (Montague et al., 1997). Leptin receptors are present in the choroids plexus, on the hypothalamus, ovary and at many other sites (Tartaglia et al., 1995; Jequier, 2002). Leptin also appears to inhibit the neuropeptide Y, which is an inhibitor of GnRH pulsatility. Leptin appears to serve as signal from the body fat to the brain about the adequacy of the fat stores for reproduction (Bray, 1996). Obesity is associated with the high circulating concentration of leptin and this in turn is a mechanism for the hypersecretion of LH in women with PCOS (Caro et al., 1996; Sepilian et al., 2006).

Insulin and insulin like growth factors:

Insulin can exert gonadotrophic effects either directly through the insulin receptor or through 'spill-over' on IGF-I receptors. It may also control the concentration of IGF-1, which in turn may regulate ovarian function. Insulin-induced alterations in IGF-I levels could result from direct stimulation of hepatic production or from a reduction of low-molecular weight IGF-binding protein (IGF-BP) levels. Women with HAIR-AN syndrome who undergo bilateral oophorectomy have complete resolution of their hyperandrogenism, but little improvement in their insulin resistance and hyperinsulinaemia. Furthermore, pharmacological reduction of androgen secretion has no effect on serum insulin concentrations (Suikkari, 1988). Giudice (2006) has stated the role of IGF-1 and androgens in endometrial hyperplasia, endometrial carcinoma, infertility and increased miscarriage rate. He studied that in addition to being responsive to the steroid hormones estradiol, progesterone and androgens, endometrium is also a target for insulin. Moreover, insulin like growth factors (IGFs) and their binding proteins (IGFBPs) are regulated in and act on endometrial cellular

constituents, and hyperinsulinemia down regulates hepatic IGFBP-1 resulting in elevated free IGF-1 in circulation.

Obesity and energy regulation problem in PCOS:

Body weight plays a critical role in the initiation, maintenance and successful outcome of reproductive function (Bates, 1992). In United States of America, obesity is the most costly and most common health issue (Rosenbaum et al., 1997). Up to one third of the adult women are affected, an estimated 35 million women (Dunaif, 1997). Polycystic ovary syndrome is also the most common endocrine disorder among women. Upto 50 % of the women affected with PCOS are thought to be obese (Franks, 1995). Obesity and PCOS have often been linked, and obesity has been found to exacerbate the underlying insulin resistance in PCOS (Dunaif et al., 1989). Obesity has also been linked to increased androgen production and hirsutism in women with PCOS (Balen et al., 1995). Obesity may be the penultimate condition in which the effects of heredity and environment will forever mingle. Studies in twins, adoptees, and nuclear family data suggest that 25—40% of body fat may be heritable. The risk of obesity is about two to three times higher if there is a family history of obesity. Although multiple studies show a single major gene for high body mass that appears to segregate from parents to children, an equal number of studies suggest no segregating allele. Bouchard et al., (1997) have pointed out that the most important regions of human genome that show linkage are 1p, 3p, 6p and 11q.

The medical complications associated with obesity are an increased risk of type 2 diabetes, hypertension, cardiovascular disease, osteoarthritis, gout, sleep apnea, dysfunctional uterine bleeding, and endometrial carcinoma (Legro, 1995). Eisenbruch et al., (2006) have concluded in their study that psychiatric illness may go undetected in a population of polycystic ovary patients. Although the majority of patients exhibit sub-clinical levels of psychological disturbances, emotional distress together with obesity lead to decrements in quality of life in PCOS women.

The best correlation with body fat for such a ratio is with the body mass index (BMI), which is calculated as weight in kilograms divided by height in meters squared (kg/m2). The 1985 National Institutes of Health Consensus Panel on Obesity defined

obesity as a weight gain greater than the 85% percentile. This value is 27.3 kg/m2. Approximately 40% of females of reproductive age are obese by this definition (Burton et al., 1985).

It is interesting that the first nongenetic mouse models were created in the early 1940s via iatrogenic hypothalamic brain lesions, which resulted in many of the characteristics of obesity found in mouse genetic models: hyperphagia, hyperinsulinemia, and a decreased metabolic rate (Zhang et al., 1994).

The relationship among hyperinsulinemia, obesity and PCOS is well known. Insulin levels in obese women with PCOS are higher than in their nonobese counterparts. It suggests that the presence of obesity is associated with insulin resistance. Insulin increases lipoprotein lipase activity, promoting lipid accumulation (Insler et al., 1993; Morales et al., 1996). Hyperinsulinemia and insulin resistance contribute to elevated ovarian androgens and subsequently to anovulation. After ovarian drilling, the correlation between BMI and insulin levels is lost. This might be consistent with the observation that body weight does not influence the efficiency of laproscopic ovarian drilling. It was also reported that ovarian drilling does not change the responses to OGTT (oral glucose tolerance test (Li et al., 1998; Tulandi et al., 2000).

Insulin action abnormality in PCOS:

Hyperinsulinaemia is found in 30% of slim and 75% of obese women with polycystic ovary syndrome. Despite resistance to insulin action in terms of glucose transport, increased insulin levels may cause hyperandrogenaemia by enhancement of androgen production in the ovaries where insulin acts as co-gonadotrophin(Conway et al., 1990).

Insulin plays a central role in human physiology by controlling the activity of many enzymes, particularly those involved in carbohydrate metabolism, it also acts by interacting with various other hormones. Its target tissues are fat, muscle and liver. In addition, insulin also acts on the ovaries, skin, brain, kidney and blood vessels, which all express the classic insulin receptor. Insulin effects include not only stimulation of glucose transport and inhibition of lipolysis but also stimulation of DNA and protein synthesis, stimulation of electrolyte transport across the cell membrane, enhancement of steroidogenesis, and other effects (Prelevic, 1997).

Insulin resistance is a metabolic state in which physiological concentrations of insulin produce subnormal effects on glucose homeostasis and utilization. It most commonly refers to resistance in terms of glucose transport and does not necessarily imply resistance with regard to other actions of insulin. Even though insulin resistant cells exhibit impaired glucose transport, other effects of insulin in these cells are not always equally impaired. Insulin can continue to act on its receptor on different cells either because of 'dissociation' of the insulin effects or 'differential regulation' of its receptors in different tissues. It is also possible, for example, that a second messenger system, and not tyrosine kinase, mediates effects of insulin on steroidogenesis (Poreteky, 1991)..

The first clinical description of an association between hirsutism (hyperandrogenism) and diabetes (insulin resistance) dates back to 1921 when Archard and Thiers reported on the 'diabetes of bearded women' (Archard and Thiers, 1921). More than 50 years later, Kahn et al., (1976) have shown the clinical connection between hyperandrogenaemia, insulin resistance and acanthosis nigricans, which they described as HAIR-AN syndrome. These first reported women were all severely insulin resistant, as a result of either insulin receptor mutations or other target cell defects in insulin action (type A syndrome) or autoantibodies to insulin receptors (type B syndrome).

Acanthosis nigrans is characterized by hyperpigmented and velvety lesions usually found over the nape of neck, axilla or beneath the breast. Its presence has been used as a marker of insulin resistance in general and the syndrome of hyperandrogenism, insulin resistance and acanthosis nigrans (HAIR-AN) in particular (Kahn et al., 1976).

Following facts showed that hyperinsulinemia causes hyperandrogenism (Robinson et al., 1993):

1-the administration of insulin to women with PCOS causes an increase in circulating androstenedione.

2-administration of glucose to hyperinsulinemic hyperandrogenic women result in an increase in circulating insulin and androgens.

3- weight loss decreases the level of insulin and androgen.

4- in vitro insulin stimulates human ovarian stromal androgen production.

The first report of hyperinsulinaemia in women with classical polycystic ovary syndrome was followed by many reports with similar findings. Although obesity was a confounding factor in the early reports, later studies also found hyperinsulinaemia

and impaired insulin sensitivity in a proportion of non-obese women with PCO (Burghan et al., 1980). Insulin resistance out of proportion to obesity is a uniform finding in overweight women with PCOS (Bringer et al., 1993). It seems that it is largely a consequence of increased truncal abdominal fat, even within the normal range of body mass index. Indeed, insulin sensitivity improved with normalization of truncal abdominal fat (Kiddy et al., 1992; Stankiewicz and Norman, 2006).

Several studies have suggested that circulating serum insulin and luteinizing hormone concentrations have an inverse relationship (Anttlia et al., 1991; Dale et al., 1992). Women with raised luteinizing hormone have normal serum insulin and hyperinsulinaemic women tend not to hypersecrete this hormone. The insulin-resistant PCOS subgroup is characterized by elevated serum insulin levels, markedly exaggerated insulin response to glucose challenge, slightly elevated luteinizing hormone levels, a modest response of luteinizing hormone to gonadotrophin-releasing hormone and often ovarian stromal hyperthecosis, whereas the non-insulin resistant subgroup is characterized by normal fasting insulin, near normal insulin response to a glucose challenge, markedly elevated luteinizing hormone levels, and an exaggerated luteinizing hormone response to GnRH, hyperandrogenism can he mediated by hypersecretion of either luteinizing hormone or insulin, but rarely both (Conway and Jacobs, 1993).

PCOS is associated with hyperinsulinimia and peripheral insulin resistance, both of which have been linked to dyslipidemia. Insulin acts via its specific receptor, which is a glycoprotein hetcrotetramcr. The receptor consists of two alpha subunits, which are extracellular and contain the insulin binding domain, and two beta subunits which span to the alpha subunit transmitting a signal to the beta subunit, which activates protein tyrosine kinase, an enzyme present (in the cytoplasmic domain of the beta subunit. The resulting phosphorylation of the tyrosine residues is an obligatory step of insulin action (Kahn and White, 1988).

Insulin resistance could be caused by defects at any step of insulin action. Several defects have been reported: anti receptor antibodies that prevent insulin from binding to the alpha-subunit, reduction in the number or the affinity of the binding domain of the insulin receptor, defects in the ability of the beta-subunit to be autophosphorylated in response to insulin, and decreased number of GLUT 4 (glucose transporter-4) (Moller and Flier, 1991).

Although mutations in the insulin receptor gene in women with type A syndrome of extreme insulin resistance have been documented, no such mutations were found in women with PCOS (Moller and Flier, 1991).

Although the cellular basis and molecular mechanisms of insulin resistance in classic insulin target tissues in women with PCOS are not defined, but recent studies indicates that the abnormality is a post receptor defect in the insulin signal transduction pathway between the receptor kinase and glucose transport (Talbot et al., 1996). Defects in insulin action in women with PCOS seem to be intrinsic, since they are not correlated with glycaemia, obesity, body fat distribution or sex hormone levels (Dunaif et al., 1992).

Women with PCOS have a significant decrease in adipocyte GLUT 4 (an insulin-regulated glucose transporter) content. This decrease is independent of glucose tolerance, obesity and sex hormone levels. It is not known, however, whether this defect is primary or secondary to abnormal insulin receptor signaling (Rosenbaum et al., 1993).

Colilla et al., (2001) have documented in their study that heritability of beta cell dysfunction of pancrease is likely to be a contributing factor in the predisposition to diabetes in PCOS. Zavaroni et al., (1987) and Lobo and Carmina, (2000) have suggested that hyperinsulinaemia, both fasting and postprandial, is a risk factor for the development of cardiovascular disease in diabetic and non diabetic individuals, the presumed mechanism for the association being through alterations in serum lipoprotein concentrations.

Kousta et al., (2005) suggested that insulin is an atherogenic hormone and that in addition to its role in lipid metabolism it has direct effects on the arteries which include:

1- increased formation and decreased regression of atherosclerotic plaques,

2- proliferation of smooth muscle cells,

3- enhanced cholesterol synthesis and increased low-density lipoprotein receptor activity, 4- stimulation of growth factors which are important elements of atherosclerotic plaque formation.

An increased concentration of circulating plasminogen activator inhibitor-I (an inhibitor of intravascular fibrinolysis) whose production is stimulated by insulin, and

which is another surrogate marker of risk for coronary disease has recently been reported in women with PCOS (Sampson et al., 1996; Tsanadis et al., 2002). Hyperinsulinemia reduces IGF-BP1 production thereby increasing bioavailable IGF-1 activity, elevation in insulin and bioavailable IGF-1 influencing gonadotropin secretion, adrenal androgen production and also contributing to abnormalities in lipid. Whether the increase in cytochrome P450c17ά activity in women is inherited or acquired are not known. A possible explanation for the ovary-stimulating action of insulin in women with PCOS is that the post- receptor mechanism of insulin action in the ovary is augmented in some way, perhaps by an abnormality in cytochrome P45017α activity that makes this enzyme complex more sensitive to insulin (Utiger, 1996). This hypothesis is supported by other researchers. Moghetti et al., (1996) have reported that hyperinsulinemia may stimulate cytochrome P450c17ά activity in adrenal glands of women with PCOS. Zhang et al., (1995) have shown that serine phosphorylation of the steroidogenic enzyme system cytochrome P450c17ά increases its 17, 20- lyase activity (that is its androgen biosynthesis activity). Consistent with latter study is the findings of reduced ovarian cytochrome P450c17ά activity and amelioration of hyperandrogenism after suppression of serum insulin concentration with metformin (Nestler and Jakubowitz, 1996). Legro et al., (1999) suggested that PCOS is more important risk factor than ethnicity or race for glucose intolerance in young women.

This study was envisaged to assess different clinical characteristics of polycystic ovarian syndrome in non-PCOS and PCOS women of the Pakistani families. Reproductive endocrine status and biochemical markers involved in long term complications have also been studied. The second part of study is about the epidemiology and linkage analysis regarding its genetic basis.

SUBJECT AND METHODS

SUBJECTS AND METHODS

Women diagnosed for polycystic ovarian syndrome at Prof. Dr Saad Rana's gynecological advisory center, Islamabad were interviewed to record information required for the present study. This study was carried out from November 2001 till February 2006. Extensive pedigrees were drawn following Bennet et al. (1995) for the genetic studies. Probands were interviewed to trace back the disease in each family. The pedigrees were constructed at least up to five generations depending upon that how much the proband could recollect about the disease in the members of the family. Women with menstrual problems, hirsutism, obesity and infertility were designated as polycystic ovarian syndrome (PCOS) women.

There were 121 women from six families, of which 17 women were dead and 42 were not available for interview. A total of 62 women agreed to cooperate and these were scored for required information and for further study. Generation wise details of women in each family are given in Table 1.

Before interviewing the patients written consent was taken for the participation of the study. During interview the information which was recorded included age of onset of syndrome, their menstrual history, fertility problem, height, weight, hip and waist measurement and hirsutism.

Conditions like hyperprolactinemia, adrenal hyperplasia, hypothyroidism were excluded as these can mislead the diagnosis. Menstruation was defined as normal cyclical if women had menstrual cycle of 22-35 days, oligomenorrhea if cycle ranged from 35-45 days. Secondary amenorrhea was labeled if menstruation delayed till 6 months or more (after the exclusion of pregnancy) (Campbell, 2000).

Menstrual cycle pattern of postmenopausal women were interviewed in detail and their past cycle was recorded for comparison with pre-menopausal women of the study.

Marital and fertility status of all women was also noted. Women who did not conceive after 2 year of unprotected intercourse were diagnosed as sub-fertile (Gnoth et al. 2005).

Table 1: Detailed information regarding the female members of the six families who were scored for the study and those not scored due to non-availability and those who were dead.

women.

FAMILY	GENERATION	SUBJECTS SCORED FOR STUDY		SUBJECTS NOT SCORED FOR STUDY		DEAD	
		Non-PCOS	PCOS	Non-PCOS	PCOS	Non-PCOS	PCOS
1	I	-	--	-	-	2	-
	II	-	-	-	-	2	-
	III	2, 4	6	10	-	-	-
	IV	3,9	1,7, 14, 17	5,16	8, 12	-	-
	V	-	2,6	5	4	-	-
2	I	-	-	-	-	2	-
	II	7	-	5	-	2	-
	III	17	4, 14, 16	3, 9,10,13	2,18,12	-	-
3	I	-	-	-	-	2	-
	II	8	-	4,11,12, 13,14	-	-	-
	III	6, 11	5,8,13, 17	1,2,12	-	-	21
	IV	7	1,11	3,4	-	-	-
4	II	-	-	-		2,5	-
	III	10	-	1,4,11,12	-	7	5,6
	IV	3,8,11,12	1,4,9,10,13	-	-	-	-
5	II	-	-	-	-	2,.6	-
	III	2,3,9	6,10,15	7,12,16,17	5	-	-
	IV	1,7, 11, 13	6,10, 12, 16	19-23	-	-	-
6	I	-	-	-	-	1,3,6	-
	II	6	4,9	2	-	-	-
	III	1,5,8,10,	3,7,12	11	9	-	-
	IV	2	6	-	-	-	-
TOTAL=120		28	34	34	8	13	3

Obesity was assessed by body mass index (BMI) as Kg/m^2.

$$BMI = \frac{\text{Weight in kilograms}}{(\text{Height in meters})^2}$$

It is classified into 4 categories:

1-Normal built	19-25 Kg/m^2
2-overweight	26-30 Kg/m^2
3-obese	31-40 Kg/m^2
4-very obese	> 40 Kg/m^2

Waist: hip ratio upto 0.85 was taken as gynecoid obesity and more than 0.85 was considered android obesity (Wijeyaratne et al, 2002).

Hirsutism was scored by Ferryman Gallway scoring system (Ferryman and Gallway, 1961) which quantifies the presence of terminal hair over 11 body areas i.e upper lip, chin, chest, upper back, lower back, upper abdomen, lower abdomen, arm, forearm, thigh and leg (Table 2). Skin problems like acanthosis nigrans, acne were also recorded.

Table 2: Ferriman Gallway scoring system

site	grade	Definition
Upper lip	1	Few hair at outer margin
	2	Small mustache at outer margin.
	3	Mustache extending in halfwayfrom outer margin.
	4	Mustache extending to midline
chin	1	Few scattered hairs.
	2	Scattered hair with small concentrations
	3 & 4	Complete cover with light &heavy.
chest	1	Circumaerolar hairs
	2	With midline hair in addition
	3	Fusion of these areas, with ¾ cover.
	4	Complete cover
Upper back	1	Few scattered hair
	2	Rather more, still scattered
	3 &4	Complete cover with light and heavy.
Lower back	1	Sacral tuft of hair
	2	With some lateral extension.
	3	3/4 cover
	4	Complete cover
Upper abdomen	1	Few midline hair
	2	Rather more, still midline
	3 & 4	Half and full cover
Lower abdomen	1	Few midline hair
	2	Midline streak of hair
	3	Midline band of hairs.
	4	Inverted V shaped growth.
arm	1	Sparse growth affecting not more than ¼ limb surface.
	2	More than this, cover still incomplete.
	3 & 4	Complete cover light and heavy
forearm	1, 2	Complete cover of dorsal surface.
	3, 4	2 grades of light & 2 grades of heavy hair
thigh	1,2,3,4	As for arm
leg	1,2,3,4	As for forearm

(Ferriman and Galway, 1961)

Arm is the area from shoulder to elbow, forearm is from elbow to hand. Likewise, thigh is from hip to knee and leg is from knee to foot.

Ultrasonography

Ultrasonography was done by using Toshiba Ultrasound Diagnostic System model SSA-220A with transabdominal probe of 3.75 MHz frequency and vaginal probe of 6 MHz frequency. Transabdominal scan was done for unmarried and transvaginal scan was done for married women. Detailed ultrasound regarding the size of ovaries, the number and size of follicles, ovarian cysts, stromal thickness, uterine size and endometrial thickness and any other pelvic pathology was done. Sonographically, polycystic ovaries were defined as enlarged ovaries with 8-10 follicles of 2-8 mm in diameter arranged on the periphery of ovaries and increased stromal thickness and density. The follicular or cyst number was established by scanning each ovary from inner to outer margin in longitudinal section. Stromal thickness of 4 mm was considered normal. Ovarian volume of 9.5 cm^3 was taken as normal. A normal ratio of stromal/area thickness ratio was 0.34. The following parameters were evaluated on sonography:

1- Number of 2-8 mm in diameter follicles in each ovary was counted and their mean was calculated.

2- Stromal area thickness: it was evaluated by outlining with the caliper the peripheral profile of the stroma, identified by a central area slight hyperechoic with respect to the other ovarian area (photograph 1).

3- Ovarian volume: it was estimated according to the formula ½ (AxBxC) where A is the longitudinal diameter, B the anteroposterior diameter and C the transverse diameter of the ovary.

4- Ovarian area: it was calculated by outlining with caliper the external limits of the ovary in the maximum plane section and area is given by the machine automatically.

5- Stromal/total area ratio (S/A). (Fulghesu et al.2001)

Blood sampling was done after an overnight fast on day two of the regular menstrual cycle and on random basis of irregular cycle. It was done by using butterfly cannula of 20G size and venepuncture from antecubital vein was done. Fifteen ml blood was drawn. Out of this 6 ml was kept for biochemical and endocrine analysis in sterile vacutainer tube and 9 ml was stored in sterile vacutainer tubes containing potassium EDTA prepared for the purpose of DNA extraction.

Assessment of Biochemical parameters

To evaluate biochemical status of these families, serum cholesterol, High density lipoproteins (HDL), Low density lipoproteins (LDL), Triglycerides (TG) and fasting blood sugar levels were quantified. Normal values are given in Table 3. Coefficient of variation during the assay and between the assays is given in Table No 4.

Enzymatic in vitro assay for the direct determination of these tests were done by using Roche/Hitache kit and Roche clinical chemistry analyzer. Principle and procedure of these tests were as follows:

Triglycerides (TG):

Principle: For the rapid and complete hydrolysis of TG to glycerol, lipoprotein lipase from microorganisms was used. It was followed by the oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide produced then reacts with 4-aminophenazone and 4-chlorophenol under the catalytic action of peroxidase to form a red dyestuff (trinder endpoint reaction).

Reagents of the kit:

Reagent 1:

Pipes buffer {piperazine-n,n'bis(2-ethanesulfonic acid)}	pH 6.8	50 mmol/l
Mg^{2+}		40 mmol/l
Sodium cholate		0.20 mmol/l
ATP		≥0.13 mmol/l
4-chlorophenol		4.7 mmol/l
potassium hexacyanoferrate		1 µmol/l
fatty alcohol polyglycol ether		0.65 %
lipoprotein lipase (pseudomonas spec.)		≥ 5 U/ml
glycerokinase (Bacillus stearothermophilus)		≥0.19 U/ml
glycerol phosphate oxidase (E.coli)		≥2.5 U/ml
peroxidase (horseradish)		≥0.1 U/ml

Protocol:

Wavelength	700 nm
Pathlength	1 cm
Reading	against air
Temperature	37°C
Reaction	3 minutes

Reagents were brought to working temperature before use.

3 µl of serum was pipetted into the tube and 250 µl of reagent 1 was mixed in it. The analyzer automatically calculated the analyte concentration.

Conversion factor: mg/dl x 0.0113=mmol/l

Mmol/l x 88.5=mg/dl.

High density lipoproteins (HDL):

Principle: Precipitation based method was used for HDL estimation. HDL was first separated by precipitating apoprotein B containing lipoprotein from serum by using combination of a polyanion and a divalent cation, such as dextran sulphate/ magnesium chloride or phophotungstate/magnesium chloride. Several approaches for the direct measurement of HDL in serum has been proposed, including the use of magnetically responsive particles such as polyanion metal combinations and the use of polyethylene glycol (PEG) with anti-apoprotein B and anti apoprotein CIII antibodies. This automated method for the direct determination of HDL in the serum and plasma uses PEG modified enzymes and dextran sulphate.

Reagents of the kit:

Reagent 1: (Dextran sulfate/buffer)

MOPS buffer (3 morpholinopropanesulfonic acid) pH 7.0	19.1 mmol/l
Dextran sulfate	0.5 g/l
Magnesium sulfate heptahydrate	2 g/l
HSDA	0.3 g/l
Ascorbate oxidase (Eupenicillium sp.recombinant)	≥3 kU/l
Peroxidase	≥ 10 kU/l

Reagent 2: (PEG enzyme/4-amino-antipyrine/buffer)

PIPES buffer (piperazine 1,4-bis(2-ethanesulfonic acid)	9.9 mmol/l
PEG cholesterol esterase (pseudomonas spec)	≥0.2 kU/l
PEG cholesterol oxidase (streptomyces sp.recombinant)	≥7.6 kU/l
Peroxidase	≥20 kU/l
4-aminoantipyrine	0.5 g/l

Protocol:

Wavelength	700 nm
Pathlength	1 cm
Reading	against air

Temperature	37°C
Reaction	3 minutes

Reagents were brought to working temperature before use.

3 µl of serum was pipetted into the tube and 250 µl of reagent 1 was mixed in it. Then 83 µl of reagent 2 was added. The analyzer automatically calculated the analyte concentration.

Conversion factor: mg/dl x 0.026=mmol/l

Mmol/l x 38.66=mg/dl.

Low density lipoprotein (LDL)

Principle : A detergent was included in the enzymatic method for cholesterol determination (cholesterol esterase cholesterol oxidase coupling reaction), the relative reactivity was in this order: HDL<chylomicron<VLDL<LDL. The combination of sugar compound with detergent enables the selective determination of LDL cholesterol in the serum.

Reagents of the kit:

Reagent 1:

MOPS buffer (3 morpholinopropanesulfonic acid)	20.1 mmol/l	pH 6.5
HSDA	0.96 mmol/l	
Ascorbate oxidase (eupencillium spe recombinant)	50 µKat/l	
Peroxidase	≥ 167 µKat/l	

Reagent 2

MOPS buffer (3 morpholinopropanesulfonic acid)	20.1 mmol/l	pH 6.8
$MgSO_4$ 7 H_2O	8.11 mmol/l	
4 aminoantipyrine	2.46 mmol/l	
Cholesterol esterase (pseudomonas spec)	≥ 50 µKat/l	
Cholesterol oxidase (Brevibacterium spec recombinant)	≥ 33 µKat/l	
Peroxidase	≥ 333 µKat/l	

Protocol:

Wavelength	700 nm
Pathlength	1 cm
Reading	against air
Temperature	37°C
Reaction	3 minutes

Reagents were brought to working temperature before use.

3 µl of serum was pipetted into the tube and 250 µl of reagent 1 was mixed in it. Then 83 µl of reagent 2 was added. The analyzer automatically calculated the analyte concentration.

Conversion factor: mg/dl x 0.0259=mmol/l

Mmol/l x 38.66=mg/dl.

Cholesterol:

Principle:

Cholesterol is determined enzymatically using cholesterol esterase and cholesterol oxidase.

Reagents of the kit:

Reagent 1:

PIPES buffer (piperazine 1,4-bis(2-ethanesulfonic acid)	pH6.8	75 mmol/l
Mg^{2+}		10 mmol/l
Sodium cholate		0.20 mmol/l
4-aminophenazone		≥0.15 mmol/l
phenol		4.2 mmol/l
fatty alcohol polyglycol ether		1%
cholesterol esterase (pseudomonas)		≥0.5 U/ml
cholesterol oxidase (E.coli)		≥0.15 U/ml
peroxidase		≥0.25 U/ml

Protocol:

Wavelength	700 nm
Pathlength	1 cm
Reading	against air
Temperature	37°C
Reaction	3 minutes

Reagents were brought to working temperature before use.

3 µl of serum was pipetted into the tube and 250 µl of reagent 1 was mixed in it. The analyzer automatically calculated the analyte concentration.

Conversion factor: mg/dl x 0.0259=mmol/l

Mmol/l x 38.66=mg/dl.

Blood glucose levels:

Principle:

Glucose 6-phosphate-dehydrogenase oxidizes glucose-6-phosphate in the presence of NADP to gluconate-6-phosphate. No other carbohydrate was oxidized by this method. The level was measured photometrically.

Reagents of the kit:

Reagent 1:

TRIS buffer	pH 7.8	100 mmol/l
Mg^{2+}		4 mmol/l
ATP		≥1.7 mmol/l
NADH		≥1 mmol/l

Reagent 2:

HEPES buffer	pH 7	30 mml/l
Mg^{2+}		4 mmol/l
HK (yeast)		≥8.3 U/ml
G-6-PDH (E.coli)		≥15 U/ml

Protocol:

Wavelength	405 nm
Pathlength	1 cm
Reading	against air
Temperature	37°C
Reaction	3 minutes

Reagents were brought to working temperature before use.

3 µl of serum was pipetted into the tube and 250 µl of reagent 1 was mixed in it. Then 50 µl of reagent 2 was added in it. The analyzer automatically calculated the analyte concentration.

Conversion factor: mg/dl x 0.0555=mmol/l

Mmol/l x 38.66=mg/dl.

Table 3: Normal values of different biochemical tests:

Biochemical test	Result (normal range)
Cholesterol	< 200 mg/dl
Low density lipoproteins (LDL)	< 100 mg/dl
High density lipoproteins(HDL)	> 65 mg/dl
Triglycerides(TG)	< 200 mg/dl
Blood glucose levels	Fasting: < 100 mg/dl, Random: 140 mg/dl

Table 4: Sensitivity, Inter and intra assay coefficient of variation of different biochemical parameters.

Biochemical parameter	Sensitivity	Inter %	Intra %
Triglyceride	4 mg/dl	1.5	1.8
HDL	3 mg/dl	0.9	1.85
LDL	3 mg/dl	0.71	1.20
Cholesterol	3 mg/dl	0.8	1.7
Fasting blood sugar	2 mg/dl	1.1	2.9

Endocrine analysis was done for serum FSH, LH, Testosterone, Prolactin, Estradiol and insulin levels. This was done by enzyme immunoassay (EIA) kits (Serono Diagnostic, Italy). Procedure and principle of assay is different for steroid and protein hormones and explained as following:

Protein Assays:

Quantitative determination of protein hormones (FSH, LH, Prolactin and insulin) by serozyme EIA, incorporating two high affinity monoclonal antibodies into an immunoenzymatic system (magnetic solid phase). The assay procedure involved two steps:

Immunological Step:

Hormone (Ag) present in the sample, standard or control was reacted in the test tubes with mixture of two monoclonal antibodies. An antibody conjugated with an enzyme (Ab1) quickly attached to one site on hormone molecule. A second monoclonal antibody linked to fluoescein (Ab2) bound at a second site on the hormone molecule, resulted in the formation of Ab1-Ag-Ab2 complex. Following incubation, an antiflorescein coupled to a magnetic solid phase was added in excess. This readily and specifically bound to the Ab1-Ag-Ab2 complex and was sedimented on a magnetic field.

