MOLECULAR INVESTIGATION AND DETECTION OF PROTEIN BIOMARKER/S IN POLYCYSTIC OVARY SYNDROME

A THESIS SUBMITTED TO

THE UNIVERSITY OF THE PUNJAB

IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY IN MEDICINE (PHYSIOLOGY)

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UNIVERSITY OF THE PUNJAB
LAHORE, PAKISTAN
2015
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ACKNOWLEDGEMENTS

“Praise be to Allah The Cherisher and Sustainer of the Worlds”

First, and foremost, I pay all my praises to Al-Mighty ALLAH, the Most Merciful, the most beneficent, for enabling me to carry out and complete this project.

I wish to express my deepest gratitude to my supervisor, Dr. Nabila Roohi for taking a keen interest in this PhD project which is an International/global problem (Infertility/PCOS). Dr. Nabila Roohi, is a dynamic and devoted personality. She has been extremely sincere in solving technical and administrative hurdles and diligently crossed all the obstacles. The proteomic analysis of plasma samples of PCOS by 2-DPAGE could not have been materialized without personal interest and painstaking efforts of Dr Nabila Roohi in the Physiology Lab of the Department. She proved to be an excellent supervisor and morale booster at times of frustration on account of technical hurdles.

I am grateful to Prof Dr Zafar Ullah Khan, Vice Chancellor, King Edward Medical University, Lahore who approved the project for funding and purchase of kits for hormonal analysis. The ex-Director of Centre of Nuclear Medicine (CENUM), Dr. Muhammad Naeem Qureshi at Mayo Hospital, Lahore, provided all the laboratory facilities specially access to RIA and IRMA equipments for hormonal estimations at CENUM. Dr. Shan Elahi was extremely helpful in storage of samples, and expert technical assistance in performing the hormonal measurements.

The selection of patients, collection of blood samples and ultrasound diagnosis of PCOS patients was only possible by the strong facilitation of Professor Dr. Farah, Professor of Obstetrics and Gynaecology at Lady Wallingdon Hospital, Lahore and Dr Saima MS of Lady Aitchison Hospital, Lahore at their Infertility Clinics of these Hospitals. Dr Imran Waheed, Director Abdullah Diagnostic Clinic, who is a foreign qualified Sonologist took keen interest in selection of infertile patients on account of PCOS. He was quite instrumental in following the First Degree Relatives (FDR) of PCOS, thus helped the author to develop FDR group to study the early diagnosis of PCOS and detection of protein biomarkers in this group of normal teens of relatives of PCOS. My thanks are owed to Dr Syed Muhammad Zubair, who physically labored and toiled hard to go to Infertility Clinics at the above mentioned Hospitals and convinced the patients to give samples of blood to investigate their problem of being infertile. Dr. Imrana Ehsan with a high degree of ethics took the history of female patients, filled the proforma, drew the
blood samples after their consent; and also provided the results of hormonal analysis to the normal/subjects at the Physiology Department of King Edward Medical University Lahore.

In fact, the whole faculty of Physiology Department of King Edward Medical University, Lahore coordinated with devotion and sincerity in achieving completion with perfection of this project, specially Dr Muhammad Shafique, and Dr. Imrana Ehsan.

I am highly obliged to the Fellows and Scholars of Physiology lab, University of the Punjab who extended every facility, help and guidance, especially in 2 D PAGE experiments. The faculty of Physiology was found to be free of professional bias and instrumental in the completion of this study/project.

Mr Maqsood Hussain, Lab Attendant deserves full marks for his services for the guest scholars in addition to the regular scholars and fellows.

I am greatly indebted to Mr Babar Ali, Laboratory Assistant of the Physiology Department of King Edward Medical University, Lahore for his extraordinary care in storage and preservation of blood samples drawn for both hormonal analysis and subsequent investigations of 2-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE).

I have all the regards for Mr. Javaid Iqbal, stenographer to the undersigned for the speed and efficiency with which he typed this manuscript; he is an expert in computer typing and has good knowledge of medical terminology. I am extremely thankful for his in time completion of this report.

In the end, I cannot forget the Information Technology services of Mr. Nasir Aziz, Data Entry Operator (DEO) of Mohtarma Benazir Bhutto Shaheed Medical College (M-B-B-S MC), Mirpur, Azad State of Jammu and Kashmir (AJ&K). He has revised, spell-checked and used his skills in finalizing this manuscript. He deserves “Shabash” for his devotion, dedication and truthfulness. I am highly obliged for his sense of commitment.

(Dr. Muhammad Akram)
CHAPTER 1

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is the commonest female endocrine entity/disorder in the developed world, Pakistan being no exception to it. It is a multifactorial and most debatable reproductive endocrinal disorder in the young females with highly controversial pathophysiology (Norman et al., 2007).

The National Institutes of Health (NIH) criteria 1990 include excess of androgens and oligo-ovulation, as the diagnostic criteria. However, 20-25% of regularly ovulating women have PCOS on ultrasound examination. The abnormal ovarian morphology that resembles with PCOS but it is not necessary for diagnosis. Moreover, recent reports indicate that ovarian morphology is no longer an indispensable diagnostic criterion of PCOS (Artini et al., 2010).

The endocrine Society, however, recommended that PCOS can be diagnosed if the adult women presents with two of the following features i.e., i) excess production of androgens, ii) anovulation and iii) pearl-sized cysts found in the ovaries (Legro et al., 2013).

PCOS was initially recognised in women who presented with irregular menstrual cycle and excessive hair growth on the body. These women had different degrees of infertility. It was hypothesized that failure of release of ovum from the ovarian follicle resulted in the rise of precursor hormones like testosterone, and androstenedione (Stein and Leventhal, 1935). It was presumed that on account of thickened ovarian capsule, the ovum was not released from the follicle causing anovulation and resultant amenorrhoea.

The surgeons resorted to a surgical procedure called as ovarian wedge resection. A part of the capsule is resected for release of the ovum. As a result of ovarian surgery all of the hormones of ovary decreased suddenly. Moreover, rise in pituitary gonadotrophins was also observed, thus restoring the normal feed-back system. Consequently, the normal folliculogenesis was encountered (Hendrik’s et al., 2007; Blank et al., 2006). This surgical resection is not done routinely these days. Nevertheless, some surgeons/researchers are still performing ovarian resection via laparoscope to induce ovulation.

The process of ovulation depends upon coordination between hypothalamus, hypophysis cerebri/pituitary gland and ovaries. Nevertheless, the hypothalamo-hypophyseal system integration is the primary determinant of ovulation in women (Blank et al., 2006). It has been reported that women who suffer from PCOS have constantly abnormal gonadotrophin secretion (Taylor et al., 1997). The PCOS is related with increased luteinizing hormone (LH) secretion from pituitary gland. The follicle stimulating hormone (FSH) levels are found to be on the lower side (Rebar et al., 1976).

The fully developed PCOS patient has a history of less than 6 ovulations per year. The cycles are prolonged usually greater than 35 days. The typical male type hair distribution along with hair loss is characteristic feature of PCOS. There is accompanying obesity, hyperandrogenemia and acne. On ultrasound examination there are multiple immature follicles. Morphologically, ultrasound examination of the ovary in PCOS may present only few small immature follicles (cysts). Some patients have cysts that may give rise to garland type of appearance to the ovary. Whereas, in other patients immature follicles (cysts) are so numerous in the ovary that it
resembles a honeycomb. On account of the typical PCOS heterogeneity, the syndrome is yet not exactly known (Belosi et al., 2006). It has, however, been reported that the number and size of immature ovarian follicles is quite significant. It has been concluded by some authors that 2-5 mm immature follicles are probably involved in the arrest of follicular growth. Moreover, the age of the patient and fasting insulin levels have important bearing on the terminal-follicle growth. It has been conjectured to be on account of exaggerated physiological inhibition from the pool of 2-5 mm follicular number and size (Dewailly et al., 2007).

The recent understanding of the mechanism of PCOS includes insulin resistance besides alterations in menstrual cycle, disturbed LH:FSH ratio and anovulation. Banaszewska et al. (2003) have reported that insulin resistance and consequent hyperinsulinaemia as occurs in maturity onset diabetes mellitus, is an important factor that may develop PCOS in women. At present the hypothalamo-hypophyseal-ovarian (H-H-O) axis and its relationship with insulin resistance is yet to be understood. It is relevant to know the role of gonadotrophins and their relationship with insulin resistance.

The underlying mechanism of genesis of polycystic ovary syndrome is unclear. Recently it has been interpreted that the principal cause of PCOS may be hyperinsulinaemia that occurs on account of insulin resistance. It has been hypothesized that ovarian tissue has insulin receptors and insulin itself stimulates ovarian steroids which lead to hyperandrogenism (Barbeiri 1986; Ehrman et al., 1995). Some workers blame that insulin resistance and accompanying hyperinsulinism leads to increase in intra-ovarian hyperandrogenism. The hyperandrogenism prevents elevation of FSH that finally results in anovulation (Jonard and Dewailly, 2004).

Insulin resistance has been linked with excess of androgens in PCOS patients. Moreover, insulin resistance is labeled as one of the important diagnostic criteria of PCOS. On the contrary some PCOS patients do not have features of insulin resistance. As a matter of fact insulin resistance has been blamed to be a vital factor in the causation of (Ojaniemi and Pugeat, 2006).

The basic mechanism of development of PCOS in women is not known. However, features like insulin resistance, obesity, excess of androgens in the blood and derranged gonadotrophin secretions are concerned in its causation. Hyperinsulinaemia has been reported to cause hyperandrogenism. Moreover, insulin may stimulate androgen level by the follicular cells in collaboration with LH. It has been reported that the main cause of failure to ovulate is inadequate release of FSH (Guzick, 2004).

Recarbarren et al. (2008) have reported that obesity and insulin resistance are heritable.

Moreover, PCOS has been labeled as a familial disease (Azziz, 2008). According to Laven (2007), genes have little role to play in the aetiology of PCOS. Some authors have reported that both genes and environment may be implicated in the aetiology of PCOS. Furthermore, poor diet and sedentary habits may lead to obesity that can deteriorate PCOS in some patients. However, the role of infectious agents and toxins is doubtful (DiamantiKandarkis et al., 2006). Despite the discrepancies in definition, diagnostic criteria, the role of gonadotrophins in the pathogenesis of PCOS, ethnic and climatic/environmental predisposition of the syndrome; the bulk of etiological and clinical data available in the literature serves to provide only a framework for studying genesis of PCOS in Pakistani women. Therefore, original investigations of various endocrine and presentation parameters are warranted to understand the patho-physiology of the syndrome in local women. Over the last few years there has been an increased recognition and importance of hormonal changes in the manifestation of PCOS. The current work was, therefore, planned to
systematically examine the specific endocrinal changes and hormonal interactions in Pakistani cohorts of PCOS patients.

Clinical application of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is important for identifying specific proteins in healthy individuals and in patients with selected known diseases. Electrophoretograms exposed certain reference protein profiles in the selected clinical disorders. Abnormal profiles were detected in the form of “abnormal spots”. It has been found that some diseases are associated with specific ‘spot patterns’ on the protein maps of the patients. Similarly, regulatory proteins have been identified in diseases like leukaemia, Ewing sarcoma and Alzheimer’s disease (Chrambach et al., 1993).

The electrophoretic protein patterns have been recognized for different human fluids such as plasma, amniotic fluid and cerebro-spinal fluid respectively (Anderson and Anderson, 1991; Hughes et al., 1992; Yun et al., 1992). Recently, seminal plasma of men was studied by 2-D PAGE. These authors reported that electrophoresis can be helpful for studying seminal plasma proteins in azosperma. They suggested further research for identification of these markers in the diagnosis of impaired spermatogenesis (Starita-Geribaldi, 2001).

On account of rising incidence of PCOS, unknown aetiology and pathogenesis; and scarcity of work in Pakistani women, it was considered imperative to investigate the syndrome in Pakistani population, and to find out any correlations amongst various parameters in PCOS. The study has been planned:

1. To find out the role of gonadotrophins, LH:FSH ratio, hyperinsulinaemia and hyperandrogenism in PCOS.
2. To determine whether or not there exists any correlation between hyperinsulinaemia and hyperandrogenism in PCOS.
3. To detect/investigate the presence of any specific protein(s) in sera of patients with PCOS.

The author, therefore, considered imperative to probe appropriate molecular diagnosis by applying 2-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE) that may lead to discover novel proteins associated with PCOS. This might be helpful in understanding the underlying etiology and facilitating early diagnosis to prevent early life complications like cardio-vascular disorders and endometrial cancer.
CHAPTER 2

LITERATURE REVIEW

PCOS is described as a heterogenous condition and got evolved from polycystic ovary disease to polycystic ovary syndrome. It is an ovarian disorder characterized by hyperandrogenism, oligo-ovulation and polycystic ovaries. The classical features of PCOS however, are hirsutism, irregular menstrual cycles, obesity and typical immature, small ovarian follicles. The diagnostic criteria for PCOS is chronic anovulation/oligo-ovulation with evidence of clinical hyperandrogenism i.e. hirsutism, acne and male type baldness. The biochemical criterion is raised serum androgens. The other causes of hyperandrogenemia must be ruled out for the diagnosis of PCOS. Abnormal ovarian morphology/folliculogenesis is inadequate to make a diagnosis of PCOS. It is on account of the fact that 20-25% of regularly ovulating women have PCOS on ultrasound examination. Therefore; abnormal ovarian morphology is consistent with PCOS but not essential for diagnosis. It is the most common reproductive endocrinal disorder in the females with highly controversial pathophysiology (Legro, 2009).

Recent reports indicate that ovarian morphology is no longer an essential requirement for diagnosis of PCOS (Ferry, 2010). There is no doubt that PCOS is a syndrome i.e. collection of signs and symptoms. Furthermore, there is no single test that can be considered as essentially diagnostic. It has been mentioned that a just and comprehensive definition is essential to enhance future research (Azziz et al., 2005). PCOS is continuously generating controversy in its definition and pathophysiology (Balen and Michelmore, 2002; Azziz, 2006; Franks, 2006).

It has been reported that ambiguities in the definition of PCOS and diagnostic criteria are still generating controversies and a clear-cut definition is indispensable to successfully probe the syndrome for knowing the pathogenesis (Azziz, 2006).

PCOS is described as a heterogenous disorder and comprises a variety of potential signs and symptoms. Uptil now, there is no universally accepted clinical definition of PCOS (Legro, 2003). Therefore, the attention is shifting towards assessment of increased health risks of PCOS in the later life (Fauser, 2008).

It was observed that major factors responsible for generating features of PCOS are hyperandrogenism and oligo-ovulation. Others have defined PCOS as a disorder of androgen excess (Zawadski and Dunaif, 1992). Subsequently PCOS is recognized as having two of the following features (ESHRE/ASRM, 2004):

1. oligo-ovulation or anovulation
2. clinical or biochemical signs of hyperandrogenism
3. polycystic ovaries.

Some researchers described PCOS on the appearance of immature follicles on ultrasound examination. These multiple immature pre-antral follicles having a size of 2-8 mm that makes a “black-pearl necklace” sign (Franks, 1995). It has been proposed that typical ovarian picture on ultrasound examination along with menstrual disorders and signs of hyperandrogenism are good indicators of PCOS. However, hormonal parameters are not essential for the diagnosis
According to Balen et al. (2003), the echographic pattern of ovary for the diagnosis of PCOS is defined as presence of 12 or more follicles in which ovary measuring 2-9 mm in diameter and/or increased ovarian volume more than 10 cm³. It was also recognized that other androgen excess disorders should be excluded before declaring the diagnosis of PCOS (ESHRE/ASRM, 2004).

2.1 Diagnostic Criteria

The diagnostic criteria for PCOS as suggested in the Rotterdam conference sponsored by European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine (ESHRE/ASRM) in 2004, include oligo-ovulation/or anovulation, androgen excess, and clinical features of high androgen levels based on polycystic ovary (PCO) morphology. The diagnosis is made by anovulation, hyperandrogenism and multiple cysts in ovary. The experts yet differ about the importance of each feature (Athanasia et al., 2008). The endocrine Society, however, recommended that PCOS can be diagnosed if the adult women presents with two of the following features i.e., i) excess production of androgens, ii) anovulation and iii) pearl-sized cysts found in the ovaries (Legro et al., 2013).

Thus if all the three criteria are present the PCOS is regarded as severe (Norman et al., 2007). When there is positive history of anovulation and clinical signs of hyperandrogenemia was the criteria for the diagnosis of PCOS before 2003 (Zawadski and Dunaif, 1992).

With advancement of sensitive hormonal assays, hyper-androgenemia became the most important diagnostic criterion (Raj, 1978). Moreover, the ultrasound examination made easier the study of ovarian morphology as the diagnostic feature (Adams et al., 1986). As the insulin resistance is being labelled as one of the etiologic factor, insulin levels have become a part of the diagnostic evaluation of PCOS (Legro et al., 1998).

The classical presentation of PCOS comprises of amenorrhoea or oligomenorrhoea with hyperandrogenism. However, presenting symptoms vary widely; including anovulation without hirsutism and hirsutism with regular cycles. The diagnosis of PCOS is made principally on hormonal analysis i.e. serum testosterone, LH and FSH. It has been suggested that on account of high risk of glucose tolerance or frank diabetes, and women complaining of irregular/anovulation/oligo-ovulation along with PCOS should undergo glucose tolerance test. The researcher further suggested that circulating insulin levels are not necessarily required (Franks, 2010).

According to Rotterdam ESHRE sponsored PCOS consensus workshop group 2003, PCOS remains a syndrome. There is no single criterion for clinical diagnosis.

