ANTI-MÜLLERIAN HORMONE (AMH) AS A USEFUL MARKER IN DIAGNOSIS OF POLYCYSTIC OVARY SYNDROME

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Abstract

The mechanism underlying anovulation in the polycystic ovary syndrome (PCOS) remains unclear, although an excessive number of small antral follicles at ultrasound scans and discrepancies with selected follicles sustain the hypothesis of altered follicular development. Anti-Müllerian (AMH) hormone is a member of TGF-b super family of growth factors produced by granulosa cells of pre- and small-antral follicle. The 2 to 3 fold increase in the number of growing follicles in the ovary from PCOS women is reflected by an increase in serum concentration of AMH and thus, this hormone may be a good marker of PCOS.

Aim. This study was intended to implement ultra-sensitive ELISA measurement of serum AMH from PCOS women and search for a potential correlation with clinical and laboratory parameters.

Subjects and methods. Sera from patients with PCOS (n = 42) and control women (n = 22) were used for ELISA measurement of AMH (AMH-EIA, Beckman Coulter) with sensitivity of 0.7 pmol/L.

Results. We found a serum concentration of AMH almost 3 folds higher in patients with PCOS compared to controls (73.7 ± 7.5 vs. 25.7 ± 3.9 pmol/L, P < 0.0001). Differences were even higher in lean subjects. A positive correlation was found between total testosterone and LH levels, but not with serum FSH or insulin. Moreover, AMH concentration was correlated to more hyperandrogenic PCOS and with amenorrhea, and thus to the severity of the syndrome.

Conclusion. Measurement of serum AMH may be used as a valuable marker for PCOS to confirm diagnosis and evaluate the extent of follicular dysfunction in relation with hyperandrogenism and menstrual disturbances.

Key words: PCOS, AMH, Hyperandrogenism.

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common cause of infertility which affects 12% of women at reproductive age (1-3). Major features include chronic anovulation, clinical and biochemical signs of hyperandrogenism (HA) and polycystic appearance of the ovaries at ultrasound examination. Although ignored for a long time, PCOS is also associated with subtle alterations in the follicular growth (4, 5). The common denominator of the broad spectrum of clinical conditions with PCO (including PCOS) is the anovulation, which results in non selection of the dominant follicle. Recently, it was shown that in PCOS non selection of follicles is responsible for accumulation (stockpiling) of growing follicles, particularly of small antral follicles (2-5 mm), while follicles of 6-9 mm (selected) remain unchanged (5-7). This discrepancy between the fate of small and selected antral follicles substantiate the theory of follicular arrest in PCOS which was further supported by the variation in the concentration of AMH.

AMH or Müllerian inhibiting substance (MIS) is a member of the transforming growth factors b family (8). Initially thought as inducing uniquely the regression of Müllerian ducts, AMH is now recognized as an active hormone in adult life, which is secreted by granulosa cells of growing follicle (9). The main actions of AMH include inhibition of primordial follicles recruitment and decreased responsiveness to FSH of growing follicles (10-12). Experimental evidence for the role of AMH was coming from AMH-null mice where there is a 3-fold increase in small non atretic growing follicles as well as an increased sensitivity to FSH (10-13). Being secreted by small antral follicles, systemic AMH levels reflect the size of follicle pool (14-16). Moreover, escaping from the control of gonadotropins and being invariable during menstrual cycle (17,18), serum AMH may be a good marker for follicular dysfunction in PCOS (14, 19-21). Indeed previous investigations have demonstrated in PCOS a 2 to 3 fold increase of AMH levels and production per follicle as well as synergistic effects with androgens (19, 22, 23). The relationship with insulin resistance, obesity and improvement of infertility under treatment with antidiabetic drugs (metformin or thiazolidindiones) remains however elusive in literature (24, 25).

As a first step for implementation AMH measurement in the clinical laboratory for diagnosis of PCOS, in this study we have used an ultra-sensitive ELISA method to investigate AMH concentration in a group of lean PCOS patients compared to controls. In addition, we included a small number of obese PCOS and searched for a potential correlation with the severity of PCOS based on levels of testosterone and the degree of menstrual disturbances.

SUBJECTS AND METHODS

Subjects. The study was performed in the Department of Endocrinology at “C.I. Parhon” Institute of Endocrinology. PCOS population (n = 42) was composed of
unrelated Caucasian women recruited in accordance to Helsinki Declaration (as revised in 1983) and after informed consent. Protocol was approved by the Ethical Committee of “C. Davila” University of Medicine and Pharmacy, Bucharest, Romania.