Enzymatic Step:

After decanting, and washing the sedimented complex, all tubes were incubated with the substrate solution, which bound to Ab1-Ag-Ab2 complex. The enzyme/ substrate reaction was ended by the addition of the stop reagent and intensity of the color developed was measured photometrically by using serozyme 1 (serono). Intensity of the colour was directly proportional to the concentration of the hormone in the sample.

Steroid assay:

In quantitative determination of the steroid hormones (estradiol and testosterone) by EIA, a high affinity polyclonal antibody for these hormones was used which incorporate magnetic solid phase separation. The assay was performed in 2 steps:

Immunological step:

Hormone (Ag) present in the sample, standard or control competes with a fixed amount of hormone derivative (conjugated to an enzyme Ag*) for binding to a limited amount of fluorescein labeled polyclonal antibody (Ab). This resulted in the formation of Ag-Ab+Ag*-Ab and free Ag*+Ag. After incubation anti-florescein

coupled to a magnetic solid phase was added in excess. This rapidly and specifically bound to the hormone derivative antibody complex (Ag*-Ab) and was sedimented. This was followed by washing.

Enzymatic step:

After decanting and washing the sediment, all tubes were incubated with an enzyme substrate solution, which bound to the Ag*-Ab complex. The enzyme reaction was ended by the addition of the stop solution. The intensity of the colour develop was measured photometrically. Intensity of the colour was inversely proportional to the concentration of the hormone present in the sample.

EIA procedure for the Hormone assay:

Serum sample (0.05-0.15 ml in disposable round bottomed (12x 75 mm) polystyrene test tubes were incubated with 0.2 ml of the enzyme conjugate (florescein labeled rabbit polyclonal antibody and bovine alkaline phosphatase labeled mouse monoclonal antibody to the protein hormone in the tris buffer with sheep, horse and bovine serum protein at 37°C in a clean water bath. The incubation time varied with the type of hormone to be measured i.e. 15min for FSH, LH, prolactin and insulin while 20 min for estradiol and testosterone without derivative and again incubate 20 minutes with serozyme hormone derivative. After incubation, 0.2 ml of thoroughly mixed separation reagent (sheep antiserum to florescein, covalently bound to magnetizable particles in tris buffer containing bovine serum albumin and sodium azide) was added to each tube and incubated for 5 minutes for each hormone at 37 C in water bath. Washing followed these incubations. The tube rak was fixed on a magnetic separator and particles were allowed to sediment for 2 minutes magnetically. The supernatant was decanted and 0.5 ml of diluted wash buffer solution (a surfactant and preservative in the tris buffer) was added to each tube. A thorough mixing was performed to assure good assay performance. The rack of the tubes was again fixed on a separator and particles allowed to settle down. This washing was repeated. The tubes were removed from magnetic base and 0.3 ml of serozyme substrate (phenolphthalein monophosphate, an enzyme co-factor) was dispensed into each tube including the blanks. The tubes were shaken and incubated for 15 minutes except for estradiol and testosterone assay where incubation time was 20 minute. After thi last incubation, 1 ml of stop solution (sodium hydroxide and chelating agent in the buffer solution pH > 10) was added into each tube including the blanks. The rack containing the tubes was fixed to the magnetic separator and

particles were allowed to settle for at least 10 minutes. Tubes were then read at wavelength of 550 nm against the reagent blank on serozyme 1 spectrophotometer (serono). The hormone concentration in each sample was determined by interpolating from the standard curve. The normal ranges of different hormones are given in Table-5. Sensitivity and inter-assay and intra-assay coefficient of variation of different hormones is given in Table 6.

Statistical analysis:

Data was analyzed with the help of SPSS version 10.1. Mean±SEM was calculated for each parameter. Independent t test was applied to see the comparison of the means between different groups.

Ratio of LH to FSH was also taken and considered to be normal if < than 2.5. Similiarly, ratio of serum insulin with fasting blood sugar levels of 6 was taken as normal.

Table 5: Normal ranges of different hormones in early follicular phase of the menstrual cycle and in postmenopausal period.

Hormone	Early follicular phase	postmenopausal women
Serum FSH	3.2-10 mIU/ml	>20 mIU/ml
Serum LH	1.2-10 mIU/ml	>20 mIU/ml
Serum estradiol	12-48 pg/ml	<10 pg/ml
Serum testosterone	> 50 ng/ml, >25 in adolescent	>35
Serum prolactin	3.3-24 ng/ml	
Serum fasting insulin	> 10 μIU/μl	

Table 6: Sensitivity, Inter and Intra assay coefficient of variation of different hormones.

Hormones	Sensitivity	Inter-assay %	Intra-assay %
FSH	1.0 mIU/ml	9	3
LH	1.0 mIU/ml	9	2
Estradiol	10.0 pg/ml	9	3.5
Testosterone	10 ng/ml	6	3
Prolactin	1.0 ng/ml	10	4
Insulin	2 μIU/μl	7	3

RESULTS

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In the present study, 6 families were studied which comprised 120 women. Of the 120 women only 62 women were available for the present study. On the basis of ultrasound, out of 62 selected women, 34 (55 %) women were diagnosed for PCOS and the remaining 28 (45 %) were non-PCOS. The details about distribution of PCOS and non-PCOS women in the six families in different generations are given in Table 7 (Fig 1-6).

Both PCOS and non-PCOS women were classified regarding their reproductive status, i.e. pre-menopausal and postmenopausal state (Table 8). There were 47 pre-menopausal women, of which 28 (59.57 %) were PCOS and 19 (40.42 %) were non-PCOS. There were 15 postmenopausal women of which 6 were diagnosed for PCOS and 9 were non-PCOS.

First part of the study consists of clinical characteristics, sonographic, endocrine and biochemical parameters of PCOS and non-PCOS women. The second part of the study is based on epidemiological and molecular parameters of these families.

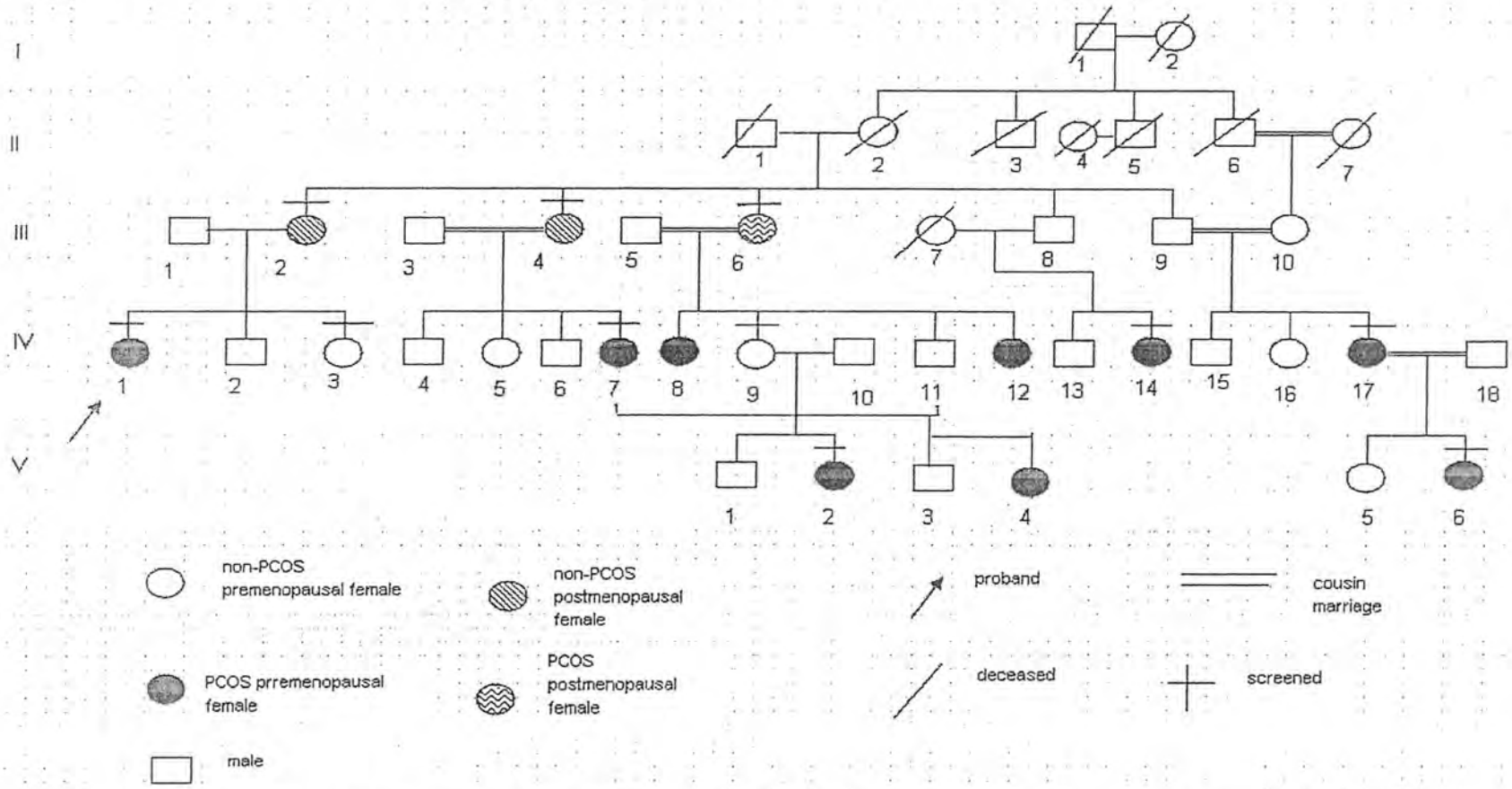

Fig 1: Pedigree of family No. 1 showing females who were screened by history, examination, pelvic ultrasound, endocrine and biochemical testing. Information regaring PCOS status in generation I and II not known.

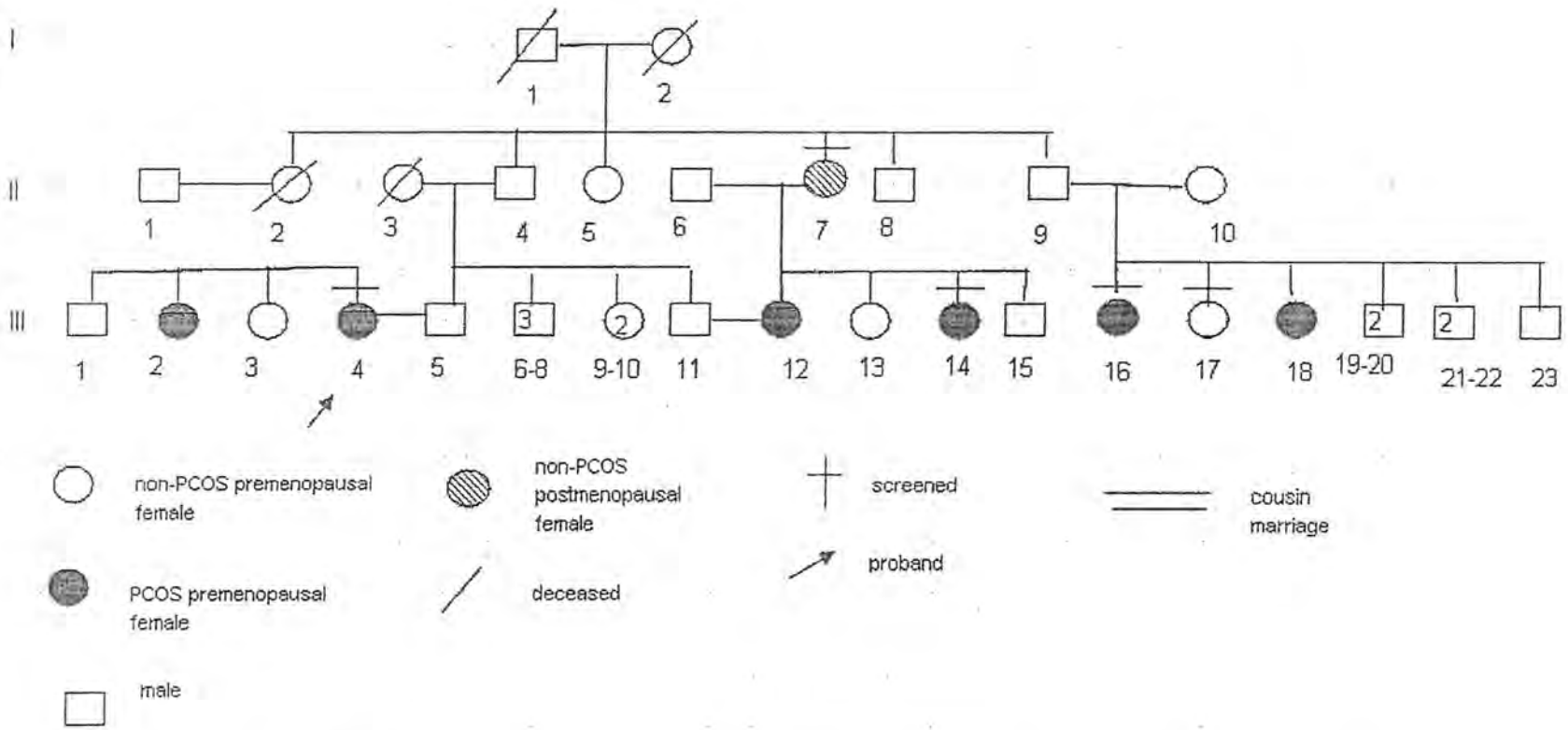

Fig 2: Pedigree of family 2 showing females screened by history, examination, ovarian ultrasound, and biochemical and endocrine parameters. Information regarding PCOS state in generation I was not known

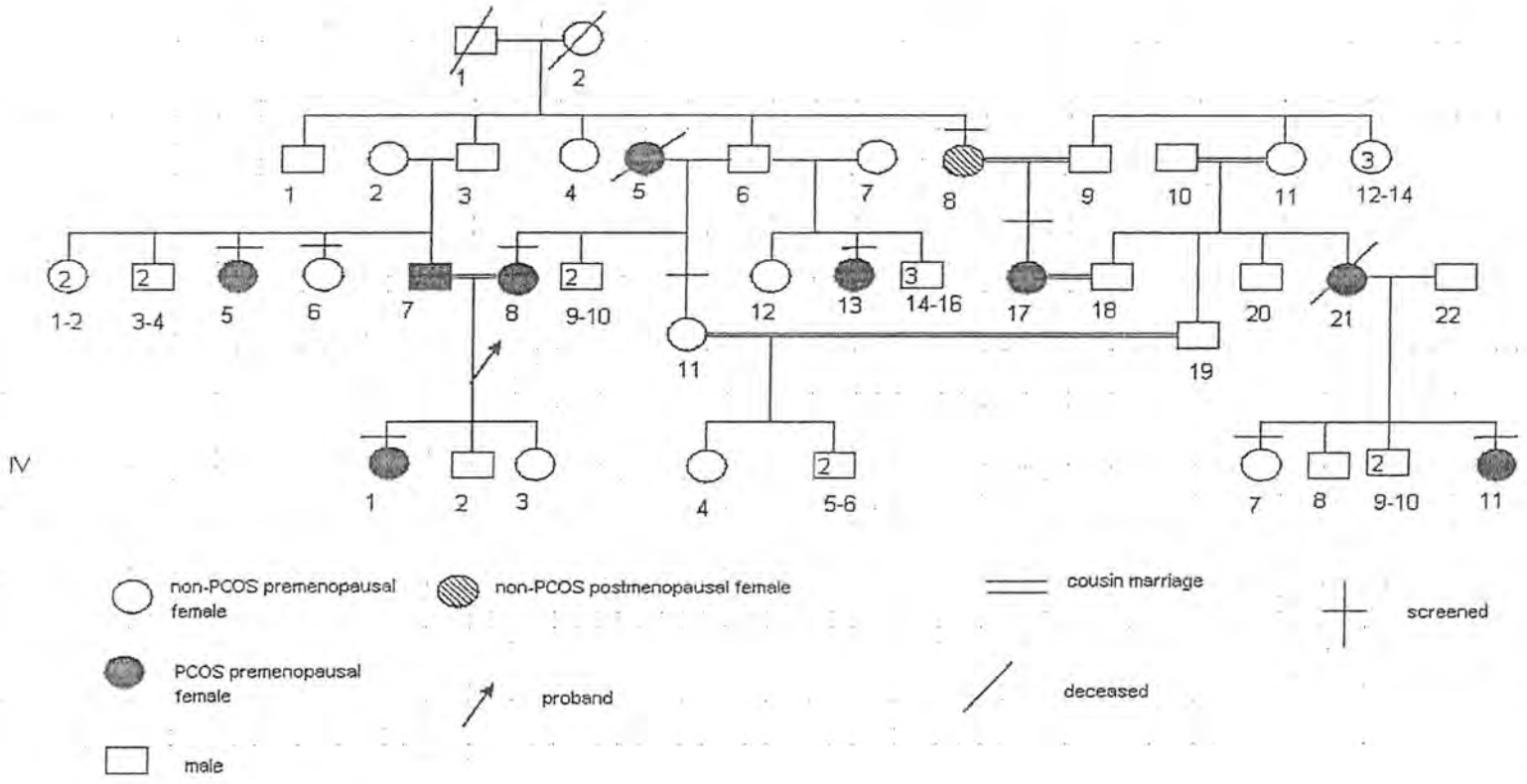

Fig 3: Pedigree of family No. 3 showing females who were screened by history, examination, pelvic ultrasound, endocrine and biochemical testing. Information regarding PCOS state was not known in generation I.

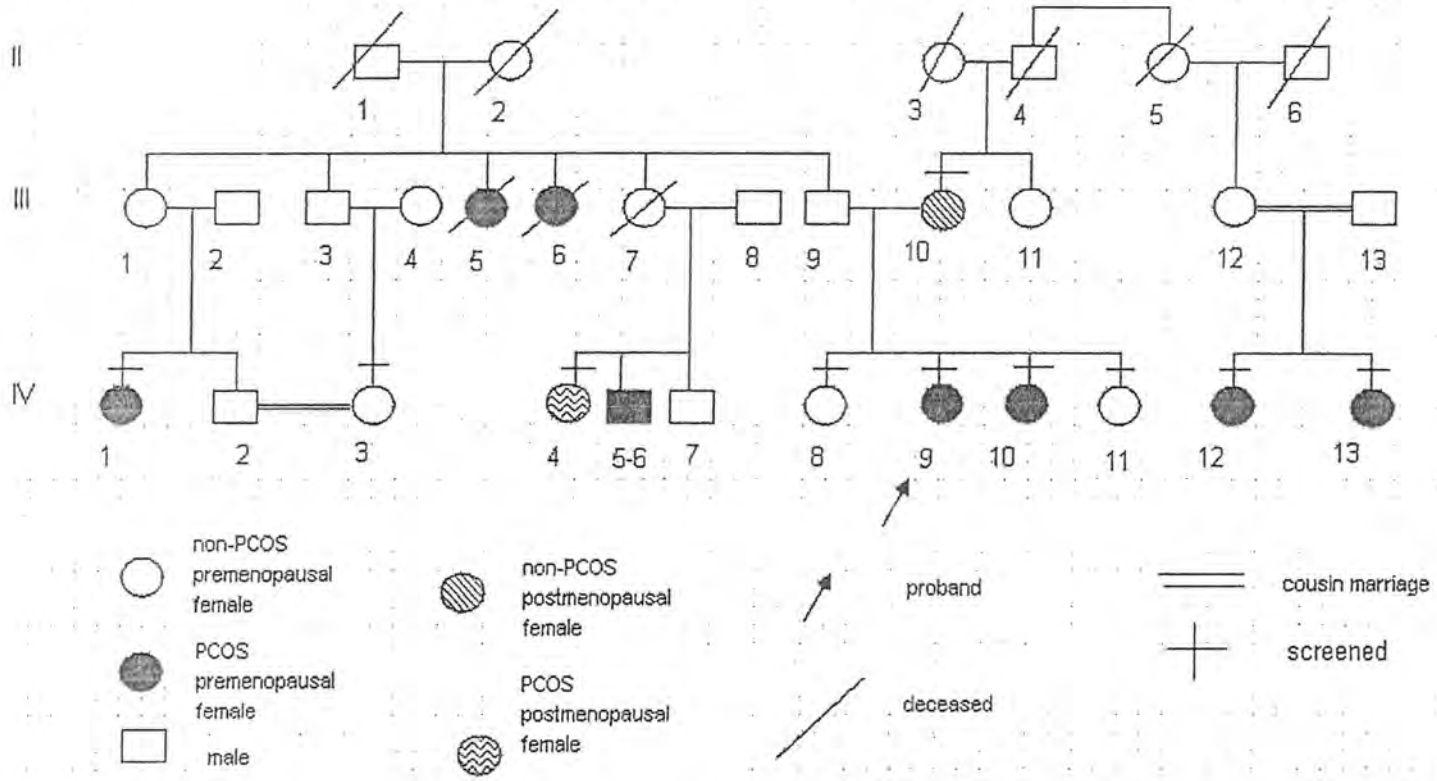

Fig 4: Pedigree of family No. 4 showing females who were screened by history, examination, pelvic ultrasound, endocrine and biochemical testing. Information regarding PCOS state in generation II was not known.

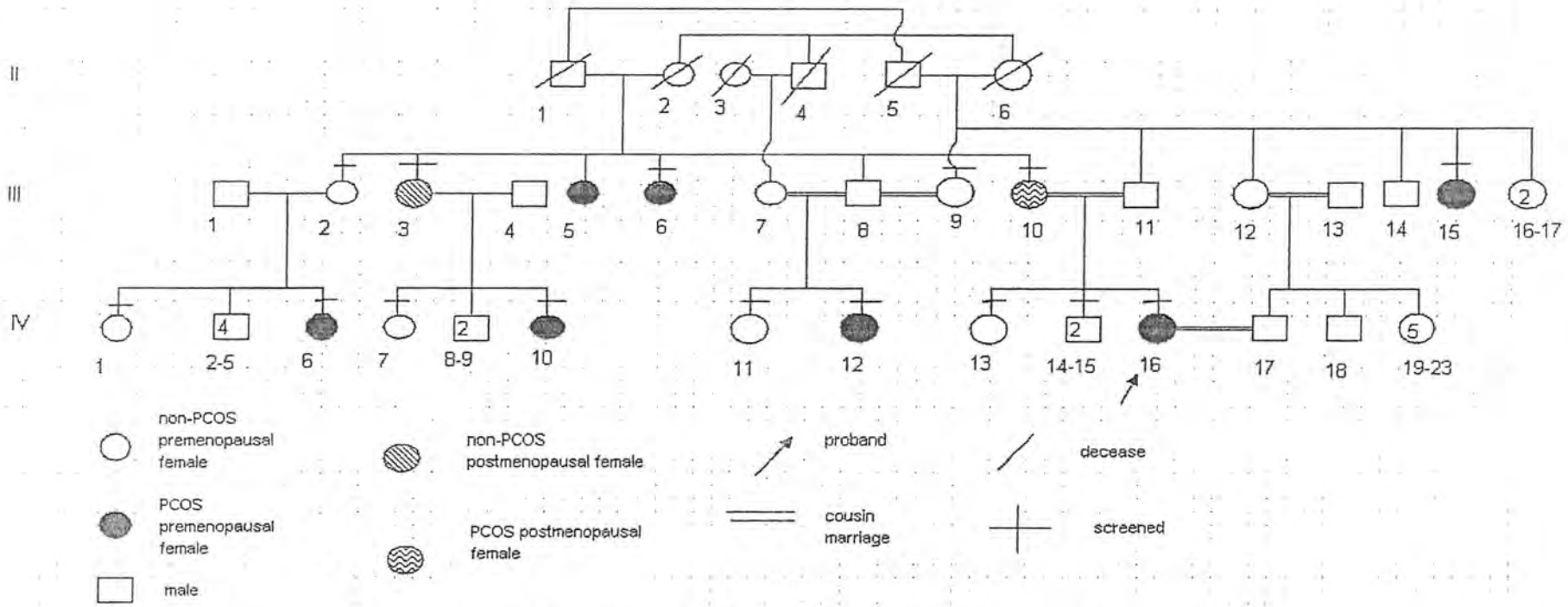

Fig 5: Pedigree of family No 5 showing females who were screened by history, examination, pelvic ultrasound, endocrine and biochemical testing. Information regarding PCOS state in generation II was not known.

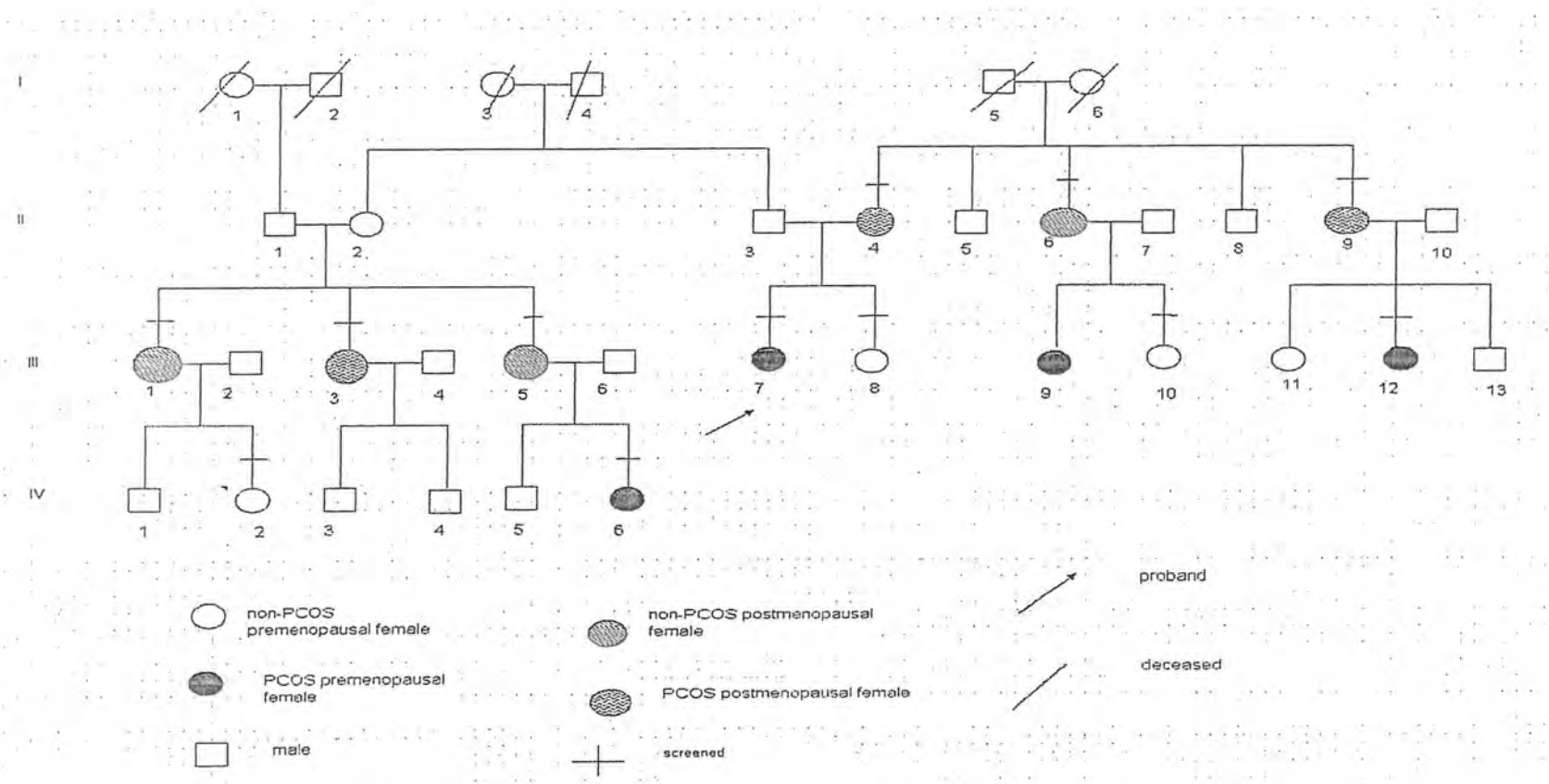

Fig 6: Pedigree of family No. 6 showing females screened by history, examination, pelvic ultrasound, endocrine and biochemical testing. Information regarding PCOS state in generation 1 was not known.

Table 7: Non-PCOS and PCOS women (diagnosed by Rotterdam criteria 2003) who were screened from the six families.

Family No.	Generation	Position in generation.	
		Non-PCOS women	PCOS women
1	III	2, 4	6
	IV	3, 9	1, 7, 14, 17
	V	-	2, 6
2	II	7	-
	III	17	4, 14, 16
3	II	8	-
	III	6, 11	5,8,13,17
	IV	7	1, 11
4	III	10	-
	IV	3, 8, 11, 12	1,4, 9, 10, 13
5	III	2, 3, 9	6, 10, 15
	IV	1, 7, 11, 13.	6, 10, 12, 16
6	II	6	4, 9
	III	1, 5, 8, 10	3, 7, 9
	IV	2.	6

Subjects mentioned in the table were screened from the families, rest were not included in the study.