In order to establish the diagnosis of PCOS, it is essential to exclude other disorders that resemble PCOS. It is also necessary to exclude hypogonadotrophic hypogonadism which is a central ovarian dysfunction. Moreover, the Rotterdam 2003 group participants emphasized that routine measurement of prolactin should be performed in hyperandrogenic patients. This is necessary to exclude hyperprolactinaemia.

It has been mentioned that ovarian morphology is not diagnostic of PCOS because this morphologic pattern is also found in childhood and adolescence (Bridges et al., 1993; Battaglia et al., 2002). The same pattern of ovary is also found in menopausal women (Birdsall and Farquhar,
1996; Dahlgren et al., 1992). Moreover, this ovarian pattern is also reported in hyperandrogenism in the absence of regular menstrual history (Ibanez et al., 2008; Norman et al., 1995; Carmina and Lobo, 2001).

2.2 ONSET OF PCOS

The features of PCOS begin around menarche (Franks, 2002), but may appear after puberty as a result of environmental factors like weight gain. Premature puberty may result on account of early secretion of adrenal steroids, may be a predictor of the disease (Ibanez et al., 2001). In addition, the abnormal intrauterine environment may be responsible in the pathogenesis of the syndrome (Eisner et al., 2003; Eisner et al., 2002; Abbot et al., 2002).

It has been experimented that ovarian theca cells in affected women convert androgenic precursors to testosterone as compared with normal theca cells (Nelson et al., 1999).

The frequency of the stimulus of hypothalamic gonadotrophin-releasing hormone (GnRH) determines, in part, the relative proportion of luteinizing hormone and follicle-stimulating hormone synthesized within the gonadotropes. Increased pulse frequency of hypothalamic gonadotrophin releasing hormone (GnRH) favours transcription of the β-subunit of luteinizing hormone over the β-subunit of follicle-stimulating hormone; conversely, decreased pulse frequency of GnRH favours transcription of the β-subunit of follicle-stimulating hormone, which decreases the ratio of luteinizing hormone to follicle-stimulating hormone (Hasenloder et al., 1991). The women with the polycystic ovary syndrome appear to have an increased luteinizing hormone pulse frequency, it has been inferred that the pulse frequency of GnRH must be accelerated in the syndrome. It is not clear whether this accelerated pulse frequency is due to an intrinsic abnormality in the GnRH pulse generator or is caused by the relatively low levels of progesterone resulting from infrequent ovulatory events. Since progestins slow the GnRH pulse generator, low circulating progestin levels in women with the polycystic ovary syndrome may lead to an acceleration in the pulsatility of GnRH, increased levels of luteinizing hormone, and overproduction of ovarian androgens (Eagleson et al., 2000).

The polycystic ovary syndrome remains one of the most common hormonal disorders in women, with a prevalence estimated between 5 and 10 percent. Variance in prevalence among populations may reflect the effect of ethnic origin, race, and other environmental factors on the phenotype (Knochenhauer, 1998; Asuncion et al., 2000).

2.3 PATHOPHYSIOLOGY OF PCOS

The fundamental abnormality in PCOS remains unknown, but growing evidence indicates that it includes insulin resistance, androgen excess and abnormal gonadotrophin dynamics (Guzick, 1998). Recent data suggest that the principal disorder in PCOS is insulin resistance, that results into hyperinsulinemia that stimulates ovarian androgen production (Nestler and Strauss, 1991; Nestler, 1994.)

In patients of PCOS the conversion of follicular androstenedione to estradiol suggests disordered aromatization. Moreover, aromatase gene mutation has been reported to cause a form of the syndrome (Conter et al., 1994).

2.4 INAPPROPRIATE GONADOTROPHIN SECRETION
LH plays an important role in the development and functioning of the reproductive system. The measurement of LH is significant for detecting the integrity of pituitary-ovarian axis, diagnosis of reproductive disorders and monitoring the anti-fertility programmes (Vandan et al., 2005).

Gonadotrophin abnormalities in PCOS include elevated levels of testosterone and LH; or high levels of LH-to-FSH ratio, an augmented LH pulse frequency and deranged diurnal rhythm of LH. The prominently elevated serum LH levels are present in a considerable proportion of women with the syndrome but are not crucial for its diagnosis (Dunaif et al., 1992).

It has been reported that inappropriate secretion of LH, is a characteristic feature of PCOS (Rebar et al., 1976). Initially an excess of LH was first detected in the urine (Keetle et al., 1957). Abnormal levels of LH secretion were later on found in the blood (McArthur et al., 1958). The subsequent studies documented an elevated level of LH: FSH ratio (Yen et al., 1970).

One of the factors implicated in the genesis of PCOS is impairment of the action of FSH. Moreover, LH that rises prematurely has been predicted to prevent the selection of dominant follicle (Jonard and Dewailly, 2004).

Abnormal levels of pituitary gonadotrophins i.e. follicle stimulating hormone (FSH) and luteinizng (LH) has also been labeled to participate in the pathophysiology of PCOS (Guzick, 1998). In addition to abnormal gonadotrophins, elevated levels of testosterone and abnormal LH:FSH ratio have been reported in women suffering from PCOS. Further, LH:FSH ratio of 3:1 is diagnostic feature of PCOS. It has been mentioned by Ehrmann, (2005) that FSH may regulate production of aromatase activity of granulosa cells of ovarian follicles. Thus, FSH determines the amount of synthesis of oestrogen from the androgen precursors. The elevated LH levels as compared to FSH may stimulate the ovary to start synthesizing excess of androgens. It has been summarized by some researchers that LH:FSH ratio is not a characteristic feature of all the patients of PCOS. Rather, it is true for a small group of women i.e. less than 50% PCOS patients have altered LH:FSH ratio (Banaszewska et al., 2003).

A recent study on LH:FSH ratio regarding pathophysiology of PCOS reports that this ratio should not be considered as the diagnostic criteria. These authors have suggested that diagnosis of PCOS is based upon clinical picture and concomitant hyperandrogenaemia. Nevertheless, other causes of hyperandrogenaemia should be ruled out (Alnakash and AitTae’e, 2007).

Moran et al. (2003) have hypothesized that LH, FSH, LH:FSH ratio are not related with insulin resistance. Nevertheless, insulin resistance can influence gonadotrophins with long-standing stimulation, especially in obese patients.

In another study it has been reported that insulin resistance has been observed in nonobese patient suffering from PCOS. Moreover, insulin resistance is also related with raised levels of LH hormone and free and unbound testosterone. It has therefore, been suggested that insulin resistance needs to be treated at an early stage. Consequently, it may decrease the risks of potential complications at a later stage of life in these women having PCOS. Furthermore, these authors described/observed a strong relationship between excessive insulin, excess of androgen and low sex hormone binding globulin (SHBG). In addition, new vessel growth in the ovarian stroma has been considered to be on account of hyperinsulinaemia (Toprak et al., 2001).
It has been blamed by certain authors that several factors in PCOS disturb the normal folliculogenesis. The theca interna cells and granulosa cells dysregulation play main role in abnormal follicular growth. The oocyte may be involved in causing increased formation of follicles. FSH is important in proliferation and survival of granulosa cells (GC). Moreover, FSH recruits the dominant follicle in normal folliculogenesis. It has been hypothesized that lofty levels of inhibin B are released by the candidate follicles. While dominant follicle produces estradiol and inhibin A, that consequents to inhibition of FSH release from anterior pituitary (Zelznik and Fairchild-Benyo, 1994). In PCOS the intercycle rise of FSH is prevented on account of excessive release of inhibin B. Inhibin B ultimately suppresses FSH production by negative feedback system.

2.5 THE PREMATURE ACTION OF LH IN PCOS

It is reported that granulosa cells (GC) build up their own receptors for LH in the middle to late follicular phase (Erickson et al., 1979). It has been hypothesized that premature action of luteinizing hormone on granulosa cells of the follicle may arrest its growth. (Franks et al., 1998).

Insulin enhances the ability of granulosa cells to respond to luteinizing hormone. These authors have suggested that raised insulin level may be labeled for arrest of maturation and subsequent anovulation (Willis et al., 1996). It has been reported that insulin resistance at the level of GC is the actual culprit (Coffler et al., 2003a). Nevertheless hyperinsulinism may be a secondary cause of anovulation in PCOS that can deteriorate the follicular arrest. However, hyperinsulinism may be a secondary cause of anovulation in PCOS that nonspecifically worsens the follicular arrest (Jonard and Dewailly, 2004).

2.6 WHY FOLLICULAR ARREST IS INCONSTANT

In fact, oligo-ovulation rather than anovulation is the real feature of PCOS. At times, a dominant follicle escapes all inhibitory influences and matures to release the ovum. Moreover, some patients of PCOS ovulate regularly despite simultaneous presence of clinical features and excess of androgens (Carmina and Lobo, 2001). It has been reported that premature LH receptors developed in GC of immature follicles are found only in those patients of PCOS who don’t ovulate (Willis et al., 1998). It has been further hypothesized that excess of small immature follicles exert relatively less blocking effect on the pool of selectable follicles (Jonard and Dewailly, 2004).

The LH:FSH ratio of more than 2 presents the best combination of sensitivity and specificity. It was concluded that LH:FSH ratio is a significant and important diagnostic criterion in detecting PCOS women presenting with oligomenorrhea or anovulation. The authors have further hypothesized that BMI and LH may provide insights in the etiopathogenesis of PCOS (Ming, 2009).

It has been reported that LH is elevated in PCOS in relation to FSH. However, LH in turn is influenced by BMI. Researchers have investigated to determine the effect of BMI on neuroendocrine disorder in PCOS. These authors have recorded that both LH and GnRH are raised in PCOS; but BMI does not influence hypothalamic function. The response of pituitary to administration of GnRH is found to be inversely related to BMI in PCOS. It was hence, concluded that BMI can influence the secretion/elevation of LH through pituitary. The rise in LH on account of increased BMI is attributed to pituitary in women suffering from PCOS (Pagan et al., 2006).
Blank et al. (2006) reported that excess of LH enhances hyperandrogenism via ovarian stimulation. It was considered on account of the known effect of LH on theca cells of the ovarian follicles; thus LH has the primary role in PCOS. Some researchers have concluded that LH dysregulation is not primary; but it may be secondary to some other ovarian factor. It has been claimed to be due to abnormal secretion of androstenedione produced by ovarian theca cells in PCOS (Suhail, 2008).

It has been reported that androstenedione may be the cause of LH rise in patients of PCOS. It is known that absence of physiological levels of estradiol facilitates elevation of gonadotrophins in both men and women (Carani et al., 1997). It was thus suggested that estradiol rather than androgens modify GnRH feed-back. It has been hypothesized that some ovarian factor, probably androstenedione may enhance un-coupling of hypothalamic GnRH secretion from the effect of estradiol; thus accelerating the activity of this pulse generator. It has also been reported that LH level was raised along with androstenedione in all the women studied. Consequently, androstenedione may be responsible for uncoupling of GnRH from inhibition by estradiol (Suhail, 2008).

2.7 ANTI-MULLERIAN HORMONE IN PCOS

Anti-Mullerian Hormone (AMH) is produced by granulosa cells (GC) of the ovary. The hormone makes its appearance in the blood in the 36th week of gestation and remains positive until cessation of menstrual cycles in the women. The highest expression of the hormone is from small antral follicles. The concentration of AMH gradually falls with the progression of follicular growth. AMH has been observed to play an inhibitory role in the growth of ovarian follicles. It not only decreases primordial follicle initiation but also decreases follicle sensitivity to FSH by inhibiting aromatase. AMH has therefore, been a focus of study in PCOS. It has thus been hypothesized that it may contribute towards anovulation, a characteristic finding in PCOS (Pellat et al., 2010). AMH values were compared amongst overweight and normal weight women with four different phenotypes of PCOS and healthy control subjects. AMH levels were significantly raised in all the four PCOS groups compared with the control group. Elevated LH concentration has been found to be the most significant link between PCOS-associated disorders of ovulation and raised AMH concentration in these patients (Athanasia et al., 2008). A positive correlation has been observed between serum AMH levels and the number of antral follicles in women with PCOS. The raised AMH concentration in the blood may be associated with PCOS and its diagnosis. Moreover, AMH can also be helpful in establishing sub-classification of PCOS that is the most controversial and heterogeneous syndrome (Jenny et al., 2006).

2.8 HYPERANDROGENISM

The adrenals and ovaries add to circulating androgens in women. The adrenal androgens, like dehydro-epiandrosterone (DHEA), are relatively weak in action as compared to ovarian testosterone. The adrenal DHEA and ovarian androstenedione may serve as prohormones and heightens the androgens in the blood. Androstenedione is the solitary circulating androgen that is higher in premenopausal women (Legro, 2003). It has been mentioned that circulating testosterone was found to be the best representative of hyperandrogenism, chronic anovulation and polycystic ovaries (Robinson et al., 1992). Hyperandrogenemia is not accepted universally as an indicator of androgen excess. It has been reported that hirsutism was found in 50% women who have PCOS (Moran et al., 1994). Chronic anovulation on account of hyperandrogenemia was found only in 50-60% of women (Azziz et al., 2001). Hirsutism which is a clinical
manifestation of hyperandrogenemia is accompanied by normal circulating androgen levels (Lobo et al., 1983).

2.9 PHYSIOLOGICAL HYPERANDROGENISM OF PUBERTY

At the onset of puberty, the gonadotrophin hormones especially LH is only found to be raised during sleep. Subsequently, it is found increased in the day time; accompanied by appearance of increased androgens in plasma. There is a concomitant enlargement of ovaries along with visible multiple follicles on ultrasound examination.

The initial menstrual cycles in adolescent girls have been found to be without ovulation. These cycles have a characteristically raised serum levels of testosterone, androstenedione and LH. At this stage there is slight degree of hyperandrogenic tendency in these adolescent girls. Within a passage of 1-2 years, the menstrual cycles become regular. However, in some girls these abnormalities and menstrual cycles get worsened, and start mimicking features of PCOS; along with hirsutism, anovulation, increased androgens, raised LH levels and increased ovarian volume. It has been commented that development of acne and irregular periods in adolescent girls was a part of normal ovarian development along with hyperandrogenism of PCOS. Moreover, lean subjects were found to be more prone to develop insulin resistance and dyslipidaemia. The researchers concluded that adolescent girls with hyperandrogenism were at risk of developing metabolic disturbances. They further reported that these girls should be investigated for metabolic abnormalities irrespective of their age, or BMI (Huppert et al., 2004).

PCOS is commonly accompanied by infertility and insulin resistance; which may be associated with frank type 2 diabetes mellitus or metabolic syndrome. The metabolic syndrome is concerned with a variety of findings like central obesity, hypertension, glucose intolerance and dyslipidemia (Eckel et al., 2005). The metabolic syndrome may also present with an increased risk of endometrial cancer (Ehrmann, 2005; Rotterdam ESHRE/ARSM, 2004).

It has been observed that most of the patients suffering from PCOS have obesity and insulin resistance. Obesity is presumed to cause insulin resistance. In order to determine whether or not insulin resistance, in the absence of obesity and acanthosis nigricans can generate the features of PCOS; non-obese patients of PCOS were selected for study. The results were compared with healthy control subjects matched for age and weight. It was concluded that insulin resistance in non-obese patients of PCOS is positively related with the syndrome. It was further hypothesized that insulin resistance is positively related with serum LH and testosterone levels in the blood. It is, therefore, imperative to treat the features developed on account of insulin-resistance. It seems mandatory to decrease the insulin-resistance at an early stage to prevent subsequent cardiovascular risks in the later part of life of the patients of PCOS (Toprak et al., 2001).

Metabolic syndrome has been reported to be closely related to PCOS on account of a common underlying etiologic factor i.e. insulin resistance. It has been postulated that metabolic syndrome is characterized by visceral obesity and insulin resistance. These two characteristics are further associated with atherosclerosis and cardio-vascular disease (Doalle, 2004; Isomaa, 2001). It has been reported that cardiovascular risk factors are invariably present in women with PCOS (Tallbot et al., 2004; Carmina et al., 2005).
The major criterion for the diagnosis of metabolic syndrome in PCOS is that adopted by the American National Cholesterol Panel/Adult Treatment Panel III (ATP-III). These criteria are presence of increased waist circumference, high blood pressure, raised fasting blood glucose, low serum HDL cholesterol and increased triglycerides. Applying these criteria, the prevalence of metabolic syndrome (MBS) in PCOS has been described to be very high i.e., 43-46% (Gluek et al., 2003; Apridonidze, 2005). This prevalence of MBS in PCOS is based upon abnormal lipids and increased waist circumference. In conclusion, it appears that MBS is much more common in PCOS as compared with general population in women of the same age group (Carmina et al., 2006).

2.10 ROLE OF ANDROGEN

The luteinizing hormone has been reported to regulate the formation of androgens by theca cells of ovary. While FSH is blamed to control the aromatase activity in granulosa cells of ovarian follicles. Consequently, aromatase activity leads to enhanced biosynthesis of estrogens from precursor hormone i.e., androgens. It has thus been hypothesized that rise in LH level as compared to FSH culminates into excessive synthesis of androgens by the ovary (Ehrmann, 2005).

Hyperandrogenism is also related with PCOS. It has been mentioned that in PCOS, 17hydroxyprogesterone (17 OHP) levels are raised above normal. This is on account of administration of GnRH (gonadotrophin releasing hormone) agonists like nafarelin. It has been concluded that at least 50% of women who have oligomenorrhea, hirsutism, and acne give abnormal response to GnRH. This clearly shows that these features of PCOS are closely related with excess of androgens that are produced by ovary (Ehrmann et al., 1992).

PCOS has been related with excess of insulin in the blood/hyperinsulinaemia and is currently being regarded as central pathogenic feature of the syndrome. Hyperinsulinaemia is also related with androstenedione. Moreover, androstenedione was found to be significantly elevated and sex hormone binding globulin (SHBG) was significantly lesser in patients of PCOS, who had hyperinsulinaemia. Consequently, it is concluded that hyperinsulinaemia, hyperandrogenemia and low SHBG are strong correlates of each other on account of a common factor i.e. insulin resistance. (Loverro et al., 2001).

