INTRODUCTION

PCOD is the most prevalent endocrine disorder among women of reproductive age (14-17% prevalence in women of fertile age) and is the most common cause of an ovulatory infertility[1,2]. It is a syndrome, characterized by chronic anovulation, insulin resistance, hyperandrogenic symptoms and increased prevalence of metabolic syndrome [1,2]. It has many significant short-term and long-term implications for patients such as oligomenorrhea, amenorrhea, infertility, diabetes mellitus, cardiovascular disease, increased risk of endometrial cancer, and excessive body hair (hirsutism)[3,4] No single test is diagnostic, and multiple diagnostic criteria exist in the literature, which are similar in principle; although not being uniform[1,3]. In this study the diagnosis was based on the Rotterdam criteria [3].

Anti-Müllerian hormone (AMH) is secreted mainly by the granulosa cells of small-antral follicles (4–8 mm size) and is a biomarker of ovarian follicular reserve. AMH expression and secretion is initiated as soon as primordial follicles are recruited to grow into small preantral follicles and its highest expression occurs in pre antral and small antral follicles. It is involved in the regulation of follicle growth initiation and in the threshold for follicle FSH sensitivity [4]. AMH is an indicator of ovarian reserve and serum AMH concentration is strongly correlated with the number of growing follicles since it represents AMH secretion from all developing follicles. There is a very
good correlation between serum AMH levels and ultrasonographic measure of the antral follicular count (AFC). Anti-Mullerian Hormone seems to be a reliable predictor of antral follicle count, independent of polycystic ovary syndrome diagnosis or ovarian morphology [5-7].

PCOD is characterized by an increased number of follicles at all growing stages, especially the pre-antral and small antral follicles (2-9 mm in diameter), those which primarily produce AMH. Thus AMH level, as a reflection of the stock of pre-antral and small antral follicles, is 2-4 fold higher in women with PCOD than in healthy women [8]. AMH, which plays a pathophysiological role in PCOD, has been suggested to be a potential objective diagnostic and treatment monitoring biomarker to replace subjective transvaginal ultrasonography for PCOD diagnosis and management [3, 4, 9]. Even though serum AMH would be theoretically more accurate than antral follicle count (AFC), as it reflects the excess of small follicles non-visible on ultrasound it is still considered premature to make this diagnostic transition because AMH can be influenced by many factors and due to existing controversies in AMH assay and standardization[2,4]. Obesity is often associated with a significantly lower level of serum AMH, but not in all studies. There is also a controversy regarding the influence of hormonal contraception: as per some authors, combined estrogen progestin does not change AMH serum levels whereas others have recently reported a decrease of 29 to 50 % that could be explained by the suppression of gonadotropin secretion [2,4]. This study is designed to study the levels of AMH and factors affecting it in PCOD patients and healthy females attending our hospital located in a rural area of Haryana. It will also provide preliminary data on AMH levels in healthy females specific to our laboratory.

METHODOLOGY

The present case control study was conducted in the Department of Biochemistry, BPS Govt Medical College for Women, Khapur, Sonepat in collaboration with Department of Obstetrics & Genecology. Fifty (50) females in the age group 18 – 40 years diagnosed with polycystic ovarian disorder were taken as study group. Fifty ages matched healthy females (18-40 years age) were included as controls. Informed consent was taken from all cases and controls enrolled on the attached consent form. Ethical clearance was taken from Institutional Ethical Committee. PCOD was diagnosed or excluded based on the Rotterdam criteria [3] according to which 2 out of following 3 are required along with exclusion of other aetiologies (congenital adrenal hyperplasias, androgen-secreting tumours, Cushing’s syndrome):

• Oligo- and/or anovulation
• Clinical and/or biochemical signs of hyperandrogenism
• Polycystic ovaries