Table 8: Distribution of pre-menopausal and postmenopausal screened women of the six families in different generations.

Family No.	Generation	Position in generation.			
		Pre-menopausal women		Postmenopausal women	
		Non-PCOS	PCOS	Non-PCOS	PCOS
1	III	-	-	2, 4	6
	IV	3, 9	1, 7, 14,	-	-
	V	17		-	-
		-	2, 6		
2	II	-	-	7	-
	III	17	4, 14, 16		
3	II	-	-	8	-
	III	6, 11	5, 8, 13,	-	-
	IV	17		-	-
		7	1, 11		
4	III	-	-	10	-
	IV	3, 8, 11,12	1, 9,	-	4
		10,13			
5	III	2, 9	6, 15	3	10
	IV	1, 7, 11, 13	6, 10,	-	-
		12, 16			
6	II	-	-	6	4, 9
	III	8, 10	7, 12	1, 5	3
	IV	2	6	-	-

Subjects mentioned in the table were screened from the families, rest were not included in the study.

Clinical characteristics

Clinical characteristics of PCOS and non-PCOS women in relation to pre-menopausal and postmenopausal state are shown in Table 9. When all PCOS and non-PCOS women were taken together, there was no appreciable difference in mean age, duration of marriage, age of menarche, weight, height, hip, waist measurements, BMI and waist: hip ratio (Table 9). In the pre-menopausal state, PCOS and non-PCOS women also show no significant differences in the above variables. A similar situation was seen with PCOS and non-PCOS women in postmenopausal state.

Postmenopausal PCOS women were significantly aged ($P<0.001$) compared to pre-menopausal women. Similarly, mean duration of age ($P<0.001$) and mean waist: hip ratio ($P<0.01$) in postmenopausal PCOS women are significantly higher compared to that of pre-menopausal PCOS women.

Different menstrual cycle patterns of non-PCOS and PCOS women in relation to pre- and postmenopausal state are shown in Table 10. The higher percentage (n=19; 67.81 %) is of non-PCOS with regular menstrual cycles compared to PCOS women (n=5; 14.7 %) giving highly significant difference ($P< 0.001$). Oligomenorrhea was seen in (n=18; 52.90 %) and secondary amenorrhea in (n=11; 32.40 %) of PCOS women. Non-PCOS women with oligomenorrhea were (n=9; 32.19 %) but not significantly different from PCOS women ($P> 0.05$). Secondary amenorrhea was not observed in any of the non-PCOS women. In this way statistically no difference was observed in the irregular menstrual cycle pattern of non-PCOS and PCOS women ($P>0.05$).

Non-PCOS and PCOS women were further studied in the pre-menopausal and postmenopausal states. It was observed that greater percentage (n=12; 63.15 %) of pre-menopausal non-PCOS women had regular cycles whereas fewer PCOS women (n=2; 7.14 %) were with regular cycle ($P<0.001$). Oligomenorrhea and secondary amenorrhea was found respectively more in PCOS women i.e (n=15; 53.58 %) and (n=11; 39.28 %) compared to non-PCOS women showing no significant difference for these variables.

Among postmenopausal women, their past history showed that in non-PCOS (77.70 %; 22.3 %) and PCOS (66.6 %; 33.4 %) women show no significant difference for regular menstrual cycles and oligomenorrhea, respectively. Secondary amenorrhea was not observed both in non-PCOS and PCOS. Menstrual cycle patterns of pre-menopausal and

postmenopausal women were also compared. It was observed that more postmenopausal PCOS women (n=4; 66.60 %) gave history of regular cycle compared to pre-menopausal PCOS women (n=2; 7.14 %, P< 0.001). There was no significant (P>0.05) difference in pre-menopausal (PCOS and non-PCOS) women compared to postmenopausal (PCOS and non-PCOS) women for oligomenorrhea.

Table 9: Profile of clinical characteristics of PCOS and non-PCOS women.

Clinical characteristics	Total no of cases (62)		Pre-menopausal (47)(75 %)		Post-menopausal (15) (25 %)	
	Non-PCOS (28) (45 %)	PCOS/ (34)(55%)	Non-PCOS (19)(40 %)	PCOS (28) (60%)	Non-PCOS (9)(60 %)	PCOS (6) (40%)
Age (years) at presentation	35.2±2.7	33.1±2.4	26.8±1.8	28.5±1.9	53.5±2.7^{d***}	56.8±2.6^{e***}
Duration of marriage (years)	13.4±2.6	11.3±2.3	5.3±1.5	7.0±1.6	31.8±2.3^{d***}	35.1±2.7^{e***}
Age at menarche (years)	13.9±0.31	12.8±0.609	13.8±0.35	12.8±.73	14.1±0.70	13.3±0.7
Weight (kilograms)	66.3±2.16	68.7±2.7	64.3±2.6	69±2.9	71±4.1	65.3±6.8
Height (meter^2)	2.43±0.02	2.45±0.02	2.4±0.03	2.46±0.02	2.4±0.04	2.38±0.03
Hip (inches)	40.2±0.77	40.6±0.72	39.4±0.9	40.3±0.8	41.6±1.5	40.1±1.5
Waist (inches)	32.6±0.89	33.6±0.99	31±1	33±1.0	35.6±1.4^{d*}	36.1±2.4
BMI(Kg/m2)	27.29±0.86	27.95±1.07	26.1±0.9	28±1.1	29.5±1.57^{d*}	27.5±2.9
Waist:hip ratio	0.8123±0.016	0.821±0.015	0.78±0.01	0.81±0.01	0.85±0.03^{d*}	0.89±0.03^{e*}

Number in parenthesis indicate number of subjects.
*P<0.05=significant; **P<0.01=highly significant;***P<0.001=very highly significant
a=total; non-PCOS vs PCOS
b=pre-menopausal; non-PCOS vs PCOS
c=postmenopausal; non-PCOS vs PCOS
d=non-PCOS; premenopausal vs postmenopausal
e=PCOS; pre-menopausal vs postmenopausal

Table 10: Different menstrual cycle patterns of the women.

Menstrual cycle	Total no of cases (62)				Pre-menopausal (47)				Post-menopausal (15)			
	Non-PCOS		PCOS		Non-PCOS		PCOS		Non-PCOS		PCOS	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Regular (days 21-35)	19	67.81	5	14.70[a***]	12	63.16	2	7.14[b***]	7	77.70	4	66.60[c**]
Oligomenorrhea (35 days-6 month)	9	32.19	18	52.90	7	36.84	15	53.58	2	22.30	2	33.40
Sec amenorrhea >than 6 months	-	-	11	32.40	-	-	11	39.28	-	-	-	-
Total	28	100	34	100	19	100	28	100	9	100	6	100

Number in parenthesis indicate number of subjects.
*P<0.05=significant; **P<0.01=highly significant;***P<0.001=very highly significant
a=total; non-PCOS vs PCOS
b=pre-menopausal; non-PCOS vs PCOS
c=postmenopausal; non-PCOS vs PCOS
d=non-PCOS; premenopausal vs postmenopausal
e=PCOS; pre-menopausal vs postmenopausal

Marital and fertility status of the women in this study is shown in Table 11a. The results showed that (n=22; 78.56 %) of non-PCOS and (n=22; 64.71 %) of PCOS women were married (P>0.05). Among non-PCOS women (n=6; 21.42 %) and PCOS women (n=12; 35.3 %) were unmarried (P>0.05). All unmarried women were in their pre-menopausal state.

All married women were subdivided into fertile and sub-fertile group. Greater number of non-PCOS women (n=17; 60.71 %) were fertile compared to (n=5; 14.71 %) of PCOS women (P<0.001). Sub-fertility (where conception was delayed for more than 2 years; primary and secondary both) was found more in PCOS women (n=17; 50 %) compared to (n=5; 17.86 %) of the non-PCOS women (p< 0.01). The data shows that PCOS condition is strongly associated with unmarried women compared to married fertile women ($X^2_{(1)}$ =7.82; P=0.0052) (Table 11b).

Likewise, (n=10; 52.64 %) of non-PCOS pre-menopausal women and (n=1; 3.57 %) of pre-menopausal PCOS were found fertile (P<0.000). In (n=15; 53.57 %) of pre-menopausal PCOS women sub-fertility was observed compared to (n=3; 15.78 %) of non-PCOS women (P<0.01). Among postmenopausal women, sub-fertility was observed in (n=3; 50 %) of PCOS women and in (n=2; 22.3 %) non-PCOS women (p>0.05). Among non-PCOS postmenopausal women (n=7; 77.7 %) and (n=3; 50 %) of PCOS women were fertile (P<0.01).

Pre-menopausal fertile non-PCOS women (n=10; 52.64 %) show no significant difference compared to those in postmenopausal phase (n=7; 77.7 %). Sub-fertility was significantly low (P=<0.05) in postmenopausal PCOS women compared to those in pre-menopausal phase.

Table 11a: Marital and fertility status of Non-PCOS and PCOS women.

Marital and fertility status	Total no of cases (62)				Pre-menopausal (47)				Post-menopausal (15)			
	Non-PCOS		PCOS		Non-PCOS		PCOS		Non-PCOS		PCOS	
A=Married (44; 70.97 %)	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
i =Fertile	17	60.71	5	14.71[a***]	10	52.64	1	3.57[b***]	7	77.70	3	50[c**]
ii= Subfertile	5	17.86	17	50.00[a**]	3	15.78	15	53.57[b**]	2	22.30	3	50[c*]
B=Unmarried (18; 29.03 %)	6	21.43	12	35.29	6	31.68	12	42.86	-	-	-	-
Total	28	100	34	100	19	100	28	100	9	100	6	100

Number in parenthesis indicate number of subjects.
*P<0.05=significant; **P<0.01=highly significant;***P<0.001=very highly significant
a=total; non-PCOS vs PCOS
b=pre-menopausal; non-PCOS vs PCOS
c=postmenopausal; non-PCOS vs PCOS
d=non-PCOS; premenopausal vs postmenopausal
e=PCOS; pre-menopausal vs postmenopausal

Table 11b: Relationship between married and unmarried non-PCOS and PCOS women.

	Non-PCOS	PCOS
Married	**17**	**5**
Unmarried	**6**	**12**
	$X^2_{(1)}$ =7.82; P=0.0052	

Body mass index (BMI) and waist: hip ratio in non-PCOS and PCOS women is given in Table 12. Distribution of non-PCOS and PCOS women in categories of normal BMI, overweight and obese is shown in Table 12. Only one woman (2.94 %) who has BMI > 40 Kg/m^2 was very obese and she was pre-menopausal PCOS woman. All the BMI categories for non-PCOS and PCOS women show no significant difference in pre- and postmenopausal women.

Waist: hip ratio was categorized into two groups > 0.85 and < 0.85 waist: hip ratio. Both non-PCOS and PCOS women showed no significant difference in waist: hip ratio. A similar picture is seen in their pre- and postmenopausal phases

Table 12: Body mass index and waist: hip ratio of the Non-PCOS and PCOS women.

Body mass index BMI(Kg/m^2)	Total no of cases (62)				Pre-menopausal (47)				Post-menopausal (15)			
	Non-PCOS		PCOS		Non-PCOS		PCOS		Non-PCOS		PCOS	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Normal (19-24.9)	10	35.72	10	29.41	8	42.11	8	28.57	2	22.22	2	33.4
Overweight (25-29.9)	11	39.28	10	29.41	8	42.11	7	25	3	33.33	3	50
Obese (30-39.9)	7	25	13	38.24	3	15.78	12	42.86	4	44.45	1	16.6
Very obese (> than 40)	-	-	1	2.94	-	-	1	3.57	-	-	-	-
Waist:hip ratio												
Ratio > 0.85	10	35.8	14	41.2	6	31.5	10	35.8	4	44.5	4	66.6
Ratio <0.85	18	64.2	20	58.8	13	68.5	18	64.2	5	55.5[d*]	2	33.4[e*]
Total	28	100	34	100	19	100	28	100	9	100	6	100

Number in parenthesis indicate number of subjects.
*P<0.05=significant; **P<0.01=highly significant;***P<0.001=very highly significant
a=total; Non-PCOS vs PCOS
b=pre-menopausal; Non-PCOS vs PCOS
c=postmenopausal; Non-PCOS vs PCOS
d= Non-PCOS ; premenopausal vs postmenopausal
e=PCOS; pre-menopausal vs postmenopausal

Indicators of PCOS like hirsutism, acne and acanthosis nigrans are given in Table 13.

Hirsutism was graded according to the severity into normal, mild, moderate and severe. Severe hirsutism was found in only two pre-menopausal PCOS woman. In the total number of subjects, moderate hirsutism was observed in (n=16; 47 %) of PCOS women as compared to (n=2; 7.14 %) of non-PCOS women (P<0.001). Among the non-PCOS women (n=18; 64.28 %) have mild hirsutism as compared to (n=12; 35.2 %) of PCOS women (P<0.001).

Moderate hirsutism was significantly higher in (n=15; 53.58 %) pre-menopausal PCOS women and (n=2; 10.53 %) of non-PCOS pre-menopausal women (P<0.001). Mild hirsutism was, however, significantly higher in (n=16; 84.21 %) non-PCOS and (n=9; 32.14 %) PCOS pre-menopausal women (P<0.001).

Mild hirsutism was seen in (n=2; 22.3 %) postmenopausal non-PCOS women and in (n=3; 50 %) postmenopausal PCOS women. Only one postmenopausal woman showed moderate hirsutism who had PCOS. More pre-menopausal non-PCOS women have no as well as mild hirsutism than postmenopausal non-PCOS women (P<0.01). Pre-menopausal PCOS women (n=9; 32.14 %) have mild hirsutism compared to postmenopausal PCOS women (n=3; 50 %) (P<0.01).

Distribution of skin problems like acne and acanthosis nigrans in non-PCOS women as well as PCOS women in their pre- and postmenopausal state do not how any appreciable difference in the distribution of these skin problems.

Among 14 PCOS women having acanthosis nigrans, 12 women i.e. 42.87 % also had menstrual problem and sub-fertility in addition to acanthosis nigrans

Table 13: Grading of hirsutism and other skin problems of the women.

Hirsutism(Ferryman gallway score)	Total no of cases (62)				Pre-menopausal (47)				Post-menopausal (15)			
	Non-PCOS		PCOS		Non-PCOS		PCOS		Non-PCOS		PCOS	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Normal (<8)	8	28.58	4	11.76	1	5.26	2	7.14	7	77.7^{d**}	2	33.3
Mild (9-16)	18	64.28	12	35.2	16	84.21	9	32.14^{b***}	2	22.3^{d**}	3	50^{e**}
Moderate (17-24)	2	7.14	16	47^{a***}	2	10.53	15	53.58^{b**}	-	-	1	16.7
Severe (25-32)	-	-	2	5.8	-	-	2	7.14	-	-	-	-
Other skin problem												
Acne	4	14.2	5	14.7	3	15.78	5	18	1	11.2	-	-
Acanthosis nigran	9	32.3	18	52.9	6	31.57	14	50	3	33.3	4	66.7
nil	15	53.5	11	39.3	10	52.63	9	32	5	55.5	2	33.3
Total	28	100	34	100	19	100	28	100	9	100	6	100

Number in parenthesis indicate number of subjects

*P<0.05=significant; **P<0.01=highly significant;***P<0.001=very highly significant

a=total; Non-PCOS vs PCOS

b=pre-menopausal; Non-PCOS vs PCOS

c=postmenopausal; Non-PCOS vs PCOS

d= Non-PCOS ; pre-menopausal vs postmenopausal

e=PCOS; pre-menopausal vs postmenopausal

Age of onset of symptoms of PCOS in the study.

Age of onset of symptoms of PCOS were arranged in groups according to their age (Table 14). Mean age of onset in older females was 30.75±1.41 years. In younger females, it was 19.25±0.62 years and in youngest females, 13.63±1.31 years. The present data showed that in most recent generation, the age of onset of symptoms of PCOS was at a very early age. This age of onset is nearer to age of menarche in this generation. Student t-test was applied for comparison of mean age of onset of symptoms. This indicates that in younger females (older females vs younger females) mean age was significantly reduced compared to older women ($t_{(18)}$= 7.51; p=< 0.001). Youngest females also shows highly significant reduction in age of onset of symptoms ($t_{(18)}$=3.90; p=<0.001). In the present data, it has been observed that mean age of onset of symptoms was significantly reduced as it is traced from older generation to the most recent ones

Table 14: Mean age (years) of onset of clinical features of PCOS in women in different generations.

Groups	Age of onset (years)
Older PCOS females	30.75±1.41
Younger PCOS females	19.25±0.62
Youngest PCOS females	13.63±1.31
Student t-test	
Older vs younger	$t_{(18)}$=7.51, p=<0.001
Older vs youngest	$T_{(14)}$=8.92, p=<0.001
younger vs youngest	$t_{(18)}$=3.90, p=<0.001

Sonographic parameters of ovary in non-PCOS and PCOS subjects:

Details of sonographic findings are given in Table 15. The mean number of the follicles of 2-8 mm in diameter ($P<0.001$), stromal thickness (mm) ($P<0.001$) and ovarian volume (mm^3) ($P<0.01$) was significantly greater in PCOS women compared to non-PCOS women.

Similarly, in pre-menopausal PCOS women mean number of follicles of 2-8 mm in diameter ($P<0.001$), stromal thickness ($P<0.001$) and ovarian volume ($P<0.01$) was significantly greater compared to non-PCOS women. Stromal/total area thickness was significantly greater in pre-menopausal PCOS women than in non-PCOS pre-menopausal women ($P<0.05$).

In postmenopausal women mean ovarian volume ($P<0.05$) and mean stromal thickness ($P<0.05$) is significantly less than in pre-menopausal women. Similarly, in postmenopausal PCOS women, mean number of follicles (2-8 mm in diameter) is highly significantly lessen ($P<0.001$) than in pre-menopausal PCOS.

Table 15: Sonographic parameters of ovary in non-PCOS and PCOS women.

SONOGRAPHIC FINDINGS	Total no of cases (62)		Pre-menopausal (47)		Post-menopausal (15)	
	Non-PCOS (28)	PCOS (34)	Non-PCOS (19)	PCOS (28)	Non-PCOS (9)	PCOS (6)
Number of (2-8 mm diameter) follicles in each ovary	6.6±0.77	12.8±1.0[a***]	8.0±0.87	15.1±0.66[b***]	2.4±0.9[d***]	4.6±1.7[e***]
Stromal thickness (mm)	6±0.38	8.7±0.57[a***]	5.9±0.5	9.7±0.47[b***]	5.58±0.91	5.8±1.2[e*]
Ovarian volume mm^3	9.88±1	17.1±1.8[a**]	11.1±1.3	20.2±1.69[b***]	5.6±1.3[d**]	5.5±3.2[e**]
Stromal/total area thickness	0.83±0.04	0.94±0.06	0.76±0.06	0.96±0.05[b*]	0.89±0.07	1±0.25

Number in parenthesis indicate number of subjects.
*P<0.05=significant; **P<0.01=highly significant;***P<0.001=very highly significant
a=total; Non-PCOS vs PCOS
b=pre-menopausal; Non-PCOS vs PCOS
c=postmenopausal; Non-PCOS vs PCOS
d= Non-PCOS ; premenopausal vs postmenopausal
e=PCOS; pre-menopausal vs postmenopausal

Endocrine parameters of the non-PCOS and PCOS women:

An overall profile of serum FSH, LH, PRL, E2, T and insulin in total number of non-PCOS and PCOS women showed no appreciable difference in their mean levels of concentration (Table 16). The same trends are reflected in non-PCOS and PCOS women in their pre-menopausal and postmenopausal states for serum FSH, prolactin, estradiol and testosterone levels. Serum LH and fasting insulin were significantly more in pre-menopausal PCOS women compared to non-PCOS pre-menopausal women ($P<0.05$). Both non-PCOS and PCOS women in their postmenopausal state show significantly higher levels in serum FSH ($P<0.001$), serum LH ($P<0.05$) and low serum estradiol ($P<0.01$) compared to these in their pre-menopausal states. The only difference we see is in the levels of serum insulin which becomes significantly low ($P<0.001$) in non-PCOS postmenopausal women compared to their counterparts in pre-menopausal state. However, significant increase in mean concentration of serum LH was observed in pre-menopausal PCOS women compared to pre-menopausal non-PCOS women ($P<0.05$).

LH: FSH ratio of PCOS and non-PCOS women is given in Table 17. Significantly more PCOS women (32.4 %) have LH: FSH ratio higher than 2.5 index compared to 10.7 % of non-PCOS women ($P<0.05$).

Table 16: Serum concentration of gonadotropin, prolactin, estradiol, testosterone and insulin hormones of non-PCOS and PCOS women.

Endocrine parameters	Total no of cases (62)		Pre-menopausal (47)		Post-menopausal (15)	
	Non-PCOS (28)	PCO (34)	Non-PCOS (19)	PCO (28)	Non-PCOS (9)	PCO (6)
Serum FSH (mIU/ml	14.7±3.75	10.8±2.2	5.6±0.83	5.5±0.79	34.3±8.5^{d***}	34.8±5^{e***}
Serum LH (mIU/ml	10.2±2.4	10.34±1.47	4.4±0.85	8.8±1.48^{b*}	22.77±5.4^{d***}	16.5±4.2^{e*}
Serum Prolactin (ng/ml)	13.3±1.8	18.64±3.0	15.5±2.4	20.8±3.5	9.8±1.5	8.9±1.8
Serum estradiol (pg/ml)	62.6±9.6	75.2±10.3	79.3±12	86.2±11	30.3±2.7^{d*}	19±5.7^{e**}
Serum testosterone (ng/ml)	44.6±2.7	41.9±3.3	46±4.6	43.6±4.0	37.8±5.7	39.3±5.8
Serum insulin (uIU/ul)	12.8±0.5	11.89±0.4	13.8±0.54	12±0.46^{b*}	10.7±0.86^{d**}	10.8±1.3

Number in parenthesis indicate number of subjects.
*P<0.05=significant;**P<0.01=highly significant;***P<0.001=very highly significant
a=total; Non-PCOS vs PCOS
b=pre-menopausal; Non-PCOS vs PCOS
c=postmenopausal; Non-PCOS vs PCOS
d= Non-PCOS ;pre-menopaupsal vs postmenopausal
e=PCOS; pre-menopausal vs postmenopausal

Table 17: LH: FSH ratio of non-PCOS and PCOS women.

LH:FSH ratio	Total no of cases (62)				Pre-menopausal (47)				Post-menopausal (15)			
	Non-PCOS		PCOS		Non-PCOS		PCOS		Non-PCOS		PCOS	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Ratio< than 2.5	25	89.3	23	67.6[a*]	16	84.2	17	60.7	9	100	6	100
Ratio>than 2.5	3	10.7	11	32.4	3	15.8	11	39.3	-	-	-	-
Total	28	100	34	100	19	100	28	100	9	100	6	100

Number in parenthesis indicate number of subjects.
*P <0.05
a=total; Non-PCOS vs PCOS
b=pre-menopausal; Non-PCOS vs PCOS
c=postmenopausal; Non-PCOS vs PCOS

Biochemical parameters of the non-PCOS and PCOS women:

Mean concentration of biochemical parameters like cholesterol, triglyceride, HDL, LDL and fasting blood sugar were measured (Table 18) in non-PCOS as well as PCOS women. An overall profile in these women and in their pre- and postmenopausal states showed no appreciable difference in the concentrations of these biochemical parameters. Non-PCOS postmenopausal women have significantly more triglyceride and fasting bloods sugar levels compared to non-PCOS pre-menopausal women ($P<0.05$). Triglyceride levels of more than 200 mg/dl were found in 4 PCOS women i.e. 11.76 % and HDL levels less than 40 mg/dl in 19 PCOS women i.e. 55.88 % in this study.

Table 18: Different biochemical parameters of the non-PCOS and PCOS women.

Biochemical parameters	Total no of cases (62)		Pre-menopausal (47)		Post-menopausal (15)	
	Non-PCOS (28)	PCOS (34)	Non-PCOS (19)	PCOS (28)	Non-PCOS (9)	PCOS (6)
Cholesterol (mg/ml)	160.3±6.1	159.4±3.8	152±5.3	158±4.3	174±14.7	166.1±9.3
Triglyceride (mg/ml)	129±7	161.4±17.3	117.9±9	155.8±20	154±6.8 [d*]	187.5±28
HDL (mg/ml)	38±1.2	37.5±1.2	40±1.4	37.4±1.38	35.1±2	37.1±3.2
LDL (mg/ml)	96±4.6	94.08±3.8	92.5±5.2	92.8±3.7	107±11	92±10
Fasting blood sugar (FBS) (mg/dl)	92.7±9.9	97±7.9	75.5±2	92±7.8	127.8±27.4 [d*]	124±24.8
FBS:Insulin ratio	8.5±1.79	9.1±1.1	5.7±0.39	8.2±1.1	14.6±5.1	13±3.7

Number in parenthesis indicate number of subjects.
*P<0.05=significant; **P<0.01=highly significant;***P<0.001=very highly significant
a=total; Non-PCOS vs PCOS
b=pre-menopausal; Non-PCOS vs PCOS
c=postmenopausal; Non-PCOS vs PCOS
d= Non-PCOS ; premenopausal vs postmenopausal
e=PCOS; pre-menopausal vs postmenopausal

Prevalence of other problems in families of PCOS of the study.

Records of history of PCOS and non-PCOS women showed the presence of diabetes mellitus type 2, hypertension, ovarian carcinoma and gilbert syndrome in their families. Hypertension was found in 17.7 % of PCOS women. Gilbert syndrome which is a liver problem was found in one PCOS female member of the study. It was also seen that two PCOS females died of ovarian carcinoma in these families (Table 19).

It was seen that diabetes mellitus type 2 was found in 6 PCOS and 5 non-PCOS women. There is no difference in prevalence of diabetes mellitus among PCOS and non-PCOS women. Among non-PCOS women 17.8 % were hypertensive. Any association of PCOS to other diseases was not observed in this study.

Table 19: Prevalence of associated diseases in the study.

Total no of cases (62)	Diabetes mellitus		Hypertension		Ovarian cancer	Gilbert syndrome
	Present No %	Absent No %	Present No. %	Absent No. %	No %	No %
Non-PCOS (28)	5 17.8	23 82.2	5 17.8	23 82.2	- -	- -
PCOS (34)	6 17.7	28 82.3	2 5.8	32 94.2	2 5.88	1 2.94

DISCUSSION

DISCUSSION

In the present study the pedigrees were studied ranging from 2-4 generations. On average there were 11 individuals per generation (including alive and dead; male and female issues). Females scored for the study were 6 per generation. Studies on this subject have been conducted on parents-sibling or a maximum of 2 generations ranging from 10-15 individuals (Frank et al., 1997; Urbanek et al., 1999). In this study, number of PCOS on the average per generation were 2.71 and non-PCOS averaged per generation were 3.88. Present study agreed with Paul et al., (2006) that large sized pedigrees with large number of PCOS and non-PCOS help in ascertaining Mendalian inheritance and also help in determining the association factor at molecular level with different markers used here. In this study non-PCOS in the pedigrees were considered as control.

The polycystic ovarian syndrome is a heterogeneous disorder investigated mainly among women in their reproductive age indicating genetic susceptibility. This syndrome is characterized by chronic anovulation, hyperandrogenism, obesity and insulin resistance (Dunaif, 1997). The present research included both pre-amd postmenopausal women for clinical and genetic study. In the literature, comparison of pre and postmenopausal states was not done. The investigators have their preferred methodology for the diagnosis of PCOS syndrome. Some preferred PCOS diagnosis through clinical symptoms and biochemical parameters i.e. raised serum LH levels or androgen levels (Urbanek et al., 1999) while others used ultrasound as sole criteria for its diagnosis (Balen et al., 1995; Kousta et al. 2005). In this study revised 2003 Rotterdam consensus for diagnostic criteria of PCOS (Fauser, 2004) was used for diagnoses of PCOS which includes clinical characteristics in combination with ultrasonography.

In this study PCOS and non-PCOS women were of matching ages in the whole sample and then in their pre- and postmenopausal states. Likewise, their age at menarche and marital duration were comparable. Their anthropometric measurements like height, weight, hip and waist circumference, body mass index and ratio of waist to hip showed no appreciable difference.

Clinical characteristics like menstrual cycle pattern, subfertility, hirsutism and obesity were analyzed in detail in this study. Menstrual cycle pattern, sub-fertility and

hirsutism were found more in pre-menopausal PCOS women compared to postmenopausal PCOS women. It was observed that secondary amenorrhea which reveals anovulation was present in PCOS women of these families. Among PCOS women 14.7 % have regular menstrual cycle. Other PCOS women had oligomenorrhea (53 %) compared to 32 % of non-PCOS women. Higher percentage of oligomenorrheic women have been reported in United Kingdom population by Govind et al. (1999). They documented 54 % oligomenorrheic and 46 % with regular menstrual cycle in women with PCOS. Another study from Netherlands showed that 76 % of PCOS women have oligomenorrhea and 24 % were amenorrheic (Van Santbrink et al, 1997). In Italian population, Crosignani and Nicolosi (2001) also reported higher percentage of oligomenorrheic PCOS women (54%).

In this study 50 % of the PCOS women and 17 % of non-PCOS women were diagnosed with fertility problem. Among PCOS women, unmarried women with PCOS disorder were significantly higher than married PCOS. Studies have been done to see the correlation between hormonal status and sonographic findings in normogonadotropic anovulatory infertility and found 21 % infertile women with polycystic ovaries. (Pache et al., 1993; Van Santbrink et al., 1997; Fulghesu et al., 2001).