Hyperinsulinism and hyperandrogenism are associated with PCOS. Insulin resistance has been found to co-exist in a significant number of patients with hyperandrogeninaemia. This is specifically true in patients with positive family history of type 2 diabetes mellitus. Nevertheless, hyperinsulinaemia may occur in type 1 diabetes mellitus. It may be on account of the fact that these patients of type-1 diabetes are treated with insulin. It has also been observed that type-1 diabetics also manifest hyperandrogenaemia (Garcia –Romero et al., 2006).

It has been reported that obesity is associated with hyperactivity of hypothalamo-pituitary-adrenal (HPA) axis (Ivana et al., 2010). It was further hypothesized that PCOS may have multiple pathologies. The PCOS is a highly complicated disorder and there is no single pathologic factor (Oussama, 2008).

It has been experimentally studied in rats that ovulation can be induced with bilateral section of superior ovarian nerve (SON). Moreover, cutting the SON results in asymmetric hormonal secretion by both ovaries in the animal. The typical PCOS was induced in rat by administration of estradiol valerate (EV) with a single dose. EV-induced PCOS is related with increased peripheral sympathetic discharge. It was observed that none of the EV-treated animals ovulated; while oil-
treated animals ovulated normally. All EV-treated animals developed features of PCOS. In oil-
treated animals, incidence of ovulation was higher in the unilaterally sectioned and/or
denervated ovary. The experiment supports the notion of neural interaction between the
ovaries. It was consequently suggested that some neural mechanism develops the control of
ovulation in the innervated ovary (Dominguez et al., 2008).

It has most recently been suggested that chronic infections may increase the risk of infertility. In
this regard Helicobacter pylori (H Pylori) have been blamed as one of the newest pathological
factor. The PCOS and H Pylori infection has been found to be coincidental. It has, therefore, been
added as one of the latest microorganism that may cause PCOS (Irfan et al., 2009).

Recently it has been hypothesized that the function of resistance vessels can be modified by
PCOS. The study was to determine the role of excess of androgens, obesity and insulin resistance
in the modulation of vascular function. It was concluded that PCOS women who were obese; had
greatly decreased vascular smooth muscle compared to non-obese subjects. Finally, it was
reported that obesity and insulin have significant influence on smooth muscle of blood vessels as
compared with hyperandrogenism (Dokras et al., 2006).

Differences in anthropometry, hormone levels and insulin resistance have been recognized in
PCOS in the Korean Women. It has been concluded that patients diagnosed as PCOS do not
suffer from concomitant hyperandrogenism. It is further reported that these Korean women are
not prone to develop metabolic syndrome and cardiovascular complications. Therefore, PCOS in
Korean women population may prove to be mild phenotype of PCOS (Chae et al., 2008).

PCOS is a multifactorial and controversial endocrine disorder in women in young age. It is being
strongly associated with type 2 diabetes mellitus and hyper-insulinaemia.

The mode of presentation of PCOS was studied and it has been reported that ovarian
overproduction of androgen is on account of hyperinsulinism. Furthermore, it has been labelled
that raised insulin levels are described as an important etiologic factor in the production of
PCOS. (Ayisha et al., 2005).

2.11 BMI IN PCOS

Women with PCOS are at risk of impaired glucose tolerance (IGT), type 2 diabetes mellitus, and
gestational diabetes (Ehrmann, 2005 and Ehrmann et al., 1995). The specific defect is increased
serine and decreased tyrosine phosphorylation of the insulin receptor.

The women suffering from PCOS are hyperinsulinemic (Ehrmann, 2005). Hyperinsulinemia
worsens and insulin sensitizers improve ovarian dysfunction and hyperandrogenemia in PCOS.
Therefore, assessment of glucose homeostasis by an oral glucose tolerance test (OGTT) has
become a regular practice (Qin and Rosenfield, 1998). The interpretation of glucose intolerance
has changed in recent years. The fasting glucose cut off for diabetes has reduced from 140 to
126 mg/dl and the new term, impaired fasting glucose (IFG), has been introduced for the values
between 110 and 125 mg/dl. In 2003, the normal fasting glucose was reduced to 100 mg/dl
(Genuth et al., 2003).

It has been concluded that patients of PCOS develop impaired fasting glucose tolerance (IFG).
Moreover, these patients are also at risk of impaired glucose tolerance (IGT). The increase in
cardiac risk factors and free androgen levels precede the overt glucose intolerance. (Kasim-
Karakas et al., 2004).
Insulin resistance increases with weight gain as observed by stepwise increase in BMI in the normal glucose tolerance test (NGT).

The relationship between hyperandrogenism and hyperinsulinaemia was studied in PCOS. The body mass index (BMI) was higher, and free testosterone index was raised. Similarly, the values of dehydroepiandrosterone sulphate (DHEAS), testosterone, 17hydroxyprogesterone (17 OHP) and androstenedione were all found to be raised as compared with normal controls. All parameters concerned with insulinaemia both basal and maximum peak values were on the higher side. Importantly, in patients with hyperinsulinaemia, free testosterone index was highly raised. Nevertheless, gonadotrophin levels were not affected and were found to be within the normal range. These researchers presented a typical hormonal picture in PCOS on account of markedly elevated hyperinsulinaemia (Vidal-Ping and Munoz-Torres, 1994).

It has been reported that excess of insulin in the blood can be considered as one of the pathogenic agent in PCOS (Dunaif, 1997). Other studies do not coincide and the role of hyperinsulinaemia in PCOS remains controversial (Webber et al., 2002).

Despite the factors blamed so far in the pathogenesis of PCOS; there is no definite criterion for the diagnosis of syndrome. The diagnosis is based on a group of clinical signs and symptoms. These clinical features vary from patient to patient and from investigator to investigator. PCOS was strictly defined for the first time in 1990 in the National Institute of Health conference. Zawadzki and Dunaif, (1992) nominated a triad comprising of hyperandrogenism, oligo-ovulation and ruling out other conditions that mimic PCOS.

Nevertheless, Welt et al. (2006) have reported that even these strict criteria may vary in severity and presentation of clinical features. These authors have mentioned that the clinical features also vary with ethnicity and environment. They have suggested cross population studies to verify the main differences in different ethnic groups in the syndrome. In order to standardize the diagnosis, number of studies were carried out, consequently leading to greater insight into the pathology of the disease (Nestler et al., 1998; Azziz et al., 2001).

It has been reported that hyperandrogenism and multiple immature follicles in the ovary are the cardinal signs of PCOS. The clinical features include oligo-ovulation, signs of excessive androgens in blood and obesity. Further, it has been observed that some women who have characteristic features of PCOS but may have regular menstrual cycles along with hyperandrogenism (Adams et al., 1986; Carmina and Lobo, 2001). The Rotterdam ESHRE/ASRM-PCOS consensus workshop 2004 concluded that PCOS remains a syndrome and no single parameter like hyperandrogenaemia is a diagnostic feature. Furthermore, the PCOS regarding its definition is still creating controversies (Balen and Michelmore, 2002; Azziz, 2006; Franks, 2006).

Clinically speaking, the patients of PCOS may present with wide variety of features like infertility, obesity, type 2 diabetes, dyslipidaemia and other systemic features e.g. cardiovascular disease and endometrial cancer (Azziz et al., 2005).

The androgen excess guideline (AEG) task force has mentioned four key features of PCOS. These are ovulatory dysfunction, hyperandrogenemia, signs and symptoms of hyperandrogenism and typical cysts are found on on ultrasound examination of ovaries. The androgen excess in PCOS patients has been found in 60-80 %, mainly in the form of free testosterone.

Researchers have reported menstrual dysfunction in PCOS. At the same time 20% patients of PCOS present with normal menstrual cycles and oligo-ovulation (Azziz et al., 2004).
Many authors have reported that polycystic ovaries were found in approximately 75% of women who are clinically diagnosed as suffering from polycystic ovarian syndrome (Jonard et al., 2003). Nevertheless, the disease cannot be diagnosed merely on the basis of appearance of the ovary (Jonard et al., 2005). It has been mentioned that PCOS can be diagnosed only when one ovary has volume of more than 10 cm$^3$ or more than 12 follicles having a diameter of 2-9 mm (The ROTTERDAM ESHRE/ASRM-PCOS Consensus workshop group, 2004). However, Balen et al., 2003 have reported that 10-30% patients do not manifest polycystic ovaries on ultrasound. Moreover, 20% women do not consent for transvaginal ultrasonography. Furthermore, the clinicians depend upon radiologists who may not be familiar with the clinical diagnosis (Farquhar et al., 1994).

2.12 OVARIAN MORPHOLOGY IN PCOS

The ovarian picture on ultrasound is not consistent with clinical disorder of PCOS. It is also found in childhood, adolescent, and in menopausal women (Battaglia et al., 2002; Bridges et al., 1993; Dahlgren et al., 1992).

The evidence of polycystic ovarian morphology is not essential for the diagnosis of the syndrome. Nevertheless, if signs and symptoms of infertility and menstrual disturbance do not exist, the ovarian morphology that is consistent with PCOS becomes a criterion for diagnosis (Rotterdam ESHRE/ARSM, 2004; Azziz, 2005).

It has been reported that volume and follicle number in the ovaries decrease with age in women. The decrease was observed longitudinally and found to be more pronounced in PCOS than controls. This age-based criterion can be used for defining polycystic ovarian morphology. It is possible to use these criteria to distinguish PCOS in women over the age of 40 years (Alsamarai et al., 2009).

PCOS has been described as one of the most widely studied and disputed/controversial topic in reproductive endocrinology. The signs of hyperandrogenism in lean/non-obese women with normal ovulatory cycles contrasting with severe hirsutism, obesity, oligomenorrhea or amenorrhea represent somersault of the clinical spectrum of PCOS. The heterogeneity in the clinical and endocrinial features remains a confounding factor in the diagnostic investigations of the syndrome. A large amount of literature review and experimental data could not guide to the pathogenesis of PCOS (Battaglia et al., 2002). Some 30% women who have normal menstrual cycles and normal androgen levels in the blood have polycystic ovaries (Legro, 2003; Farquhar et al., 1994; Koivunen et al., 1999). It has been reported that the polycystic ovaries can be independent risk factor to develope ovarian hyperstimulation syndrome, especially after ovulation induction (Enskog et al., 1999). It is important to note that on the basis of morphology of the ovary, the patient should not be labelled as a case of PCOS (Louks et al., 2000). It has, therefore, been suggested that ovarian morphology cannot be employed as gold standard for the diagnosis of PCOS (Koskinen et al., 1996).

“The excess of small follicles appears as the salient and constant feature of PCOS. Pathological studies have shown that the pool of growing primary and secondary follicles is 2-3 fold that of normal ovaries, while the pool of primordial follicle is normal. The hyperandrogenism of PCOS is designated as the main culprit for this follicle excess” (Jonard and Dewailly, 2004).

It has been reported that ethnicity also determines the ovarian morphology. Maximum ovarian volumes and the number of follicles observed in a single plane on ultrasound were smaller in Icelandic than Boston subjects with PCOS; and were even smaller in Icelandic control than
Icelandic PCOS subjects. There was no difference in the proportion of ovaries in the Icelandic and Boston PCOS groups, meeting the criteria for PCO morphology. However, there were more normal ovaries in the Icelandic group when compared with Boston group. (Farquhar et al., 1994; Carmina et al., 1997; Gilling-Smith, 1997)

Insulin resistance (IR) and hyperinsulinemia are common characteristics of PCOS; and are associated with the pathophysiology of the condition (Diamanti-Kandarakis and Papavassiliou, 2006, Poretsky et al., 1999).

Evidence exists for altered adipocyte function in PCOS with altered secretion of adipokines such as adiponectin. The high molecular weight (HMW) adiponectin has been hypothesized to influence PCOS (Carmina et al., 2005). A large group of women with PCOS was compared with BMI–matched controls. In order to explore the variables other than IR and BMI that may regulate HMW adiponectin in PCOS. These authors reported that HMW adiponectin is selectively decreased in women suffering from PCOS irrespective of BMI and insulin resistance (O’Connor et al., 2010).

The diagnosis of PCOS is highly controversial (Azziz 2006, Franks 2006). Many authors over the world have made attempts to evolve standard criteria to develop unanimity of diagnosis of PCOS (Zawadzki and Dunaif, 1992; Rotterdam ESHRE/ASRM-sponsored PCOS Consensus Workshop Group 2004; Azziz et al., 2006; Catherine et al., 2006). The study of plasma proteomics of PCOS by 2-Dimensional Gel Electrophoresis (2-D PAGE) is becoming popular in establishing molecular diagnosis of PCOS. This is on account of the fact that proteomic analysis of plasma provides direct pictures of cellular activities and changes in various organs of the body including ovaries. Moreover plasma or serum sampling is the easiest approach for proteomic analysis of PCOS (Catherine et al., 2006). Further, plasma proteomics of PCOS can be of great help in getting each individual’s molecular profile. It has been reported that plasma proteomic biomarkers of PCOS are highly significant to understand the mechanism of genesis of PCOS. A biomarker explains and indicates underlying pathological/inflammatory process (Biomarkers Definitions Working Group, 2001). According to some authors proteomic analysis of PCOS for molecular diagnosis is in very preliminary stage; yet it has the potential to detect and explore proteins that can guide us to the underlying pathology (Atiomio et al., 2009). Moreover, proteomic techniques applied to investigate PCOS have revealed number of processes related to oxidative stress, inflammatory changes and iron metabolism involved in the pathogenesis of features characteristic of PCOS (Insensner and Escobar-Morreale, 2011). Nevertheless, reproducibility of proteomic studies, analysis of data its interpretation and biomarkers specific to PCOS is yet to be developed (Zhao et al., 2008).

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY SAMPLE
The study recruited a total of 204 subjects and patients. Fifty normal, healthy subjects with age ranging 18-45 years were randomly selected as a reference population for comparative purposes (Control). They were healthy, non-pregnant, non-lactating and non-smoking for the previous two years and had regular menstrual cycles.

One hundred and five patients of PCOS were selected from the infertility clinics at Lady Wallingdon Hospital, Lady Aitchison Hospital, Abdullah diagnostic Clinic and Private Community based Family Planning Clinic in the city of Lahore. A third group comprising of 25 subjects who were the first degree relatives (FDR) of the patients of PCOS, were also selected for investigating hormonal profile and electrophoretic protein patterns. The participation rate was 77% in controls (n=50), 62% in PCO patients (n=65) and 50% (n=25) in FDR of the patients. All of the participants, i.e., controls, PCO patients and FDR of patients belonged to the middle socio-economic status. The study proposal was approved by the Ethical Review Committee (ERC) of King Edward Medical University and Mayo Hospital, Lahore.

The objective of the study was explained to each women (including controls, PCOS patients and FDR of the patients), and a written consent was obtained. All of the participants were provided with the results of all the investigations i.e., ovarian morphology (sonograms) records and hormonal estimations.

A detailed history of past gynecological events of each patient was noted on a proforma devised for this study (Annex I). History of hypertension, diabetes mellitus, menstrual irregularities, weight gain, malignancy of genito-urinary tract was recorded. Detailed menstrual history including date of onset of first menstrual period, last menstrual period, duration of length of cycles, history of missed periods, or complete loss of cycles was recorded. Each woman was examined for the presence of signs of acne and hair growth on the body and face region. Women with hepatic, renal or with evident endocrine disorders, history of immunosuppressive therapy or on any form of drug treatment were excluded from the study.

The ultrasound diagnosis of PCOS was made on the basis of ESHRE/ASRM criteria, 2004 i.e., 10 or more cystic follicles/ovary, varying in size from 2-9 mm or ovarian volume of 10 cms in maximal diameter.

3.2 BLOOD SAMPLING

Blood sample (5 - 7 ml) of each patient was drawn from cubital vein puncture, with least possible stress, by registered technicians; using good quality sterilized disposable syringes (Becton Dickinson Private Limited). The blood samples were drawn at 10.00 a.m. to 11.00 a.m in the morning considering the diurnal hormonal variations. The collected blood samples were immediately transferred from the syringe to the centrifuge tubes after removal of the needle to avoid possibility of haemolysis and allowed to coagulate at room temperature. Subsequently the
sera were separated by centrifugation (2000xg) for 5 minutes at room temperature with a centrifuge (Model NF 1215, Nuve, Turkey). The collected sera were divided into multiple aliquots and stored in the labeled plastic vials at 80°C until used for analyses.

3.3 ANALYTICAL PROCEDURES

Serum LH, FSH, insulin, testosterone, androstenedione, prolactin, growth hormone (GH) were analysed by hormonal assay kits in all of the samples. Electrophoretic protein profiles were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in blood samples selected on the basis of hormonal variations. The hormonal analyses were performed at radio-immuno-assay (RIA) laboratories, Centre for Nuclear Medicine (CENUM), Mayo Hospital, Lahore. The protein profiles of controls, PCOS patients and FDRs were performed at University of the Punjab. Serum androstenedione and testosterone were determined by competitive radioimmunoassay (RIA) while serum LH, FSH, prolactin, insulin and GH were determined by a ‘sandwich type’ immunoradiometric assay (IRMA) by using commercial kits of Immunotech Inc. (Beckman Coulter, Czech Republic and France).

Commercially derived control sera of low, medium and high concentrations were included in every run for determination of assay reliability. All assays were carried out in duplicate, and were performed in batches to eliminate variability within assays, and RIA and IRMA results were expressed at less than 10% cumulative variation (CV) of imprecision profile. The electrophoretic protein profiles were studied by using the method of Laemli (1970) for one dimensional and Garfin (2003) for two dimensional gel electrophoresis.