All PCOS patients fulfilled 2003 revised Rotterdam diagnosis criteria (26) as two of the following: 1) prolonged oligo-ovulation (6 or fewer menses per year) or anovulation 2) clinical hirsutism defined by Feriman-Gallowey score > 7, acne, androgenic alopecia and/or biochemical signs of HA and 3) PCO morphology on ultrasound examination. Biological HA was defined as total testosterone > 2.4 mmol/L or 0.69 ng/ml. Cushing’s syndrome, non-classical adrenal 21-hydroxylase deficiency, hyperprolactinemia or androgen-secreting neoplasms were excluded. Subjects were maintained on free diet and standard OGTT (75 g glucose) was performed in PCOS patients. Glucose intolerance (IGT) and impaired fasting glucose (IFG) were defined by 2006 American Diabetes Association (ADA) criteria (27). Insulin resistance was evaluated by Homeostasis Model Assessment (HOMAIR) index. For this study we selected from a total population of PCOS (n = 183) a group of lean PCOS patients (n = 37). Several obese (n = 5) women were also studied as pilot experiment.

Control population (n = 18) was composed of women consulting in the Obstetrics and Gynecology Department of the same University Hospital of normal reproductive status with regular menses, proven fertility (at least one child) and with no clinical or biochemical signs of HA (4 control subjects with high values of total testosterone were excluded). Subjects consulting for gestational diabetes or diabetic patients were also excluded.

Hormonal immunoassays. All assays were done at the research laboratory of the University of Medicine and Pharmacy, Bucharest. Blood samples were collected during the second to fifth day of a spontaneous menstrual cycle or during amenorrhea, after an overnight fast and stored at -20 °C. Serum AMH was measured in duplicate using ultra-sensitive ELISA (AMH-EIA, Beckman Coulter, Villepinte, France). Intra-assay coefficient of variation was 12%. The detection limit was 0.7 pmol/L. Insulin was measured by radioimmunoassay (Adaltis RIA/PEG, Rome, Italy) with a sensitivity of 1 mIU/ml and 29% cross-reactivity with proinsulin. Testosterone, LH, FSH and PRL measurements were performed Beckman Access ® Immunoassay system (Beckman-Coulter, Chaska, MI, USA). Plasma glucose was measured by glucose-oxidized method on a Hitachi 912.

Data and statistical analysis. Numerical variables were analyzed with the Mann-Whitney and Kruskal-Wallis nonparametric statistical test and expressed as mean ± SEM or median (25°-75°). Nominal variables were analyzed with Chi² test. Significant values were considered at nominal p < 0.05. Relation between AMH and various parameters was evaluated by linear regression or by ANOVA (2a level was set at 5%). Multiple regression analysis was performed by logistic regression using the descendent method. All tests were performed using StatView 5.0 and SAS (Abacus Concepts, Berkeley, CA).
RESULTS

Clinical characteristics of PCOS. All patients with PCOS received diagnosis based on 2 Rotterdam criteria (26). A proportion of 86% of women displayed chronic anovulation and signs of HA. In remaining patients diagnosis was completed by ultrasound scan. Most of patients (61%) presented clinical hirsutism while a proportion of 45% showed a biological elevated total testosterone level (> 0.69 ng/ml). Amenorrhea was detected in 60% of patients and oligomenorrhea in 35%. Other major clinical and laboratory data of lean patients and controls are indicated in Table 1.

PCOS women were younger and with a normal fasting glucose, but significantly elevated 2 h glucose during OGTT. Fasting insulin was slightly elevated in PCOS (1.3 fold) but non significant. A similar nonsignificant increase was observed for HOMAIR index. Overweight (BMI > 27 kg/m²) was detected in 25% of PCOS patients. As we indicated above, we also studied a small group of obese PCOS (n = 5) with BMI of 38.8 ± 2.4 (mean ± SEM). Endocrine parameters of these obese PCOS were similar. However, they displayed higher 2 h glucose (6.5 ± 1.3 mmol/L), fasting insulin (29.7 ± 9.4) and HOMAIR index of 6.6 ± 2.4, which indicate more insulin resistance. This group also displayed a total testosterone level at 0.66 ± 0.2 ng/ml.

Hormonal assay of AMH. The main finding of our study was the high concentration of serum AMH in PCOS compared to controls (Figure 1, left panel).