Women both PCOS as well as non-PCOS in this study have body mass index in overweight category i.e 27.2 and 27.9 Kg/m^2 respectively. Ratio between waist and hip measurements were also comparable in both groups. Details of body mass index showed that there was only one very obese woman who was a pre-menopausal PCOS. Wijeyaratne et al., (2002) have documented that World Health Organization redefined obesity in Asian Indians and considered BMI more than 27 as class 1 obesity. Obesity was not significantly different in PCOS women compared to non-PCOS women in American population (Legro et al., 1998a; Legro et al., 1998b), Italian population (Fulghesu et al., 2001; Battaglia et al., 2002), British population (Govind et al., 1999) and from Netherlands population (Van Santbrink et al., 1997). Gambinri et al., (2002) have reported that 50 % of PCOS women were overweight or obese. Present study shows 42 % of PCOS women were overweight. Joan et al (2006) were of the view that Asian PCOS women had a lowest prevalence of obesity whereas Blacks and Hispanics had the highest.

An increased waist circumference is typically highly correlated with hyperinsulinemia and is thought to reflect an increase in the proportion of total body fat that is deposited

in the visceral compartment compared with subcutaneous space (Kabir et al., 2005; Norris et al., 2005). Vrbikova et al., (2002) have reported that in both lean and o' ese PCOS women central obesity was more comparable to healthy control women.

Moderate hirsutism was significantly higher in PCOS women compared to non-PCOS women whereas acanthosis nigrans was seen in 52.5 % of PCOS women in this investigation. Joans et al., (2006) observed that 11.2 % of women were diagnosed for infertility, menstrual irregularity, acanthosis nigrans or insulin resistance. These features in this study were observed among 42.87 % PCOS women. Carmina et al. (2006) also observed higher percentages of PCOS women had acne, hirsutism, alopecia and obesity. Similar researches also showed the higher prevalence of hirsutism in Asian women than in Caucasians (Wijeyaratne et al., 2002; Zagar et al., 2002). Charnvises et al. (2005) has stressed upon the importance of acanthosis nigrans as a clinical predictor in development of abnormal glucose tolerance test in women with PCOS.

PCOS appears to have its origin during adolescence and is thought to be associated with increase in weight gain during puberty (Balen and Dunger, 1995). Obesity is most common health issue worldwide. It affects female more than males (Legro et al., 1998). History of older PCOS women in the present study showed that menstrual problems, hirsutism and obesity appear after 30 years of age compared to their grand-daughters who developed these symptoms at the time of menarche (13.63±1.31 years).

Sonography in the present study was carried out to observe different ovarian morphometric changes in PCOS women. There was highly significant difference in mean number of 2-8 mm follicles, stromal thickness and ovarian volume of PCOS women compared to non-PCOS women in total sample and in pre-menopausal group. According to Bucket et al., (2003) increased stromal volume is a feature of PCOS women and ovarian volume is good for quantification of stromal volume from clinical point of view. Van Santibink et al., (1997) indicated that sonographic test for prediction of hyperandrogenicity had increased mean ovarian volume. Similiarly, Govind et al., (1999), Crosignani and Nicolosi (2001) and Fulghesu et al., (2001) observed significantly increased ovarian volume in PCOS women than in non-PCOS women. Pache et al., (1993) showed that ovarian volume and stromal echogenicity correlated significantly with LH levels. Wijeyaratne et al., (2002) have found greater mean ovarian volume in South Asian PCOS women compared to Caucasian PCOS

women. Carmina et al., (2005) reported highly significant increase in ovarian size in patients with classical PCOS than in women with the other androgenic disorders. Sielfen et al., (2003) has documented no statistically significant difference in ovarian volume between obese and non-obese PCOS women. Legro et al., (2005) has found no significant difference in ovarian volume between PCOS and control women in relation to parameters for glucose metabolism and reproductive hormone levels.

Number of follicles in both non-PCOS and PCOS postmenopausal women were low in this study. Elting et al., (2001, 2003) found that there were few ovarian follicles in older women with PCOS whose cycles have become regular, implying a resolution of the PCO morphology. Similarly, large cross sectional studies showed fewer ovarian follicles with age in women with PCOS (Bili et al., 2001). Unfortunately, there are few natural history studies of changes in ovarian morphology with age, in either women with PCOS or the general population (Erdem et al., 2002).

This study showed no association between polycystic ovarian syndrome and insulin resistance when pre-menopausal and postmenopausal women were compared. Mean fasting insulin levels were more than 10 μU/μl in PCOS and non-PCOS women irrespective of their reproductive status and the same was observed in their pre-menopausal and postmenopausal states. Perloe (2000) suggested that fasting insulin level greater than 10 μU/μl in PCOS women and non-PCOS women was considered as insulin resistant. Najmabadi et al., (1997) have found positive association between polycystic ovarian syndrome and insulin resistance. A modest correlation between ovarian volume and insulin resistance was observed by Pache et al., (1993). Avvad et al., (2001) also reported no significant difference in glucose and insulin parameters of PCOS and control women. Wijeyaratne et al., (2002) observed that fasting insulin levels were higher in South Asian PCOS women than in Caucasian PCOS women. Kaufman et al., (2006) reported that Mexican American women with PCOS were significantly more insulin resistant than their white counterparts.

Legro et al., (2005) and Oppermann et al., (2003) found a modest association between raised LH to FSH ratio in women with polycystic ovarian morphology. In this study LH: FSH ratio (> 2.5) was not significantly higher in PCOS women than in non-PCOS women. Fauser (2004) and Legro et al., (2005) also indicated elevated ratio of LH/FSH in PCOS women. Pre-menopausal PCOS women have significantly higher LH levels compared to non-PCOS women. There was no appreciable difference in serum total testosterone levels among non-PCOS and PCOS women in this study.

Fauser (2004) indicated that serum testosterone might not be sensitive marker of androgen excess. Elevated serum LH concentrations in PCOS women was reported by Van Santibink et al., (1997) and Laven et al., (2002). Govind et al (1999) reported in United Kingdom population that there was no significant difference in serum LH and serum testosterone levels of PCOS and non-PCOS sisters from proband families. Fulghesu et al., (2001) has observed significant difference in serum testosterone levels between PCOS and control women and non-significant difference in serum LH levels. Legro et al., (2002) reported in United States population (Boston) significant difference in serum LH, testosterone, insulin levels of PCOS women and their non-PCOS sisters.

Serum estradiol levels were significantly higher in pre-menopausal women compared to postmenopausal women. Present study included all women with normal prolactin levels. Glucose: insulin ratio significantly decreased in that study. But no significant difference in fasting blood sugar levels was observed. In this study no significant difference was observed in fasting blood sugar levels and glucose: insulin ratio of PCOS and non-PCOS women of Pakistani families.

Different biochemical parameters studied for PCOS and non-PCOS women are given in Table 18 which comprise cholesterol, triglyceride, HDL, LDL fasting blood sugar and FBS/insulin ratio. An overall profile shows no difference for these parameters in PCOS and non-PCOS women. Non-PCOS women in postmenopausal state showed significantly high concentrations of triglycerides compared to pre-menopausal state ($P<0.05$). Joan et al., (2005) identified individuals with triglyceride levels greater than 200 mg/dl and HDL cholesterol levels less than 40 mg/dl for dyslipedemia. In this study triglyceride levels were more than 200 mg/dl in 11.76 % (n=4) and HDL < 40 mg/dl were in 55.88 % (n=19) PCOS women. Sam et al (2005) reported significantly higher triglyceride levels in affected sisters of PCOS women than in sisters of normal women. Essa and Nestler (2006) reported that in PCOS women with metabolic syndrome have decreased HDL cholesterol levels. Ehermann et al., (2005) observed PCOS women with metabolic syndrome having HDL levels less than 50 mg/dl in 66 % and triglyceride levels more than 150 mg/dl in 32 % women. Meirow et al; (1996) have suggested that dyslipidemia in PCOS patients may occur irrespective of insulin resistance, because both insulin resistant women and those who did not exhibit insulin resistance had similar lipid abnormalities.

Carmina and Lobo (2001) and Adams (2004) have suggested that women with unrecognized reproductive or metabolic abnormalities, consistence with the observation that many women with polycystic ovary morphology have, however subtle, of androgen excess and insulin resistance with more detailed testing.

Webber (2003) and Taponen et al., (2004) have supported this hypothesis that polycystic ovaries are intrinsically abnormal and may be the ovarian morphologic consequence of intrinsic defects in follicular development and steroidogenesis.

The present study showed that diabetes mellitus 2 was equally prevalent in PCOS and non-PCOS women i.e 17 %. Shera et al., (1999) have given the prevalence of type 2 diabetes mellitus in 10.6 % of urban and 4.8 % of rural Pakistani women. Wijeyaratne et al., (2002) have reported that greater prevalence of type 2 diabetes mellitus in the first and second degree relatives of PCOS South Asian women due to greater degree of consanguinity. The prevalence of type 2 diabetes mellitus in obese PCOS women lies between 11 and 38 % (Dunaif., 1993; Dunaif and Fingood, 1996; Holte, 1996). However, Dahlgren et al., (1992) indicated that 13 % prevalence of diabetes mellitus in postmenopausal PCOS women compared to 2 % of general population- a seven fold increase in risk. Although ovarian carcinoma is related to anovulatory PCOS (Schildkraut et al., 1996), there were two PCOS women having ovarian carcinoma who died during chemotherapy.

This study suggests that PCOS can be predicted reliably with the help of clinical features, sonography, endocrine and biochemical parameters at the adolescent stage. It is the syndrome which really affects physical, mental and social wellbeing of women and make them ill. So, with proper guidance and counseling to PCOS women and members of PCOS families may help in the prevention of long term complications like cardiovascular, metabolic abnormalities and endometrial carcinoma. In postmenopausal women, information regarding the PCOS condition helps in evaluating the gynecological malignancies like endometrial, ovarian and breast carcinomas and dyslipidemia.

CHAPTER 2

INTRODUCTION

INTRODUCTION

Epidemiology

A number of investigations have been performed over the last 20 years which have drawn attention to the phenomenon of familial clustering of cases of polycystic ovary syndrome (Cooper et al., 1968; Ferriman and Purdie 1979; Givens, 1988; Hague et al., 1988; Lunde et al., 1989; Carey et al, 1993). Detailed analysis of these has been carried out in two excellent reviews by Simpson, 1992 and Legro, 1995. They suggested that there is no unequivocal method of diagnosis, it is not surprising that the criteria used to identify probands and affected family members vary considerably between studies. A further confounding factor is that identification of affected family members was made by direct clinical observation in some studies, by questionnaire alone in some and by a combination of the two in others. Carey et al., (1993) have done the St Mary's family studies which relied principally on direct interviews and observations of relatives rather than on indirect evidence from questionnaires. Assignment of affected status was made on the basis of ultrasound evidence of polycystic ovaries in the women. Although the diagnosis of polycystic ovaries was made ultrasonographically, 92% of affected female family members had at least one clinical (hirsutism, acne, menstrual disturbance) or biochemical feature (raised serum testosterone, LH) of polycystic ovary syndrome. The segregation ratio, expressed as the percentage of affected subjects in each generation was found to be 51%, i.e. consistent with an autosomal dominant mode of inheritance (Franks et al., 1997).

PCOS has been occasionally reported in identical twins (McDonough et al., 1972; Hutton and Clark, 1984). An Australian study found a 50% incidence of PCOS in 34 female twin pairs studied. The high degree of discordance in sonographic ovarian imaging between twins suggests a complex inheritance pathway and/or an important role of environmental factors in the genetic transmission mechanism (Johnfar et al., 1995). Crosignani and Nicolosi, (2001) have suggested the high prevalence of PCOS among twins may be explained by factors acting in prenatal life.

The major mechanisms responsible for several complex human diseases are poorly understood. At least four major obstacles hinder the identification of the major defect in these conditions. The first is the lack of recognition that these are syndromes and not individual diseases; the second is the variety of possible etiologies responsible for

disease; the third is the complex interplay of several physiological systems that regulate the final clinical manifestation of disease: and the fourth is the interaction between environment and disease (Odunsi and Kidd, 1999). For genetic diseases that are not associated with obvious structural rearrangements of chromosomes, the causative gene(s) can be localized by genetic linkage analysis in families segregating for the disease phenotype. Genetic linkage is tile phenomenon whereby loci appear to be transmitted together rather than independently to offspring. For a complex disorder, analysis involves testing whether an allele at a marker locus is preferentially transmitted along with the disorder through a family. If there is statistically significant co-transmission of marker and disorder, the explanation is that sonic genetic variant in the chromosomal segment around the marker is contributing to the development of the disorder (Odunsi and Kidd, 1999). There is evidence that PCOS is clustere' in families and is inherited as a complex trait, like osteoporosis and hypertension, where environmental factors interact with genetic susceptibility to produce the phenotype (Legro, 1995). However, studies in families have revealed that in PCOS the clinical phenotype is very heterogeneous in females, and in males, obligate carriers appear to have varying degrees of premature male pattern baldness (Carey et al., 1993; Heinonen et al., 2001). Gharani et al., (1997) have suggested that although PCOS is a familial disorder, with a risk to siblings of 50 % but mode of transmission has not been agreed yet.

Molecular studies

More than 60 years since PCOS was first recognized as a common entity, clinicians have entertained the notion that PCOS is a genetic disease. However, the exploration of the genetics of PCOS has been hampered by several factors. First, PCOS is associated with infertility and low fecundity. Thus, it is rare to find large pedigrees with multiple affected women with whom to perform linkage analysis. Second, assigning phenotypes to premenarchal girls and postmenopausal women is not straightforward, a problem that also limits the use of pedigrees. Third, there has been an ongoing debate over disease phenotypes. The larger the number of distinct phenotypes within the affected category, the more complex the genetic analysis and the greater the likelihood that investigators using different diagnostic criteria will arrive at different conclusions. Fourth, although a male phenotype has been

postulated, there are no rigorously established clinical or biochemical features that can be used to identify PCOS males. This makes formal segregation analysis as well as genetic linkage studies more difficult. Fifth, the lack of animals that spontaneously develop a PCOS-like phenotype, especially mice, precludes the use of powerful tools of genetic mapping (Legro and Strauss, 2002).

The analysis by Urbenak et al., (1999) covered the possible candidate genes that may be involved in the pathways of steroid hormone metabolism and action, gonadotropic action, obesity and energy regulation, and insulin action. Given the heterogeneous nature of its clinical and biochemical features, it has been suggested that PCOS represents a range of disorders rather than a single entity (Simpson, 1992).

Primers are designed to amplify these possibly polymorphic sites STRPs (short tandem repeat polymorphisms) and SNPs (single nucleotide polymorphism) that are molecularly within or adjacent to the candidate gene. Segregation of these loci is examined in the families and contribute valuable information on allele sharing. With proper checks and controls, one can directly go to the molecular region of candidate gene to find markers (Odunsi and Kidd, 1999).

Power estimates assume that the marker and disease-susceptibility locus are linked and that the marker is informative. The effects of recombination can be reduced by multimarker analysis whereas the effects of non-informativeness can be reduced by using highly polymorphic markers, The closer the polymorphism is to the disease gene, the less likely is recombination between the two at meiosis, and therefore the two are more likely to be inherited together. High-density genetic maps that facilitate positional cloning projects have been generated by using STRPs (Weissenbach et al., 1993; Murry et al., 1994). Urbanek et al., (1999) observed 28 of the 37 candidate genes analyzed that there is at least one polymorphic marker within I centiMorgan (cM) of the candidate gene, and for the remainder, the markers are 1-4 cM from the candidate gene. This proximity improves the power of the study because recombination is likely to be minimal. The difficulty with whole-genome scans is the level of significance required to adequately compensate for the multiple independent and partially independent tests carried out. Although the required significance levels can be determined, the sample sizes can be prohibitive (Kruglyak and Lander, 1995).

The power to detect association by using a marker depends on several factors:

strength of the linkage disequilibrium between marker and disease, disease predisposing alleles, the recombination fraction between the disease and marker loci,

the increase in risk attributable to the particular susceptibility locus under consideration, and the penetrances of the different disease locus genotypes (Odunsi and Kidd, 1999).

Several genes involved in the biosynthesis of androgen, and action of insulin and gonadotropin have been examined as candidate genes for PCOS (Conway et al., 1994; Talbot et al., 1996;Gharani et al., 1997; Waterworth et al., 1997; Moller and Flier, 1998; Franks, 1999; Dunaif, 1999). These genes include those for cholesterol side chain cleavage enzyme (CYP 11 A), 17 alpha hydroxylase 17-20 lyase (CYP 17), insulin, insulin receptor, and LH. CYP 11 A and insulin gene, variable number of tandem repeats (VNTR), has been proposed as genetic factors contributing to PCOS. However, neither of them has been widely accepted as a major cause for this syndrome (Goordazi and Azziz, 2006).

Genes Involved in Steroid Hormone Synthesis and Action

The first enzyme in the synthesis of the steroid hormones, including adrenal and ovarian androgens, is P450scc, which is encoded by the CYP11A gene. This mitochondrial enzyme catalyzes the cleavage of the 20-22 side chain of the cholesterol, which is then converted into pregnanolone. This initial step in the steroidogenesis also requires transport of the cholesterol into the mitochondria, which is mediated by the steroidogenic acute regulatory proteins and is under the control of ACTH and LH in the adrenal and ovary, respectively. Any mutation in CYP 11A resulting in gain of the function of P450scc might result in increased androgen secretion by the adrenal and the ovary, respectively. Hyperandrogenaemia is seen both in women with PCOS and men with premature male pattern baldness suggesting an underlying disorder of androgen biosynthesis or metabolism (Franks, 1989; Franks, 1991; Stephen et al., 1993). Androgens are synthesized by the adrenals, the theca cell layer of the developing ovarian follicle and the testicular Leydig cells. Both scalp hair loss and hirsutism are known to be mediated by androgens (Hamilton, 1942; Lobo, 1991). The sensitivity of the hair follicle to androgens is dependent on a number of factors, such as serum concentrations of bioavailable androgens and the presence and number of androgen receptors (Barth, 1988; Randall et al., 1992).

Retinoids especially retinol augment steroid production in PCOS theca cells is associated with increased gene expression of several steroidogenic enzymes important for androgen biosynthesis, including CYP11a, CYP 17, and (3hydroxysteroidogenase

β2) 3 HSD β2 (Nelson et al., 1999; Weikenheisser et al., 2005). Recent microarray analysis comparing normal and PCOS theca cells has revealed that PCOS theca cells have a gene expression profile that is distinct from normal theca cells (Wood et al., 2003; Wickenheisser et al., 2005).

The major circulating androgen is testosterone in PCOS. Its biosynthesis requires androgenic 17 βhydroxy steroid dehydrogenase activity, namely 3 and 5 17 HSD. The 17 HSD gene is mainly expressed in testes, where it is essential for sexual differentiation and development but it is not expressed in the adrenal gland or ovary. On the other hand the 17 βhydroxy steroid dehydrogenase 5 gene is widely expressed and is found in the ovary and adrenal glands. Human HSD 17β 5 is composed of nine exons spanning 16 kb and is located on chromosome 10p14, 15. An activating HSD 17β5 variant in hyperthecosis form of PCOS is caused by profound type B insulin resistance (Oin et al., 2005).

Abnormalities in ovarian steroidogenesis, particularly androgen production, are a prominent feature of PCOS (Crossgnanil and Nicolosi, 2001). There has been intensive interest in the regulation of genes encoding the gateway to androgen synthesis.

CYP11A Gene

Gharani et al., (1997) examined 20 families with multiple affected women and found evidence for a weak linkage to the CYP11A locus, that encodes the cholesterol side-chain cleavage enzyme and total serum testosterone levels. Although, no regulatory role has been assigned to this polymorphism in terms of CYP11A gene transcription and allelic variants of this have a role in the hyperandrogenemia of PCOS.

CYPI7 Gene

No association with the phenotype of premature pubarche, hirsutism, and oligomenorrhea in adolescence was found with CYPI7 variants and mutations (Witchel et al., 1998). This enzyme plays a critical role in androgen synthesis.

CYP2I Gene

Mutations in the CYP2I gene that encodes the 21-hydroxylase enzyme are responsible

for the major forms of congenital adrenal hyperplasia, a disorder that can mimic the PCOS phenotype (Legro et al., 2002).

Androgen Receptor

Urbanek et al., (1999) failed to find evidence for association of trinucleotide (CAG) repeat polymorphism in the X-linked androgen receptor gene with PCOS. Mifsud et al., (2000) have noted an association between short CAG repeat lengths and anovulation in women with polycystic ovaries and low testosterone levels due to increased androgen action mediated by the short androgen receptor alleles.

Sex Hormone—Binding Globulin (SGBG)

A woman presented with nondetectable sex hormone binding globulin (SHBG), regular menses, and hirsutism, which progressed to marked hyperandrogenemism during pregnancy. She had two novel mutations in each copy of the SHBG gene (Hogeveen et al., 2002).Gharani et al., (1997) has found an association with D15S520, which is located in the promoter region of CYP11A and exclude linkage with CYPI9. Likewise Carey et al., (1994) found no evidence for linkage between CYPI 7 and PCOS/HA.

Genes Involved in insulin action

The common occurrence of insulin resistance and pancreatic β-cell dysfunction in association with PCOS and the increased risk for development of type II diabetes mellitus is now well recognized (Prelevac, 1997).

Insulin Receptor

Polycystic ovary syndrome have genetic defect in transduction of the insulin signals distal to the insulin receptors. Mutations in the insulin receptor gene cause severe insulin resistance (type A syndrome) associated with acanthosis nigricans and PCOS. However, genes near the insulin receptor may contribute to PCOS. Hyperinsulenimia compensatory to insulin resistance is a common feature of the obese and non obese women with PCOS. Hyperinsulinemia may play a role in causation of hyperandrogenism, that is, increased ovarian androgen production (androstenedion and testosterone) from ovarian stromal cells. It also acts on the liver to decrease sex

hormone binding globulin (SHBG), thereby increasing free testosterone levels in the blood (Kiddy et al., 1992)

Linkage or association has been found between a marker (D19S884 at l9pl3.3) that is located 2 megabases centromeric from the insulin receptor and PCOS (Urbanek et al., 2005; Florez, 2005).

Insulin Gene

Waterworth et al., (1997) concluded that they had discovered strong linkage and association between alleles at the insulin gene 5' VNTR and PCOS.

Resistin, a protein hormone that is secreted by adipocytes and believed to modulate glucose tolerance and insulin action, is a plausible candidate susceptibility gene for PCOS because of both its function and map location. Many of PCOS patients in addition to being hyperandrogenic, are also insulin resistant and/or obese, resistin might be expected to play a role in PCOS (Steppan et al., 2001). The human resistin gene maps to chromosome 19p13.3 and is located between INSR (insulin receptor) and D19S884, ~470 kb from INSR. The location of human resistin gene strongly supported its possible importance in the etiology of PCOS. It is also unlikely that the variation in resistin gene accounts for the strong association between D19S884 allele and PCOS. Instead, this association is more likely due to some other gene or genetic element in the region of D19S884 (Urbanek et al., 2003).

Genes involved in energy regulation and obesity

Genome-wide scan and multipoint linkage analysis identified a locus on 2p2l that showed strong evidence of linkage with serum leptin levels (lod score 4.95) (Comuzzle et al., 1997). The candidate gene in this region, which includes such genes as glucokinase regulatory protein (QCKR) and proopiomelancortin (POMC), has not been identified to date. Bouchard, (1997) has documented multiple family studies that show linkage with the chromosome 2 region where leptin is located including studies of Pima Indians (Norman et al., 1997), the Quebec Family Study (Borecki et al., 1994), the Paris Cohort of Obese Sibling study (Clement et al., 1996), the University of Pennsylvania Family Obesity Study (Reed et al., 1996), and the San Antonio Family Diabetes Study (Duggirala et al., 1996)

It is also possible that other neuroendocrine factors associated with obesity may have accounted for the putative differences in ovulatory status between PCOS and

hyperandrogenemic sisters. Legro et al., (2002) have shown that circulating leptin levels do not differ in PCOS and weight-comparable control women. They observed that obesity modifies the phenotype in PCOS families. Whether this is environmental or reflects the interaction of additional genes will require further investigation.

The leptin receptor is a member of the cytokine family of receptors and has several splice variants. The leptin receptor has been cloned in the human. A mutation in the human receptor gene has been identified. Homozygous affected patients present with early onset morbid obesity and no pubertal development. In addition, their secretion of growth hormone and thyrotropin is reduced (Clement et al., 1998).

Both PCOS and obesity are common disorders with a complex phenotype. Both are presumably heterogeneous in etiology. Most obesity mutations identified to date are inherited in an autosomal recessive fashion. Therefore, it is unlikely that such mutations, even when identified in a human population, could explain only a fraction of the cases that make up the high prevalence of both of these disorders. Although mouse models of single gene defects causing obesity contain many similar aspects of the PCOS phenotype such as obesity and subfecundity (Legro, 1999).

Genes Involved in Gonadotropin Action and Regulation

Abnormalities in gonadotropin secretion, particularly LH, are characteristic of PCOS. Because LH plays a permissive role in driving thecal androgen production, there has been interest in exploring genes related to the regulation of LH secretion, LH bioactivity, and LH action (Legro et al., 2002).

Dopamine Receptor Genes

Dopamine inhibits GnRH and prolactin secretion. Polymorphisms have been identified in the dopamine D2 and D3 receptor genes. Homozygosity for the rare allele (allele 2) of the D3 receptor has been associated with PCOS and clomiphene resistance in Hispanic women (Legro et al., 1995).

Follistatin

Follistatin (FST) is a single chain glycosylated polypeptide that can bind to activin with high affinity and neutralize its biological action of stimulating the secretion of FSH and increasing FSH beta mRNA levels (Carroll et al., 1989; Rivier and Vale,

1991; Shimonaka et al., 1991) and may therefore arrest folliculogenesis (Liao et al., 2000).

This cell-associated protein was also found to accelerate the uptake of activin into pituitary cells, leading to an increase in its degradation by lysosomal enzymes and thus playing a role in activin clearance system (Hashimoto et al., 1997).

FST may modulate the granulosa cell function in an autocrine/paracrine fashion through binding and neutralization of activin action, thus likely to favour the process of luteinization and atresia. (Mather et al., 1997; Guo et al., 1998). Activin enhances FSH induced aromatase activity (Xiao et al., 1990, 1991).

Follistatin (FST) and follistatin like 3 (FST L3) are structurally related proteins that bind and neutralize activin and are closely related members of Transforming growth factor (TGF) beta superfamily.Three follistatin isoforms (FST 288, FST 303, FST 315) are produced from the follistatin gene that primarily secreted proteins. FST L3 is secreted, but is also observed within the nucleus of numerous cell lines, primary granulosa cells, and tissue sections. FST is found in cytoplasm of some cells, including granulosa cells, but not in the nucleus. These observations suggest that biosynthesis and intracellular trafficking of FST and FST L3 are differentially regulated (Saito et al., 2005).

Odunsi and Kidd, (1999) were unable to detect any mutation of the activating or inhibiting type in the entire coding region of follistatin gene in 64 patients of PCOS.

Extensive sequencing of the follistatin gene identified variants at 17 sites, but none of these seem to be likely etiological agents in PCOS. No common variants were detected in the coding region of follistatin. The exon 6 variant, which is not translated, and the other closely linked polymorphic sites outside the follistatin gene are tested by TDT (transmission disequilibrium test) for association and linkage. Even at exon 6 which gave strongest evidence did not remain significant after multiple testing (Urbanek et al., 2000).

Roldan et al., (2004) suggests that both linkage and association studies are valid tools for the study of genetics of PCOS. The present study was carried out to study genetic basis of this heterogeneous condition by linkage analysis.

SUBJECT AND METHODS

SUBJECT AND METHODS

The mode of data collection is given in chapter 1. Epidemiological analysis and study at molecular level of the syndrome are given in chapter 2. Blood samples were taken from both PCOS and non-PCOS women in a family. The samples were further processed for the extraction of DNA. The methodology followed is as follows:

DNA Extraction from blood

In a 15 ml sterile falcon tube, 4 ml peripheral whole blood was taken. In it 12 ml RBC (red blood cells) lysis buffer was added and incubated at room temperature for one minute. Tube was inverted several times during incubation. Then it was centrifuged for 3 minutes at 4500 rpm at room temperature. Supernatant was discarded and tube was blotted on an absorbent paper. Again 4ml of RBC lysis buffer was added to this and all steps were repeated. It was then vortex vigorously for 15 seconds to resuspend the pellet. 400 µl of nuclear lysis buffer (NLB) was added for digestion overnight at 55° C along with the addition of 30 µl of 20 % SDS (sodium dodecyl sulphate) and 20 µl of protein kinase in it. Next morning, 3 ml TE (tris EDTA) and equal volume of phenol was added in it and tube was inverted for 5 minutes for mixing purpose. This mixture was then centrifuged at 4500 rpm for 10 minutes. The aqueous phase (upper one) was again collected in a new tube. 2 ml of TE was added in remaining organic phase and above steps repeated. Equal volume of chloroform/isoamyl alcohol (24:1) was added in it and centrifuge at 4500 rpm for 10 minutes. Again aqueous phase was collected in a new tube and one tenth volume of sodium acetate and two volume of ethanol added. Tube was inverted several times and DNA was precipitated. It was centrifuged at 4500 rpm for 10 minutes, supernatant was discarded and pellet was washed with 5 ml of 70 % cold ethanol. It was again centrifuged for 10 minutes, supernatant discarded and resuspend the pellet in TE.

DNA dissolving buffer which contains 10 mM Tris (pH 8.0) and 0.1 mM EDTA was used for dissolving the precipitated form of DNA.

Then DNA dilution was made. The stock DNA was diluted to 40-50 ng/µl for PCR amplification(1:10). Micropipetting was carried out using adjustable micropipettors with disposable tips ranging from 10 µl to 1000 µl of the upper volume limit.