3.4 HANDLING OF SAMPLES

- All precautionary measures were taken considering as the serum samples were potentially capable of transmitting hepatitis or AIDs. All waste was discarded.
- Eating, drinking, smoking and use of cosmetics were not allowed during the process of sample handling. The pipeting of radio-active material/solutions was strictly not done by mouth. The gloves and lab overalls were used to avoid contact with radio-active material.

3.5 STATISTICAL ANALYSIS

Statistical analysis was performed using Graph Pad Prism version 5.00 for windows Graph Pad software, San Diego, California, USA. Data obtained from each parameter of the study was presented as mean±SEM. The data was analysed statistically using one-way analysis of variance (ANOVA). Tukey’s Post-Hoc Multiple comparison test was employed for comparing variations amongst different groups of the study.
HORMONAL ASSAYS

3.6 MEASUREMENT OF LH, FSH, INSULIN, PROLACTIN AND GROWTH HORMONE

These hormones were measured by immune-radiometric assay (IRMA) with the help of respective kits provided by Immunotech SAS (Czech Republic) on gamma camera model PC-RIA.MAS (Stratec Inc, Germany).

3.6.1 Principle
The immuno-radio-metric assay (IRMA) is a sandwich-type assay. The principle of the assay is to use mouse monoclonal antibodies directed against two different epitopes of respective hormones. Samples or calibrators are incubated in tubes, which are coated with the first monoclonal antibody in the presence of the second \(^{125}\text{I}\)-labeled monoclonal antibody. The contents of tubes were aspirated and rinsed after incubation and finally the bound radioactivity was measured. The values of relevant hormonal estimations were calculated by interpolation from the standard curve. The radioactivity bound is directly proportionate to the concentrations of concerned hormones in the samples.

3.6.2 Reagents provided
The following reagents were provided in the kits used for hormonal analysis:

- Anti-LH monoclonal antibody coated tubes: 2 x 50 tubes,
- \(^{125}\text{I}\)-labeled monoclonal antiLH, FSH,
- Insulin, Prolactin and Growth hormone antibodies for each hormone: one 5.5 ml vial.
- The vial contains 370 KBq (Kilo Buequral), at the date of manufacture, of \(^{125}\text{I}\)-labeled immunoglobulins in buffer with bovine serum albumin, sodium azide and a dye.
- Calibrators: six vials, the calibrator vials contain from 0 - 180 IU/L of LH in horse serum with sodium azide. The exact concentration is indicated on each vial.
- Control serum: one vial, the vial contains relevant hormones lyophihlized in human serum.
- Wash solution: one 50 ml vial, concentrated solution has to be diluted before use.

All reagents of the kit were stored at 2-8°C.

3.6.3 Preparation of reagents
All reagents were allowed to acquire the room temperature. The calibrators and control serum were reconstituted with distilled water according to the instruction on the label. After 30 minutes of reconstitution, the contents of the vials were mixed gently and stored at 2-8°C. The wash solution was prepared by pouring the contents of the vial into 950 ml of distilled water. It was homogenized and stored at 2-8°C.

3.6.4 Assay procedure:
Series of antibody coated tubes for the respective hormones were numbered in duplicate. A measured quantity i.e. 100 ml of calibrator control or sample was added in respective tubes. 50 µl
of the tracer was added to each tube and contents were thoroughly mixed. Two tubes were prepared separately containing only 50 µl of tracer in order to obtain total cpm (T). The tubes were incubated at 18-25°C while shaking (at >350 rpm) for 90 minutes. The contents of the tubes were aspirated carefully and washed with 2 ml of wash solution twice except the 2 tubes for total cpm.

The radioactivity of calibrator, control and each sample tube (bound counts) and separated 2 tubes (total counts) was counted for 1 minute by gamma counts. The results were recorded, as obtained from the standard curve by interpolation in case of each hormone i.e. LH, FSH, Insulin, Prolactin and Growth hormone. The standard curve serves to determine the concentration of each hormone in samples measured at the same time as the calibrators.

3.7 TESTOSTERONE AND ANDROSTENEDIONE

These two hormones were measured by Radio-Immuno-Assay (RIA) with the help of RIA testosterone and androstenedione kits provided by Immunotech SAS (France) on gamma camera model PC-RIA.MAS (Stratec Inc, Germany).

3.7.1 Principle
Testosterone and androstenedione were measured by radio-immuno-assay which is a competitive assay. The samples or calibrators are incubated with $^{125}$I-labeled testosterone and androstenedione respectively in antibody-coated tubes. The liquid contents of tubes were aspirated after incubation, and the bound radioactivity was determined with a gamma counter. A standard curve is drawn and unknown values are obtained from curve by interpolation.

3.7.2 Reagents used
- Anti-testosterone and anti-androstenedione antibody-coated tubes: 2 x 50 tubes for each hormone, $^{125}$I-labeled testosterone tracer: one 55 ml vial. The vial contains 185KBq (Kilo Bacqural) of $^{125}$I-labeled testosterone in liquid form containing gelatin and a dye.
- Calibrators: six 0.5 ml vials. The calibrator vials contain from 0 - 20 ng/ml (0 - 69 nM) of testosterone and androstenedione from 0-15 ng/ml (0-52 nM) of ∆4androstenedione in buffer with bovine serum albumin and sodium azide respectively.
- Human serum with sodium azide.
Control serum: one 0.5 ml vial

All reagents of the kit were stored at 2-8°C.

3.7.3 Assay procedure

All reagents and samples were allowed to acquire room temperature used for the measurement of testosterone and androstenedione. The series of antibody coated tubes were numbered in duplicate. 50 µl of calibrator, control or sample was added in respective tubes. 500 µl of tracer was added to each tube and was thoroughly mixed. Two tubes were prepared separately containing only 500 µl of tracer in order to obtain total cpm (T). Tubes were covered and incubated at 37°C in water bath for 3 hours. The contents of the tubes were aspirated carefully except the 2 tubes separately prepared for total cpm (T). The radioactivity of each calibrator, control and sample tubes (bound counts) and separately prepared two tubes (total counts) was counted for 1 minute by gamma camera. The results were obtained from the standard curve by interpolation. The standard curve was employed to determine the concentrations of testosterone and androstenedione in samples as well as the calibrators.

3.8 ONE DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

Taking into account the importance of Gel Electrophoresis in the detection of proteins that may be associated with patients of PCOS, SDS-PAGE was performed.

Serum protein fractions were analyzed by SDS-PAGE using the method of Laemmli (1970). The gel is prepared by polymerizing acrylamide \((\text{CH}_2=\text{CH.CO.NH}_2)\) and a small quantity of cross linking reagent, methylene-bisacrylamide \((\text{CH}_2=\text{CH.CO.NH}_2)\). \(\text{CH}_2\) (bis), in the presence of a catalyst ammonium persulfate. Tetramethyl-ethylene diamine (TEMED) is also present to initiate and control the polymerization. Sodium dodecyl sulfate [\(\text{CH}_3(\text{CH}_2)_{10}\text{CH}_2\text{OSO}_3\text{Na}^+\)] abbreviated as SDS is added as a detergent that readily binds to proteins. At pH 7.0, in the presence of 1% W/V SDS and dithiothreitol (DTT), proteins dissociate into their subunits and bind large quantities of the detergent. This binding masks the natural charge of the protein giving a constant charge to mass ratio. The larger the molecule, the greater is the charge. Thus the electrophoretic mobility of the complex depends on the size (molecular weight) of the protein. Molecular weights of unknown proteins can therefore be determined by comparing their mobilities with a series of protein standards.

The sieving effect of polyacrylamide is important in this technique and the range of molecular weights that can be separated on a particular gel depends on the pore size of the gel. The amount of cross linking and hence the pore size in a gel can be varied by simply altering the amount of acrylamide-bisacrylamide solution to make different percentages of gels.

3.8.1 Reagents Preparation

Acrylamide-Bis-acrylamide (30%)

After weighing 29 g of acrylamide and 1 g of bis-acrylamide in a flask and dissolved in distilled water with magnetic stirrer. The volume was made up to 100 ml. It was stored in a reagent bottle at 4°C, and wrapped with aluminium foil to avoid exposure to light thus preventing any reaction.

1.5 M Tris-HCl (pH 8.8)
Then 18.17 g of trizma base [Tris (hydroxymethyl) amino methane] was measured in a flask and 
dissolved in distilled water on a magnetic stirrer. The pH was adjusted to 8.8 with 1 N HCl. The 
final volume was made up to 100 ml by adding distilled water and was stored at 4°C in a reagent 
bottle.

1.0M Tris-HCl (pH 6.8)
In order to prepare 1.0M Tris-HCl, 12.11 g of trizma base was weighed and dissolved in distilled 
water on a magnetic stirrer. The pH was brought to 6.8 with 1 N HCl. Final volume was made up 
to 100 ml and stored at 4°C in a reagent bottle.

SDS (10%)
Ten gram (10 g) of SDS was weighed and dissolved in distilled water on a magnetic stirrer. The 
final volume was made up to 100 ml and stored in a reagent bottle at room temperature.

Electrophoresis Buffer
Took 3.02 g of Tris, 18.8 g of glycine and 1 g of SDS and dissolved in distilled water. The volme 
was made up to 1 liter and stored at room temperature.

Fixative Solution
In order to make fixative solution 30 ml of absolute ethanol, 10 ml of glacial acetic acid were 
taken in a graduated cylinder and 60 ml of distilled water was added to it. Final volume was thus 
made up to 100 ml. Colloidal Coomassie Staining Solution

To prepare Colloidal Coomassie Staining Solution (CCSS) 100 g of ammonium sulfate 
(NH4)2SO4 was dissolved in 200 ml distilled water usig magnetic stirrer. Then 100 ml of 85% 
orthophosphoric acid was added in ammonium sulfate solution then 1.2 g of Coomassie Brilliant 
Blue G-250 was added to it. The solution thus prepared was thoroughly dissolved on magnetic 
stirrer for one hour. The final volume was made up to 800 ml with distilled water and stored in a 
reagent bottle at room temperature. Methanol was added in 1:4 ratios to stain the gel.

Destaining Solution
A measured amount of 50 ml methanol and 70 ml of acetic acid was taken in a graduated cylinder 
and distilled water was added to make the final volume up to 1 litre. The solution was stored 
after mixing at room temperature.

Low Molecular Weight Markers For 12% Gel
PageRuler™ Unstained Protein Ladder (Fermentas, Catalog#SM0661 (5μl) was used as standard. 
It was a mixture of 14 recombinant, highly purified proteins from 10 kDa to 200 kDa. 
Approximately 0.02-0.05 mg/ml of each protein was present in the following storage buffer: 62.5 
mM Tris-H3PO4 (pH 7.5 at 25°C), 1 mM EDTA, 2% SDS, 0.1 M DTT, 1 mM NaN3, 0.01% 
bromophenol blue and 33% glycerol. It was stored at -20°C.

Gel Assembly
Mini protean tetra electrophoresis cell (Bio-Rad, Catalog#165-3301) was used to run the SDS-
PAGE with PowerPacTM HV Power Supply (Bio-Rad, Catalog#164-5056). Casting frame was 
placed upright with the pressure cams in the open position and facing forward on a flat surface. A 
short plate was placed on top of the spacer oriented upside. The two glass plates were slided into 
the casting frame with short plate facing the front of the frame. When the glass plates were in 
place, pressure cams were engaged to secure the glass cassette sandwich in the casting frame.
Casting frame was placed in the casting stand by positioning the casting frame onto the casting gasket while engaging the spring-loaded lever of the casting stand onto the spacer plate.

**Casting Polyacrylamide Gel**
The glass plate was marked at the level at which the resolving gel is to be poured and a comb was placed completely into the assembled gel cassette and marked 1cm below the comb teeth. Resolving gel monomer solution was prepared by combining all reagents except APS and TEMED which were added at the time of pouring. After adding APS and TEMED, solution was poured to the mark using disposable plastic pipette. Immediately, monomer solution was overlaid with water; added slowly to prevent mixing. Gel was polymerized within 45 minutes to 1 hour. Gel surface was rinsed completely with distilled water and dried the top of the resolving gel with filter paper before pouring the stacking gel. Stacking gel monomer solution was prepared and poured between the glass plates until the top of the short plate was reached. Comb was inserted between the spacers and stacking gel was allowed to polymerize for 30-45 minutes. After polymerization, comb was removed and wells were rinsed thoroughly with distilled water or running buffer.

**Electrophoresis Module Assembly**
Clamping frame was set to the open position on a clean flat surface and gel cassette was placed (with the short plate facing inward) onto the gel support at an angle of 30º tilting away from the center of clamping frame. Second gel was placed on the other side of the clamping frame and both gels were gently pulled towards each other creating a functioning assembly. Electrode assembly was used to run two gels simultaneously.

**Gel Buffer System**
In this experiment, discontinuous buffer system was used i.e. different buffer ions were present in the gel and electrode reservoirs. Proteins were denatured by heating in buffer containing SDS and DTT. The resultant denatured polypeptides appeared as a rod-like shape and a uniform charge-to-mass ratio proportional to their molecular weights.

**Placement of Electrode Assemblies in Mini-Protean Tetra Tank**
Electrode assembly was placed in the back position of the cell and lower chamber of the tank was filled with buffer to the indicated level (550 ml for 2 gels and 680 ml for 4 gels).

Required total buffer volume in the upper and lower chamber was 700 ml for 2 gels.

**Sample Loading**
Upper chamber of the assembly was filled with buffer up to just under the edge of the outer gel plate. Samples were loaded slowly to settle evenly on the bottom of the wells with a Hamilton syringe or a pipette using gel loading tips.

**Mini-Protean Tetra Tank Assembly**
Mini-protean tetra tank lid was placed and aligned with the color coded banana plugs and jacks. The correct orientation was made by matching the jacks on the lid with the banana plugs on the electrode assembly. At this point, lid was firmly pressed down with thumbs using even pressure, till the lid securely and tightly positioned on the tank.

**Power Conditions**
Power was applied to the mini-protean tetra cell through PowerPacTM HV power supply. The process of electrophoresis was started and gel was run at 60 V for 1 hour for best stacking. The
voltage was increased to 120 V for resolving gel. For mini protean tetra cell, the minimum run time was approximately 2:30 hours for 12% SDS-PAGE.

**Gel Removal**

After electrophoresis was completed, power supply was turned off and electric leads were disconnected. Tank lid was removed and electrode assembly was lifted carefully. The running buffer was poured and the arms of the assembly were opened to remove the gel cassettes. The gel was removed from gel cassette by separating the two glass plates.

Finally the gel was picked carefully and placed in the fixative solution.

**Fixation**

Gel was fixed in fixative solution for 4 hours to overnight.

**Staining**

After fixation, gel was transferred to staining solution. Then methanol was added afresh to the staining solution for characteristic granules formation. The gel was stained overnight with constant agitation on an orbital shaker.

**Destaining**

After staining the gel, it was transferred to destaining solution. Once the gel was destained with constant agitation, a clear background became visible. Consequently, the protein fractions appeared to the naked eye in the form of blue colored bands.

**Image Storing and Photography**

Once the protein fractions appeared in the form of blue coloured bands, the gel was photographed and its image was saved with Gene Genius Bio-imaging gel documentation system.

**Quantification of Protein Fractions**

Total Lab Quant that provided the data of molecular weight and density of each protein fraction was used to quantify the separated proteins. Ultimately, the band percentage displayed by each protein fraction was recorded.

**Statistical Analysis**

The protein fractions in the gels were presented as Mean±SEM (standard error of mean).

The data obtained was analyzed statistically using one-way analysis of variance (ANOVA). P-values of less than 0.05 (p< 0.05) were considered statistically significant.

### 3.9 TWO DIMENSIONAL GEL ELECTROPHORESIS (2-DE)

The samples were further analyzed through two-dimensional gel electrophoresis to determine the expression of protein fractions that could employ as biomarkers. The technique consisted of two steps, first iso-electric focusing (IEF) and second SDS-PAGE. In IEF, soluble proteins were
separated according to their iso-electric point (pI) and then migrated into the SDS-PAGE where they resolved according to their molecular weights.

The experimental sequence of 2-Dimensional Electrophoresis (2-DE) is as under:

1. Sample preparation
2. IPG strip rehydration
3. Iso-electric focusing
4. IPG strip equilibration
5. SDS-PAGE
6. Visualization
7. Analysis

3.9.1 Reagents Preparation

**Rehydration Buffer**

<table>
<thead>
<tr>
<th>Description</th>
<th>Final Concentrations</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>7 M</td>
<td>4.20 g</td>
</tr>
<tr>
<td>Thiourea</td>
<td>2 M</td>
<td>1.52 g</td>
</tr>
<tr>
<td>CHAPS</td>
<td>1.2% (w/v)</td>
<td>0.12 g</td>
</tr>
<tr>
<td>DTT</td>
<td>20 mM</td>
<td>0.031 g</td>
</tr>
<tr>
<td>Ampholyte (Bio-Lyte 3/10 ampholyte, 100X, 1 ml)</td>
<td>0.25% (v/v)</td>
<td>25 μl</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.005% (w/v)</td>
<td>0.005 g</td>
</tr>
<tr>
<td>Deionized water</td>
<td></td>
<td>up to 10 ml</td>
</tr>
</tbody>
</table>

It was stored in 2 ml aliquots at -20°C.