Table 1. Clinical and laboratory features of lean women with PCOS and controls investigated for AMH hormonal level

<table>
<thead>
<tr>
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<th>Controls (n = 18)</th>
<th>PCOS (n = 37)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>29.1 ± 1.2</td>
<td>22.1 ± 0.6</td>
<td>0.0001</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>23.4 ± 0.7</td>
<td>22.1 ± 0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Waist/Hip ratio</td>
<td>0.76 ± 0.01</td>
<td>0.79 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>ND</td>
<td>14.01 ± 2.8</td>
<td>NA</td>
</tr>
<tr>
<td>FSH ( U/l)</td>
<td>ND</td>
<td>6.3 ± 0.4</td>
<td>NA</td>
</tr>
<tr>
<td>TT (ng/ml)</td>
<td>0.4 ± 0.04</td>
<td>0.75 ± 0.1</td>
<td>0.004</td>
</tr>
<tr>
<td>Fast glucose (mmol/L)</td>
<td>4.8 ± 0.3</td>
<td>4.8 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>2-h glucose (mmol/L)</td>
<td>3.1 ± 0.4</td>
<td>5.5 ± 0.2</td>
<td>0.001</td>
</tr>
<tr>
<td>Fast insulin (U/ml)</td>
<td>6.8 ± 1.2</td>
<td>9.16 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>HOMAIR</td>
<td>1.96 ± 0.4</td>
<td>2.1 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>55.1 ± 1</td>
<td>184.7 ± 19.01</td>
<td>0.013</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>88.1 ± 10.0</td>
<td>86.6 ± 14.9</td>
<td>NS</td>
</tr>
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</table>
Antimullerian hormone in PCOS

The mean (± SEM) values were of 73.7 ± 7.5 pmol/L in PCOS patients and 25.7 ± 3.9 pmol/L in controls. There was a clear relationship between AMH levels and obesity. Thus, the highest values were found in lean PCOS with AMH of 92.5 ± 10.7 pmol/L compared to only 54.1 ± 8.9 pmol/L in overweight patients and 48.8 ± 17.4 pmol/L in obese PCOS (P < 0.0003, Kruskal-Wallis). Differences between lean PCOS and lean controls were highly significant (P < 0.0001, Mann-Whitney). A similar level of significance of higher AMH levels was detected in PCOS with or without elevated total testosterone levels (P < 0.0039 and 0.001, respectively). Thus, high serum AMH seems to characterize the entire PCOS population.

Next, we investigated in more detail the potential correlation to hormonal levels or other PCOS features. No correlation was found with insulin levels, HOMA or the presence of insulin resistance (data not shown). No significant correlation was found with LH or FSH levels, although positive and negative trends were defined, respectively. For instance, LH levels were correlated with AMH in linear regression with P < 0.07, r² = 0.1. Similar significance was obtained in ANOVA with P < 0.02, with an interaction factor of 0.6. The relation was more significant in patients without HA (P < 0.03), but in HA patients this relationship was no more valid. Interestingly, in ANOVA, FSH had an independent effect since the significance of the relationship between AMH and LH in the presence of FSH increased to P < 0.0005, interaction factor 0.8.

A significant positive correlation was found between AMH and total testosterone (Figure 1, right panel). In linear regression this correlation has reached
statistical significance (P < 0.032, adjusted r² of 0.14). In ANOVA, values of AMH were correlated to testosterone with P < 0.032 with an interaction factor of 0.57. To better understand the correlation with HA, we stratified PCOS patients as function of biological total testosterone (cut-off of 0.69ng/ml). As shown in Figure 2, left panel, differences in numerical AMH values (63.4 ± 9.4 vs. 98.3 ± 13.5) were significant between two groups (P < 0.019 Mann-Whitney). In logistic regression model, AMH was associated with HA group with P < 0.0075, but OR was close to 1 (1.1 95%CI 1.0-1.1) indicating a poor direct relationship. Interestingly, in the same multivariate model, independent effects were noticed for 2 h glucose values in HA group (P < 0.02, OR 2.2 95%CI [1.5-5.2]) and for FSH (P < 0.01, OR 1.8, 95%CI [1.04-3.06], but again, no effect was detected for LH levels. When patients were stratified as function of the ovulatory status, PCOS with amenorrhea displayed mean levels of AMH of 105.6 ± 17.5 while those with oligomenorrhea showed values of 83.2 ± 15.0 pmol/L (Figure 2, right panel).