Linkage analysis with 40 primers was carried out. Polymerase chain reaction (PCR) was done after DNA extraction from whole peripheral blood. Polyacrylamide gel electrophoresis was followed for this purpose. Primers were selected on the basis of 4 different etiologic pathways involving the polycystic ovarian syndrome. These were regarding the gonadotropic, steroid, insulin and obesity related control of ovarian function. These primers are enlisted in Table 20.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed in 0.2 ml tubes (Axygen USA) containing 25 µl total reaction mixture. The reaction mixture was prepared by adding 1 µl sample DNA dilution, 2.5 µl 10 X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 1.5 mM $MgCl_2$), 0.5 µl dNTPs (10 mM), 0.3 µl of each forward and reverse primer (1.0 µM) and 0.3 µl Taq DNA polymerase (1 u) in 20.1 µl PCR water. The reaction mixture was centrifuged for few seconds for thorough mixing.

The reaction mixture was taken through thermocycling conditions consisting: 5 minutes of 90 °C for template denaturation followed by 40 cycles of amplification each consisting of 3 steps; one minute at 90 °C for DNA denaturation into single strands; one minute at 55-57 °C for primers to hybridize or "anneal" to their complementary sequences on either side of the target sequence; and one minute at 72 °C for extension of complementary DNA strand from each primer. Final 10 minutes at 72 °C for Taq polymerase to synthesize any unextended strands left.

PCR was performed using Gene Amp PCR system 2400 and Gene Amp PCR system 9700 thermocyclers (Perkin Elmer, USA).

Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out to analyze the amplified DNA samples. Two percent w/v agarose gel was prepared by melting 1 gram of agarose in 50 ml of 1 X TBE (tris borate EDTA) buffer in microwave oven. Five microlitre ethidium bromide solution (10 mg/ml) was added to stain DNA.

DNA samples were mixed with DNA loading dye containing 0.25 % bromophenol blue prepared in 40 % sucrose solution. Electrophoresis was performed at 100 volts (50 mA) for half an hour in 1X TBE running buffer. After electrophoresis amplified product was detected by placing the gel on UV-Transilluminators (Life Technology, USA).

10 X TBE was made with 0.89 M Tris, 0.025 M Borate and EDTA at 8.3 pH.

Polyacrylamide Gel Electrophoresis

The amplified PCR products were resolved on 8 % non-denaturing polyacrylamide gel. Reagents were mixed in a flask and polyacrylamide gel solution was poured between two glass plates held apart by spacers of 1.5 mm thickness. After inserting the comb, gel was allowed to polymerize for 20-30 minutes at room temperature. Amplified products were mixed with loading dye containing 0.25 % bromophenol blue prepared in 40 % sucrose solution and loaded into the wells. Electrophoresis was carried out at 100 volts for 90 minutes and the gel was stained with ethidium bromide (10 mg/ml) solution for visualization on UV Transilluminator. Gel was photographed by using Digital camera DC 120 (Kodak, USA).

Composition of 8 % Polyacrylamide Gel

13.5 ml 30 % Acrylamide solution (29 g acrylamide, 1g N, N Methylene-bis-acrylamide).

5 ml 10X TBE

0.35 ml 10 % Ammonium persulphate

17.5 µl TEMED

31.13 ml distilled water

GENETIC AND PRIMER DATABASE ANALYSIS

Analysis of microsatellite markers for the known loci as enlisted in Table 20 was performed by PCR; the amplified products were resolved in 8 % standard non-denaturing polyacrylamide gel as described above. Microsatellite markers were visualized by placing the ethidium bromide stained gel on UV transilluminator and genotypes were assigned by visual inspection.). Microsatellite markers mapped by cooperative human linkage centre (CHLC) were obtained from research genetics, Inc. (USA). The cytogenetic location of these markers as well as the length of the

amplified products was obtained from Genome Data Base homepage (www.gdb.org) and Marshfield Medical Center (www.marshmed.org.gov/genetics/).

Homozygosity and heterozygosity was recorded and alleles were numbered in relation to the origin.

This study was envisaged to look at two aspects i) Clinical Study and ii) Genetics of Polycystic Ovarian Syndrome. In the Genetic part we wanted to see the Mendelian Inheritance of the trait, and that how different Microsatellite markers segregate for particular loci, especially of these markers which of them show an association with the disease based on heterozygosity and homozygosity of respective alleles in PCOS and Non-PCOS individuals. The objective was to correlate the markers (indicating association) with the clinical features of Polycystic Ovarian Syndrome patients. Chi-squared test was applied to find out association.

Table 20: Genotype panel for PCOS 37 candidate genes.

Marker locus	Gene symbol	Candidate gene	Distance in centimorgans(cM)	Chromosome location
Steroid hormone				
DIS514	HSD3ß1+2	3ßHydroxysteroid dehydrogenase, type I &II	< 1	1p31.1
D8S1821	STAR	Steroidogenic acute regulatory protein	<2	8p11.2
D9S1809	HSD17ß3	17ßhydroxysteroid dehydrogenase type III	<1	9q22
D10S192	CYP 17	Cyp 17cytochrome P450 17 áhydroxylase /17,20 desmolase	<1	10q24.3
D15S519	CYP11 A	CYP 11A cytochrome P450 side chain cleavage enzyme	0	15q 23-24
D15S520	CYP 11 A	CYP 11A cytochrome P450 side chain cleavage enzyme	0	15q 23-24
D17S934	HSD17ß1	17ßhydroxysteroid dehydrogenase type I	<2	17q11-21
HSD17ß2	HSD17ß1I	17ßhydroxysteroid dehydrogenase typeII	0	16q24.2
Gonadotropin action				
D2S2335	ACTR1	Activin receptor 1	<1	12q13,12
D2S1352	LHCGR	Luteinizing hormone/chorioniogonadotropic receptor	<2	2p21
D2S293	INHB ß	Inhibin ß B	<2	2cen-2q13
D2S163	INHA	Inhibin A	<1	2q33.34
D3S1298	ACTR2 ß	Activin receptor 2 ß	<1	3q22.2
D5S623	FS	Follistatin	<0.5	5q14
D5S822	FS	Follistatin	<1	5p14
INHBA	INHBA	inhibin ßA	2	7p13-15
D5S474	FS	Follistatin	<2	5p14
D12S1691	INHC	Inhibin C	<1	12q13
D12S347	ACTR1	Activin receptor 1	<1	12q13.12
D17S1353	SHBG	Sex hormone binding globulin	<1	17p13.2
D18S474	MADH4	Mothers against decapentaplegic homolog 4	<1	18q21
Obesity and energy regulation				
D18S64	MC4R	Melanocortin 4 receptor	<3	18q21.32
D7S1875	OB	Leptin	0.2	7q31.3-32.1
D1S198	OBR	Leptin receptor	0.5	1q31
D2S131	POMC	proopiomelanocortin	<1	2p23
D11S911	UCP2&3	Uncoupling protein 2 &3	<4	11q13
Insulin action				
IGF 1	IGF1	Insulin like growth factor 1	0	12q22-23
IGF-1R	IGF1R	Insulin like growth factor receptor	0	15q25-26
D7S519	IGFBP 1&3	Insulin like growth hormone binding protein 1 &3	1	7p13-7p12
INSR	INSR	Insulin receptor	0	19p13.3
D19S216	INSR	Insulin receptor	4.2	19p13.3
D19S884	INSR	Insulin receptor	1.2	19p13.3
D19S922	INSR	Insulin receptor	1.2	19p13.3
D19S391	INSR	Insulin receptor	3.6	19p13.2
D19S865	INSR	Insulin receptor	7.2	19p13.2
D19S906	INSR	Insulin receptor	11	19p13.2
D19S840	INSR	Insulin receptor	14	19p13.2
D19S212	INSL3	Leydig Insulin like protein 3	<1	19p13.1
D19S410	INSL3	Leydig insulin like protein 3	<1	19p13.1
D2S2647	IRS1	Insulin receptor substrate 1	0	2q36-37
D3S1263	PPARG	Peroxisome proliferators activated receptor gamma	<0.2	3p25-24.2

RESULTS

Epidemiological study

Family 1

In the first generation individuals I-1 and I-2 were dead and no information was available regarding PCOS. They have one daughter II-2 and three sons II-3, II-5 and II-6. All of them were dead when the members of the families were contacted. Their status regarding the PCOS was also not known. Individual II-6 was married to his cousin II-7. They have one non-PCOS daughter III-10. Individual II-2 was married to II-1. They have three daughters III-2, III-4, III-6 and 2 sons III-8 and III-9. Out of three daughters, III-6 was diagnosed for PCOS. (Fig 1; chapter 1)

In the third generation, III-2 was married to a man III-1. They have 2 daughters IV-1 (PCOS, the proband), IV-3 (non-PCOS) and one son IV-2. Individual III-4 was married to III-3, they have two daughters IV-5 (non-PCOS) and IV-7 (PCOS) and 2 sons IV-4 and IV-6. The individual III-6 (PCOS) was married to a man III-5. They have got three daughters IV-8 (PCOS), IV-9 (non-PCOS) and IV-12 (PCOS) and one son IV-11. A man III-8 was married to a non-PCOS woman III-7. They have got one PCOS daughter IV-14 and one son IV-13. Another man III-9 was married to a non-PCOS III-10. They have 2 daughters IV-16 (non-PCOS) and IV-17 (PCOS woman) and have one son IV-15.

In the fourth generation, IV-7 (PCOS) was married to her cousin IV-11. They have one daughter V-4 (PCOS) and one son V-3. A non-PCOS woman IV-9 was married to a man IV-10. They have one PCOS daughter, V-1 and son V-2. One PCOS woman IV-17 was married to her cousin IV-18. They have two daughters V-5 (non-PCOS) and V-6 (PCOS).

The pedigree indicated the recessive mode of inheritance for PCOS. The pedigrees showed that out of eight marriages, there were five marriages in which the wives were non-PCOS (III-1 x III-2, III-3 x III-4, III-7 x III-8, III-9 x III-10, IV-9 x IV-10) but in three marriages the wives were PCOS (III-5 x III-6, IV-7 x IV-11, IV-17 x IV-18). Six marriages were consanguinous (III-3 x III-4, III-5 x III-6, III-9 x III-10, II-6 x II-7, IV-7 x IV-11, IV-17 x IV-18). It is expected of this marriage type that this will encourage homozygosity for PCOS allele.

Family 2

In individuals in generation I, I-1 and I-2 presence or absence of PCOS condition was not known as they were dead at the time of interviewing members of this family. They have six children i.e. three daughters II-2, II-5, II-7 and three sons II-4, II-8 and II-9. Their daughter II-2 was married to II-1. II-2 was non-PCOS. They have four children, one son III-1 and three daughters, III-2, III-3, III-4 (proband). Of these, III-2 and III-4 were suffering from PCOS (Fig 2; chapter 1).

In generation II, a man II-4 was married to a non-PCOS woman II-3, they have got five sons III-5, III-11, III-6, III-7, III-8 and two daughters III-9 and III-10 who were non-PCOS. Daughter II-7 was married to II-6 and they have three daughters III-12, III-13, III-14 and one son III-15. Of these III-12 and III-14 were suffering from PCOS.

Individual II-9 was married to a non-PCO woman II-10. They have got three daughters, III-16, III-17, III-18 and two sons III-19 and III-20. In these III-16 and III-18 were PCOS patients.

In the couples, II-1 x II-2; II-6 x II-7 and II-9 x II-10, the wives were non-PCOS but they have got PCOS daughters. This indicates that wives and husbands were in heterozygous condition and the disorder was controlled by a recessive gene. The pattern of inheritance in this family indicates recessive inheritance of PCOS in these patients.

Family 3

In generation I, individuals I-1 and I-2 were of unknown status regarding PCOS condition. They have got two normal daughters (II-4 and II-8) and three sons (II-1, II-3 and II-6) (Fig 3; chapter 1).

In generation II, an individual II-3 was married to non-PCOS woman II-2. They have got four daughters (III-1, III-2, III-5, III-6). Daughter (III-5) was diagnosed as PCOS patient. A male (II-6) was married to PCOS woman (II-5) and they have got one non-PCOS daughter (III-11), one PCOS daughter (III-8, proband) and two sons (III-9 and III-10). From his second marriage with a non-PCOS woman (II-7), he has one non-PCOS daughter (III-12) and one PCOS daughter (III-13) and three sons (III-14-16). A non-PCOS woman (II-8) was married to her cousin (II-9). They have got one PCOS daughter (III-17). A man (II-10) married to non-PCOS woman (II-11). They have got one PCOS daughter (III-21), three sons (III-18, III-19 and III-20).

In the third generation, individual III-7 was married to his PCOS cousin III-8. They have got one non-PCOS daughter (IV-3) and one PCOS daughter (IV-1) and one son (IV-2). Non-PCOS woman (III-11) was married to her cousin (III-19). They have got one non-PCOS daughter (IV-4) and two sons (IV-5 and IV-6). Another PCOS woman (III-17) was married to her cousin (III-18), but they have no issue. One woman (III-21) diagnosed as PCOS patient (died due to ovarian cancer) was married to (III-22) and they have one non-PCOS daughter (IV-7) and one PCOS daughter (IV-11) and three sons IV-8, IV-9 and IV-10.

The recessive mode of inheritance for PCOS was ascertained in this family.

Family 4

In generation 1I, individual with unknown status (II-2) was married to II-1 and they have got two non-PCOS daughters (III-1 and III-7) and two PCOS daughters (III-5 and III-6). Both PCOS daughters (III-5 and 6 were dead at the time when family was studied. They were not married (Fig 4; chapter 1).

Individual III-1 was married to III-2. They have one PCOS daughter IVI-1 and one son (IV-2). A PCOS women (III-4) was married to III-3. They have one non-PCOS daughter (IV-3) who was married to her cousin (IV-2). They were issueless. A man III-8 was married to non-PCOS woman (III-7) and they have got one PCOS daughter (IV-4) and three sons IV 5-7. A non-PCOS women (III-10) was married to III-9. They have two non-PCOS daughters (IV-8 and IV-11) and two PCOS daughters (IV-9 proband and IV-10). A non-PCOS woman III-12 was married to her cousin (III-13). They have got one PCOS daughter (IV-13) and non-PCOS daughter (IV-13). The mode of inheritance ascertained for PCOS in this family was autosomal recessive.

Family 5

In generation I, husbands and wives in the marriages I-1 x I-2 and I-3 x I-4 were of unknown status regarding PCOS disorder. The couple I-1 x I-2 has two sons (II-1 and II-5) and the other couple I-3 x I-4 has two daughters II-2 and II-6 and one son II-4 (Fig 5; chapter 1). Since they were dead their status regarding PCOS disorder was not known. However, couple (II-1 and II-2) has three PCOS daughters (III-5, III-6 and III-10) which indicates that possibly II-2 was suffering from this disorder or she was carrier for this. This couple has two more non-PCOS daughters (III-2, III-3), and one son (III-8). Marriage between II-5 and II-6 shows they have one PCOS daughter (III-15) and four non-PCOS daughters (III-9, III-12, III-16, III-17) and two sons (III-1 and III-14).

In the third generation, III-2 was married to III-1 and they have one non-PCOS daughter (IV-1) and one PCOS daughter (IV-6) and four sons (IV-2-5). Woman III-3 was married to III-4 man. They have two sons (IV-8, IV-9), one non-PCOS daughter (IV-1) and one PCOS daughter IV-10. A non-PCOS woman (III-7) was married to her cousin (III-8). They have one non-PCOS daughter (IV-11) and one PCOS daughter (IV-12). PCOS woman (III-10) was married to her affected cousin (III-11). They have one PCOS daughters (IV-16, proband) and one non- PCOS daughter (IV-13) and 2 sons (IV-14 and IV-15).

Individual III-12 (non-PCOS woman) was married to III-13. They have two sons (IV-17 and IV-18) and five non- PCOS daughters (IV-19-23).

Family 6

In generation I, the marriage between I-1 x I-2 resulted in one son II-1. In marriage between I-3 x I-4, they had one non-PCOS daughter (II-2) and one son (II-3). The marriage I-5 x I-6 resulted in two PCOS daughters II-4 and II-9, one non-PCOS daughter (II-6) and two sons (II-5 and II-8) (Fig 6; chapter 1).

In generation II, the couples II-1 x II-2 (deceased) had one PCOS daughter III-3 and two non-PCOS daughters (III-1 and III-5). The individual II-3 was married to II-4 (PCOS) and they have one non-PCOS daughter (III-8) and one PCOS daughter (III-7, proband). A non-PCOS woman (II-6) was married to II-7 and they have one PCOS daughter (III-10) and one non-PCOS daughter (III-9). In a marriage between II-9 (PCOS women) and II-10, they had two non-PCOS) daughters (III-11, 12) and one son (III-13).

In the third generation a non-PCOS woman (III-1) married to man (III-2). They had one son (IV-1) and one non-PCOS daughter (IV-2). A PCOS woman (III-3) was married to man (III-4) and they had two sons (IV-3 and IV-4). In a marriage between III-5 (non-PCOS) and III-6, they had one son (IV-5) and one PCOS daughter (IV-6).

The marriages contracted in the whole pedigree indicate recessive mode of Mendelian inheritance.

Mendelian mode of inheritance

The six pedigrees proceeded for the present study indicated recessive Mendelian inheritance. Two types of marriages were observed in these pedigrees (i) a marriage between PCOS x normal and (ii) between normal x non-PCOS. In both the marriage types, data were assembled regarding the inheritance of PCOS and non-PCOS condition in the offspring.

Table 21 shows the Goodness of fit to 1:1 phenotype ratio in marriages between PCOS x Normal individuals. The test of significance shows an agreement to 1:1 Mendelian phenotypic ratio ($X^2_{(1)}$=2.78; P= >0.05) .

Table 22 shows the Goodness of fit to 3: 1 Mendalian phenotypic ratio where marriages were contracted between non-PCOS x normal individuals. These results also show an agreement to 3: 1 Mendalian ratio. ($X^2_{(1)}$=2.76; P=> 0.05).

Table 21: Goodness of fit to 1:1 Mendelian ratio in marriages between non-PCOS and PCOS individuals.

Number of families	Marriage type Male X female	Offspring Non-PCOS PCOS
6	Normal x PCOS	Observed number 19 10
		Phenotypic ratio 1 1
		Expected number 14.5 14.5
		$X^2_{(1)}$=2.78 P= >0.05

Table 22: Goodness of fit to 3:1 Mendelian ratio in marriages between non-PCOS and PCOS individuals.

Number of families	Marriage type Male X female	Offspring Non-PCOS PCOS
6	Normal x non-PCOS	Observed number 70 33
		Phenotypic ratio 3 1
		Expected number 77.5 25.75
		$X^2_{(1)}$=2.76 P=> 0.05

Association with microsatellite markers

Family 1

The blood samples were taken from PCOS women (III-6, IV-1, IV-7, IV-14, IV-17, V-2, V-4) and non-PCOS women (III-2, III-4, IV-3, IV-9). DNA was extracted from the blood samples. The samples were numbered as given to the individuals in the pedigree. The markers used for chromosome No. 1, No. 2, No. 5, No. 10, No. 12, No. 15, No 19 were D1S514, D2S1352, D2S293, D2S2335, D2S163, D3S1298, D5S822, INHBA, D8S1821, D10192, D12S347, D12S1691, D15S519, HSD 17 β2, D17S1353, D18S474, D19S216, D19S905, INSR, D19S922, D19S391, D19S865, D19S906, D19S410 and D19S212 respectively.

Co-segregation of the markers with different loci showed homozygosity as well as heterozygosity for both non-PCOS and PCOS individuals.

Of the markers used here, homozygosity at allele 1 was observed with markers D1S514, D2S293, D2S2335, D2S163, D3S1298, D8S1821, D12S347, D12S1691, D17S1353, INSR, D19S922, D19S410 AND 19S212. In the case of marker D19S212 genes segregating with it show homozygosity for allele 1 in the five PCOS individuals out of seven. Of the remaining two, one is homozygous for allele 2 and the other (V-2) is heterozygous for allele 1 and 2 (1-2). Among non- PCO women, one of them (IV-3) is homozygous for allele 1. Significant association of allele 1 with marker D19S212 was not observed but the results suggest presence of ($X^2_{(1)}$ =2.25; P >0.10) a weak association in the transmission of allele 1 with marker D19S212 (Table 23).

Table 23: Genotypes, homozygosity and heterozygosity at different loci in PCOS and non-PCOS women in family 1.

Marker	Status PCOS women	Genotype	Homozygosity No.	Status Non-PCOS women	Genotype	Homozygosity No.
DIS514	III-6, IV-1, IV-17, V-2, V-6 IV-7, IV-14	2-2(1) 1-1(4) 1-2(2)	2-2(1) 1-1 (4)	IV-9 III-2,III-4, IV-3	2-2(1) 1-1(3)	2-2(1) 1-1 (3)
D2S1352	III-6, IV-7 IV-1, IV-14, V-6 IV-17, , V-2	1-1(2) 1-3(3) 1-2(2)	1-1(2)	IV-3 III-2,III-4, IV-9	1-3(1) 1-2(3)	
D2S293	IV-7, IV-17, V-2, III-6, IV-1, IV-14, V-6	1-1(3) 1-3(2) 1-2(2)	1-1(3)	III-4, IV-3, IV-9, III-2	1-1(3) 1-3(1)	1-1(3)
D2S2335	IV-1, III-6, IV-14 IV-7, IV-17, V-2, V-6	- 1-1(2) 1-2(4)	1-1(2)	III-2,III-4, IV-9 IV-3	1-2(3) 1-1(1)	1-1(1)
D2S163	III-6, IV-1, IV-7, IV-17, IV-14, V-2, V-6	1-1(4) 1-2(3)	1-1(4)	III-2,III-4, IV-3 IV-9	1-1(3) 1-2(1)	1-1(3)
D3S1298	III-6, IV-1, IV-14, IV-17, V-2,V-6 IV-7,	1-1(6) 1-2(1)	1-1 (6)	III-2,III-4, IV-9 IV-3	1-1(3) 1-2(1)	1-1 (3)
D5S822	III-6, IV-7, IV-17, V-2, IV-1, IV-14, V-6	- 1-1(3) 1-2(3)	1-1(3)	III-2,III-4, IV-3, IV-9	1-2(4)	
INHBA	III-6, IV-1, IV- 7,IV-14, IV-17, V- 2, V-6	1-2(7)		III-2,III-4, IV-3, IV-9	1-2(4)	
D8S1821	III-6, IV-1, IV-	1-1(7)	1-1(7)	III-2,III-4, IV-3,	1-1(4)	1-1(4)

	7,IV-14, IV-17, V-2, V-6			IV-9		
D10S192	IV-17, III-6, V-2, V-6 IV-1, IV-7, IV-14,	- 1-3(3) 1-2(3)		III-4, IV-3 IV-9,III-2	1-2(2) 1-3(2)	
D12S347	III-6, IV-1, IV-7, V-2, V-6 IV-14, IV-17,	2-3(5) 1-1(2)	1-1(2)	IV-9 III-2 III-4, IV-3	2-3(1) 1-1(1) -	1-1(1)
D12S1691	IV-1, IV-14, III-6, IV-7, IV-17, V-6 V-2,	1-1(2) 1-2(4) -	1-1(2)	III-4, IV-3, IV-9 III-2	1-1(3) 1-2(1) -	1-1(3)
D15S519	IV-1, IV-7, , V-2, III-6, IV-14, IV-17, V-6	1-1(3) 1-2(4)	1-1(3)	IV-9 III-2,III-4, IV-3	1-3(1) 1-2(3)	
HSD17ß2	III-6, IV-1, IV-7,IV-14, IV-17, V-2, V-6	1-2(7)		III-2,III-4, IV-3, IV-9	1-2(4)	
D17S1353	III-6, IV-7,IV-14, V-2, IV-1, IV-17, V-6	1-1(4) 1-2(3)	1-1(4)	III-2 III-4, IV-3, IV-9	1-2(1) 1-1(3)	1-1(3)
D18S474	III-6, IV-1, IV-7, IV-14, IV-17, V-2, V-6	1-2(7)		III-2,III-4, IV-3, IV-9	1-2(4)	
D19S216	III-6, IV-1, IV-7, IV-14, IV-17, V-2, V-6	1-3(6) 1-1(1)	 1-1(1)	III-2, III-4, IV-3, IV-9	1-3(1) 1-2(2) 1-1(1)	 1-1(1)
D19S905	IV-14 III-6, IV-1, IV-7, IV-17, V-2, V-6	1-1(1) 1-2(6)	1-1(1)	III-4, IV-3, III-2, IV-9,	- 1-1(1) 1-2(2)	 1-1(1)

INSR	III-6, IV-1, V-6 IV-7, IV-14, IV-17, V-2,	1-1(3) 1-2(4)	1-1(3)	III-2,III-4, IV-9 IV-3	1-2(3) 1-1(1)	1-1(1)
D19S922	III-6, IV-1, , IV-14, V-2, V-6 IV-7, IV-17	1-1(3) 1-3(2) 1-2(2)	1-1(3)	III-2,III-4, IV-3 IV-9	1-1(3) 1-2(1)	1-1(3)
D19S391	IV-17, V-6 III-6, IV-1, IV-7, IV-14, V-2	1-1(2) 1-2(5)	1-1(2)	III-2,III-4, IV-3, IV-9,	1-2(4)	
D19S865	III-6, IV-1, IV-14, IV-17, V-2 IV-7, , V-6	1-3(5) 1-2(2)		III-2,III-4, IV-3, IV-9,	1-3(4)	
D19S906	III-6, IV-1, IV-7, IV-14, IV-17, V-2, V-6	1-2(6) 1-3(1)		III-2, IV-3, IV-9, III-4,	1-2(3) -	
D19S410	III-6, IV-1, IV-17, IV-7, IV-14, V-2, V-6	1-1(3) 1-2(4)	1-1(3)	III-2,III-4, IV-3, IV-9,	1-1(4)	1-1(4)
D19S212	III-6, IV-1, IV-7, IV-14, IV-17, V-6 V-2	1-1(5) 2-2(1) 1-2(1)	1-1(5) 2-2(1)	IV-3, III-2,III-4, IV-9,	1-1(1) 1-2(2) -	1-1(1)

Numbers in parenthesis indicate the number of women.

Family 2

In family 2 the individuals II-7, III-4, III-14, III-16, III-17 were processed for molecular studies. DNA samples were numbered as was shown in pedigree of family 2 (Fig 2; chapter 1).

All the known loci for PCOS were excluded with the microsatellite markers for the confirmation of association. Individual II-7 (non-PCOS) was heterozygous for alleles 1 and 2 (1-2) with markers D3S1298, D5S822, D15S520, D1S514, D2S1352, D2S293, D2S2335 and D2S163. Individual III-17 was homozygous for allele 1 with markers D1S514, D2S2335, D2S1352 but heterozygous for allele 1 and 2 with markers D2S293, D3S1298, D5S822 and D15S520. This individual is heterozygous for allele 1 and 2 with marker D2S163.

With marker D2S1352 all the PCOS individuals (III-4, III-14 and III-16) and non-PCOS (III-17) showed homozygosity for allele 1. Similar is the case with marker D2S2335 in these PCOS and non-PCOS individuals. In the third generation PCOS as well as non-PCOS individuals have heterozygosity for allele 1 and 2 with marker D15S520 (Table 24). In the individuals with PCOS, genes segregating with marker D5S822 show homozygosity for allele 1 and significant strong association was observed at this gene locus ($X^2_{(1)}$ =4.89; P<0.05).

All the PCOS individuals III-4, III-14, III-16 showed homozygosity at allele 1 (1-1) with marker D5S822 but individuals who were non-PCOS II-7, III-17 were heterozygous for allele 1 and 2 (1-2).

Table 24: Genotypes, homozygosity and heterozygosity at different loci in PCOS and non-PCOS women in family 2.

Marker	Status PCOS women	Genotype	Homozygosity No.	Status Non-PCOS women	Genotype	Homozygosity No.
DIS514	III-14, III-16 III-4,	1-1(2) 1-2(1)	1-1 (2)	III-17 II-7	1-1(1) 1-2(1)	1-1 (1)
D2S1352	III-14, III-16,III-4	1-1(3)	1-1(3)	III-17,II-7	1-1(2)	1-1(2)
D2S293	III-14 III-16,III-4	1-1(1) 1-2(2)	1-1(1)	III-17,II-7	1-2(2)	
D2S2335	III-14, III-16,III-4	1-1(3)	1-1(3)	III-17,II-7	1-1(2)	1-1(2)
D2S163	III-16,III-4 III-14	1-3(2) 1-2(1)	- -	II-7 III-17	1-3(1) 1-2(1)	- -
D3S1298	III-14,III-4 III-16	1-1(2) -	1-1(2) -	III-17,II-7	1-2(2)	-
D5S822	III-14, III-16,III-4	1-1(3)	1-1(3)	III-17,II-7	1-2(2)	-
D15S520	III-14, III-16,III-4	1-2(3)		III-17,II-7	1-2(2)	-

Numbers in parenthesis indicate the number of women.

Family 3

Blood samples of II-8, III-5, III-6, III-8, III-11, III-13, III-17, IV-1, IV-7, IV-11 were taken and processed for molecular studies. The blood samples were numbered as shown in pedigree (Fig 3).

Known loci for PCOS were excluded for microsatellite markers. Markers used were D2S131, D2S1352, D2S293, D2S2335, D2S163, D3S1298, D5S822, D5S474, INHB A, D8S1821, D10S192, D11S911, D12S1691, D12S347, IGF-1, D15S519, D15S520, HSD 17 B2, D17S934, D19S884, D19S391, D19S865, D19S840, D19S212 (Table 25).

All PCOS women and non-PCOS women are heterozyguous at allele 1 (1-2) within marker D2S131, D8S1821, D12S1691, D12S347, IGF-1, D15S520, D17S934, D19S391, D19S840 and D19S212. Both PCOS women and non-PCOS women show nearly equal distribution of homozygous individuals for allele 1 with markers D2S1352, D2S293, D2S2335, D2S163, D3S1298, D5S822, D5S474, INHBA, D10S192, D15S519, HSD17 B 2, D19S865.