Hirsutism was assessed using the modified Ferriman and Gallwey score (mF-G score) system [10] (described in case sheet), and clinical hyperandrogenism (HA) was defined as an mF-G score of 6 or greater. Biochemical HA was assessed as testosterone >0.68 ng/mL or free Testosterone >1.72 pg/mL [3, 11]. Polycystic ovaries were diagnosed on ultrasonography (USG) as presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter, and/or increased ovarian volume (>10 ml)[3,4]. Subjects with thyroid abnormalities, hyperprolactinemia, other endocrine abnormalities, other causes of infertility, other acute or chronic diseases, history of calcium/ vitamin D supplementation and history of long term medication use were excluded. Relevant clinical history was taken, and physical examination was performed in all study and control group patients. All patients and controls were subjected to anthropometric measurements, routine as well as special lab investigations and ultrasonography. Anthropometry included measurement of weight, height, BMI and waist circumference measurement. AMH levels along with routine investigations (hemoglobin, hemogram, peripheral smear, liver and kidney function tests, uric acid and lipid profile, fasting and PP glucose, HbA1c, etc.) were performed in all subjects. Reproductive and other hormones like LH, FSH, testosterone etc. were analyzed as required for evaluating inclusion and exclusion criteria. Hormones and AMH were measured by ELISA. Appropriate internal and external quality controls were run before analyzing samples.

Specimen Collection

Fasting blood sample was collected. For measurement of reproductive hormones fasting sample was taken between days 2 and 4 of a menstrual cycle or during a spontaneous bleeding episode. Serum was separated by centrifugation of the sample and used for the assays (sample were analysed the same day or stored at 2-80 C for 1day, and at -200C if storage was required for more than 1 day). Effort was made to carry out all investigations on same day of sample collection minimizing the need for sample storage. Appropriate quality controls were carried out for all investigations.

DATA & STATISTICAL ANALYSIS

SPSS ver. 20 was used for various statistical analyses. Comparison of data between groups was done using Mann Whitney test for quantitative data and Chi-square test for qualitative data. Spearman’s correlation coefficient (r) formula was used to assess correlations.

OBSERVATIONS

The observations are summarized in table 1. BMI and waist circumference of PCOD patients was significantly higher than control group (BMI: 27.13±2.99 vs 22.44±1.99 kg/m2; p<0.001, Waist Circumference: 81.28±11.19 vs 62.26±8.70 cm; p<0.001). Values of fasting blood sugar (96.6±11.3 vs
74.0±6.7 mg/dL, p<0.001) and HbA1c (5.9±1.44 vs 5.0±0.66, p<0.001) were significantly higher in PCOD patients than in control group subjects. Values of triglycerides (197.0±65.5 vs 139.2±29.8 mg/dL, p<0.001), total cholesterol (212.5±38.5 vs 189.2±21.8 mg/dL, p<0.001), LDL-cholesterol (142.4±35.7 vs 92.5±16.2 mg/dL, p<0.001)) were significantly higher in PCOD patients than in control group subjects. Levels of HDL-cholesterol were significantly lower in PCOD patients (37.3±5.7 vs 51.2±8.9 mg/dL, p<0.001). The prevalence of dyslipidemia was significantly higher in PCOD patients than in healthy controls for all lipid parameters (figure 1). AMH levels are significantly higher in PCOD patients (12.0±8.93 vs 5.35±1.80 ng/mL, p<0.001, figure 2). Area under ROC curve for AMH was 0.766 with p <0.001. On ROC analysis a cut-off of 7.1 ng/mL yielded 89.8 % specificity and 64.6% sensitivity for PCOD. While a level of 8.3 ng/mL results in 99.8% specificity and 54.2% sensitivity (figure 3). Combined with the clinical symptoms and signs levels above 6.8 ng/mL were able correctly diagnose PCOD in all patients diagnosed by the Rotterdam criteria. Thus along with clinical correlations AMH values can avoid the need of subjective ultrasonography. AMH levels correlated with HbA1c (r=0.261, p<0.001) in PCOD patients (figure 4). In this study no correlation was found between BMI or Waist circumference and AMH levels in either the healthy controls or PCOD patients. Further levels of AMH were statistically similar in subjects using OCP or not in both PCOD patient as well as healthy subject group. Oral Contraceptives were used by 54% patients and 46% healthy controls. The distribution is similar (table 1).