Figure 2. Concentrations of AMH in PCOS patients with hyperandrogenism (HA) and infertility. Median (25%-75%) and 10%-90% 

Taken together, these data indicate that PCOS is associated with high levels of AMH in lean individuals and with a positive correlation to levels of testosterone and LH and the severity of PCOS, all indicating that hormonal measurement of AMH in the serum may contribute to the positive diagnosis of PCOS.
**Antimullerian hormone in PCOS**

**DISCUSSION**

In this paper we demonstrated that serum concentration of AMH was significantly increased in a group of lean patients with PCOS compared to weight matched control women, reinforcing the importance of implementation of this hormonal investigation for clinical diagnosis of PCOS. Moreover, our data suggest that elevated AMH levels correlated with the severity of PCOS and the degree of HA.

Implementation of AMH measurement in the clinical laboratory was based on the initial observation that patients with anovulatory infertility and lean phenotype (BMI < 27 kg/m²) respecting the 3rd Rotterdam criterion at ultrasound scan (> 12 follicles/ovarian section) display in fact an excess of small antral follicles (2-5 mm), though total testosterone levels may remain at the upper limit of controls. In this case, AMH measurement can confirm the PCOS diagnosis. Moreover, AMH levels may be used in this diagnosis in women for whom trans-vaginal ultrasound scanning can not be performed, such as *virgo* patients.

Data from the literature indicated that in PCO there is an excess of small antral follicles (2-5 mm) compared to follicles of 6-9 mm (selected follicles). The number of selected follicles is negatively correlated with BMI and insulin concentration (21, 28). Therefore, based on the negative effect of obesity on follicular maturation (29) and excess of follicularity in overweight women, we performed this study in lean PCOS. A limited number of obese patients was however studied who demonstrated lower values of AMH than lean PCOS. AMH is considered a good biological marker of number of small antral follicles in both normal and PCOS women (20, 28). The pattern of expression of AMH is limited to growing unselected follicles, which are increased in PCOS (30). For this reason, AMH may be considered as a "surrogate" of follicle number in PCOS (28). Our data confirmed these findings and, accordingly, 2 to 3 fold increase in AMH level reflects the increase to the same extent of small antral follicles.

Although with a small number of patients, we have found a correlation between the BMI and AMH levels in both overweight and obese women. These data are in contradiction with a recent report where such a correlation was not found in PCOS, but in control subjects (21). We do not have a definitive explanation for this situation and further studies on larger sample size are necessary to elucidate this aspect.

In PCOS patients we found a correlation between serum concentrations AMH and LH as well as total testosterone. Moreover, AMH level varied as function of the presence of amenorrhea or oligomenorrhea in infertile women. Taken together, these data suggest that AMH level is correlated to HA and the severity of infertility. These data are concordant to previous studies which suggest that AMH would have a distinct effect from HA, acting perhaps through inhibition of FSH-dependent cyclic recruitment and decreasing aromatase activity (31-34). Higher concentrations of AMH in amenorrheic compared to oligomenorrheic women suggest, not only a potential role of AMH in pathogenesis of anovulation, but may reflect a more severe
alteration of follicular development and dysfunction of granulosa cells in patients with amenorrhea compared to those with oligomenorrhea (35). These considerations may suggest that serum levels of AMH can be used for a further classification of PCOS. This aspect is important, since, compared to other serum markers, AMH is not under the influence of gonadotropic hormones, does not vary through the menstrual cycle and thus reflects better the number of follicular pool (15, 36-38), the degree of maturation (39) and even the sensitivity to the action of FSH (16).

The mechanisms involved in AMH action in anovulation are largely unknown. One major hypothesis is the decreased sensitivity of follicles under FSH action (12, 14, 32). This would contribute to abnormal selection of dominant follicle (15, 40) and the decrease in the activity of aromatase (41), which is inversely correlated with HA (36). The increase in the concentration of AMH in follicular fluid and serum may be the result of excess of antral follicles (14) and/or high production of AMH by granulosa cells (42) and would be involved in the arrest of follicular development through negative action of FSH during selection (19, 21). Thus, tonic increase of AMH may be involved in the arrest of follicular development and would explain efficiency of therapeutic intervention through an increased concentration of FSH during selection (31).

In conclusion, our data confirm increased serum concentrations of AMH in PCOS and favor further use as a criterion for severity of PCOS in relation with HA and menstrual disturbances. Understanding the physiological role of AMH would give a new insight into further monitoring of follicular pool as part of the process of ovarian aging (14, 15), extent of follicular dysfunction in PCOS and hypothalamic amenorrhea as well as a marker for ovarian response during in vitro fertilization (37). Finally, measurement of AMH in women with clinical and biochemical signs of HA may be useful for diagnosis of PCOS in the absence of ultrasound scan and may be employed as surrogate of the number of antral follicles.

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Antimullerian hormone in PCOS


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Antimullerian hormone in PCOS


Laura Leonte et al.