All the microsatellite markers were non informative regarding association at PCOS loci. Genes segregating with different markers do not show association of allele 1 with any of the markers. The data for this family showed that homozygosity for allele 1 has an even distribution among PCOS as well as non-PCOS women.

Table 25: Genotypes, homozygosity and heterozygosity at different loci in PCOS and non-PCOS women in family 3.

Marker	Status PCOS women	Genotype	Homozygosity No.	Status Non- PCOS women	Genotype	Homozygosity No.
D2S131	III-5, III-8, III-13, III-17, 1V-1, IV-11	1-2 (6)		II-8,III-6,III-11,IV-7	1-2(4)	
D2S1352	III-5, III-8, III-13, 1V-1, IV-11	1-1(6)	1-1(6)	II-8,III-6,III-11,IV-7	1-1(4)	1-1(4)
D2S293	III-5, III-8, III-13, 1V-1, IV-11	1-1(6)	1-1(6)	II-8,III-6,III-11,IV-7	1-1(4)	1-1(4)
D2S2335	III-5, III-8, III-13, IV-11 1V-1,III-17	1-2(4) 1-1(2)	1-1(2)	II-8, IV-7 III-6,III-11,	1-2(2) 1-1(2)	1-1(2)
D2S163	III-5, III-8, III-13, III-17, 1V-1, IV-11	1-1(6)	1-1(6)	II-8,III-6,III-11,IV-7	1-1(4)	1-1(4)
D3S1298	III-5, III-13, III-17, 1V-1, IV-11 III-8	1-1(5) 1-2(1)	1-1(5)	II-8, III-11 III-6, IV-7	1-1(2) 1-2(2)	1-1(2)
D5S822	III-8, III-13, III-17, , IV-11 III-5, 1V-1	1-2(4) 1-1(2)	- 1-1(2)	II-8, III-11,IV-7 III-6	1-2(3) 1-1(1)	1-1(1)
D5S474	III-5,III-8, III-13, III-17, 1V-1, IV-11	1-1(6)	1-1(6)	II-8,III-6,III-11,IV-7	1-1(4)	1-1(4)
INHBA	III-5,III-8, III-13, III-17, 1V-1, IV-11	1-1(6)	1-1(6)	II-8,III-6,III-11,IV-7	1-1(4)	1-1(4)

D8S1821	III-5, III-13, IV-11 III-8, III-17, 1V-1	1-2(3) 1-3(3)	- -	III-6, IV-7 II-8, III-11,	1-1(2) 1-3(2)	1-1(2)
D10S192	III-8, III-13, III-17, IV-11 III-5, 1V-1	1-2(4) 1-1(2)	- 1-1(2)	III-11,IV-7 II-8,III-6,	1-2(2) 1-1(2)	 1-1(2)
D11S911	III-5, III-17, IV-11 III-8, III-13, 1V-1	1-2(3) 2-3(3)	- -	III-6, IV-7 II-8 III-11	1-2(2) 2-3(1) -	
D12S1691	III-5, III-8, III-13, III-17, 1V-1, IV-11	1-2(6)		II-8,III-6,III-11,IV-7	1-2(4)	
D12S347	III-5, III-8, III-13, III-17, 1V-1, IV-11	1-2(6)		II-8,III-6,III-11,IV-7	1-2(4)	
IGF-1	III-5, III-8, III-13, III-17, 1V-1, IV-11	1-2(6)		II-8,III-6,III-11,IV-7	1-2(4)	
D15S519	III-5,III-8, III-17, III-13, 1V-1, IV-11	1-2(3) 1-1(3)	 1-1(3)	III-6,III-11 IV-7,II-8	1-2(2) 1-1(2)	 1-1(2)
D15S520	III-5, III-17, IV-11 III-8, III-13,1V-1	2-2(3) 1-2(3)	2-2(3) -	II-8,III-6,III-11,IV-7	1-2(4)	
HSD17ß2	III-5, III-8, III-13, III-17, 1V-1, IV-11	1-1(6)	1-1(6)	II-8,III-6,III-11,IV-7	1-1(4)	1-1(4)
D17S934	III-5, III-8, III-13, III-17, 1V-1, IV-11	1-2(6)		II-8,III-6,III-11,IV-7	1-2(4)	
D19S884	III-5, III-8, , 1V-1 III-13, IV-11 III-17	1-1(3) 1-2(2) 2-2(1)	1-1(3) - 2-2(1)	II-8 III-6,III-11,IV-7	1-1(1) 1-2(3)	1-1(1)
D19S391	1V-1 III-8, III-13, III-17, IV-11	1-3(1) 1-2(4)	- -	IV-7 II-8 III-6,III-11,	1-3(1) 1-2(1) 1-1(2)	 1-1(2)

	III-5	1-1(1)	1-1(1)			
D19S865	III-5, III-8, IV-11 III-13, III-17 1V-1	1-1(3) 1-2(2) -	1-1(3) - -	II-8,III-6,III-11,IV-7	1-1(4)	1-1(4)
D19S840	III-5 1V-1 III-8, III-13, III-17, IV-11	- 1-2(1) 2-2(4)	- - 2-2(4)	IV-7 II-8,III-6,III-11	- 1-2(3)	
D19S212	III-5, III-8, III-13, III-17, 1V-1, IV-11	1-2(6)		II-8,III-6,III-11,IV-7	1-2(4)	

Numbers in parenthesis indicate the number of women.

Family 4

Known PCOS loci were excluded with microsatellite markers; D1S514, D2S131, D2S1352, D2S293, D2S163, D3S1263, D3S1298, D5S822, D5S474, D5S623, D7S519, D8S1821, D10S192, IGF-1, D15S519, D18S474, D19S391, D19S865, D19S906, D19S212, D19S840 (Table 26).

The blood samples of III-10, IV-1, IV-3, IV-4, IV-8, IV-9, 10, 11, 12 and 13 were taken and processed for molecular studies.

In family 4 (Table 26) genes segregating with all the markers, except marker D2S293, do not show any signs of association of allele1 with any of the markers. However, with marker D2S293 a weak association with allele1 was observed ($X^2_{(1)}$ =2.85; P=>0.05). In case of the other markers homozygosity and hetrozygosity at allele 1 seems to be evenly distributed (Table 26).

Table 26: Genotypes, homozygosity and heterozygosity at different loci in PCOS and non-PCOS women in family 4.

Marker	Status PCOS women	Genotype	Homozygosity No.	Status Non- PCOS women	Genotype	Homozygosity No.
DIS514	IV-1, IV-4, IV-9, IV-10, IV-13	1-2(5)	-	III-11,IV-3, IV-8, IV-11, IV-12	1-2(5)	-
D2S131	IV-1, IV-4, IV-9 IV-10, IV-13	1-2 (3) 1-1(2)	1-1(2)	IV-3, IV-8, IV-11, IV-12 III-11	1-2 (4) - 1-1(1)	- - 1-1(1)
D2S1352	IV-1, IV-4, IV-9, IV-10, IV-13	1-2(5)	-	III-11,IV-3, IV-8, IV-11, IV-12	1-2(5)	-
D2S293	IV-1, IV-9, IV-10 IV-4, IV-13	1-2 (3) 1-1(2)	1-1(2)	III-11,IV-3, IV-8, IV-11 IV-12	1-2 (4) - 1-1(1)	- - 1-1(1)
D2S163	IV-1, IV-4, IV-9, IV-10,IV-13	1-2(5)	-	III-11,IV-3, IV-8, IV-11, IV-12	1-2(5)	-
D3S1263	IV-1, IV-4, , IV-10, IV-13 IV-9	1-3 (5) 1-2 (1)	-	IV-3, IV-11, IV-12 III-11, IV-8,	1-3 (3) 1-2(2)	- -
D3S1298	IV-1, IV-4, IV-9, IV-10, IV-13	1-2(5)	-	III-11,IV-3, IV-8, IV-11, IV-12	1-2(5)	-
D5S822	IV-1, , IV-9 IV-4, IV-13 IV-10	1-2 (2) 2-2 (2) 1-1(1)	- 2-2 (2) 1-1(1)	III-11,IV-3, IV-11 IV-8, IV-12	1-2 (3) 2-2 (2)	- 2-2 (2)
D5S623	IV-1, IV-4, IV-9, IV-10, IV-13	1-2(5)	-	III-11,IV-3, IV-8, IV-11, IV-12	1-2(5)	-
D5S474	IV-1, IV-10,IV-13 IV-4, IV-9	1-2(3) 1-1(2)	1-1(2)	III-11,IV-3, IV-11, IV-12 IV-8	1-2(4) 1-1(1)	1-1(1)
D7S519	IV-1, IV-4, IV-10,IV-13 IV-9	1-2 (4) 1-1 (1)	1-1 (1)	III-11, IV-11 IV-3, IV-8, IV-12	1-2(2) 1-1 (3)	1-1 (3)

INHBA	IV-1, IV-4, IV-9, IV-10,IV-13	2-2(5)	2-2(5)	III-11,IV-3, IV-8, IV-11, IV-12	2-2(5)	2-2(5)
D8S1821	IV-1 IV-4, IV-10,IV-13 IV-9	- 1-2(3) 1-1(1)	- - 1-1(1)	III-11, IV-11, IV-12 IV-3, IV-8	1-2(3) 1-1(2)	- - 1-1(2)
D10S192	IV-1, IV-4, IV-10,IV-13 IV-9	1-2(4) 1-1(1)	 1-1(1)	III-11,IV-3, IV-8, IV-11 IV-12	1-2(4) 1-1(1)	- - 1-1(1)
IGF-1	IV-1, IV-4, ,IV-13 IV-9, IV-10	1-2(3) 1-1(2)	 1-1(2)	III-11,IV-3, IV-8, IV-11 IV-12	1-1(4) 1-2(1)	1-1(4)
D15S519	IV-1, IV-9 IV-4, IV-10,IV-13	1-3(2) 1-1(3)	 1-1(4)	III-11,IV-3 IV-8, IV-11, IV-12	1-2(2) 1-1(3)	 1-1(3)
D18S474	IV-9 IV-1, IV-4, IV-10, IV-13	- 1-1(4)	 1-1(4)	III-11,IV-3, IV-11, IV-12 IV-8	1-2(4) 1-1(1)	 1-1(1)
D19S391	IV-1 IV-4 IV-9, IV-13 IV-10	1-3(1) 1-4(1) 1-2(2) 1-1(1)	- - - 1-1(1)	IV-3, IV-8 IV-11 III-11 IV-12	1-3(2) 1-4(1) 1-2(1) 1-1 (1)	- - - 1-1 (1)
D19S865	IV-1, IV-10,IV-13 IV-4, IV-9	1-1(3) 1-2(2) -	1-1(3) - -	IV-3, IV-8, IV-12 IV-11 III-11	1-1(3) 1-2(1) 2-2(1)	1-1(3) - -
D19S840	IV-4, IV-9, IV-10, IV-13 IV-1	1-1(3) 1-2(1) 1-3(1)	1-1(3) - -	III-11, IV-8, IV-11 IV-3, IV-12 -	1-1(3) 1-2(2) -	1-1(3) - -
D19S212	IV-1, IV-4, IV-9, IV-10, IV-13	1-2(5)	1-2(5)	III-11,IV-3, IV-8, IV-11, IV-12,	1-2(5)	1-2(5)

Numbers in parenthesis indicate the number of women.

Family 5

Blood samples were collected from eight PCOS and six non- PCOS women and DNA extraction was done from these blood samples. Known loci were excluded by using the following microsatellite markers: D2S1352, D2S293, D2S163, D5S822, D5S474, D12S1691, D15S519, D18S474 (Table 27).

All markers used here were non-informative regarding allele association with any of these markers. Co-segregation of markers with different loci showed homozygosity as well as heterozygosity for both PCOS and non-PCOS women, hence results were non-informative. There was only one marker D12S1691 which showed homozygosity for all PCOS as well non-PCOS women.

In family 5 (Table 27) genes segregating with different markers do show homozygosity for allele 1 in PCOS women but in the case of marker D15S519, five PCOS women III-6, III-10, IV-10, IV-12, IV-16, out of seven show homozygosity for allele1 and in non-PCOS women all of them (III-2, III-3, III-9, IV-11, V-7, V-11, V-13) show heterozygosity for allele 1. In PCOS women a strong association of homozygosity for allele 1 with marker D15S519 has been observed ($X^2_{(1)}$ =7.78; P= < 0.01).

Table 27: Genotypes, homozygosity and heterozygosity at different loci in PCOS and non-PCOS women in family 5.

Marker	Status PCOS women	Genotype	Homozygosity No.	Status Non- PCOS women	Genotype	Homozygosity No.
D2S1352	III-10 IV-10, IV-16 III-6, IV-6, IV-12 III-15	- 1-1(2) 1-2(3) 2-2(1)	 1-1(2) - 2-2(1)	IV-11 III-2, III-9, IV-7, IV-13 III-3 IV-1	- 1-1(4) 1-2(1) 1-3(1)	- 1-1(4) - -
D2S293	III-10 III-6, III-15, IV-6, IV-10, IV-12 IV-16	1-1(1) 1-3(5) 1-2(1)	1-1(1) - -	IV-11 III-2, III-9 III-3, IV-7, IV-13 IV-1	1-1(1) 1-3(2) 1-2(3) -	1-1(1) - - -
D2S163	III-6, IV-6, IV-10 III-10, III-15, IV-12, IV-16	1-1(3) 1-2(4)	1-1(3) -	III-2, III-9, IV-1, IV-7, IV-13 III-3, IV-11	1-2(5) 1-3(2)	- -
D5S822	III-15, IV-6 III-6, III-10, IV-10, IV-12, IV-16	1-2(2) 1-1(5)	- 1-1(5)	III-2, III-9, IV-7 ,IV-11, IV-13 III-3, , IV-1	1-2(5) 1-1(2)	- 1-1(2)
D5S474	III-6, III-10, IV-6, IV-10, IV-12, IV-16 III-15	1-1(6) 1-2(1)	1-1(6)	III-2,III-3, IV-13 IV-1 IV-11 III-9, IV-7	1-1(3) 1-2(2) 1-3(2)	1-1(3)
D12S1691	III-6, III-10, III-15, IV-6, IV-10, IV-12, IV-16	1-1(7)	1-1(7)	III-2,III-3, III-9, IV-1IV-7, IV-11, IV-13	1-1(7)	1-1(7)

D15S519	III-15 IV-6 III-6, III-10, IV-10, IV-12, IV-16	1-2(1) 1-3(1) 1-1(5)	- - 1-1(5)	III-2,III-3, III-9, IV-1IV-7, IV-11, IV-13	1-3(7)	
D18S474	III-15, IV-6 III-6 IV-12 III-10, IV-10, IV-16	- 1-2(1) 1-3(1) 1-1(3)	 1-1(3)	IV-1 III-2, III-9, IV-11 III-3, IV-7, IV-13	- 1-2(3) 1-1(3)	 1-1(3)

Numbers in parenthesis indicate the number of women.

Family 6

The blood samples of the woman with PCO (II-4, II-9, III-3, III-7, III-10, IV-6) and non-PCOS woman (II-6, III-1, III-5, III-8, III-12, IV-2) were taken and DNA was extracted. The samples were numbered as given to the individuals in the pedigree. (Fig 6) The markers used were D1S514, D2S131, D2S293, D2S1352, D2S2335, D3S1263; D7S519, D11S911, D12S347, D12S1691, D15S519, D15S520, D17S1353, D17S934, D18S64, D18S474, D19S216, D19S884 and D19S391.

Co-segregation of D1S514 with the locus showed homozygosity (1-1) at allele 1 for these PCOS women (II-9, III-10) but heterozygosity (1-2) was seen at this locus in these PCOS women (II-4, III-3 and IV-6) as was seen in the non-PCOS women except one non-PCOS women (III-8) who was homozygous at this locus.

All of the PCOS women were heterozygous for D2S131 and D2S1352 at allele 1 except one (III-7) who was homozygous for D2S1352 at allele 1. PCOS women (II-4, II-9, III-3, III-7, IV-6) were homozygous for marker D2S2335 at allele 1, but others were heterozygous at this allele. There was amplification problem with other markers of chromosome No.2.

Markers for chromosome No. 3 were non informative. Similiarly, markers for chromosome No.7 and 11 were not informative in terms of consistency for homozygosity and heterozygosity at allele 1. Markers for chromosome No.12, 15, 17 were also non informative. Marker D18S64 showed homozygosity at this locus for both PCOS and non-PCOS women. Co segregation of this marker at this locus was informative and indicated association of PCOS with this locus.

Markers for chromosome No. 19 D19S216 and D19S884 were non-informative, D19S391 which was 28.8 centimorgan from insulin receptor gene has given homozygous 1-1 pattern with all PCOS (II-4, II-9, III-3, III-7, IV-6) and some of the non-PCOS (II-6, IV-2).

In family 6 (Table 28) genes segregating with different markers show homozygosity and heterozygosity evenly distributed. However, a weak association of homozygosity for allele 1 with marker D19S391 was seen ($X^2_{(1)}$ =3.45; P= ≥0.05).

Table 28: Genotypes, homozygosity and heterozygosity at different loci in PCOS and non-PCOS women in family 6.

Marker	Status PCOS women	Genotype	Homozygosity No.	Status Non- PCOS women	Genotype	Homozygosity No.
DIS514	II-9, III-10 II-4, III-3, IV-6 III-7 -	1-1(2) 1-2(3) - -	1-1 (2) - - -	III-8 III-12 IV-2 II-6, III-1, III-5	1-1(1) 2-2(1) 1-3(1) 1-2(3)	1-1 (1) - - -
D2S131	II-4, II-9, III-3, III-10 III-7, IV-6	1-3(4) 1-2(2)	- -	III-1, III-5, III-12, IV-2 II-6, III-8	1-3(4) 1-2(2)	- -
D2S1352	III-7, IV-6 II-4, III-3, III-10 II-9	1-1(2) 1-2(4) -	1-1(2) - -	IV-2 II-6, III-1, III-5, III-8, III-12	1-1(1) 1-2(5) -	1-1(1) - -
D2S2335	II-4, II-9, III-3, III-7, IV-6 III-10	1-1(5) 1-2(1)	1-1(5) -	II-6, III-1, III-8 III-5, III-12, IV-2	1-1(3) 1-2(3)	1-1(3) -
D2S293	II-4, II-9, III-3, III-7, III-10, IV-6	1-2(6)	-	II-6, III-1, III-5, III-8, III-12, IV-2	1-2(6)	-
D3S1263	II-4, IV-6 II-9, III-3, III-10 III-7	1-1(2) 1-2(3) -	1-1(2) - -	II-6, III-1, III-5, III-8, III-12, IV-2	1-2(6)	-
D7S519	III-3 II-4, II-9, III-7, III-10 IV-6	1-1(1) 1-2(4) 2-2(1)	1-1(1) - 2-2(1)	III-1, III-5, III-8, III-12, IV-2,II-6	1-1(4) 1-2(2)	1-1(4) -
D11S911	III-10 II-4, III-7 III-3 II-9, IV-6	- 1-3(2) 1-2(1) 1-1(2)	- - - 1-1(2)	III-12 II-6, III-5, III-8 III-1 IV-2	- 1-3(3) 1-2(1) 1-4(1)	- - - -
D12S347	II-4, II-9, , IV-6	1-2(3)	-	II-6	-	-

	III-3, III-7, III-10 -	2-2(3) -	2-2(3) -	III-1, III-5, III-8 III-12, IV-2	1-2(3) 2-2(2)	- 2-2(2)
D12S1691	II-4, II-9, III-3, III-7, III-10, IV-6	1-2(6)	-	II-6, III-1, III-5, III-8, III-12, IV-2	1-2(6)	-
D15S520	II-9, III-7, II-4, III-10, IV-6 III-3	1-1(2) 1-2(3) -	1-1(2) - -	II-6, III-8, III-1, III-5, III-12, IV-2	1-1(2) 1-2(4)	1-1(2) -
D15S519	II-4, II-9, III-3, III-7 III-10, IV-6	1-2(4) 1-1(2)	- 1-1(2)	III-5, III-8, III-12 IV-2 II-6, III-1	1-2(3) 1-1(1) 1-3(2)	- 1-1(1) -
HSD17ß2	II-4, II-9, III-3, III-7, III-10, IV-6	1-2(6)	-	II-6, III-1, III-5, III-8, III-12, IV-2	1-2(6)	-
D17S1353	II-4, II-9, III-3, III-7, III-10, IV-6	1-2(6)	-	II-6, III-1, III-5, III-8, III-12, IV-2	1-2(6)	-
D17S934	II-4, II-9, III-3, III-7, III-10, IV-6	1-1(6)	1-1(6)	II-6, III-1, III-5, III-8, III-12, IV-2	1-1(6)	1-1(6)
D18S64	II-4, II-9, III-3, III-7, III-10, IV-6	1-1(6)	1-1(6)	II-6, III-1, III-5, III-8, III-12, IV-2	1-1(6)	1-1(6)
D18S474	II-4, II-9, III-3, III-7, III-10, IV-6	1-2(6)	-	II-6, III-1, III-5, III-8, III-12, IV-2	1-2(6)	-
D19S216	II-9, III-7, IV-6 II-4 III-3, III-10	1-3(3) 1-1(1) 1-2(2)	- 1-1(1) -	IV-2 III-8 II-6, III-1, III-5, III-12,	1-3(1) 1-1(1) 1-2(4)	- 1-1(1) -
D19S884	II-4, II-9, , III-7, III-10, III-3, IV-6	1-2(4) 1-1(2)	- 1-1(2)	II-6, III-1, III-8, IV-2 III-5, III-12	1-2(4) 1-1(2)	- 1-1(2)
D19S391	II-4, II-9, III-3, III-7, IV-6 III-10	1-1(5) -	1-1(5)	II-6, III-12, IV-2 III-1, III-5, III-8,	1-1(3) 1-2(3)	1-1(3) -

Numbers in parenthesis indicate the number of women.

Relationship between clinical aspects and findings at molecular level.

Strong association of allele 1 with marker D15S519 (family 5) and marker D5S822 (family 2) was observed. Weak association of allele 1 with markers D19S391 (family 6) and D19S212 (family 1) was seen.

In family 5 candidate gene for marker locus D15S519 is CYP 11A (cytochrome P 450 side chain cleavage enzyme). This enzyme is involved in the synthesis of the steroid hormones, including adrenal and ovarian. Any mutation in CYP 11a results in increased androgen secretion by the adrenal and the ovary. The major circulating androgen is testosterone in PCOS. Its biosynthesis requires androgenic 17 β hydroxy steroid dehydrogenase activity, namely 3 and 5 17 β hydroxy steroid dehydrogenase. The 17 HSD gene is mainly expressed in testes, where it is essential for sexual differentiation and development, but it is not expressed in the adrenal glands or ovary. On the other hand, 17 β hydroxy steroid dehydrogenase 5 gene is widely expressed and is found in ovary and adrenal glands. An activating HSD 17 β 5 variant in hyperthecosis form of PCOS is caused by profound β insulin resistance.

In this study, mean levels of testosterone (60.5±7.4) were the highest in PCOS women (in pre-menopausal state) in family 5 where strong association of allele 1 with markers locus D15S519 was seen compared to PCOS women in remaining five families. Likewise, mean insulin levels (14 ±0.75) in PCOS women (in pre-menopausal state) in family 5 were the highest compared to that in PCOS women in other families. Regression analysis of variance of concentration of testosterone in PCOS women showed a very highly significant increase in testosterone (b=6.63±0.49; F=182.2; P=0.0002) in pre-menopausal state. Among non-PCOS women significant increase in testosterone (b=4.75±0.72; F=43.31; P=0.0028) was also seen but this is less highly significant increase compared to PCOS women. It is suggested that the reason for significant levels of testosterone in non-PCOS women could be that they may be carriers for the syndrome/gene because they belong to PCOS families. However, this could be due to some genetic disposition of genetic influence in the family.

In PCOS women, mean concentration of insulin was also very highly significant (b=0.68±0.11; F=37.31; P=0.0036) compared to non-PCOS women (b=0.92±0.24; F=14.94; P=0.018).

A similar picture was also seen with mean concentration of LH which is very highly significant in PCOS women (b=0.68±0.11; F=37.31; P=0.0036) compared to non-PCOS women (b=0.92±0.24; F=14.94; P=0.018).

In family 2 strong association with marker D5S822 suggests that the candidate gene for PCOS disorder in this family is follistatin (FS). Follistatin is a single chain glycosylated polypeptide. This stimulates the secretion of FSH and may therefore arrest folliculogenesis (Rivier et al. 1991). Follistatin is expressed in many tissues including the ovary, pituitary, adrenal cortex and pancrease.

A follistatin gene mutation in PCOS patients may play a role in the functional impairment of FSH-granulosa cell axis (Urbenak et al. 1999). These authors are of the view that overexpression of follistatin will be expected to lead to increased ovarian androgen production and reduction in circulating FSH levels which are the characteristics of PCOS.

Various studies have shown that in PCOS women, LH pulse is usually higher in frequency and amplitude due to high GnRH pulstile release by the hypothalamus. Hypersecretion of LH occurs in 40 % of the women with PCOS. Several hypotheses have suggested to explain this hypersecretion of LH. These include increased pulse frequency of GnRH, increased pituitary gland sensitivity to GnRH, hyperinsulinemic stimulation of pituitary gland and disturbances of ovarian steroid feedback (Balen et al. 1993). The ovary secretes an additional factor, called inhibin. Inhibin suppresses FSH output by the pituitary and provides a further possible mechanism in control of FSH secretion. High levels of inhibin have been found in PCOS and this provides an additional means by which FSH is reduced (Ehremann et al. 1992). In family 2, mean testosterone levels (50.3±8.9) and mean LH levels (21.2±4.8) are very high. The characteristics of follistatin gene regarding increased secretion of androgens and hypersecretion of LH are in line with characteristics of PCOS women in family 2 where these women have high levels of testosterone and that of LH. Characteristics of family 5 and family 2 as a result indicate that candidate gene for the former family is CYP 11a and for the latter family candidate gene is follistatin (FS). Both the candidate genes arrest folliculogenesis, this is what we observe in the case of PCOS disorder.

Regarding the clinical features, PCOS women in family2 are mainly oligomenorrhea and secondary amenorrhea, overweight and obese, but one of them is normal for this

feature (III-14;Fig 2). In family 2, hirsutism grades are moderate and mild for PCOS and also they are subfertile except one who is unmarried family 2; III-14).

In family 5 majority of PCOS women are oligomenorrheic and one of them is with secondary amenorrhea. They also show obesity and overweight character. Hirsutism grade varies from mild to moderate.

This has been investigated that PCOS disorder is heterogeneous in mode of inheritance (Adams et al., 1986; Hull, 1987). Because of heterogeneous nature of PCOS, it has been suggested that it represents a range of disorders rather than a single entity (Simpson, 1992). This is what has been observed in family 5 and family 2 that candidate gene responsible for PCOS disorder is not one but these may be two different genes. This is suggested that different candidate genes controlling this disorder in different families.

Weak association of PCOS disorder with allele 1 was seen in family 1 (D19S212) and family 6 (D19S391). Here the candidate gene in the former family is INSL-3 (leydig insulin like protein 3) and in latter candidate gene is INSR (insulin receptor). In these two families, PCOS seems to be related to hyperinsulinemia. A possible explanation for the ovary stimulating actions of insulin in women with PCOS is that the post-receptor mechanism of insulin action in the ovary is augmented in some way perhaps by abnormality in cytochrome P 450c 17α activity that makes this enzyme complex more sensitive to insulin (Utiger, 1996).

Increased activity of cytochrome P 450c 17α in response to hyperinsulinemia causes hyperandrogenism in PCOS. Cytochrome P 450c 17α is bifunctional enzyme having both 17 α hydroxylase and 17, 20 lyase activity required for biosynthesis of androgens. This is current opinion that hyperinsulinemia in response to insulin resistance increases the activity of Cytochrome P 450c 17α which causes hyper-androgenemia. Whether the increase in Cytochrome P 450c 17α activity in women is inherited or acquired are not known. Other theory proposed is that women who have PCOS have a defect in Cytochrome P 450c 17α which makes enzyme susceptible to over stimulation by insulin.

DISCUSSION

DISCUSSION

This study was carried out on 6 large consanguineous families. Female members in these families were diagnosed for PCO syndrome. PCOS in these families was ascertained as recessive Mendelian mode of inheritance. There is no general agreement on mode of inheritance of PCOS. Different workers have suggested different mendelian modes of inheritance. Cooper et al., (1968), Ferriman and Purdie (1979), Lunde et al., (1989) and Carey et al., (1993) have investigated that segregation analysis results were consistent with autosomal dominant inheritance. Givens, (1988) have suggested an X-linked mode of inheritance of PCOS. The mode of inheritance of PCOS has not been firmly established. Although some researchers support a single dominant gene with high penetrance (Carey et al., 1993; Legro et al., 1998; Govind et al., 1999), others do not (Jahanfar et al., 1995). It has not been possible to establish conclusively the role of any particular gene or region. The lack of progress reflects in part the difficulties recognized in the analysis of complex genetic diseases, including heterogeneity of the PCOS phenotype, the likely contribution of multiple genes, and the uncertain role of the environment (Urbanek et al., 2005).

Prevalence of polycystic ovaries among siblings was too high to be explained by a simple dominant model as was indicated by Hague et al., (1988). Franks et al., (1997) observed that familial clustering of PCOS cases suggests that genetic factors play an important part in its etiology. They suggested an autosomal dominant trait in families with several cases of PCOS. Further detailed analysis of large families by them cast doubt on the mode of inheritance. They are of the opinion that autosomal dominant inheritance remains possible but a more complex aetiology seems more likely (Legro et al., 1995).