### Table-1: Summary of observations in PCOD patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>PCOD Patients (n=50)</th>
<th>Healthy controls (n=50)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>27.0±6.12</td>
<td>28.2±6.32</td>
<td>Not significant</td>
</tr>
<tr>
<td>BMI</td>
<td>27.1±2.99</td>
<td>22.4±1.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>81.2±11.19</td>
<td>62.2±8.70</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OCP use</td>
<td>27 (54%)</td>
<td>24 (46%)</td>
<td>Not significant</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>197.0±65.5</td>
<td>139.2±29.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>212.5±38.5</td>
<td>189.2±21.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>37.3±5.7</td>
<td>51.2±8.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>142.4±35.7</td>
<td>92.5±16.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting Blood sugar (mg/dL)</td>
<td>96.6±11.3</td>
<td>74.0±6.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.9±1.44</td>
<td>5.0±0.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG &gt; 150 mg/dL</td>
<td>70%</td>
<td>42%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TC &gt;200 mg/dL</td>
<td>62%</td>
<td>26%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-C &lt;40mg/dL</td>
<td>68%</td>
<td>10%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C &gt;130 mg/dL</td>
<td>60%</td>
<td>4%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AMH (ng/mL)</td>
<td>12.0±8.93</td>
<td>5.35±1.80</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

#Mann-Whitney test for quantitative variables and chi-square test for proportions

**Fig-1: Comparison of dyslipidemia in PCOD patients and healthy controls**
DISCUSSION

Age, BMI and Waist circumference

The age distribution in case and control group was comparable (table 1) as age matched healthy controls were enrolled. BMI and waist circumference were significantly higher in PCOD patients (table 1). The long-term implications for patients such as insulin resistance, diabetes mellitus, cardiovascular disease,
increased risk of endometrial cancer are shared with long term implications of obesity [4]. Insulin resistance is at the core pathophysiology of PCOD and is directly related to obesity [1]. The increasing epidemic of obesity could be driving up the prevalence of PCOD, as obesity worsens the endocrine and metabolic profile of PCOD [4]. Metabolic disorders are often associated to PCOD (up to 50 %), including an increased rate of insulin resistance, regardless of obesity [12]. Though PCOD may not be always associated with obesity and both lean and obese variants of PCOD exist, obesity worsens the endocrine and metabolic problems occurring in PCOD [13]. Weight loss is an integral and critical part of PCOD treatment [11].

Sugar & Lipid profile

Values of fasting blood sugar and HbA1c were significantly higher in study than in control group subjects (Table 1). Even though the value of fasting blood sugar was higher in PCOD patient’s values were within the reference ranges in most patients. However 64% patients had elevated HbA1c (>5.7%). This may be due to the insulin resistance associated with PCOD. Values of triglycerides, total cholesterol and LDL-cholesterol were significantly higher and values of HDL-cholesterol were significantly lower (table 1) in PCOD subjects than in control group subjects. The prevalence of dyslipidemia was significantly higher in study group than control group for all the lipid parameters (table 1). This metabolic derangement contributes to increased cardiovascular risk and is a consequence of the insulin resistance associated with PCOD. The observed dyslipidemia could be due to higher BMI and waist circumference of PCOD patients in this study. Increased adipose tissue secretes more adipokines and pro-inflammatory factors like resistin, TNF-α, IL-6, free fatty acids etc. which promote insulin resistance which in turn leads to dyslipidemia [14-16]. Kumar et al. reported similar findings as reards to lipid parameters and fasting blood sugar [17].

AMH

AMH levels are significantly higher in PCOD patients (table 1). There is a very good correlation between serum AMH levels and ultra-sonographic measure of the antral follicular count (AFC) as circulating AMH is mostly produced by granulosa cells of follicles from 2 to 9 mm in diameter. PCOD is characterized by an increased number of follicles at all growing stages, especially the pre-antral and small antral follicles (2-9 mm in diameter), those which primarily produce AMH. Thus AMH level, as a reflection of the stock of pre-antral and small antral follicles, is 2-4 fold higher in women with PCOD than in healthy women [8]. Production of AMH by granulosa cells was found in vitro to be 75-fold higher in anovulatory PCOD and 20-fold higher in normo ovulatory PCOD than in normal ovaries [18]. This suggests increased serum AMH levels in PCOD would also reflect an intrinsic dysregulation of the granulosa cells, in which AMH, itself, could be involved since an over expression of the AMH receptor type II (AMHRII) has also been demonstrated [11]. Increased LH pulsatility is an important pathophysiological feature in many cases of polycystic ovary syndrome (PCOD), the most common cause of female infertility, in which circulating AMH levels are also often elevated. However, the origin of this dysregulation remains unknown. It has been hypothesized that the AMH-dependent regulation of GnRH release could be involved in the pathophysiology of fertility and could hold therapeutic potential for treating PCOD [19].