**Equilibration buffer**

<table>
<thead>
<tr>
<th>Description</th>
<th>Final Concentrations</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>6 M</td>
<td>18.02 g</td>
</tr>
<tr>
<td>Tris-HCl, pH 8.8</td>
<td>50 Mm</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>2% (w/v)</td>
<td>1 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>30% (v/v)</td>
<td>15 ml</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.002% (w/v)</td>
<td>5 mg</td>
</tr>
</tbody>
</table>

**SDS-PAGE Equilibration Buffer 1 (with DTT)**

Equilibration buffer 1 was made by dissolving 0.08 g DTT in 4 ml of equilibration buffer achieving final 2% (w/v) DTT concentration. **SDS-PAGE Equilibration Buffer 2 (with IAA)**

Equilibration buffer 2 was made by dissolving 0.1 g IAA in 4 ml of equilibration buffer achieving final 2.5% (w/v) IAA concentration.
0.5% Agarose Sealing Solution

<table>
<thead>
<tr>
<th>Description</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS electrophoresis buffer</td>
<td>1X (0.302 g Tris, 1.88 g glycine and 0.1 g SDS in 100 ml deionized water)</td>
<td>100 ml</td>
</tr>
<tr>
<td>Agarose</td>
<td>0.5%</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.002% (w/v)</td>
<td>200 μl</td>
</tr>
</tbody>
</table>

Agarose and bromophenol blue were dissolved in Tris-Glycine-SDS buffer on heating stirrer. The solution was not allowed to boil over. It was stored at room temperature.

3.9.2 ISO-ELECTRIC FOCUSING (IEF)
The first-dimension separation procedure involved the following steps:

- Sample preparation
- IPG strip rehydration
- Sample application
- Iso-electric focusing

As the IPG strips were provided dry, rehydration was required with appropriate additives prior to IEF. For a well-focused first-dimension separation, sample proteins were completely disaggregated and fully solubilized. IEF was performed on a flat-bed system at very high voltages with active temperature control. Strip was rehydrated in PROTEAN® IEF focusing tray (Bio-Rad, Catalog#163-4010). The ReadystripTM IPG strip(Bio-Rad, Catalog#163-2007) with a strip length of 17 cm (strip length 17.8 cm, gel length 17.1 cm, strip width 3.3 mm and gel thickness 0.5 mm) and linear pH interval of 3-10 was used for an overview of total protein distribution. The PROTEAN® IEF cell (Bio-Rad, Catalog#165-4000) was programmed for active rehydration and transition automatically to focusing run.

Sample Preparation
Plasma sample was thawed; vortexed and protein concentration was determined by Bradford assay. Protein concentration of 250 μg (sample volume 300 μl) was loaded on 17 cm IPG strip for proper protein separation.

IPG Strip Rehydration and Sample Application
The sample was prepared in rehydration buffer. The sample volume of 300 μl was dispensed as a line along the periphery of a channel in an IEF focusing tray. After protein sample had been loaded, the cover sheet from the IPG strip was peeled off using forceps and strip was gently placed with gel side down onto the sample in IEF focusing tray so that the acidic (marked with “+”) end was at the anode of the IEF cell. It was ensured that the gel made contact with the electrodes and no air bubble was trapped beneath the IPG strip. The IPG strip was soaked with samples at room temperature and the mineral oil (2-3 ml) was overlaid on the strip to prevent evaporation during rehydration process. The focusing tray was covered with lid and placed in the PROTEAN IEF cell. Rehydration was done under active condition at 50 V for at least 12 hours. Focusing conditions were set according to sample composition, complexity and IPG pH range.
Following rehydration, the voltage was maintained at 250 V for 15 minutes (S1) and rapidly attained 10,000 V in three hours (S2). During Step 3, the voltage was linearly increased for another 3 hours attaining the final 40-60000 V/hr, determining the ramp of the process.

After IEF had been completed, the IPG strip was removed from the focusing tray and transferred with gel side up into a new clean, dry disposable equilibration tray according to the length of the IPG strip. The IPG strip was held vertically with forceps to let the mineral oil be drained for at least 5 seconds before transfer.

3.9.3 SECOND DIMENSION/SDS-PAGE
SDS-PAGE for second dimension consisted of four steps:

- Assembling the gel plates and preparation of the gel
- Equilibrating the IPG strip in SDS equilibration buffer
- Placing the equilibrated IPG strip on the SDS gel
- Electrophoresis

Glass Plates Assembling
PROTEAN II XL Cell (Bio-Rad, Catalog#165-3188) was used for second dimension SDS-PAGE. The longer glass plate was laid down and two spacers of equal thickness were placed along the rectangular plate. The shorter glass plate was placed on top of the spacers so that the bottom ends of the spacers and glass plates were aligned. The screws on the clamp assembly were loosened and glass plate sandwich was gently slid into the clamp assembly. The screws of the clamp assembly were tightened and clamp assembly was placed into the alignment slot of the casting stand. The screws were loosened to allow the plates and spacers to set firmly against the casting stand base. All the screws were gently tightened and clamp assembly was transferred to one of the casting slots in the casting stand.

Preparation and Casting the Gel
By using the following reagents, 50 ml of 12% resolving gel was prepared.

<table>
<thead>
<tr>
<th>12% Resolving Gel</th>
<th>Distilled Water (ml)</th>
<th>30% Acrylamide/Bisacrylamide (ml)</th>
<th>1.5M Tris (pH 8.8) (ml)</th>
<th>10% SDS (μl)</th>
<th>10% Ammonium persulfate (APS) (μl)</th>
<th>TEMED (μl)</th>
<th>Final volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16.5</td>
<td>20</td>
<td>12.5</td>
<td>500</td>
<td>500</td>
<td>20</td>
<td>50</td>
</tr>
</tbody>
</table>

The gel mixture was poured into the assembled glass plates on its top, avoiding air bubbles and a comb was inserted in the gel. The gel was allowed to polymerize for one hour. The comb was removed and gel surface was rinsed with de-ionized water.

IPG Strip Equilibration
The IPG strip was equilibrated in SDS equilibration buffers prior to the second dimension. The two step equilibrations ensured the reduction and alkylation of cysteine which minimized/eliminated vertical streaking. The equilibration buffer 1 and 2 were prepared with 2%
DTT and 2.5% IAA respectively. Mineral oil was removed from the IPG strip by placing it (the gel is upside) on to a piece of dry filter paper and blotting with a second piece of wet filter paper. The equilibration buffer 1 was placed in an equilibration tray and the blotted IPG strip was transferred (gel side should be up) in it. The strip was equilibrated with DTT for 10 minutes with constant shaking. IPG strip was blotted after incubation, holding vertically and transferred to equilibration buffer 2 and the tray was returned to orbital shaker for 10 minutes. During the process of incubation, 0.5% agarose solution was melted in a microwave oven. At the end of incubation period, strip was blotted again and was washed briefly by dipping in 100 ml glass cylinder with 1X TGS buffer.

**Transferring the Strip on SDS-PAGE**

Excess of buffer was blotted from equilibrated IPG strip and the strip was placed on the PAGE with the plastic side towards the back plate. The IPG strip was carefully pushed into the well by using forceps, avoiding any air bubbles beneath the IPG strip. Agarose solution was overlaid into the IPG well over the strip and allowed to solidify for 5 minutes. The gel was mounted into the electrophoresis cell and marker proteins (15 to 20 μl) were loaded on the “+” end of the IPG strip.

**Electrophoresis**

The reservoir was filled with 1X TGS running buffer and lid was placed on the electrophoresis cell; and power was turned on. The migration of the bromophenol blue, present in the overlay agarose solution, was used to monitor the progress of the electrophoresis run. The gel was initially run at 16 mA for one hour and increased to 35 mA for the rest of gel.

**Fixation**

Following the completion of electrophoresis, the electric supply was switched off and gel assembly was lifted out of the chamber. The plates forming the gel assembly were carefully separated from each other with the help of spatula. The gel was carefully picked up and kept in fixative solution overnight.

**Staining**

The staining solution was prepared by mixing 80 ml of staining solution and 20 ml of methanol. Subsequently the gel was kept in it after fixation. Characteristic granules were appeared. Gel was stained overnight with constant shaking.

**Destaining**

The stained gel was shifted to to the destaining solution and destained with constant agitation until protein spots became visible with a transparent background.

**Analysis**

The gels were analyzed through Ludesi REDFIN and Swiss 2-D page database. Different proteins were identified in patients of PCOS and their expression was compared with controls group.
RESULTS

Table 4.1 presents the analysis of basic parameters in age-matched groups comprising of normal control subjects (n=50), patients of PCOS (n=65) and first degree relatives (FDR, n=25) of PCOS patients. Significant alterations were observed in comparable groups as indicated by one way ANOVA at P<0.0001 and confidence interval of 99.9% (Table 4.2).

For multiple comparisons Tukey’s post-HOC analysis was performed.

4.1 BODY MASS INDEX (BMI)

The body mass index in the control group had a mean value of 20.81±0.42 Kg/m² The BMI in PCO group was significantly high and showed a 26% rise (26.23±0.55) as compared with control group (P<0.0001 and CI of 99.9%). The FDR group depicted 23% significant decrease (20.11±0.43) in the BMI as compared with PCO group (P<0.0001 and CI of 99.9%). There was no significant difference in the control Vs FDR group (Fig. 4.1).

![Figure 4.1: Average body mass index (kg/m²) of comparable groups.](image)

*** Significance at P<0.001 (CI 99.9 %) in comparison to control

4.2 HORMONAL ANALYSES

4.2.1 LUTEINIZING HORMONE (LH)

The average levels (mean±SEM) of LH in the control, PCO and FDR groups were 6.39±0.88, 38.11±3.11 and 5.09±0.69 respectively. The LH was elevated significantly in the PCO group as compared with control group indicating 60% rise (P<0.0001 and CI of 99.9%). The LH level fell significantly in the FDR group demonstrating 87% decrease compared with PCO group (P<0.0001 and CI of 99.9%). The FDR group LH values were insignificant as compared with normal control group (Fig. 4.2).

![Figure 4.2: Average luteinizing hormone levels.](image)
4.2.2 FOLLICLE STIMULATING HORMONE (FSH)

The mean FSH values were $5.54 \pm 0.38$, $3.74 \pm 0.25$, and $3.62 \pm 0.27$ in the control, PCO and FDR groups respectively. The FSH estimations were significantly lowered in PCO and FDR groups as compared with controls. There was 33% fall in PCO group and 35% fall in the FDR group respectively ($P<0.0001$ and CI of 99.9%) in the FSH values (Fig. 4.3).

4.2.3 LH: FSH RATIO
The ratio of LH: FSH was observed to be 1.12±0.11, 21.53±5.83 and 1.68±0.26 in the control, PCO and FDR groups respectively. The LH: FSH ratio in PCO Vs controls was observed to be significantly higher (P<0.01 and CI of 99%). The FDR group Vs PCO group also showed significantly lower ratio (P<0.05 and CI of 95%). The ratio of LH: FSH was non-significant in the FDR Vs control group (4.4).

4.2.4 INSULIN

The estimated mean levels of insulin were 7.22±0.61 in the control group, 43.48±4.09 in the PCO patients and 4.82±0.46 in the FDR group. The insulin in the PCO Vs control was significantly elevated. The PCO group manifested a very high mean value of 43.48±4.09 indicating five-fold rise in this group having (P<0.001 and CI of 99.9%). The FDR group showed a significant fall (P<0.001 and CI of 99.9%) depicting 89% decrease compared with PCO group. The FDR group Vs control group demonstrated no significant difference (Fig. 4.5).
4.2.5 TESTOSTERONE

Testosterone estimations in control, PCO and FDR groups were found to be 0.64±0.04, 1.19±0.05 and 0.38±0.04 respectively. The testosterone levels in the PCO group were highly significantly raised, depicting 86% elevation as compared with control group (P<0.001 and CI of 99.9%). There was 68% decrease in the testosterone values in the FDR group in comparison with PCO group. This indicated 96% significant fall (P<0.001 and CI of 99.9%) in the FDR group. The testosterone levels also showed a significant fall in the FDR group in comparison with control group (P<0.01 and CI of 99%). It demonstrated 40% decrease in the testosterone (Fig. 4.6).

![Bar chart showing testosterone levels](image)

**Fig. 4.6.** Average levels of testosterone in comparable groups.

4.2.6 ANDROSTENEDIONE

The average androstenedione estimation was found to be 2.11±0.09, 5.55±0.28 and 1.29±0.17 in the control, PCO and FDR groups respectively. The androstenedione was greatly
Fig. 4.7. Average levels of androstenedione in comparable groups.

*** Significance at P<0.001 (CI 99.9 %) in comparison to control

*** Significance at P<0.001 (CI 99.9 %) in comparison to PCO

elevated in the PCO group as compared to the controls indicating a very highly significant rise of 163% (P<0.0001 and CI of 99.9%). There was significant decrease of 76% (P<0.0001 and CI of 99.9%) in the FDR group as compared with PCO group. There was no significant difference in normal controls and FDR group (Fig. 4.7). A significant correlation was observed between LH and androstenedione (P<0.05 and CI of 95%).

4.2.7 GROWTH HORMONE (GH)

The mean GH estimations were found to be 3.20±0.52, 3.46±0.83 and 1.86±0.37 in the control, PCO and FDR groups respectively. These values in the controls Vs PCO group were non significant at 11%. Similarly mean values in FDR Vs PCO and FDR Vs control groups were non-significant respectively at 46% and 42% (Fig. 4.8).

Fig. 4.8. Average levels of growth hormone in comparable groups.

4.2.8 PROLACTIN
The prolactin measurements revealed to be 3.99±0.51, 9.75±0.71 and 2.71±0.22 in the control, PCO and FDR groups. The values of prolactin in control Vs PCO group was significantly raised in PCO patients indicating 45% elevation of prolactin in this group(P<0.001 and CI of 99.9%). The FDR group showed significantly higher values of prolactin Vs controls manifesting 48% rise (Fig. 4.9).

Fig. 4.9. Average levels of prolactin in comparable groups.
*** Significance at P<0.001 (CI 99.9 %) in comparison to control
*** Significance at P<0.001 (CI 99.9 %) in comparison to PCO

4.2.9 CORRELATION OF LH WITH TESTOSTERONE AND ANDROSTENEDIONE

The LH and testosterone correlation was found to be non-significant (r=0.068, P=0.637 NS). The LH and androstenedione was highly significantly correlated (r=0.375, P=0.007,CI of 99%).
Table 4.1. Summary statistics of categorical variables in different cohorts of study.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control group</th>
<th>PCOS group</th>
<th>FDR group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (yrs)</td>
<td>19.67</td>
<td>26.71</td>
<td>19.86</td>
</tr>
<tr>
<td>Amenorrhea (%)</td>
<td>Nil</td>
<td>75.38</td>
<td>Nil</td>
</tr>
<tr>
<td>Acne (%)</td>
<td>18.00</td>
<td>32.31</td>
<td>16.00</td>
</tr>
<tr>
<td>Hair growth (%)</td>
<td>10.00</td>
<td>63.08</td>
<td>05.71</td>
</tr>
<tr>
<td>Obesity (%)</td>
<td>06.00</td>
<td>81.54</td>
<td>08.00</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>Nil</td>
<td>10.77</td>
<td>Nil</td>
</tr>
<tr>
<td>Mean age of menarche (yrs)</td>
<td>13.05</td>
<td>13.85</td>
<td>16.00</td>
</tr>
<tr>
<td>Family H/O infertility (%)</td>
<td>Nil</td>
<td>26.15</td>
<td>100.0</td>
</tr>
<tr>
<td>Family H/O hypertension (%)</td>
<td>34.00</td>
<td>44.62</td>
<td>36.00</td>
</tr>
<tr>
<td>Family H/O diabetes (%)</td>
<td>32.00</td>
<td>32.31</td>
<td>32.00</td>
</tr>
</tbody>
</table>

FDR - First degree relatives of PCOS patients. H/O - History of.
Table 4.2. BMI and hormonal variables in control group, PCOS patients and their first degree relatives (FDR).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls</th>
<th>PCOS group</th>
<th>FDR group</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>20.81±0.42</td>
<td>26.23±0.55*</td>
<td>20.11±0.43 Δ</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>LH (IU/L)</td>
<td>6.39±0.88</td>
<td>38.11±3.11*</td>
<td>5.09±0.69 Δ</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>FSH (IU/L)</td>
<td>5.54±0.38</td>
<td>3.74±0.25*</td>
<td>3.62±0.27*</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>LH:FSH</td>
<td>1.12±0.11</td>
<td>21.53±5.83*</td>
<td>1.68±0.26 Δ</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Insulin (µIU/mL)</td>
<td>7.22±0.61</td>
<td>43.48±4.09*</td>
<td>4.82±0.46 Δ</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Testosterone (ng/mL)</td>
<td>0.64±0.04</td>
<td>1.19±0.05*</td>
<td>0.38±0.04* Δ</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Androstendione (ng/mL)</td>
<td>2.11±0.09</td>
<td>5.54±0.28*</td>
<td>1.28±0.16 Δ</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Growth hormone(mIU/L)</td>
<td>3.20±0.52</td>
<td>3.46±0.83</td>
<td>1.86±0.37</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>Prolactin (ng/mL)</td>
<td>3.99±0.51</td>
<td>9.75±0.71*</td>
<td>2.71±0.22 Δ</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

* Significant in comparison to control group
Δ Significant in comparison to PCOS group
Values are mean ± SEM

4.3 ONE DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS
The electrophoretic results in the form of appearance/disappearance of protein fractions in healthy subjects and PCOS patients are presented (Fig. 4.10-4.29). Band percentages expressed by each of the fractions are analyzed in comparable subjects (Tables 4.3).
4.3.1 CONTROL GROUP

An overall view of protein profile resulted in the detection of seven high molecular weight protein fractions ranging between 181-13kDa.

Among these, protein fractions of 181kDa (ranging between 3.88-6.74%) was first in expression. Its band percent was 6.74, 5.59, 6.60, 5.74, 6.10, 3.88, 4.52 % in subject no. 18 (Fig. 4.10).