In the present study, 44 (34.6 %) women out of 122 were diagnosed for PCOS which is quite a high incidence compared to other studies. The clinical features in PCOS women studied were menstrual irregularities, hirsutism, obesity and sub-fertility. Incidence of PCOS women in different populations has been documented by various researchers. Goodarzi and Azziz (2006) have documented 6.5-8 % prevalence of PCOS in unselected women of reproductive age. Rodin et al., (1998) has reported the prevalence of PCOS in 52 % of Indian subcontinent Asian women because they observed significant association between PCOS and menstrual irregularity, infertility

and body hair distribution. Lowe et al., (2005) documented 17 % bilateral and 6 % unilateral PCO in Australian and Caucasian women. However, in this study, all women were with bilateral polycystic ovaries as observed. Lam et al., (2005) observed 95.6 % incidence of PCOS in women living in China complaining of menstrual problem, hirsutism or infertility. Carmina et al., (2006) based on metabolic syndrome in PCOS observed that 43-46 % of women were diagnosed for PCOS. However, incidence of PCOS was less in Italian women because metabolic syndrome was much lower than in USA.

The PCOS phenotype is complex, and genetic analysis will necessarily require an understanding of the possible physiologic mechanisms of the disease to search for candidate genes. Although the exact mechanism for the development of PCOS is not known, evidence indicates that alterations in the endocrine, paracrine, and autocrine control of ovarian folliculogenesis are involved (Odunsi and Kidd, 1999). This study also showed association of PCOS with follistatin in family 2.

Several studies have found evidence of familial aggregation of PCOS, supporting a genetic contribution to its etiology. (Cooper et al., 1968; Givens et al., 1988; Legro et al., 1998). More than 50 candidate genes have been considered or studied (Urbanek et al., 2002). Several pathways have been implicated in the etiology of PCOS. These include the metabolic or regulatory pathways of steroid hormone synthesis (Carey et al., 1994; Gharani et al., 1997), regulatory pathways of gonadotropin action (Franks, 1995), the insulin-signaling pathway (Dunaif et al., 1992; 1995; Ciaraldi et al., 1992), and pathways regulating body weight (Kiddy et al., 1992). In this study markers used were related to metabolic and regulatory pathways of gonadotropin action, steroid hormone, insulin action, energy regulation and obesity. Several genes from these pathways have been tested as candidate genes for PCOS (Gharani et al., 1997; Carey et al. 1994; Conway et al., 1994; Talbott et al., 1996; Sorbara et al., 1994; Taylor et al. 1992; Krook et al. 1994; 1996). In particular, in the insulin receptor gene *(INSR),* mutations have been identified in several rare syndromes that were characterized by hyperandrogenism and insulin-resistant diabetes mellitus. (Taylor et al., 1992; Krook et al., 1994; 1996). Although mutation analysis, linkage studies, and case control association studies have been carried out by workers with these candidate genes, evidence that any of them play a role in PCOS has not been replicated widely and is still inconclusive. These uncertainties are common in 'complex' genetic diseases, where identifying the contributing genes is made difficult by likely genetic

heterogeneity, environmental contributions, and multiple etiologies (Diamanti-Kandarakis et al., 2006).

Although several loci have been proposed as PCOS genes including CYP 11 A, insulin gene and a region near insulin receptor, evidence supporting linkage is not overwhelming (Franks et al., 1999;2000). The strongest case can be made for the region near insulin receptor gene, as it has been identified in 2 separate studies. However, the responsible gene at chromosome 19p13.3 remains to be identified (Dunaif, 2006; Franks, 2006).

The present study showed strong association of PCOS locus at chromosome 5 with marker D5S822 ($P<0.05$) in family 2 for allele 1. Urbanek et al., (2000) carried out their study in families from European descent (n=90),Caribbean (n=5), Mexican descent (n=2) African-american descent (n=1) and Asian-indian descent (n=1). They tested the association between PCOS and the alleles for 4 markers (D5S474, D5S822, D5S623 and the SNP in exon 6). In 249 familes were 324 affected individuals. The 2 markers with the largest X^2 values were the exon 6 variant (allele 1, $X^2$5; P=0.025) and D5S623 (allele 11, X^2=4.26; P=0.039) in TDT analysis. They compared the expression level of follistatin between PCOS and control women. No substantial difference in follistatin expression between PCOS and control women was found.

In family 5 highly significant association of PCOS locus with marker D15S519 at chromosome 15 for allele 1 was found in this study ($P<0.01$). Gharani et al., (1997) indicated that linkage results for CYP 11a provide support for the involvement of this gene in the etiology of PCOS/MPB. Their association data demonstrates that allele variants of CYP 11a mediate the development of hyperandrogenemia, which in turn is associated with PCOS and hirsutism (Diamanti-Kandarakis et al., 2000).

Franks et al., (2001) demonstrated the identification of follistatin gene as a potential disease locus. They indicated that CYP 11a appears to be a major susceptibility gene at least populations studied in U.K.

Linkage and association have been confirmed to be associated with both PCOS and testosterone levels in women with PCOS to CYP 11 A variant (Weikenheisser et al., 2000). Legro et al., (2002) has studied CYP 11A as nominally significant in their study. The strongest effect in transmission test was observed in the INSR region with marker D19S884 at allele 5 (P=0.02), but this was not significant after correction. While San Millan et al., (2001) have studied that CYP 11a does not play any

significant role in the pathogenesis of hirsutism and Hyperandrogenism in women from Spain.

Present study revealed a weak association of PCOS locus at chromosome 19 with marker D19S391 ($P \geq 0.05$) was seen in family 6 for allele 1. Very weak association of PCOS locus was observed in family 1 ($P > 0.1$) with marker D19S212 for allele 1.

Tucci et al., (2001) found that insulin receptor gene marker D19S884 was significantly associated with PCOS ($P = 0.001$). They suggested that a susceptibility gene for PCOS was located on chromosome 19 p13.3 in the insulin receptor gene region remains to be determined if this susceptibility gene is the insulin receptor gene itself or closely located gene. They are of the opinion that insulin stimulates androgen secretion from ovarian stroma. It is likely that INSR function in the ovary is involved in the gender susceptibility of PCOS. Urbanek et al., (2005) has clarified marker marker interactions by testing STR (D19S922) that also showed nominal evidence for association. When the transformation disequilibrium test (TDT) is conditioned on individuals with the wild type allele at D19S922, it still reveals highly significant transmission distortion for the D19S884 A8 allele, suggesting that the association signals arises from the latter. Florez, (2005) has concluded that an D19S884 allele show significant evidence for linkage and association specific to phenotype PCOS/HA

Screening of the insulin receptor gene was undertaken by Conway et al., (1994) taking into consideration tyrosine kinase domain of the insulin receptor gene in 22 patients, but no abnormalities were found. In another study (Talbot et al., 1996) molecular scanning of entire coding region of the gene was performed in 24 hyperinsulinaemic subjects with PCOS and no significant mutations were detected. They concluded that mutations of the insulin receptor gene are therefore unlikely to be a major cause of insulin resistance in PCOS. Dunaif and Thomas (2001) gave similar opinion in their study.

To date, candidate genetic loci, including CYP 17, CYP 11A, CYP 19, follistatin, insulin receptor, have been investigated by mutation detection linkage, and case control association studies. However, traditional linkage analyses have been confounded by the extreme phenotypic heterogeneity (Xita et al., 2002). In the search for the PCOS genes, several candidate genetic loci have been evaluated. No clear causal variant in the follistatin gene was identified upon sequencing its promoter and

coding regions and subsequent linkage and association studies yielded negative results. (Urbanek et al., 2000; Liao et al., 2000; Tucci et al., 2001).

There is vigorous debate about whether PCOS is fundamentally a neuroendocrine or ovarian disorder (Ehrmann et al., 1995). Evidence is accumulating in favor of the latter. Thus, polycystic ovary syndrome seems to usually arise as a complex genetic disorder in which an intrinsic ovarian genetic trait interacts with other congenital or cellular environmental factors to cause abnormal regulation (dysregulation) of steroidogenesis (Nelson et al., 1999).

Legro et al., (2002) concluded that different findings are consistent with the concept that a gene or several genes are linked to PCOS susceptibility. Because, mutations/genotypes associated with PCOS are rare, and their full impact on the phenotype are incompletely understood. The treatment implications for individually identified genetic variants is uncertain and must be addressed on a case by case basis.

REFERENCES

REFERENCES

Abbott DH, Dumesic DA, Franks S (2002). Developmental origin of polycystic ovary syndrome: a hypothesis. J Endocrinol; 174 (1): 1-5.

Adams J, Frank S and Polson D (1985). Multifollicular ovaries: clinical and endocrine features and response to pulsatile gonadotropin releasing hormone. Lancet; 2: 1375-1378.

Adams J., Polson D W and Franks S.(1986) Prevalence of polycystic ovaries in women with anovulation and idiopathic hirsutism. B. M. J.293; 355-359.

Adams JM, Taylor AE, Crowley Jr WF, Hall JE (2004). Polycystic ovarian morphology with regular ovulatory cycles: insight into pathophysiology of the polycystic ovarian syndrome. J Clin Endocrinol Metab;89: 4343-50.

Anttila L, Ding YQ, Ruutiainen K, Erkkola R, Irjala K, Huhtaniemi I (1991). Clinical features and circulating gonadotropin, insulin and androgen interactions in women with polycystic ovarian disease. Fertil Steril; 55: 1057-61.

Archard C, Thiers J (1921). Hirsuties and its link with glycolytic insufficiency (diabetes of bearded women) (in French). Bull Acad Natl Med; 86:51-85.

Ardaens Y, Robert Y, Lemaitre L, Fossati P, Dewailly D (1991). Polycystic ovary disease: contribution of vaginal endosonography and reassessment of ultrasonic diagnosis. Fertil Steril; 55: 1062-8.

Avvad CK, Holeuwerger R, Silva VC, Bordallo MA, Breitenbach MM (2001). Menstrual irregularity in the first postmenarchal years: an early clinical sign of polycystic ovary syndrome in adolescence. Gynecol Endocrinol; 15(3): 170-7.

Balen A H, Tan S L, and Jacobs H S (1993). Hypersecretion of luteinizing hormone-a significant cause of infertility and miscarriage. Br J Obstet Gynecol; 100: 1082-89.

Balen A H (1995). Polycystic ovary syndrome: spectrum of disorder in 1741 patients. Hum Reprod 10: 2107-2111.

Balen AH and Dungers D (1995). Pubertal maturation of the internal genitalia. Ultrasound in Obstetrics and Gynecology; 6: 164-5.

Balen AH, Conway G S, Kaltsas G, Techatrraisisak K, Manning P J, West C et al., (1995). Polycystic ovary syndrome: the spectrum of the disorder in 1741 patients. Hum Reprod; 8: 2107-11.

Balen AH, Conway G S, Kaltsas G, Techatrraisisak K, Manning P J, West C et al., (1995). Polycystic ovary syndrome: the spectrum of the disorder in 1741 patients. Hum reprod; 8: 2107-11.

Barbieri RL, Smith S, and Rayan K J 1988). The role of hyperinsulinemia in the pathogenesis of ovarian hyperandrogenism. Fertil steril; 50: 197-212.

Barnes R B (1998). The pathogenesis of polycystic ovary syndrome: lessons from ovarian stimulation studies. J Endocrinol. Invest; 21: 567-579.

Barth A H (1988). Alopecia and hirsuties: current concept in pathogenesis and management. Drugs;35: 83-91.

Bates W (1992). Nutritional aspects of infertility. In: Hammond MG, Talbert LM (eds). Infertility: A practical guide for the physician 3rd ed. London, Blackwell scientifics: 182-195.

Battaglia C, Regnani G, Mancini F, Iughetti L, Flamigni C and Venturoli S (2002). Polycystic ovaries in childhood: a common finding in daughters of PCOS patients. A pilot study. Human Reproduction; 17: 771-776.

Bennet R L, Sceplnhaus K A, Urich S B, O'Sulivan C K, Resta R G, Lochner-Doyle D, Markel D S, Vincent V, Sananish J (1995). Recommendations for standardized pedigree nomenclature. Am J human genetics; 6: 745-52.

Bernard D J, Burns K H, Haupt B, Matzuk MM and woodruff T K (2003). Normal reproductive function in INH BP/ P120 deficient mice. Mole cell boil; 23: 4882-4891.

Bili H, Laven J, Imani B, Eijkemans M J, Fauser BC (2001). Age related differences in features associated with polycystic ovary syndrome in normogonadotropic oligomenorrhic infertile women of reproductive years. Eur J Endocrinol;145: 749-55.

Borecki I B, Rice T, Pérusse L, Bouchard C, Rao D C (1994). An exploratory investigation of genetic linkage with body composition and fatness phenotypes: the Québec Family Study. Obes. Res.; 2:213-219

Bouchard C (1997). Genetics of human obesity: recent results from linkage studies. J Nutr.; 127: 1887-90.

Bray G A (1996). Leptin and Leptinomania. Lancet; 348: 140-41.

Bridge NA, Cooke A, Healy M J R, Hindmergh P C and Brooke C G. (1993). Standards for the ovarian volume in children and puberty. Fertil Steril; 60: 456-60.

Bringer J, Lefebvre P, Boulet F, Grigorescue F, Renard E, Hedan B, Orsetti A, Jaffiol C (1993). Body composition and regional fat distribution in polycystic ovary syndrome. Relationship to hormonal and metabolic profiles. Ann N Y Acad Sci; 687: 115-23.

Britzow T L, Lehtovirta M T, Vainamo U ((1996). The effect of weight reduction on gonadotropin, insulin and androgen metabolism in hyperandrogenic overweight infertile women. Hum Reprod; 11: 47-48.

Bucket WM, Bouzayen R, Watkin K L, Tulandi T and Tan S L (2003). Ovarian stromal echogenecity in women with normal and polycystic ovaries. Hum Reprod; 18: 598-603.

Burghen G A, Given J R, Kitabachi A E (1980). Correlation of hyperandrogenism with hyperinsulinism in polycystic ovarian disease. J Clin Endocrinol Metabol; 50: 113-16.

Burger C W, Korsen T, Van Kessel H, Van Dop P A and Schoemaker J (1985). Pulsatile luteinizing hormone patterns in the follicular phase of the menstrual cycle, polycystic ovary disease and non PCOD amenorrehea. J Clin Endocrinol Metab; 61: 1126-32.

Burger L L, Dalkin A C, Aylor K W, Workman L J, Haisenleder D J and Marshall J C (2001). Regulation of gonadotropin subunit primary transcripts reveals differential roles of GnRH and inhibin. Endocrinology; 142: 3435-3442.

Burton B T, Foster W R, Hirsh J and Itallie V (1985). Health implications of obesity: an NIH consensus development conference. Int J Obesity; 9: 155-70.

Campbell S, Monga A (ed)(2000). Disorders of the menstrual cycle. In Gynecology by ten teachers; 17th edition: 47-63.

Carey AH, Chan KL, Short F, White D, Williamson R, Franks S (1993). Evidence for a single gene effect causing polycystic ovaries and male pattern baldness. Clin Endocrinol ; 38: 653-8.

Carey A H, Waterworth D, Patel K, White D, Little J, Novelli P (1994). Polycystic ovaries and premature male pattern baldness are associated with one allele of steroid metabolism gene CYP 17. Hum Mol Genet; 3: 1873-6.

Carmina E, Lobo RA, (2001). Polycystic ovaries in hirsute women with normal menses. Am J Med;111: 602-6.

Carmina E, Orio F, Palomba S, Longo RA, Lombardi G, Lobo R A (2005). Ovarian size and blood flow in women with polycystic ovary syndrome (PCOS) and their correlations with some endocrine parameters. Fertil Steril; 84: 413-19.

Carmina E, Napoli N, Longo RA, Rini GB, Lobo RA (2006). Metabolic syndrome in polycystic ovary syndrome (PCOS): lower prevalence in southern Italy than in USA and the influence of criteria for the diagnosis of PCOS. Eur J Endocrinol; 154 (1): 141-5.

Carmina E, Rosato F, Janni M Rizzo and R A Longo (2006). Relative prevalence of different androgen excess disorders in 950 women referred because of clinical hyperandrogenism. Endocrinol Metab; 91: 2-6.

Caro J F, Kolaczynski J W and Nyce M R (1996). Decreased cerebrospinal fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance. Lancet; 348: 159-61.

Carroll R S, Corrigan A Z, Gharib S D et al, (1989). Inhibin, activin and follistatin: regulation of follicle stimulating hormone messenger ribonucleic acid levels. Mol Endocrinol; 3: 1969-76.

Chapman S C, Bernard D J, Jelen J, and Wooduff J K (2002). Properties of inhibin binding to beta glycan, INH B/P120 and activin type 2 receptors. Mol Cell Endocrinol; 196: 79-83.

Charnvises K, Weerakiet S, Tingthanatikul Y, Wansumrith S, Chanprasertyothin S, Rojanasakul A (2005). Acanthosis nigrans: clinical predictor of abnormal glucose tolerance in Asian women with polycystic ovary syndrome. Gynecol Endocrinol; 21: 161-4.

Ciaraldi T P, el-Roeiy A, Mader Z, Reichart D, Olefsky J M, Yen S S (1992). Cellular mechanisms in insulin resistance in polycystic ovarian syndrome. J Clin Endocrinol Metab;75: 577-83.

Clement K, Garner C, Hager J, Philippi A, LeDuc C, Carey A, Harris T J, Jury C, Cardon L R, Basdevant A, Demenais F, Guy-Grand B, North M, Froguel P (1996). Indication for linkage of the human OB gene region with extreme obesity. Diabetes; 45:687-690

Clement K C, Vaisse N, Lahlou A (1998). A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature; 392: 398-401.

Colilla S, Cox NJ, Ehrmann D A (2001). Heritability of insulin secretion and insulin action in women with polycystic ovary syndrome and first degree relatives. J Clin Endocrinol Metab;86 (5): 2027-31.

Comuzzie A G, Hixon J E, Almary L, Mitchell B D, Mahaney M C, Dyer T D, Stern M P, McCluer J W, Blangero J (1997). A major quantitative trait locus determining serum leptin levels and fat mass is located on human chromosome 2. Nat. Genet; 15: 273-76.

Conway GS, Jacobs HS, Holly JMP, Wass JAH (1990). Effects of luteinizing hormone, insulin, insulin like growth factor-1, and insulin like growth factor protein 1 in polycystic ovary syndrome. Clin Endocrinol; 33: 593-603.

Conway G S and Jacobs HS (1993). Clinical implications of hyperinsulinemia in women. Clin Endocrinol; 39: 623-32.

Conway G S, Avey C, and Rumsby G (1994). The tyrosine kinase domain of the insulin receptor gene is normal in women with hyperinsulinemia and PCOS.Hum Reprod; 9:1681-1683.

Conway G S, Avey C, Rumsby G (1994). The tyrosine kinase domain of insulin receptor gene is normal in women with hyperinsulinemia and polycystic ovary syndrome. Hum Reprod; 9: 1681-83.

Cooper HE, Spellacy WN, Prem KA, Kohen WD, (1968). Hereditary factors in stein leventhal syndrome. Am J Obstet Gynecol;100: 371-387.

Cresswell, J.L., Barker, D P., Osmond, C.et al. (1997) Fetal growth, length of gestation, and polycystic ovaries in adult life. Lancet; 350, 1131—1135.

Crosignani P G and Nicolosi A E (2001). Polycystic ovarian disease: heritability and heterogeneity.Hum Reprod; 7: 3-7.

Crum C P, Lester S C, Cotran R S (2003). The female genital system and breast. In: Robbins Basic Pathology; 7th edition: 679-718.

Dale P O, Tanbo T, Vaaler S, Abyholm T (1992). Body weight, hyperinsuliemia and gonadotropins in polycystic ovary syndrome: evidence of two distinct populations. Fertil Steril; 58: 487-91.

Dalgren E, Johannson S, Lindstedt G (1992). Women with polycystic ovary syndrome wedge resected in 1956 to 1965: a long term follow up focusing on natural history and circulating hormones. Fertil Steril; 57: 505-13.

Devane W G, Czekala M N, Judd L H and Yen S CC (1975). Circulating gonadotropins, estrogens and androgens in polycystic ovary disease. Am J Obstet Gynecol; 121: 496-500.

Diamanti-Kandarakis E, Bartzis MI, Berglie A T, Tsianateli T C, Kouli C R (2000). Microsatellite polymorphism (ttta)(n) at-528 base pairs of gene CYP 11A influences hyperandrogenemia in patients with polycystic ovary syndrome. Fertil Steril; 73: 735-41.

Diamanti-Kandarakis E, Piperi C, Spina J, Argyrakopoulou G, Papanastasiou L, Bergiele A, Panidis D (2006). Polycystic ovary syndrome: the influence of environmental and genetic factors. Hormones (Athens); 5(1): 17-34.

Duggirala R, Stern M P, Mitchell B D, Reinhart L J, Shipman P A, Uresandi O C, Chung W K, Leibel R L, Hales C N, O'Connell P, Blangero J (1996). Quantitative variation in obesity-related traits and insulin precursors linked to the OB gene region on human chromosome 7. Am. J. Human Genet; 59:694-703.

Dunaif A, KR Segal, W Futterweit and Dobrjanksy (1989). Profound peripheral insulin resistance, independent of obesity, in Polycystic ovary syndrome. Diabetes; 38: 1165-74.

Dunaif A, Segal KR, Shelly DR, Green G, Dobrjansky A, Lincholai T (1992). Evidence for distinctive and intrinsic defects in insulin action in polycystic ovary syndrome. Diabetes; 41: 1257-66.

Dunaif A (1993). Insulin resistance and ovarian dysfunction. In: Moller, D. (ed). Insulin resistance. Wiley, New york: 301-25.

Dunaif A, Xia J, Book C B (1995). Excessive insulin receptor phophorylation in culture fibroblasts and in skeletal muscle. J Clin Inves; 96: 801-10.

Dunaif A, Finegood D T(1996). Beta cell dysfunction independent of obesity in polycystic ovary syndrome. J Clin Endocrinol Metab; 81: 942-47.

Dunaif A (1997). Insulin resistance and polycystic ovary syndrome: mechanism and implications for pathogenesis. Endocrinol Rev.; 18: 774-800.

Dunaif A (1999). Insulin action in polycystic ovary syndrome. Endocrinol. Metabol Clin N Am;28: 341-59.

Dunaif A (2006). Insulin resistance in women with polycystic ovary syndrome. Fertil Steril; 86: 13-4.

Edman C D and MacDonald P C (1978). Effect of obesity on conversion of plasma androstenedione to estrone in ovulatory and anovulatory young women. Am J Obstet Gynecol; 130: 456-61.

Ehrmann D A, BarnesR B and Rosenfeld R L (1989). Hyperandrogenism, hirsutism and polycystic ovary syndrome In: Textbook of endocrinology (De Groot J, Ist ed.). W B Saunders Co., Philadelphia; 2093-2107.

Ehrmann D A, and Rosenfield R L (1992). Detection of functional ovarian hyperandrogenism in women with androgen excess. N Engl J Med; 157-62.

Ehrmann D A, Sturis J, Byrne M M, Karrison T, Rosenfield R L, Polonsky K S (1995). Insulin secretary defects in polycystic ovary syndrome. Relationship to insulin sensitivity and family history of non-insulin dependent diabetes mellitus. J Clin Invest;96: 520-27.

Ehremann DA (2005). Medical progress. Polycystic ovary syndrome. N Engl J Med;352: 1223-36.

Eisenbruch S, Benson S, Hahn S, Tan S, Mann K, Pleger K, Kimig R, Janssen O E (2006). Determinants of emotional distress in women with polycystic ovary syndrome. Hum Reprod; 21: 1092-9.

Elting MW, Korsen TJ, Schoemaker J (2001). Obesity, rather than menstrual cycle pattern or follicle cohort size, determines hyperinsulinemia, dyslipidemia and hypertension in aging women with polycystic ovary syndrome. Clin Endocrinol; 55: 767-76.

Elting MW, Kwee J, korsen TJ, Rekers Momberg LT, Schoemaker J (2003). Aging women with polycystic ovary syndrome who achieve regular menstrual cycles have a smaller follicle cohort than those who continue to have irregular cycle. Fertil Steril; 79: 1154-60.

Erdem A, Erdem M, Biberoglu K, Hayit O, Arsalan M, Gursoy R(2002). Age related changes in ovarian volume, antral follicle counts and basal FSH in women with normal reproductive health. J Reprod Med ;47: 835-39.

Erel C T, Senturk L M, Oral E and Colgar U (1998). adrenal adrenergic response 2 h ACTH stimulation test in women with PCOS. Gynaecol. Endocrinol; 12: 223-229.

Essah P A, Nestle J E (2006). Metabolic syndrome in women with polycystic ovary syndrome. Fertil Steril; 86: 18-9.

Fauser B C and D Jong (1993). Gonadotropins in polycystic ovary syndrome . Ann N Y Acad Sci; 687: 150-161.

Fauser (2004). Revised 2003 consensus on diagnostic criteria and long term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod;19 (1): 41-47.

Ferriman D, Gallway J D (1961). Clinical assessment of body hair growth in women. J Clin Endocrinol Metab; 21: 1440-47.

Ferriman D and Purdie A W (1979). The inheritance of polycystic ovarian disease and possible relationship to premature balding. Clin. Endocrinol;11: 291-300.

Filicori M (1999). The role of LH in folliculogenesis and ovulation induction. Fertil Steril; 71: 405-13.

Florez J C (2005. Genetic susceptibility for polycystic ovary syndrome on chromosome 19: advances in the genetic dissection of complex reproductive traits. The J Clin Endocrinol and Metab; 90: 6732-6734.

Franks S (1989). Polycystic ovary syndrome: a changing perspective. Clin. Endocrinol; 31: 87-91.

Franks S (1991). The ubiquitous polycystic ovary. J Endocrinol;129; 317-19.

Franks S (1995). Polycystic ovary syndrome. N Engl J Med.; 333: 853-61.

Franks S., Gharani N., Waterworth D, Batty S, Davinia W, Williamson R, McCarthy M (1997). The genetic basis of polycystic ovary syndrome. Hum reprod; 12: 2641-2648.

Franks S, Gilling-Smith C, Watson H, Willis D (1999). Insulin action in the normal and polycystic ovary. Endocrinol Metabol Clin North Am; 28: 361-78.

Franks S, Mason D, and Willis D (2000). Follicular dynamics in polycystic ovary syndrome. Mol. Cell Endocrinol; 163: 49-52.

Franks S, Gharani N, McCarthy M (2001). Candidate genes in polycystic ovary syndrome. Hum Reprod; 7: 405-10.

Franks S (2006). Candidate genes in women with polycystic ovary syndrome. Fertil Steril; 86 (1): 15.

Fujimura H, Ohsawa K, Funaba M, Murata T, Murata E, Takahashi M, Abe M and Torii K (1999). Immunological localization and ontogenetic development of inhibin alpha subunit in rat brain. J Neuroendocrinol; 11: 157-163.

Fulghesu A M, campelli M, Belosi C, Rosanna Apa, Virginia Pavone, Antonio Lanzone (2001). A new ultrasound criterium for the diagnosisof polycystic

ovary syndrome: predictivity of stroma/total area ratio. Fertil Steril;76: 326-31.

Gambineri A, Pelusi C, Vicenneti V, Pagotto U, Pasquali R (2002). Obesity and polycystic ovary syndrome. Int J Obesity Related Disorders; 26: 883-96.

Gharani N, Waterworth DM, Batty S, White D, Gilling smith C, Conway GS, Franks S, Williamson R (1997). Association of steroid synthesis gene CYP11 A with polycystic ovary syndrome and hyperandrogenism. Hum Mole Genet; 6: 397-402.

Gilling-Smith C, Willis DS, Beard RW, Franks S (1994). Hypersecretion of androstenedione by isolated theca cells from polycystic ovaries. J Clin Endocrinol Metab; 79: 1158-1165.

Giudice L C (2006). Endometrium in PCOS: implantation and predisposition to endocrine carcinoma. Best Prac Res Clin Endocrinol Metab; 20 (2): 235-44.

Givens JR (1988); . Familial polycystic ovary disease. Endocrinol Metab clin N Am; 17: 771-783.

Gnoth C, Godehardt E, Herrman P F, Friol K, Jurgen Tigges and Freund G (2005). Definition and relevance of sub-fertility and infertility. Hum Reprod; 20 (5): 1144-47.

Goodarzi M O, Azziz R (2006). Diagnosis, epidemiology and genetics of polycystic ovary syndrome. Best Prac Res Clin Endocrinol Metab; 20 (2): 193-205.

Govind A, Obhrai M S and Clayton R N (1999). Polycystic ovaries are inherited as an autosomal dominant trait: analysis of 29 polycystic ovary syndrome and 10 control families. J Clin Endocrinol Metab; 84 (1): 38-43.

Gua Q, Kumar T R, Woodruff T et al., (1998). Overexpression of mouse follistatin causes reproductive defects in transgenic mice. Mol Endocrinol;12: 96-106.

Hague W M, Adams J, Reeders S T (1988). Familial polycystic ovaries: a genetic disease? Clin Endocrinol; 29: 593-605.

Hamilton J B (1942). Male hormone stimulation is a prerequisite and an incitant in common baldness. Am j Anat; 71: 451-480.

Handelsman D J (2006). Aging in the hypothalamic-pituitary-testicular axis. In: Knobil and Neill's physiology of reproduction. Eds: Neill J D; 3rd ed: 2697-2728.

Hart R, Hickey M, Franks S (2004). Definition, prevalence and symptoms of polycystic ovary syndrome. Best Prac Res Clin Obstet Gynaecol; 18 (5): 671-83.