AMH levels decrease with treatment and levels were correlated with ovarian volume [20]. Serum AMH level is also correlated to the severity of PCOD symptoms and is higher when hyperandrogenism or oligo-ovulation is present [4]. AMH also serves as important tool in the context of managing safe superovulation during in-vitro fertilization therapy for PCOD related infertility [21].

Given its strong implication in the pathophysiology of PCOD, serum AMH could be considered the “Gold Standard” in the diagnosis of PCOD. Even though serum AMH would be theoretically more accurate than AFC, as it reflects also the excess of small follicles non-visible on ultrasound it is still considered premature to make this diagnostic transition because AMH can be influenced by many factors and due to existing controversies in AMH assay and standardization [2,4]. Obesity is often associated with a significantly lower level of serum AMH, but not in all studies. Also according to some authors, combined oestrogen progestin does not change AMH serum levels whereas others have recently reported a decrease of 29-50% [2,4]. OCP use or obesity did not affect AMH levels in PCOD patients or healthy females in our study.

Problems exist in AMH assays. There is molecular heterogeneity of the circulating AMH level with a non-cleaved biologically inactive form and a cleaved biologically active form. Also, there is variable sensitivity of the immunoassays to interference by complement C1q and C3. The stability of AMH samples during the storage is not well known. Another technical problem is the inter-laboratory variability; mainly for low values of serum AMH due to there being different ELISA immunoassays used worldwide which use different monoclonal antibody and different standards. The lack of standardization between these assays leads to absence of consensual reference values and decision thresholds in the literature [4].

Dumont et al. observed that, it is now undeniable that serum AMH is a valuable tool for the diagnosis of PCOD. As for its benefit in the treatment of PCOD, there may be an advantage in therapeutic decision support, but this needs to be confirmed by further studies. However, the current technical
difficulties to set up consensual serum AMH thresholds (stability and heterogeneity of circulating AMH, wide range of values, inter laboratory variability, different immunoassays used worldwide) may have curbed the enthusiasm of some clinicians to make it “THE” marker of PCOD [4, 22-24]. In our study area under ROC curve for AMH was 0.766 with p <0.001. On ROC analysis a cut-off of 7.1 ng/mL yielded 89.8 % specificity and 46.6% sensitivity for PCOD. While a level of 8.3 ng/mL results in 99.8% specificity and 54.2% sensitivity (figure 3). Combined with the clinical symptoms and signs levels above 6.8 ng/mL were able correctly diagnose PCOD in all patients diagnosed by the Rotterdam criteria. In literature multiple cut-off values (6.1, 6.6 and 6.8 ng/mL) have been suggested in different studies as having best sensitivity and specificity for PCOD diagnosis [22-24]. AMH cut-off value from this study is consistent with most previous studies and this encourages furthering pursuing research on reference values and inter-laboratory standardization of AMH to become more appealing to the clinician. A little higher value may be due to focus on specificity for diagnosis rather than sensitivity which is more important for screening markers.

Due to small sample size, some correlations may be difficult to assess. Use of AMH as diagnostic tool should be explored in larger number of patients to establish specific reference ranges and diagnostic cut-off and possibilities to combine its use with other biomarker / clinical marker for more accurate and rapid diagnosis.

CONCLUSIONS

PCOD patients had higher BMI and waist circumference. HbA1c and lipid profile was deranged in PCOD patients. AMH levels were significantly higher in PCOD patients correlating with glycemic derangement. Further studies in a larger sample will be helpful to establish reference ranges and diagnostic cut off for AMH levels.

REFERENCES


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