Protein fraction of 131kDa (ranging between 1.04-3.67%). This fraction exhibited a band percent of 1.19, 1.87, 1.04, 1.90, 1.95, 1.81, 3.67% in subject no. 1-8 (Fig. 4.11).

Protein fraction of 100kDa (ranging between 3.53-6.46%) was next in expression. In subject no. 1-8, the fraction covered 5.32, 6.83, 6.54, 7.28, 6.04, 6.04, 5.92 band percent, respectively (Fig. 4.12).

Protein fraction of 80kDa (ranging between 7.24-9.86%) was next to 100kDa in expression. This fraction exhibited a respective band percent of 9.15, 7.24, 8.52, 8.36, 8.43, 9.00, 9.86, in subject no. 1-8 (Fig. 4.13).

Protein fraction of 66kDa (ranging between 3.41-5.45%) was next in expression. This protein fraction exhibited band percentage of 35.66, 33.74, 36.65, 39.74, 37.89, 35.77, 38.88% in subject no. 1-8 (Fig. 4.14).

51kDa protein fraction (ranging between 11.32-16.02%) was next in expression. This fraction covered band percent of 11.32, 16.02, 15.40, 18.46, 15.99, 14.51, 11.65% in subject no.1-8 (Fig. 4.15).

Protein fraction of 32kDa (ranging between 4.70-9.98%) was next in expression. It covered 8.99, 4.70, 8.38, 7.73, 6.05, 7.06, 9.98 fraction percent in subject no. 1-8, respectively (Fig. 4.16).

Protein fraction of 29kDa (ranging between 3.10-4.63%) was next in expression. This fraction covered 4.50, 3.10, 4.48, 3.99, 3.33, 3.11, 4.63 band % in subject no. 1-8 (Fig. 4.17).

Protein fraction of 23kDa (ranging between 14.51-15.91%) was next in expression. This fraction covered 15.46, 15.73, 14.51, 14.98, 15.91, 15.47 band % in subject no. 1-8 (Fig. 4.18).

Protein fraction of 17kDa (ranging between 3.38-5.87%) was next to 23kDa in expression. This fraction covered 4.01, 5.87, 4.98, 3.38, 5.78, 4.37, 4.09 respective band % in subject no. 1-8 (Fig. 4.19).

Protein fraction of 15kDa (ranging between 0.83-1.61%) was next in expression. It covered, respectively, 1.37, 1.35, 1.52, 1.55, 1.61, 1.20, 0.83 band % in subject no. 1-8 (Fig. 4.20).

Protein fraction of 13kDa (ranging between 0.97-1.59%) was next in expression. Its fraction percent was 1.30, 1.13, 0.97, 1.59, 1.37, 1.33, 1.52, 1.13 in subject no. 1-8, respectively (Fig. 4.21).

4.3.2 PCOS PATIENTS GROUP

The serum protein profile analysis in PCOS patients resulted in the detection of twelve protein fractions resolved on 10 and 15% gel ranging between 181-13kDa.
Among these, protein fraction of 181kDa (ranging between 4.70-9.71%) was first in expression. It exhibited fraction percent of 9.71, 8.79, 7.13, 6.38, 6.97, 5.27, 7.34, 8.53, 7.52 in subject no. 9-18, respectively. Whereas, 6.22, 7.94, 4.75, 6.23, 5.78, 6.84, 6.26, 5.95, 4.70 fraction percent was observed in subject no. 19-27, respectively (Fig. 4.10).

The fraction covered by 131kDa proteins was not expressed in any PCOS patient (Fig. 4.11).

Protein fraction of 100kDa (ranging between 6.15-8.75%) was next in expression. This fraction exhibited a band percent of 8.01, 8.36, 7.95, 7.73, 8.35, 7.16, 6.15, 6.53, 6.90 in subject no. 9-18, respectively. The same fraction occupied 8.55, 8.29, 7.97, 7.32, 8.02, 7.27, 9.15, 8.75, 8.47 band % in subject no. 19-27, respectively (Fig. 4.12).

Protein fraction of 80kDa (ranging between 7.14-12.17%) was next in expression. This fraction exhibited 9.61, 10.63, 11.71, 10.15, 9.75, 9.08, 10.70, 12.17, 9.51 band % in subject no. 9-18, respectively. A fraction % of 10.86, 11.91, 7.14, 9.42, 9.51, 7.98, 7.37, 8.96, 10.34 was found in subject no. 19-27, respectively (Fig. 4.13).

Protein fraction of 66kDa (ranging between 32.13-39.92%) was next in expression. This protein fraction occupied 32.13, 39.86, 35.00, 35.56, 36.84, 33.25, 34.17, 37.41, 33.33% in subject no. 9-18, respectively. Moreover, a band % of 38.95, 39.92, 39.40, 39.68, 37.39, 37.69, 35.24, 38.04, 33.09 was observed in subject no. 19-27, respectively (Fig. 4.14). Protein fraction of 51kDa (ranging between 9.50-15.11%) was next in expression. This fraction exhibited a percentage of 15.11, 12.90, 12.48, 13.11, 14.24, 14.82, 10.66, 12.52, 11.58 in subject no. 9-18, respectively. Subject no. 19-27 showed 12.79, 14.41, 10.76, 13.66, 11.27, 11.49, 12.79, 11.63, 9.50 band percent, respectively (Fig. 4.15).

Protein fraction of 32kDa (ranging between 5.23-9.47%) was next in expression. This fraction exhibited a band % of 7.67, 6.61, 8.19, 6.50, 7.01, 5.66, 7.47, 8.75, 9.47% in subject no. 9-18, respectively. Subject no. 19-27 showed 7.20, 7.17, 7.18, 5.23, 7.64, 7.95, 7.18 fraction percent, respectively (Fig. 4.16).

Protein fraction of 29kDa (ranging between 3.37-6.07%) was next in expression. This fraction exhibited %age of 4.36, 3.52, 4.21, 4.43, 4.14, 3.37, 4.62, 4.41, 4.50 in subject no. 9-18, respectively. The same fraction occupied 3.59, 4.44, 4.50, 6.07, 5.53, 4.92, 5.66, 5.48, 4.38 band % in subject no. 19-27, respectively (Fig. 4.17).

Protein fraction of 23kDa (ranging between 11.23-13.83%) was next in expression. This fraction exhibited 12.62, 13.70, 12.58, 13.49, 11.33, 13.83, 12.31, 11.56, 12.34 band %age in subject no. 9-18, respectively. A fraction percent of 12.65, 12.28, 11.70, 12.74, 12.99, 11.09, 11.23, 11.59, 12.30 was found in subject no. 19-27, respectively (Fig. 4.18). Protein fraction of 17kDa (ranging between 0.45-3.17%) was next in expression. This protein fraction occupied 0.45, 2.52, 2.69, 7.77, 3.62, 3.00, 2.01, 2.29, 3.17 band %age in subject no. 9-18, respectively. A band percent of 2.19, 2.51, 2.28, 2.82, 2.61, 2.23, 2.80, 2.74, 2.18% was observed in subject no. 19-27, respectively (Fig. 4.19).

Protein fraction of 15kDa (ranging between 0.99-2.68%) was next in expression. This fraction covered 1.46, 1.66, 1.57, 2.65, 2.68, 1.07, 1.01, 1.13, 1.31% in subject no. 9-18, respectively. Subject no. 19-27 showed 0.99, 1.11, 1.16, 1.52, 1.05, 1.11, 1.18, 1.24, 1.59 fraction percent, respectively (Fig. 4.20).
Protein fraction of 13kDa (ranging between 0.91-2.96%) was next in expression. This fraction exhibited a percentage of 1.67, 2.09, 1.94, 2.94, 2.30, 2.03, 1.32, 1.67, 1.61 in subject no. 9-18, respectively. Subject no. 19-27 showed 1.35, 0.91, 1.36, 2.01, 2.78, 1.68, 1.51, 2.69, 2.32 band percent, respectively (Fig. 4.21).
Fig. 4.10. Band %age exhibited by 181kDa protein fraction in Control subjects and PCOS Patients showing individual variations.

Fig. 4.11. Band %age exhibited by 131kDa protein fraction in Control subjects and PCOS Patients showing individual variations.

Fig. 4.12. Band %age exhibited by 100kDa protein fraction in Control subjects and PCOS Patients showing individual variations.
Fig. 4.13. Band %age exhibited by 80kDa protein fraction in Control subjects and PCOS Patients showing individual variations.

Fig. 4.14. Band %age exhibited by 66kDa protein fraction in Control subjects and PCOS Patients showing individual variations.
Fig. 4.15. Band %age exhibited by 51kDa protein fraction in Control subjects and PCOS Patients showing individual variations.

Fig. 4.16. Band %age exhibited by 32kDa protein fraction in Control subjects and PCOS Patients showing individual variations.
Fig. 4.17. Band %age exhibited by 29kDa protein fraction in Control subjects and PCOS Patients showing individual variations.

Fig. 4.18. Band %age exhibited by 23kDa protein fraction in Control subjects and PCOS Patients showing individual variations.
Fig. 4.19. Band %age exhibited by 17kDa protein fraction in Control subjects and PCOS Patients showing individual variations.

Fig. 4.20. Band %age exhibited by 15kDa protein fraction in Control subjects and PCOS Patients showing individual variations.
Fig. 4.21. Band %age exhibited by 13kDa protein fraction in Control subjects and PCOS Patients showing individual variations.
showing individual variations.
showing individual variations.
showing individual variations.
4.3.3 GROUP COMPARISON

181kDa Protein Fraction (Table 4.4, Fig. 4.30)
The average band percentage covered by 181kDa protein fraction was found to be 5.6 ± 0.40 in control group and 6.8 ± 0.32 in PCOS patients group indicating significant (p=0.0480) increase of 21.42% in patients when compared to control subjects.

- showing individual variations.
131kDa Protein Fraction (Table 4.4, Fig. 4.31)
Average band percentage covered by 131kDa protein fraction was found to be 1.9 ± 0.32 in control group and 0.00 ± 0.00 in PCOS patients group indicating highly significant (p<0.0001) decrease of 100% in patients when compared to control subjects.

100kDa Protein Fraction (Table 4.4, Fig. 4.32)
The average band percentage covered by 100kDa protein fraction was found to be 6.3 ± 0.25 in control group, whereas, patients group, the value increased to 7.8 ± 0.19 indicating highly significant (p=0.0001) increase of 23.8% in patients when compared to control subjects.
showing individual variations.

80kDa Protein Fraction (Table 4.4, Fig. 4.33)
The average band percentage covered by 80kDa protein fraction was found to be 8.7 ± 0.31 in control group, whereas, 9.8 ± 0.34 in patients group indicating non significant (p=0.0542) increase of 12.64% in patients group when compared to control group subjects.

66kDa Protein Fraction (Table 4.4, Fig. 4.34)
The average band percentage covered by 66kDa protein fraction was found to be 37 ± 0.78 in control group, whereas, in exposed worker group, the value increased to 36 ± 0.61 indicating non significant (p=0.7148) decrease of 2.70% in patients when compared to control subjects.

51kDa Protein Fraction (Table 4.4, Fig. 4.35)
The average band percentage covered by 51kDa protein fraction was found to be 15 ± 0.96 in control group, whereas, in patients group, the value decreased to 13 ± 0.36 indicating significant (p=0.0130) decrease of 13.34% in patients when compared to control subjects.
32kDa Protein Fraction (Table 4.4, Fig. 4.36)
The average band percentage covered by 32kDa protein fraction was found to be 7.6 ± 0.68 in control group, whereas, in patients group, the value decreased to 7.4 ± 0.25 indicating a non significant (p=0.7714) decrease of 2.64% in patients as compared to control subjects.

29kDa Protein Fraction (Table 4.4, Fig. 4.37)
The average band percentage covered by 29kDa protein fraction was found to be 3.9 ± 0.26 in control group whereas in patients group, the value increased to 4.6 ± 0.17 showing individual variations.
Fig. 4.37. Average band percentage exhibited by 29kDa protein fraction in controls and patients group. Values are shown as Mean ± SEM, * representing significance at p<0.05.

indicating a significant (p=0.0448) increase of 17.94% in patients when compared to control subjects.

23kDa Protein Fraction (Table 4.4, Fig. 4.38)
The average band percentage covered by 23kDa protein fraction was found to be 15 ± 0.20 in control group, whereas, in patients, the value decreased to 12 ± 0.19 indicating highly significant (p=<0.0001) decrease of 20% in patients when compared to control subjects.

Fig. 4.38. Average band percentage exhibited by 23kDa protein fraction in controls and patients group. Values are Mean ± SEM, *** representing significance at p<0.001.

17kDa Protein Fraction (Table 4.4, Fig. 4.39)
The average band percentage covered by 17kDa protein fraction was found to be 4.6 ± 0.35 in control group, whereas, in patients group, the value decreased to 2.5 ± 0.15 indicating highly significant (p=<0.0001) decrease of 45.66% in patients when compared to control subjects.

Fig. 4.39. Average band percentage exhibited by 17kDa protein fraction in controls and patients group. Values are Mean ± SEM, *** representing significance at p<0.001.

15kDa Protein Fraction (Table 4.4, Fig. 4.40)
The average band percentage presented by 15kDa protein fraction was found to be 1.3 ± 0.10 in control group, whereas, in patients, the value increased to 1.4 ± 0.12 indicating a non significant (p=0.7353) decrease of 7.69% in patients as compared to control subjects.

showing individual variations.
**13kDa Protein Fraction (Table 4.4, Fig. 4.41)**

The average band percentage covered by 13kDa protein fraction was found to be 1.3 ± 0.085 in control group whereas in patients group, the value increased to 1.9 ± 0.14 indicating a significant (p=0.0096) increase of 46.15% in patients when compared to control subjects.

**Table 4.4. Average band percentages of various protein fractions in control and PCOS patients groups showing group comparison. Values are Mean±SEM.**

<table>
<thead>
<tr>
<th>Molecular weigh(kDa)</th>
<th>Controls Average band percentage</th>
<th>Patients</th>
<th>Percentage ↑ or ↓</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>181</td>
<td>5.6±0.40</td>
<td>6.8±0.32</td>
<td>*21.42↑</td>
<td>0.0480</td>
</tr>
<tr>
<td>131</td>
<td>1.9±0.32</td>
<td>0.0±0.00</td>
<td>***100↓</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>100</td>
<td>6.3±0.25</td>
<td>7.8±0.19</td>
<td>***23.8↑</td>
<td>0.0001</td>
</tr>
<tr>
<td>80</td>
<td>8.7±0.31</td>
<td>9.8±0.34</td>
<td>12.64↑</td>
<td>0.0542</td>
</tr>
<tr>
<td>66</td>
<td>37.0±0.78</td>
<td>36.0±0.61</td>
<td>2.7↓</td>
<td>0.7148</td>
</tr>
<tr>
<td>51</td>
<td>15.0±0.96</td>
<td>13.0±0.36</td>
<td>*13.34↓</td>
<td>0.013</td>
</tr>
</tbody>
</table>

showing individual variations.
CONTROL GROUP

On 2D gel, first spot in expression was Serotransferrin (Transferrin) with molecular weight in the range of 76-87kDa, pI (6.14-6.55) and accession number P02787. The next band/spot was serum albumin with molecular weight in the range of 66-68kDa, pI (5.67-5.58) and accession number P02768, was detected. Spot number 3 was recognized as Immunoglobulin heavy chain gamma (IGHG) with molecular weight in the range of 5155kDa, pI (6.15-9.02) and accession number P99006. Spot number 4 was identified as Haptoglobin Beta chain with molecular weight 37-44kDa, pI (4.81-5.86) and accession number P00738. Spot number 5 was identified as Alpha-1 Antitrypsin with molecular weight in the range of 53-58kDa, showing individual variations.

**4.4 TWO DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS 4.4.1**
pl (4.87-5.10) and accession number P01009. Spot number 6 was recognized as Alpha-1-acid glycoprotein with molecular weight 43-46kDa, pl (4.11-4.29) and accession number P02763. Spot number 5 was identified as Alpha-2HS-glycoprotein (Fetuin-A) with molecular weight in the range of 52-58kDa, pl (4.564.71) and accession number P02765. Spot number 8 was an unidentified protein which was present in control group. Spot number 9 was recognized as Apolipoprotein A-1 had molecular weight 23-24kDa, pl (4.99-5.48) and accession number P02647. Spot number 10 was identified as Immunoglobulin light chain (IgL) having molecular weight 2329kDa, pl (5.02-8.56) and accession number P99007. Band number 11 was said to be Haptoglobin Alpha chain had molecular weight 16-17kDa, pl (5.40-6.07) and accession number P00738. Band number 12 was recognized as Transthyretin with molecular weight 13-14kDa, pl (5.02-5.52) and accession number P02766 (Table 4.5, Fig. 4.42).

4.4.2 PCOS PATIENTS GROUP

In PCOS patients, the first spot, on 2D gel, in expression was Serotransferrin (Transferrin) with molecular weight in the range of 76-87kDa, pl (6.14-6.55) and accession number P02787. The next band/spot was serum albumin with molecular weight in the range of 6668kDa, pl (5.56-7.38) and accession number P02768, was detected. Spot number 3 was recognized as Immunoglobulin heavy chain gamma (IGHG) with molecular weight in the range of 51-55kDa, pl (6.15-9.02) and accession number P99006. Spot number 4 was identified as Haptoglobin Beta chain with molecular weight 37-44kDa, pl (4.81-5.86) and accession number P00738. Spot number 5 was identified as Alpha-1 Antitrypsin with molecular weight in the range of 53-58kDa, pl (4.87-5.10) and accession number P01009.