Hashimoto O, Nakamura T, Shoji H et al., (1997). A novel role of follistatin, an activin binding protein, in the inhibition of activin action in rat pituitary cells. Endocytotic degradation of activin and its acceleration by follistatin associated with cell surface heparin sulphate. J Biol. Chem.; 272: 13835-13842.

Heinonen S, Korhonen S, Hippelainen M, Hiltunen M, Mannermaa A and Saarikoski S (2001).Apolipoprotein E alleles in women with Polycystic ovary syndrome; Fertil and Steril 75: 878-880.

Hernandez E (1996). The ovary: Normal physiologic changes, endometriosis and metastatic tumours. In: Clinical gynecologic pathology(Atikinson F B and Atikinson H eds). W B Saunders Co., Philadelphia USA: 414-415.

Hogeveen K N, Cousin P, Pugeat M, Dewailly D, Soudan B, Hammond G L (2002). Human sex hormone-binding globulin variants associated with hyperandrogenism and ovarian dysfunction. J Clin Invest ; 109: 973-81.

Holte J (1996). Disturbances in insulin secretion and insulin sensitivity in women with polycystic ovary syndrome. Clin Endocrinol Metabol; 10: 221-47.

Hull MC (1987). Epidemiology of infertility and polycystic ovarian disease: endocrinological and demographic studies. Gynecol Endocrinol.; 1: 235-45.

Hutton C and Clark F (1984). Polycystic ovarian syndrome in identical twins. Postgrad Med J; 60: 64-65.

Ibanez L,de Zeghar F, Potau N (1999). Anovulation after precocious pubarche: early markers and time course in adolescence. J Clin Endocrinol Metab; 84: 2691-5.

Insler V, Shoham Z, Barash A, Koistinen R, Seppala M, Hen M, et al (1993). Polycystic ovaries in non obese and obese patients: possible pathophysiological mechanism based on new interpretation of facts and findings. Hum Reprod; 8: 379-84.

Jahnfar S, Edens J A, Warrens P (1995). A twin study of polycystic ovary syndrome. Fertil Steril; 63: 478-86.

Jequier E (2002). Leptin signaling, adiposity and energy balance. Ann N Y Acad Sci; 967: 379-88.

Joans C Lo, Seth L Feigenbaum, Jingrong Yang, Alice R Pressman, Joe V Selby and Alan S Go (2006). Epidemiology and adverse cardiovascular risk profile of

diagnosed polycystic ovary syndrome. J of Clinical Endocrinol and Metab; 91 (4): 1357-63.

Kabir M, Catalano KJ, Ananthnarayan S, Kim S P, Van Citters G W, Dea M K, Bergman R N (2005). Molecular evidence supporting the portal theory: a causative link between visceral adiposity and hepatic insulin resistance. Am J Physiol Endocrinol Metab; 288: 454-461.

Kahn CR, Flier JS, Bar RS, Archer JA, Gorden P, Martin MM, Roth J (1976). The syndrome of insulin resistance end acanthosis nigricans. N Engl J Med; 294:739-745.

Kahn C R and White M F (1988). The insulin receptor and the molecular mechanism of insulin action. J Clin Invest; 82: 1151-56.

Kauffman RP, Baker M, DiMarino P, Castracane V D (2006). Hyperinsulinemia and circulating dehydroepiandrosterone sulfate in white and Mexican American women with polycystic ovary syndrome. Fertil Steril; 85 (4): 1010-6.

Kent S C and Legro R S (2002). Polycystic ovary syndrome in adolescents. Adolescent medicine state of art Reviews; 13: 73-88.

Kiddy D S, Hamilton Fairly D, Bush A (1992). Improvement in endocrine and ovarian function during dietary treatment of obese women with olycystic ovary syndrome. Clin Endocrinol (oxf); 36: 105-111.

Kiddy DS, Himilton Fairley D, Bush A Short F, Amyaoku V, Reed MJ, Franks S (1992). Improvement in endocrine and ovarian function during dietary treatment of obese women with polycystic ovary syndrome. Clin Endocrinol; 36: 105-11.

Kousta E, Tolis G, Franks S (2005). Polycystic ovary syndrome. Revised diagnostic criteria and long term health consequences. Hormones; 3: 133-147.

Krook A, Kumar S, Laing I, Boulton A J, Wass J A, O'Rahilly S (1994). Molecular scanning of insulin receptor gene in syndromes of insulin resistance. Diabetes;43: 357-68.

Krook A, O'Rahilly S (1996). Homozygous mutation in the insulin resistance. Baillieres Clin Endocrinol Metab; 10: 97-122.

Kruglyak L and Lander E S (1995). Complete multipoint sib-pair analysis of quantitative and qualitative traits. Am J Hum Gene;57: 439-454.

Lam P M, Ma R C, Cheung L P, Chow C C, Chan J C, Haines C J (2005). Polycystic ovarian syndrome in Hong Kong Chinese women: patient characteristics and diagnostic criteria. Hong Kong Med J; 11 (5): 336-41.

Lane DE (2006). Polycystic ovary syndrome and its differential diagnosis.Obstet Gynecol Surv.; 61 (2): 125-35.

Laven J S, Imani B, Eijkemans M J, Fauser B C (2002). New approaches to PCOS and other forms of anovulation. Obstet Gynecol Surv; 57: 755-67.

Legro RS (1995). The genetics of polycystic ovary syndrome. Am J Med; 98: 9-15.

Legro R S, Fingood D and Dunaif A (1998a). A fasting Glucose to insulin ratio is a useful measure of insulin sensitivity in women with polycystic ovary syndrome. J Clin Endocrinol and Metab;83: 2694-98.

Legro RS, Driscoll D, Jerome F, Strauss JF and Dunaif A (1998b) evidence for a genetic basis for hyperandrogenemia in polycystic ovary syndrome. Proc Natl Acad Sci;95: 14956-960.

Legro RS, Kunselman AR Dodson WC, Dunaif A (1999). Prevalence and predictors of risks for type 2 diabetes mellitus and impaired glucose tolerance in polycystic ovary syndrome: a prospective controlled study in 254 affected women. J Clin Endocrinol Metab; 84: 165-69.

Legro R S and Strauss J F. (2002). Molecular progress in infertility: polycystic ovarian syndrome. Fertil and Steril; 78;3: 569-576.

Legro R S, Urbanek M, Kunselman AR, Leiby BE, Dunaif A (2002). Self selected women with polycystic ovary syndrome are reproductively and metabolically abnormal and untreated. Fertil Steril; 78: 51-57.

Legro R S, Lewis R , Driscol D, Wang S C and Dunaif A (2002). Insulin resistance in sisters of women with polycystic ovary syndrome: association with hyperandrogenism rather than menstrual irregularity. J of Clin Endocrinol and Metabol; 87: 2128-33.

Legro R S, Percy Chiu, Allen R, Kunselman, Christina M Bentley, William C Dodson and Andrea Dunaif (2005). Polycystic ovaries are common in women with hyperandrogenic chronic anovulation but do not predict metabolic or reproductive phenotype. J Clin Endocrinol Metab; 90: 2571-79.

Lewis T L T and Chamberlain G V P(1990). Gynecology by ten teachers, Edward Arnold Kent; 143-44.

Li TC, Saravelos H, Chow MS, Chisabingo R, Cooke ID. Factors affecting the outcome of laproscopic ovarian drilling for polycystic ovarian syndrome in women with anovulatory infertility. Br J Obstet Gynecol 1998; 105: 338-44.

Liao W X, Roy A C and Ng S C (2000). Preliminary investigation of follistatin gene mutations in women with polycystic ovary syndrome. Mole Hum Reprod;6: 587-590.

Lobo R A (1991). Hirsutism in polycystic ovary syndrome: current concepts. Clin Obstet Gynecol; 34: 817-26.

Lobo RA, Carmina E (2000). The importance of diagnosing the polycycstic ovary syndrome. Ann Intern Med; 132 (12): 989-93.

Lowe P, Kovacs G, Howlett D (2005). Incidence of polycystic ovaries and polycystic ovarian syndrome amongst women in Melbourne, Australia. Aust N Z J Obstet Gynecol; 45 (1): 17-9.

Lunde O, Magnus P, Sandvik L, Hoglo S (1989). Familial clustering in the polycystic ovarian syndrome. Gynecol Obstet Invest;28: 23-30.

MacDonough P G, Mahesh V B, Ellegood J O (1972). Steroid, follicle stimulating hormone and luteinizing hormone profiles in identical twins with polycystic ovary syndrome. Am J obstet Gynecol; 113: 1072-78.

Mao W, Li M, Chen Y, Lu C, Wang Y, Zhang X, Qiao, Wang A (2001). Study on mode of inheritance for familial polycystic ovary syndrome. Zhonghua Yi Xue Yi Chuan Xue Za Zhi; 18 (1): 21-3.

Marsden P J, Murdoch A and Taylor R (1994). Severe impairment of insulin action in adipocytes from amenorrheic subjects with PCOS. Metabolism; 43: 1536-42.

Mather J P, Moore A, and Li R H (1997). Activins, inhibins and follistatins: further thoughts on a growing family of regulators. Proc. Soc. Exp. Biol. Med; 215: 209-222.

Meirow D, Rz I, Yossepowitch O, Brzezinski A, Rosler A, Schenker J G, Berry E M (1996). Dyslipidemia in polycystic ovarian syndrome: different groups, different etiologies. Hum Reprod; 11: 1848-53.

Mifsud A, Ramirez S, Yong E L (2000). Androgen receptor gene cag trinucleotide repeats in anovulatory infertility and polycystic ovaries. J Clin Endocrinol Metab; 85: 3484-8.

Minanni L S, Cavaleiro M A, Yezozzo P D and Neto G D(1999). Analysis of gonadotropin pulsality in hirsute women with normal menstrual cycle and in women with PCOS. Fertil Steril;7: 675-83.

Moghetti P, Castello R, Negri C, Tosi F, Spiazzi G G, Brun E, Balducci R, Toscano V, Muggeo M (1996). Insulin infusion amplifies 17 alpha-hydroxy-corticosteroid intermediates response to adrenocorticotropin in hyperandrogenic women: apparent relative impairment of 17, 20-lyase activity. J Clin Endocrinol Metab; 81: 881-886.

Moller D E, Flier J S (1991). Insulin resistance: mechanisms, syndromes and implications. N Engl J Med; 325: 938-48.

Montague C T, Farooqi I S, Whitehead J P, Soos M A, Rau H, Wareham N J, Sewter C P, Digby J E, Mohammad S N, Hurst J A, Cheetham C H, Earley A R, Barnett A H, Prins J B, O' Rahlly S (1997). Congenital leptin deficiency is associated with severe early onset obesity in humans. Nature; 387: 903-908.

Morales AJ, Laughin GA, Butzow T, Maheshwari H, Baumann G, Yen SCC (1996). Insulin, somatotropic and luteinizing hormone axes in lean and obese women with polycystic ovary syndrome: common and distinct features. J Clin Endocrinol Metab; 81: 2854-64.

Murry J C, Buetow K H, Weber J L, Ludwigsen S L, Scherphir Heddema T, Manion F, Quillen J, Sheffield V C, Sunden S, Duyk G M (1994). A comprehensive human linkage map with centimorgan density. Cooperative human linkage centre (CHLC). Science; 265 (5181): 2049-54.

Najmabadi S, Wilcox JG, Acacio BD, Thornton MH, Kolb BA, Paulson RJ (1997). The significance of polycystic appearing ovaries versus normal appearing ovaries in patients with polycystic ovary syndrome. Fertil Steril;67:631-35.

Nazir F, Saeed S, Malik M, Aziz H, Aliya S and Rana S. (1999) Polycystic ovary syndrome-Diagnosis and management in fertility deprivation. Pak J Obstet Gynecol; 12: 59-70.

Nelson V L, Legro R S, Strauss J F, Mc Allister J M (1999). Augmented androgen production is a stable steroidogenic phenotype of propagated theca cells from polycystic ovaries. Mol Endocrinol; 13: 946-57.

Nestler JE, Jakubowitz DJ (1996). Decreases in ovarian cytochrome P 450c 17 alpha activity and serum free testosterone after reduction of insulin secretion in polycystic ovary syndrome. J Engl J Med; 335: 617-23.

Norman R A, Thompson D B, Foroud T, Garvey W T, Bennett P H, Bogardus C, Ravussin E and other members of the Pima Diabetes Gene group (1997). Genomewide search for genes influencing percent body fat in Pima Indians: suggestive linkage at chromosome 11q21-q22. Am. J. Hum Genet.; 60:166-173.

Norris JM, Langefeld C D, Scherzinger A L, Rich S S, Bookman E, Beck S R, Saad M F, Haffner S M, Bergman R N, Bowden D W, Wagenknecht LE (2005). Quantitative trait loci for abdominal fat and BMI in Hispanic-Americans and African-Americans: the IRAS family study. Int J Obes Relat Metab Disord;29: 67-77.

Odunsi K and Kidd K K (1999). A paradigm for finding genes for a complex human trait: polycystic ovary syndrome and follistatin. Proc Natl Acad Sci; 96: 8315-8317.

Oin K, Cox E R, Refetoff S and Rosenfield R L (2005). Identification of a functional polymorphism of the human type 5 17 βhydroxy steroid dehydrogenase gene associated with polycystic ovary syndrome. J Clin Endocrinol Metab; 91: 270-276.

Oppermann K, Fuchs SC, Spritzer PM 2003). Ovarian volume in pre- and peri-menopausal women: a population based study. Menopause;10: 209-13.

Pache TD, de Jong FH, Hop WC, Fauser BC (1993). Association between ovarian changes assessed by transvaginal sonography and clinical and endocrine signs of the polcystic ovary syndrome. Fertil Steril;59: 544-49.

Paul M, Stephen K, Azziz R (2006). Family size in women with polycystic ovary syndrome. Fertil Steril; 85: 1837-9.

Perloe et al., (2000). Polycystic ovary syndrome: treatment with insulin lowering medications. Web page.

Porcu E, Venturoli S and Magrini O (1987). Circadian variation of luteinizing hormone can have two different profiles in adolescent anovulation. J Clin Endocrinol Metab; 65: 488-93.

Poreteky L (1991). On the paradox of insulin-induced hyperandrogenism in insulin-resistant states. Endocrinol Rev;12:3-11.

Potau N., IbanezL., Rique S., Sanchez-Urafat C., deZeghar F.(1999). Pronounced adrenarche and recocious pubarche in boys. Horm Res; 51: 238-41.

Paul M, Stephen K, Azziz R (2006). Family size in women with polycystic ovary syndrome. Fertil Steril; 85: 1837-9.

Perloe et al., (2000). Polycystic ovary syndrome: treatment with insulin lowering medications. Web page.

Porcu E, Venturoli S and Magrini O (1987). Circadian variation of luteinizing hormone can have two different profiles in adolescent anovulation. J Clin Endocrinol Metab; 65: 488-93.

Poreteky L (1991). On the paradox of insulin-induced hyperandrogenism in insulin-resistant states. Endocr Rev;12:3-11.

Potau N., IbanezL., Rique S., Sanchez-Urafat C., deZeghar F.(1999). Pronounced adrenarche and recocious pubarche in boys. Horm Res; 51: 238-41.

Prelevic G M (1997). Insulin resistance in polycystic ovary syndrome. Current Opinion in Obstetric and Gynecology; 9:193-201.

Prelevic G M (1997). Insulin resistance in polycystic ovary syndrome. Curr Opin Obstet Gynecol; 9: 193-201.

Rajkovic A, Pangas S A and Matzuk M M (2006). In: Knobil and Neill's Physiology of Reproduction. Neill J D(eds). Follicular development: mouse, sheep and human models.; 3rd ed: 383-423.

Randall V A, Thorton M S, Hamada K, Messanger A G(1992). Mechanism of androgen action in cultured dermal papilla cells derived from human hair follicles in varying response to androgen in vivo. J Inves Dermatol;98; 865-915.

Reed D, Ding Y, Xu W, Cather C, Green E D, Price R A (1996). Extreme obesity may be linked to markers flanking the human OB gene. Diabetes; 45:691-694.

Rivier C, and Vale W (1991). Effects of recombinant activin A on gonadotropin secretion in the female rat. Endocrinology; 129: 2463-65.

Robinson S, Kiddy D, Gelding S V, Willis D S, Niththyananthan R, Bush A and Franks S (1993). The relationship of insulin insensitivity to menstrual pattern in women with hyperandrogenism and polycystic ovaries. Clin Endocrinol; 39: 351-55.

Rodin D A, Bano G, Bland J M, Taylor K, Nussey S S (1998). Polycystic ovaries and associated metabolic abnormalities in Indian subcontinent Asian women. Clin Endocrinol (Oxf); 49(1): 91-9.

Sampson M, Kong C, Patel A, Unwin R, Jacobs HS (1996). Ambulatory blood pressure profiles and plaminogen activator inhibitor (PAI-1) activity in lean women with and without polycystic ovary syndrome. Clin Endocrinol; 45: 623-29.

San Millan J L, Sancho J, Calvo R M, Escoban Morsaele H F (2001). Role of pentanucleotide (ttta)(n) polymorphism in promoter of CYP 11A gene in the pathogenesis of hirsutism. Fertil Steril; 75 (4): 797-802.

Sattar N, Hopkinson Z E and Greer I A (1998).Insulin sensitizing agents in polycystic ovary syndrome. Lancet, 351, 305-307.

Schildkraut J M, Schwingl P J, Bastos E, Evenoff A, Hughes C (1996). Epithelial ovarian cancer risk among women with polycystic ovary syndrome. Obstet Gynecol; 88(4): 554-9.

Schwall R H, Mason A J, Wilox J N, Bassett S G and Zelenik A J (1990). Localization of activin / inhibin subunit mRNA within the primate ovary. Mole Endocrinol; 4: 75-79.

Sepilian V P, Crochet JR, Nagamani M (2006). Serum soluble leptin receptor levels and free leptin index in women with polycystic ovary syndrome: relationship to insulin resistance and androgen. Fertil Steril; 85 (5): 1441-7.

Shamasaki M, Inouye S, Shamasaki S (1991). Follistatin binds to both activin and inhibin through the common subunit. Endocrinol; 128: 3313-3315.

Shera A S, Rafique G, Khawaja I A, Baqai S, and King H (1999). Pakistan national diabetes survey: prevalence of glucose intolerance and associated factors in Baluchistan province. Diabetes Research and Clinical Practice; 44: 49-58.

Shoham Z, Conway G S, Paul A, and Jacobs H S (1992). Polycystic ovaries in patients with hypogonadotropic hypogonadism: similarity of ovarian response to gonadotropin stimulation in patients with polycystic ovarian syndrome. Fertil Steril; 58: 37-45.

Sielfen M E, Denburg M R, Manibo A M, Lobo R A, Jaffe R, Ferin M, Levine L S and Oberfield S E (2003). Early endocrine, metabolic and sonographic characteristic of polycystic ovary syndrome (PCOS): comparison between non obese and obese adolescents. J Clin Endocrinol Metab; 88 (10): 4682-88.

Silva Rdo C, Pardini DP, Kater CE (2006). Polycystic ovary syndrome, metabolic syndrome, cardiovascular risk and the role of insulin sensitizing agents. Arg Bras Endocrinol Metabol; 50 (2): 281-90.

Simpson J L (1992). Elucidating the genetics of polycystic ovary syndrome. In: Dunaif A., Givens J R, Haseltine F P and Merriam G R (eds). Polycystic ovary syndrome. Blackwell Scientific, Oxford: 59-77.

Sorbara L R, Tang Z, Cama A, Xia J, Schenker E, Kohanski R A, Poretsky L, Koller E, Taylor S I, Dunaif A (1994). Absence of insulin receptor gene mutation in three insulin resistant women with polycystic ovary syndrome. Metabol;43: 1568-74.

Speroff L, Glass R H, and Kase N G (1994). Clinical gynecologic endocrinology and infertility. Williams and Wilkins, London; 302-320.

Stafford De, Gordon CM (2002). Adolescent androgen abnormalities. Current Opinion in Obstetrics & Gynecology; Oct.: 445-51.

Stankiewiez M, Norman R (2006). Diagnosis and management of polycystic ovary syndrome: a practical guide. Drugs; 66 (7): 903-12.

Stein I F, Leventhal M L. (1935). Amenorrhea associated with bilateral polycystic ovaries. Am. J. Obstet. Gynecol; 29: 181.

Stephen J, Carey A H, Short F, Williamson R and Franks S (1993). Premature balding the male phenotype of polycystic ovaries (PCO). J Endocrinol; 139: 031.

Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima R, Lazar MA (2001).the hormone resistin links obesity to diabetes. Nature; 409: 307-312.

Stirrat G M (1997). Fertility and Subfertility In: Aids to obstetrics and gynecology, Churchill Livingstone; 4: 202-15.

Suikkari AM, Ruutianen VA, Rutanen EM, Karonen SL, Seppala M (1988). Insulin regulates the serum levels of low molecular weight insulin like growth factor binding protein. J Clin Endocrinol Metab;66: 266-72.

Takahashi K et al., (1994). Relationship between ultrsonography and histopathological changes in polycystic ovarian syndrome. Hum Reprod; 9: 2255-2258.

Talbott J A, Bicknell E J, Rajkhowa M, Krook A, O' Rahilly S, Clayton R N (1996). Molecular scanning of insulin receptor gene in women with polycystic ovary syndrome. J Clin Endocrinol Metab;81:1979-83.

Taponen S, Ahonkallo S, Martikainen H, Koivunen R, Ruokonen A, Sovio U, Hartikainen AL, Pouta A, Laitinen J, King V, Franks S, Mc Carthy MI, Jarvelin M R (2004). Prevalence of polycystic ovaries in women with self

reported symptoms of oligomenoorhea and/or hirsutism: Northern Finland Birth Cohort 1966 study. Hum Reprod;19: 1083-88.

Tartalgia L A, Dembski M and Weng X (1995). Identification and expression cloning of a leptin receptor, Obes-Res Cell;83: 1-20.

Tsanadis G, Vartholomatos G, Korkontzelos I, Avgoustatos F, Kakosimos G, Sotiriadis A, Tatsioni A, Eleftheriou A and Lolis D (2002). Polycystic ovarian syndrome and thrombophilia. Hum Reprod;17:314-19.

Tucci S, Futterweit W, Concepcion ES, Greenberg DA, Villanueva R, Davies TF, Tomer Y (2001). Evidence for association of polycystic ovary syndrome in caucasian women with a marker at the insulin receptor locus. J Clin Endocrinol Metab;86: 446-449.

Tulandi T, Saleh A, Payne N, Jacobs H, Tan SL. Effects of laproscopic ovarian drilling on serum vascular endothelial growth factor (VEGF) and on insulin responses to oral glucose tolerance tests. Fertil Steril 2000; 74: 585-8.

Urbanek M, Legro R S, Driscoll D A, Azziz R, Ehrmann DA, Norman R J, Strauss J F, Spielman R S and Dunaif A (1999). Thirty seven candidate genes for polycystic ovary syndrome: strongest evidence for linkage is with follistatin. Proc Natl. Acad. Sci. 96; 8573-8578.

Urbanek M, Wu X, Vickery K R, Kao L C, Christenson LK, Scheneyer A, Legro R S, Driscoll DA, Strauss J F, Dunaif A, Spielman RS (2000). Allelic variants of follistatin gene in polycystic ovary syndrome. The J Clin Endocrinol Metab; 85: 4455-4461.

Urbanek M, , Spielman R S(2002). Genetic analysis of candidate genes for polycystic ovary syndrome. Curr Opin Endocrinol Diabetes;9: 492-501.

Urbanek M, Yangzhu Du, Silander K, Collins FS, Stephan C M, Strauss J F, Dunaif A, Spielman RS, Legro RS (2003). Variation on resistin gene promoter not associated with polycystic ovary syndrome. Diabetes; 52: 214-217.

Urbanek M, Woodroffe A, Ewens K G, Kandarakis ED, Legro RS, Strauss JF, Dunaif A, Spielman RS (2005). Candidate gene region for polycystic ovary syndrome on chromosome 19p13.2. J Clin Endocrinol Metabol; 90: 6623-6629.

Utiger RD (1996).Insulin and the polycystic ovary syndrome. J Engl J Med; 335: 657-58.

Van Santbrink E J P, Hop W C, Fauser B C J (1997). Classification of normogonadotropic infertility: polycystic ovaries diagnosed by ultrasound

versus endocrine characteristics of polycystic ovary syndrome. Fert Steril; 67: 452-8.

Vrbikova J, Bendlova B, Hill M, Vankova M, Vondra K, Starka L (2002). Insulin sensitivity and beta cell function in women with polycystic ovary syndrome. Diabetes; 25: 1217-22.

Wajeyaratne CN, Balen AH, Barth JH, Beichetz PE (2002). Clinical manifestations and insulin resistance in polycystic ovary syndrome (PCOS) among south Asians and Caucasians: is there a difference? Clin Endocrinol; 57: 343-50.

Waldstreicher J, Santoro N F, Hall J E, Filicori M and Crowley W F Jr (1988). Hyperfunction of hypothalamic pituitary axis in women with polycystic ovarian disease: indirect evidence for partial gonadotroph desensitization. J Clin Endocrinol Metab.;66: 165-80.

Waterworth D M, Bennett S T, Gharani N et al., (1997). Linkage and association of insulin gene VNTR regulatory polymorphism with polycystic ovary syndrome. Lancet; 349: 986-990.

Webber LJ, Stubbs S, Stark J, Trew GH, Margara R, Hardy K, Franks S (2003). Formation and early development of follicles in polycystic ovary. Lancet;362: 1017-21.

Weissenbach J, Gyapay G, Dib C, Vignal A, Morissette J, Millasseau P, Vaysseix G, Lathrop M (1993). A second generation linkage map of the human genome. Nature (London); 359: 794-801.

Welt CK (2002). The physiology and pathophysiology of inhibin, activin and follistatin in female reproduction. Current opinion Obstet Gynecol; 14 (3): 317-23.

Wickenheisser J K, Quinn P G, Nelson V L, Legro R S, Strauss J F 3rd , McAllister J M (2000). Differential activity of cytochrome P 450 alpha hydroxylase and steroidogenic acute regulatory protein gene promoters in normal and polycystic ovary syndrome theca cells. J Clin Endocrinol metab; 85: 2304-11.

Wickenheisser J K, Nelson Degrave V L, Hendricks K L, Legro RS, Strauss J F, Mc Allister J M (2005). Retinoids and retinol differentially regulate steroid biosynthesis in ovarian theca cells isolated from normal cycling women and women with polycystic ovary syndrome. J Clin Endocrinol Metab;90: 4858-4865.

Wickenheisser J K, Nelson Degrave V L, McAllister J M (2005). Dysregulation of cytochrome P 450 17 alpha hydroxylase messenger ribonucleic acid stability in theca cells isolated from women with polycystic ovary syndrome. J Clin Endocrinol Metab;90: 1720-27.

Willis D, Mason H and Gilling-Smith C (1996). Modulation by insulin of FSH and LH action in human granulosa cell of normal and polycystic ovaries. J of Endocrinol Metab; 81: 302-309.

Witchel S F, Lee P A, Suda-Hartman M, Smith R, Hoffman EP (1998). 17 alpha hydroxylase/ 17-20 lyase dysregulation is not caused by mutations in the coding regions of CYP 17. J Pediatr Adolesc Gynecol; 11: 133-7.

Wood J R, Nelson V L, Ho C, Jansen E, Wang C Y, Urbanek M, McAllister J M, Mosselman S, Strauss 3rd JF (2003). The molecular phenotype of polycystic ovary syndrome (PCOS) theca cells and new candidate PCOS genes identified by microarray analysis. J Biol Chem; 278: 26380-390.

Xiao S, Findly J K, (1991). Interaction between activin and follicle stimulating hormone suppression protein and their mechanism of action on cultured rat granulose cells. Mol Cell Endocrinol; 79: 99-107.

Xita N, Georgiou I, Tsatsoulis A (2002). The genetic basis of polycystic ovary syndrome. Eur J Endocrinol; 147 (6): 717-25.

Yen H C, Futterweit W and Thornton J C PCOD (1987). PCOD: ovarian ultrasound features in 104 patients, Radiology; 163: 111-16.

Young R L and Goldzieher J W. (1988). Clinical manifestation of polycystic ovarian disease. In: Endocrinol Metab Clin North Am (Mahajan D K ed). W B Saunders Co., London. 1988:17 (4): 560-574.

Zagar AH, Wani AI, Masoodi SR, Laway BA, Bashir MI, Salahuddin M (2002). Epidemiological and etiologic aspects of hirsutism in Kashmiri women in the Indian subcontinent. Fertil Steril;77: 674-8.

Zavaroni L, Dallagio E, Bonara E, Alpi O, Passeri M, Reaven GM (1987). Evidence that multiple risk factors for coronary artery disease exists in persons with abnormal glucose tolerance. Am J Med; 83: 609-12.

Zeleznik A J and Porl C R (2006). Control of follicular development, corpus luteum function, the maternal recognition of pregnancy and neuroendocrine regulation of the menstrual cycle in higher primates. In: Knobil and Neill's Physiology of Reproduction. Eds: Neill J D; 3rd ed: 2449-2510.

Zhang Y R, Proenca M, maffei M, Barone L, Leopold and J M Friedman (1994). Positional cloning of the mouse obese gene and its human homologue. Nature; 372: 425-32.

Zhang LH, Rodriguez H, Ohno S, Miller W L (1995). Serine phosphorylation of human P450C17 increases 17, 20 lyase activity: implications for adrenarche and polycystic ovary syndrome. Proc Natl Aca Sci; 92: 10619-623.

Zumoff B, Freeman R and Coupey S (1983). A chronobiologic abnormality in luteinizing hormone secretion in teenage girls with the polycystic ovary syndrome. N Engl J Med; 309: 1206-9.