Spot number 6 was recognized as Alpha-1-acid glycoprotein with molecular weight 4346kDa, pl (4.11-4.29) and accession number P02763. Spot number 5 was identified as Alpha-2-HS-glycoprotein (Fetuin-A) with molecular weight in the range of 52-58kDa, pl (4.56-4.71) and accession number P02765. Spot number 8 was an unidentified protein which was present in control group. Spot number 9 was recognized as Apolipoprotein A-1 had molecular weight 23-24kDa, pl (4.99-5.48) and accession number P02647. Spot number 10 was identified as Immunoglobulin light chain (IgL) having molecular weight 23-29kDa, pl (5.02-8.56) and accession number P99007. Band number 11 was said to be Haptoglobin Alpha chain had molecular weight 16-17kDa, pl (5.40-6.07) and accession number P00738. Band number 12 was recognized as Transthyretin with molecular weight 13-14kDa, pl (5.02-5.52) and accession number P02766 (Table 4.5, Fig. 4.43-4.44).

4.4.3 GROUP COMPARISON

Comparison was done between PCO’s patient and healthy women (control group) through the comparison of qualitative expression of different spots in both groups. Up to 12 different protein bands/spots were identified and their expressions were compared with that of control. Some of these bands showed an up regulation and some of them exhibited an expression of down regulation as compared to control. The bands/spots that showed up regulation in their expression were Serotransferrin (spot 1), Haptoglobin alpha chain (spot 11) and Alpha-2-HS-glycoprotein (Fetuin-A) (spot 7) showed up regulated expression in PCO’s patients as compared to control healthy females. Whereas Immunoglobulin heavy chain gamma (IGHG) (spot 3), Haptoglobin beta chain (spot 4), Alpha-1 antitrypsin (spot 5), and Alpha-1-acidic glycoprotein (spot 6), Apolipoprotein A-1 (spot 9), Immunoglobulin light chain (IgL) (spot 10) and Transthyretin (spot 12) showed down regulation in their expression when compared with showing individual variations.
control. An unidentified protein (spot 8) was also down-regulated in PCOs as compared with control group (Table 4.5, Fig. 4.42-4.44).
Fig. 4.42. Gel image of healthy control woman indicating variedly expressed protein spots (1-12) as resolved by 2D PAGE.

1: Serotransferrin (STF); 2: Albumin (ALB); 3: Immunoglobulin heavy chain (IGHG); 4: Haptoglobin β chain; 5: α-1 antitrypsin (ATR); 6: Alpha-1 acidic glycoprotein (AGP); 7: Alpha-2-HS-glycoprotein (FETUA); 8: Unidentified protein; 9: Apolipoprotein A-1 (ApoAI); 10: Immunoglobulin light chain (IGLC); 11: Haptoglobin α chain, 12: Transthyretin (TTR)

showing individual variations.
Fig. 4.43. Gel image of Polycystic Ovary Syndrome patient indicating variedly expressed protein spots (1-12) as resolved by 2D PAGE.

1: Serotransferrin (STF); 2: Albumin (ALB); 3: Immunoglobulin heavy chain (IGHG); 4: Haptoglobin β chain; 5: α-1 antitrypsin (ATR); 6: Alpha-1 acidic glycoprotein (AGP); 7: Alpha-2-HS-glycoprotein (FETUA); 8: Unidentified protein; 9: Apolipoprotein A-1 (ApoAI); 10: Immunoglobulin light chain (IGLC); 11: Haptoglobin α chain; 12: Transthyretin (TTR)

showing individual variations.
Fig. 4.44. Gel image of Polycystic Ovary Syndrome patient indicating variedly expressed protein spots (1-12) as resolved by 2D PAGE.

1: Serotransferrin (STF); 2: Albumin (ALB); 3: Immunoglobulin heavy chain (IGHG); 4: Haptoglobin β chain; 5: α-1 antitrypsin (ATR); 6: Alpha-1 acidic glycoprotein (AGP); 7: Alpha-2-HS-glycoprotein (FETUA); 8: Unidentified protein; 9: Apolipoprotein A-1 (ApoAI); 10: Immunoglobulin light chain (IGLC); 11: Haptoglobin α chain; 12: Transthyretin (TTR)

showing individual variations.
Table 4.5. Protein fractions showing variable expressions in women with PCOS as compared to controls.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein Names</th>
<th>Accession Numbers</th>
<th>Molecular Weights</th>
<th>pI</th>
<th>Expression in PCOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Serotransferrin (STF)</td>
<td>P02787</td>
<td>76-87kDa</td>
<td>6.14-6.55</td>
<td>Slightly Up-Regulated</td>
</tr>
<tr>
<td>2</td>
<td>Albumin (ALB)</td>
<td>P02768</td>
<td>66-68kDa</td>
<td>5.56-7.38</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>Immunoglobulin heavy chain (IGHG)</td>
<td>P99006</td>
<td>51-55kDa</td>
<td>6.15-9.02</td>
<td>Down-Regulated</td>
</tr>
<tr>
<td>4</td>
<td>Haptoglobin β chain</td>
<td>P00738</td>
<td>33-44kDa</td>
<td>4.81-5.86</td>
<td>Down-Regulated</td>
</tr>
<tr>
<td>5</td>
<td>α-1 antitrypsin (ATR)</td>
<td>P01009</td>
<td>53-58kDa</td>
<td>4.87-5.1</td>
<td>Down-Regulated</td>
</tr>
<tr>
<td>6</td>
<td>Alpha-1 acidic glycoprotein (AGP)</td>
<td>P02763</td>
<td>43-46kDa</td>
<td>4.11-4.29</td>
<td>Down-Regulated</td>
</tr>
<tr>
<td>7</td>
<td>Alpha-2-HSglycoprotein (FETUA)</td>
<td>P02765</td>
<td>52-58kDa</td>
<td>4.56-4.71</td>
<td>Up-Regulated</td>
</tr>
<tr>
<td>8</td>
<td>Unidentified Protein</td>
<td>------</td>
<td></td>
<td>5.8</td>
<td>Down-Regulated</td>
</tr>
<tr>
<td>10</td>
<td>Immunoglobulin light chain (IGLC)</td>
<td>P99007</td>
<td>23-29kDa</td>
<td>5.02-8.56</td>
<td>Down-Regulated</td>
</tr>
<tr>
<td>11</td>
<td>Haptoglobin α chain</td>
<td>P00738</td>
<td>16-17kDa</td>
<td>5.4-6.07</td>
<td>Up-Regulated</td>
</tr>
<tr>
<td>12</td>
<td>Transthyretin (TTR)</td>
<td>P02766</td>
<td>13-14kDa</td>
<td>5.02-5.52</td>
<td>Down-Regulated</td>
</tr>
</tbody>
</table>

showing individual variations.
CHAPTER 5
DISCUSSION

PCOS is an entity which is generating controversy regarding its pathogenesis, diagnostic criteria and therapeutic procedures (Banaszewska et al., 2003). The diagnosis of PCOS becomes difficult on account of discrepancies in diagnostic criteria; especially because of controversy in interpretation of ovarian ultrasound imaging (Cajdler-luba et al., 2010).

Over the current years, hormonal analyses are being regarded as the principal diagnostic criteria of PCOS. It has been reported that insulin appears to disrupt all components of hypothalamohypophyseal-ovarian axis; and that hyperandrogenemia is the major factor responsible for the clinical features and complications of PCOS (Rojas et al., 2014).

Nevertheless, classical presentation of PCOS comprises of oligomenorhea with clinical and biochemical evidence of hyperandrogenism; however, presenting symptoms vary widely; including anovulation with hirsutism or without hirsutism and regular cycles. Interestingly the principal criterion of diagnosis of PCOS is hormonal analysis i.e. serum LH, FSH, insulin and testosterone levels (Franks, 2010). The present study was, therefore, carried out to determine endocrine attributes of PCOS in the back drop of local, ethnic, socioeconomic and environmental elements in Pakistani population.

The results of the hormonal analysis in the present study demonstrated significantly higher levels of LH, FSH, LH:FSH ratio, insulin, testosterone, androstenedione and body mass index. Nevertheless, the hormonal profile is supportive of international studies. Similarly the LH:FSH ratio was found to be significantly higher as compared to the reported ratios. Serum LH levels were significantly elevated (P<0.001) in PCOS group as compared to control group, indicating 60% rise. The elevated LH in PCOS has also been reported earlier (Suhail, 2008). However, the elevation in LH level, in our study, was significantly intense than the reported values possibly on account of ethnic variations and environmental factors in our population. The LH levels fell significantly in the FDR group demonstrating 87% decrease compared to PCO group (P<0.001).The LH levels in FDR group were insignificant as compared to normal control group.

It has most recently been investigated that LH, FSH, testosterone and androstenedione can prove to be predictive markers of ovulation after laparoscopic ovarian drilling (LOD). The serum LH and androstenedione levels were found to be independent markers of ovulation after LOD. The PCOS patients who have elevated levels of LH prior to LOD, demonstrated good response in terms of postoperative induction of ovulation (Johannes et al., 2009). LOD markedly decreased postoperative LH and androstenedione levels depicting the effectivity of laparoscopic ovarian drilling. It was especially true for the patients who had higher androstenedione values of more than 3.26 ng/ml; and for patients with both LH concentration of 12.1 IU/L, or greater and androstenedione exceeding these cut off values. Moreover, ovarian surgery has been reported showing individual variations.
to cause rapid reduction in serum levels of all ovarian hormones i.e. initiation of folliculogenesis, and — restoration of normal feed-back to hypothalamus and pituitary, resulting in appropriate gonadotrophin secretion and normal follicular development. This reversal towards normal situation may be due to increasing FSH levels that occur on account of restoration of normal feed back system as a consequence of ovarian surgery (Hendriks et al., 2007).

The LH: FSH ratio in PCOS in comparison to control subjects was observed to be significantly higher (P<0.001) in the present study. This ratio was raised by 18–folds in the PCOS group. It has been reported that most of the women (75%) with PCOS have an elevated LH level in the early follicular phase; and 94% have an enhanced LH to FSH ratio (Taylor et al, 1997). It has, therefore, been hypothesized that gonadotrophin dysfunction plays a key role in generating anovulatory amenorrhea characteristic of PCOS women (Pagan et al, 2006).

Moreover, raised LH:FSH ratio may prove to be beneficial in revealing PCOS by gonadotrophin releasing hormone (GnRH) stimulation test. This test is suggested to be an important additional investigation in the diagnosis of PCOS based on elevated LH in serum (Cajdler-Luba, 2010). The LH:FSH ratio in PCOS demonstrates greater accuracy in prediction of PCOS than total testosterone and average ovarian volume in these women presenting with oligomenorrhea or anovulation. Further, an LH:FSH ratio of more than one presented the best combination of sensitivity and specificity (Ming et al., 2009). It, therefore, appears that hormonal analysis especially LH levels and LH:FSH ratio are reliable predictors of PCOS as observed in the current study.

On the other hand PCOS group compared to FDR group also showed significantly lower ratio (P<0.05) indicating 91% decrease in the ratio. The ratio of LH:FSH varied nonsignificantly in the FDR group in comparison to control group. The highly raised ratio may be on account of very high LH values in PCOS patients. Conversely, the FSH concentrations were significantly lower (P<0.01), indicating 33% fall in the PCOS group, contributing towards a very high LH:FSH ratio as observed in this study. The FSH also decreased in FDR group compared to controls indicating 35% fall in this group. The raised LH:FSH ratio has been regarded by most authors an important diagnostic feature in PCOS. The highly significantly raised levels of LH, and LH:FSH ratio in our study is in agreement with most of the international reports.

Moreover, raised LH:FSH ratio may prove to be beneficial in revealing PCOS by gonadotrophin releasing hormone (GnRH) stimulation test. This test is suggested to be an important additional investigation in the diagnosis of PCOS based on elevated LH in serum (Cajdler-Luba et al., 2010). In a retrospective study conducted at a tertiary academic centre, it was concluded that LH:FSH ratio is a valuable diagnostic tool in evaluating women having PCOS specially complaining of oligomenorrhea or anovulation. An LH:FSH ratio of more than one (>1) may be used as a decision threshold. It has been observed that LH:FSH ratio is a better predictor of showing individual variations.
PCOS. This ratio demonstrates greater accuracy in prediction of PCOS than total testosterone and average ovarian volume in these women presenting with oligomenorrhea or anovulation. Further, an LH:FSH ratio of more than one (>1) presented the best combination of sensitivity and specificity. It has been further reported that body mass index was negatively correlated with LH in PCOS, and no correlation was depicted in non-PCOS subjects. It was hence concluded that the link between body mass index and LH may provide clues for deeper insight in the pathophysiology of PCOS (Ming et al., 2009).

On the contrary, our study indicates a positive role of LH. The raised LH levels in PCOS patients also depicted a significantly raised BMI as compared to the control subjects. The BMI in PCOS group of patients showed a significant difference (P<0.001), indicating 26% increase in BMI. The BMI decreased significantly (P<0.001) indicating 23% fall in the FDR group as compared to PCOS group. There was no significant difference in BMI in comparison to control group.

It has been reported that women suffering from PCOS have consistent abnormalities in the gonadotrophin secretion. About 75% of these patients have elevated LH levels and 94% have elevated LH:FSH ratio (Blank et al., 2006). The overproduction of LH and consequently disturbed LH:FSH ratio is detected in 94% of PCOS patients. Until 1980s, LH:FSH ratio was considered a gold standard for the diagnosis of PCOS. Later, it was found that most of PCOS women with normal LH:FSH ratio suffered from hyperinsulinemia and obesity. Moreover, the elevated levels of LH occur more rarely in a group of patients with insulin resistance and hyperinsulinemia. It was suggested that LH:FSH ratio is not a characteristic sign of all PCOS patients (Banaszewska et al., 2003).

Most recently, raised leptin levels have been found to be associated with obesity; it has also been implicated to influence FSH, testosterone (T), obesity and PCOS (Sunita et al., 2014). It has also been observed that PCOS when associated with obesity leads to elevation of proinsulin concentrations, that is a known indicator of fertility outcomes in PCOS women. Moreover, treatment with metformin antagonises proinsulin and decreases the fertility outcomes in these patients of PCOS (Kruszyńska et al., 2014).

The LH:FSH ratio was correlated with elevated serum LH levels, insulin resistance, menstrual irregularities and obesity. It was reported that characteristic increase in LH relative to FSH, which is the commonest finding in PCOS is not a dependable variable/parameter. This is because LH is released in pulses, and a single test estimation of LH may not represent a real concentration of LH in serum. Consequently it was suggested that LH:FSH ratio may not be considered as a criterion for diagnosis of PCOS. These authors failed to find any correlation between LH:FSH ratio, BMI, menstrual pattern and hirsutism. Ultimately they disproved the notion that heavier the patient, higher the LH value; and conjectured that loss of correlation of LH with disease manifestations may add more to this mysterious syndrome (Alnakash and Al-Tae, 2007).

Sheehan (2004), in a review article has mentioned that LH:FSH ratio of more than two showing individual variations.
 (>2.0) is suggestive of PCOS; but is not highly sensitive or specific. The diagnosis of PCOS in Europe — does not require hormonal estimations in routine. This is in absolute contrast to the National Institute of Health conference held in 1990, which did not include ovarian morphology on pelvic ultrasound examination as a criterion for diagnosis of PCOS (Dunaif and Thomas, 2001). This is on account of the fact that 20% patients of PCOS do not have typical ultrasound picture consistent with its diagnosis. Nevertheless, pelvic ultrasonography is a useful evaluation tool for diagnosis of PCOS, but polycystic ovarian morphology is not specific for PCOS. The diagnosis of PCOS is made largely by ovarian morphology on ultrasound examination. It has been proposed that if four classic features of PCOS are present i.e. menstrual disturbance, hirsutism, acne or anovulatory infertility; an ultrasound assessment of ovaries is suggested. If polycystic ovarian morphology (PCOM) is detected, the diagnosis is confirmed (Homburgh, 2002). It therefore appears that hormonal analysis especially LH levels and LH: FSH ratio are reliable and good predictor of PCOS as indicated in our study.

In addition, we have observed highly significantly raised insulin levels. The PCOS group manifested a very high mean value of 43.48± 4.09 indicating 5-fold rise in this group (P<0.001). The FDR group showed a significant fall (P<0.001) depicting 89% decrease compared with PCO group. The increase in insulin levels in our study may be attributed to high levels of LH. It has been reported that insulin exerts a synergistic action along with LH. This in turn stimulates ovarian androgen synthesis (Nestler et al., 1998a).

Insulin also decreases hepatic production of sex hormone binding globulin (SHBG) resulting in higher levels of free testosterone in the blood (Dunkel et al., 1985). Insulin resistance and consequent hyperinsulinemia is a characteristic finding associated with PCOS reported by most workers. Moreover, insulin resistance is a common metabolic abnormality seen in obese and to a lesser degree in lean women with PCOS (Dunaif et al., 1989; Carmina et al., 1992; Legro et al., 1998; DeUgrate et al., 2005). It has been reported that treatment of patients of PCOS improves not only insulin resistance but also corrects hyperandrogenemia and restores normal ovarian function (Fleming et al., 2002; Ghazeeri et al., 2003; Lord et al., 2003). It has been further observed that correction of hyperandrogenemia is on account of decrease in circulating insulin levels. Moreover, it has been studied in vitro, that insulin directly influences steroid biosynthesis in the ovary (Attia et al., 2001; Mitwally et al., 2002; Mansfield et al., 2003). The highly significantly raised serum insulin levels in our study strongly support these international reports of hyperinsulinemia that contribute towards pathophysiology of PCOS. Moreover, the significantly raised LH concentrations in our study in PCOS may be on account of neuroendocrine dysfunction. It is evidenced by animal studies that hyperinsulinaemia raises basal and GnRH-stimulated LH and FSH secretion from pituitary cells in vitro (Soldani et al., 1994). Nevertheless infusions of exogenous insulin does not alter LH secretion amongst the women suffering from PCOS (Patel et al., 2003; Mehta et al., 2005). Moreover, it has been found that short-term treatment with anti-diabetic drugs like metformin does not inhibit or slow down the LH pulse frequency; despite the fact that significant improvement in hyperinsulinemia is observed in these patients (Eagleson et al., 2003). Similarly, thiazolidinedione/pioglitazone improves insulin sensitivity but does not influence or alter LH pulse patterns (Mehta et al., 2005). The body of international literature cited, suggests that hyperinsulinemia is no doubt, associated with PCOS, but does not appear to be directly linked with neuroendocrine abnormalities. Nevertheless, hyperinsulinemia may modify hypothalamic function indirectly by increasing androgen levels. The androgen levels especially testosterone and androstenedione were also significantly raised in our experiments. The testosterone levels in the PCOS group were highly significantly raised, depicting 86% elevation as compared with control group showing individual variations.
showing individual variations.

(P<0.001). There was 68% decrease in the testosterone values in the FDR group in comparison with the PCOS group. This indicated 96% significant fall (P<0.001) in the FDR group. The testosterone levels also showed a significant fall in FDR group in comparison to control group (P<0.01). It demonstrated 40% decrease in the testosterone. The rise in testosterone in our study is in accordance with reported androgen excess in PCOS. The significant fall in the testosterone levels in FDR group as compared to control group couldn’t be explained.

The androstenedione was greatly elevated in the PCO group as compared to the controls indicating a very highly significant rise of 163% (P<0.001). There was significant decrease of 76% (P<0.001) in FDR group as compared to PCO group. There was no significant difference in normal controls and FDR group.

A significant correlation (P<0.05) was observed between LH and androstenedione levels in this study. The highly significant rise in LH may potentiate hyperinsulinemia, which in turn can stimulate hyperandrogenemia. Nevertheless, hyperandrogenemia is an important variable associated with PCOS; the raised androgen level in PCOS appears to have multiple influences like raised LH levels, insulin-based stimulation of ovaries that may lead to hypersecretion of androgens (Nestler et al., 1998a; Nelson et al., 1999).

Irrespective of the cause of hyperandrogenemia in PCOS, the elevated androgens in the PCOS group in our study may be the reason of neuroendocrine disturbance. It was presumed initially that neither androgen infusions in women with PCOS nor short term therapy (01 month) with flutamide brought any change in the LH concentration and its pulsatility. Subsequently, it was found that short term treatment with flutamide, which is an androgen receptor blocking drug, restores hypothalamic sensitivity to progesterone mediated slowing of LH pulsatility in women with PCOS (Eagleson et al., 2000). Furthermore, long term treatment (06 months) with flutamide may alleviate many of the reproductive manifestations of PCOS (De Leo et al., 1998). This shows that elevated androgens have significant neuroendocrine effects. Moreover, rapid GnRH pulse frequency in PCOS is the factor that promotes excessive LH feed-forward mechanism leading to high androgen synthesis. It also affects the relative FSH deficiency that culminates into ovulatory dysfunction. It is thus summarized that elevated serum LH, elevated LH:FSH ratio and rapid GnRH pulse frequency in our study may have lead to insulin resistance. Insulin in synergy with elevated LH may increase the androgens in the blood that is characteristic of adult PCOS and adolescent hyperandrogenemia (Blank et al., 2006). We therefore, postulate that LH elevation in the blood is the central feature of PCOS. The elevated LH concentration subsequently leads to other clinical and biochemical events of the syndrome. Henceforth, it is suggested that raised LH levels, high LH:FSH ratio and hyperinsulinemia are the best indicators of PCOS.

The elevation of gonadotrophin especially LH in the patients of PCOS has been related with endocrine disruption of the reproductive neuro-endocrine system. This regulation of this system involves GnRH. GnRH has been cited as the primary stimulus for the reproductive axis i.e., hypothalamic-hypophyseal-gonadal (H-H-G) axis. LH and FSH act on the ovary and generate and generate steroid hormones (Gore, 2002).

Another important hypothesis states that sex steroid also regulate the GnRH in an indirect manner. The GnRH cells/neurons cannot express receptors for steroid hormones. It has, therefore, been hypothesized that some other neurons of the brain that can express receptors for steroid hormones are the target of the endocrine disrupting cells (EDCs). It further explains that EDCs can modify the brain neurotransmitter systems. The EDCs can modify or toxify the nor-adrenergic, serotonergic, dopaminergic and other neuroendocrine cells of the brain showing individual variations.
showing individual variations. (Dickerson and Gore, 2007; Walker and Gore, 2007). It has also been observed that all these neurons — endocrine cells can express steroid hormone receptors. Moreover, all these neurons can project to and regulate GnRH neurons. Thus, EDCs act on this linkage between neural and endocrine systems and exert their effects on the hypothalamo-pictuar-gonadal axis (Gore, 2002).

Polychlorinated biphenyls (PCBs), organochlorine pesticides like methoxychlor and chlorpyrifos have been blamed to influence GnRH cells, and cause significant changes in GnRH gene expression (Gore et al., 2002; Gore, 2002).

It has recently been reported that disposable plastic cups, cooking oil fumes and indoor decoration are environmental risk factors associated with PCOS (Huang et al., 2007).

All these data cited above, is suggestive of direct influence on GnRH cell lines. It appears that this complex and enigmatic disorder originates from within and outside the H-P-O axis. It may be conjectured/concluded that EDCs as environmental factors contribute in the pathogenesis of PCOS.

Hyperinsulinaemia is reported in women with polycystic ovary syndrome. It has been reported that administration of supplements of essential amino-acids significantly decreased total testosterone, LH and FSH. This consequently improves the clinical features of hyperandrogenism in PCOS. It appears that hyperandrogenism is related with and a consequence of hyperinsulinaemia (Unfer et al., 2008).

It has been demonstrated that environmental chemicals called as endocrine-disrupting chemicals (EDC) can modify reproductive function (Gore, 2007). The hypothalamopituitary-ovarian (HPO) axis has been reported to be influenced by environmental EDCs. (Gore, 2008). Moreover, it has been found that polychlorinated biphenyls (PCBs) and organochlorine pesticides such as methoxychlor and chlorpyrifas altered the GnRH gene expression, GnRH peptide release and morphology of the gonadotrophin cells (Gore et al., 2002; Gore, 2002).

Animal models also exhibited that GnRH neurons are targets of both natural environmental estrogens such as coumestrol, and industrial contaminants and pesticides (McGarvey et al., 2001; Gore, 2001). It has been concluded that EDCs can impair the reproductive physiology specially H-P-O-axis (Gore, 2008).

The use of oral contraceptives in the hyperandrogenic non-ovulating women, slows the LH pulse frequency but not to the extent observed in normal controls (Daniels and Berga, 1997).

Most recently, raised leptin levels have been found to be associated with obesity; it has also been implicated to influence FSH, testosterone (T), obesity and PCOS (Sunita et al., 2014). It has also been observed that PCOS when associated with obesity leads to elevation of proinsulin concentrations, that is a known indicator of fertility outcomes in PCOS women.

Moreover, treatment with metformin antagonises proinsulin and decreases the fertility outcomes in these patients of PCOS (Kruszynska et al., 2014).
This part of the present study of PCOS patients is focused on gonadotrophins, androgens and insulin levels mainly. Our data supported the renewed interest in the importance of endocrine parameters in the diagnosis of PCOS. However, attempts to predict PCOS risk in asymptomatic subjects could not be successful as the FDR group was small in number (n = 25). This was on account of decreased participation (18%) of the first degree relatives of PCOS patients who avoided investigations on account of being labeled as “infertile family”.

In the present study we have further investigated PCOS specific proteins and determined expression pattern with 2-D PAGE in order to understand the pathogenesis of PCOS. Poteomic techniques applied to investigate biomarkers of PCOS have revealed a number of processes related to oxidative stress, inflammatory changes and iron metabolism to be involved in the pathogenesis of PCOS (Insenser and Escobar-Morreale, 2012). For this purpose we analysed plasma protein profiles in normal subjects and patients of PCOS in Pakistani women. Consequently we have recognized 12 proteins, out of which 08 proteins were under-expressed and down-regulated in PCOS patients as shown in PCO table. The proteins like sero-transferrin, alpha 2-heat specific (HSP) glycoprotein and haptoglobin alpha chain were increased and upregulated in our population of patients of PCOS as compared with controls. All these proteins along with their accession number, molecular weights and isoelectric points (pI) are listed in PCO table.

Serotransferrin is known to be stress related, acute phase response molecule. Its over expression in patients of PCOS in our study can be elaborated on the basis of underlying inflammatory process (Galaziz et al., 2013). Serotransferrin is a glycoprotein that transports iron to the ovarian follicles (Briggs et al., 1999). Its up-regulation seems to be legitimate and reasonable in the present study conducted in Pakistani patients of PCOS. Moreover, overexpression of serotransferrin appears to be a compensatory change on account of anaemia of chronic inflammation. Upregulation of transferrin has also been reported in human follicular fluid (Gua and Gungxia, 2012).

Haptoglobin is a plasma alpha-2 glycoprotein formed by hepatocytes in the liver and subsequently thrown in the circulation (Bowman, 1993). The anti-inflammatory and antioxidant properties of haptoglobin justify its up-regulation and over-expression in our study. It has been suggested that haptoglobin alpha chain over-expression is a sign of reactive response to iron loss and renal injury; as a result oxidative stress gets improved and alleviated (Insenser et al., 2010). It has further been reported that haptoglobin alpha chain is over expressed in patients of PCOS as well as in type-2 diabetes mellitus (T2 DM) patients. It has been mentioned that both PCOS and T2DM are inflammatory conditions (Galaziz et al., 2013); it has, therefore, been hypothesized that haptoglobin interacts with haemoglobin and is found to be elevated on account of inflammation and oxidative stress (Zhang et al., 2004). Studies have suggested that oxidative stress, in women with PCOS, stimulates androgen-producing steroidogenic enzymes thus leading to hyperandrogenism, most notable characteristic of PCOS (Gonzalez et al., 2006).

Alpha-2-heat specific glycoprotein (alpha-2 HSG) has been reported to be over-expressed in preterm birth and PCOS in response to inflammation (Galaziz et al., 2013). Alpha-2
HSG is a receptor signaling protein and tyrosine kinase inhibitor. The levels of α-2 HSG are increased by the mechanisms such as oxidative stress, heat shock exposure, infection, inflammation and anaemia (Lindquist and Craig, 1988; Ciocca et al., 1992). Alpha-2 HSG is upregulated in our presented proteomic identification of PCOS which further potentiates the notion that PCOS is an inflammatory condition.

The proteomic map of our study in PCOS group of patients indicates under-expression of haptoglobin beta chain, alpha -1 antitrypsin (α-1 ATR), acidic glycoprotein (AGP), apolipoprotein A-1(Apo A-1), immunoglobulin light chain (IGLC) and transthyretin (TTR).

Apolipoprotein A-1(Apo A-1), in our study, is down regulated in PCOS patients as compared to healthy controls. The proteomic identification in granulosa cells of patients of PCOS also shows significant under-expression of Apo A-1. It has been claimed that the reduced level of Apo A-1 in granulosa cells may disturb normal steroidogenesis (Choi et al., 2010). It has been proposed that heat specific proteins (HSP) and Apo A-1 may be labeled as Disease Specific Proteins (DSPs) of PCOS. It is important to note that ApoA-1 is down-regulated in both PCOS and T2 DM (Galaziz et al., 2013).Hence, it is concluded that down-regulated ApoA-1 in women with PCOS is closely associated with aetiology of T2 DM and its cardiovascular complications. Transthyretin (TTR) is down-regulated in PCOS patients of the present study. TTR is a serum and cerebro-spinal fluid carrier of thyroxine and retinol. Its reduced expression may indicate the future risks of diabetes, insulin resistance and cardiovascular manifestations in these women.

Another important protein identified to be down-regulated in PCOS women of our study was alpha 1-antitrypsin (α-1 ATR). Alpha 1-antitrypsin is protease inhibitor, generally called as trypsin inhibitor. It has been reported that α-1 ATR inhibits wide range of proteases (Gettins, 2002). It protects the enzymes released from inflammatory cells like neutrophil elastase whose concentration rises many times during acute inflammation. The enzyme neutrophil elastase causes breakdown of elastic tissue thus affects elasticity in tissues. α-1 ATR was also found to be underexpressed in mice fed on dietary supplements of branched chain amino acids and analysed by 2-D PAGE (Brocca et al., 2013). The results of proteomic identification, in our study suggest the potential role of immuneregulation, inflammation and anti-oxidant activity involved in the pathogenesis of PCOS. Most recently it has been reported that PCOS gets exaggerated in pregnancy on account of low grade chronic inflammation. The Endocrine Society’s Journal of Clinical Endocrinology and Metabolism (JCEM) described; as studied by one of the authors in May, 2014 that the chronic low grade inflammation deteriorates during pregnancy.The author has blamed that PCOS is associated with hormonal alteration which may trigger inflammatory process (Palomba et al., 2014) This report further supports our impression that PCOS is chronic low grade inflammatory process. In nut-shell, the scientific community is now in a better position to comprehend the differential expressions of these biomarkers in the causation of PCOS (Galazi et al., 2013).The biomarkers of our study can/may provide a useful framework for early diagnosis of PCOS and for screening and prevention of complications in women with PCOS.

Conclusions

The endocrinological responses, in PCOS women of the present study, reinforce that the criteria of endocrinal diagnosis of the syndrome, especially gonadotrophins and LH to FSH ratio are important aids in the description of PCOS. It, however, seems mandatory that molecular investigations like protein biomarkers in PCOS, in addition to endocrinal correlations, be studied to arrive at exact and early diagnosis of the syndrome.
The altered expressions of ApoA-I, Haptoglobin and transthyretin predict that PCOS women are at a higher risk of future progression towards diabetes, insulin resistance and cardiovascular disorders, particularly, atherosclerosis. Further, the over-expressions of Serotransferrin and Haptoglobin α chain, whereas, declined expression of Haptoglobin β chain demonstrate that variations in iron metabolism, oxidative stress and low grade chronic inflammation might be the culprits in molecular pathogenesis of PCOS in women.

**SUMMARY**

Incidence of PCOS in Pakistani women is considerably increasing, however, bulk of available clinical and aetiological data serves to provide only a framework for studying its genesis. We, in our study, found that the gonadotrophins, LH to FSH ratio and androgens are dependable diagnostic parameters of PCOS. The LH:FSH ratio in PCOS group, was raised by 18-folds in this study. This highly raised ratio may be on account of very high LH and significantly lower FSH values in PCOS patients. It has, therefore, been hypothesized that gonadotrophin dysfunction plays a key role in generating anovulatory amenorrhoea characteristic of PCOS women. Moreover, raised LH:FSH ratio may prove to be beneficial in revealing PCOS by Gonadotrophin Releasing Hormone (GnRH) stimulation test. The LH:FSH ratio in PCOS demonstrates greater accuracy in prediction of PCOS than total testosterone and average ovarian volume in these women presenting with oligomenorrhea or anovulation. Further, an LH:FSH ratio of more than one presented the best combination of sensitivity and specificity. The elevated LH in PCOS has also been reported earlier. However, the elevation in LH level, in our study, was significantly intense. Henceforth, LH appears to be the most important in this regard, consequent to hyperandrogenism. In addition, we have observed highly significantly raised insulin levels in PCOS patients indicating a 5-fold rise in this group of patients; that may be attributed to high levels of LH. Moreover, the women suffering from PCOS have higher rates of obesity and hyperinsulinemia. The higher levels of insulin can be a consequence of different ethnic backgrounds. Our data supported the renewed interest in the importance of endocrine parameters in the diagnosis of PCOS.

In the present study we have further investigated PCOS specific proteins and determined expression pattern with 2-D PAGE in order to understand the pathogenesis of PCOS.

Poteomic techniques applied to investigate biomarkers of PCOS have revealed a number of processes related to oxidative stress, inflammatory changes and iron metabolism to be involved in the pathogenesis of PCOS. Moreover, proteomic identification of serum signatures seems to be a valid tool for predicting and tracking the progression of comorbidities in women with PCOS.
For this purpose we analysed plasma protein profiles in normal subjects and patients of PCOS in Pakistani women. Consequently we have recognized 12 proteins, out of which 08 proteins were under-expressed and down-regulated in PCOS patients. The proteins like serotransferrin, alpha 2-heat specific (HSP) glycoprotein and haptoglobin alpha chain were increased and upregulated in our population of patients of PCOS as compared with controls. Serotransferrin is known to be stress related, acute phase response molecule. Its over expression in patients of PCOS in our study can be elaborated on the basis of underlying inflammatory process.

The altered expressions of ApoA-I, Haptoglobin and transthyretin predict that PCOS women are at a higher risk of future progression towards diabetes, insulin resistance and cardiovascular disorders, particularly, atherosclerosis. Moreover, the over-expressions of Serotransferrin and Haptoglobin α chain, whereas, declined expression of Haptoglobin β chain demonstrate that variations in iron metabolism, oxidative stress and low grade chronic inflammation might be the culprits in molecular pathogenesis of PCOS in women. Haptoglobin is a plasma alpha-2 glycoprotein formed by hepatocytes in the liver and subsequently thrown in the circulation. The anti-inflammatory and anti-oxidant properties of haptoglobin justify its up-regulation and over-expression in our study. It is important to note that ApoA-1 is down-regulated in both PCOS and T2 DM. Hence, it is concluded that down-regulated ApoA-1 in women with PCOS is closely associated with aetiology of T2 DM and its cardiovascular complications.

CHAPTER 6

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