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To cite this article: M. Stracquadanio, L. Ciotta & M. A. Palumbo (2018) Relationship between serum anti-Mullerian hormone and intrafollicular AMH levels in PCOS women, Gynecological Endocrinology, 34:3, 223-228, DOI: 10.1080/09513590.2017.1381838

To link to this article: https://doi.org/10.1080/09513590.2017.1381838

Published online: 23 Sep 2017.

Article views: 331

Citing articles: View citing articles
PCOS: SERUM AMH AND INTRAFOLLICULAR AMH LEVELS

Relationship between serum anti-Mullerian hormone and intrafollicular AMH levels in PCOS women

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ABSTRACT
Polycystic ovary syndrome is a complex disease characterized by various endocrine disorders that are the potential cause of anovulation and hyperandrogenism. Anti-Mullerian hormone expression is suspected to be overexpressed in PCOS granulosa cells. AMH acts as a regulator of folliculogenesis: it is produced by the granulosa cells of follicles from the stage of the primary follicle to the initial formation of the antrum. Serum and intrafollicular AMH levels are elevated in patients with PCOS due to increased number of small follicles and an increased secretion within each of these small follicles. This excess of AMH is strongly suspected to play a role in the characteristic follicular arrest of PCOS, through a negative action on aromatase expression and on FSH action. Value above 5 ng/ml or 35 pmol/l might be considered as a diagnostic criterion for PCOS. The aim of our study is to demonstrate the presence of higher AMH serum levels and higher AMH intrafollicular fluid level of PCOS patients, undergone to IVF cycles, compared to normovulatory patients. The results clearly indicate that blood and intrafollicular AMH levels are significantly higher in PCOS women comparing to the normovulatory population. Serum AMH level appears to be a good predictive marker for the risk ovarian hyperstimulation syndrome: thus, its evaluation should be recommended before starting a controlled ovarian stimulation for IVF.

Introduction
Polycystic ovary syndrome (PCOS) is a complex disease characterized by various endocrine disorders that are the potential cause of anovulation and hyperandrogenism. This heterogeneous syndrome affects about 5–10% of reproductive-age female population, and it can be considered the most common endocrine disorder affecting women during reproductive life [1].

PCOS is a multifactorial polygenic disease: genes involved in insulin-resistance, genes that encode inflammatory factors and genes that regulate steroidogenesis and folliculogenesis are interested.

Chronic anovulation is often manifested as oligomenorrhea; anovulatory cycles may lead to dysfunctional uterine bleeding and decreased fertility. Cutaneous manifestations of hyperandrogenemia include hirsutism, acne, and male-pattern hair loss (androgenic alopecia), whereas acanthosis nigricans is a cutaneous marker of hyperinsulinemia [2].

Anti-Mullerian hormone (AMH) is a glycoprotein hormone with a molecular weight of 140 kDa that belongs to the superfamily of the transforming growth factor beta (TGF-β) [3].

AMH acts as a regulator of folliculogenesis: it is produced by the granulosa cells of follicles from the stage of the primary follicle to the initial formation of the antrum. Its production begins in the perinatal period, it increases gradually until puberty, remaining stable in the reproductive period and it becomes very low after the menopause [4]. It is predominantly produced by pre-antral and antral follicles (diameter <4 mm), and the production decreases during the process of final maturation and luteinization. AMH contributes to the initial recruitment and selection of the dominant follicle. AMH is produced by growing follicles in the ovary, according to two mechanisms of action:

- Inhibition of the initial recruitment of follicles [5];
- Inhibition of growth hormone (FSH-dependent) and selection of pre-antral and small antral follicles [6].

Serum AMH level has been proposed as a test of ovarian reserve: unlike other blood tests, such as FSH and estradiol, which must be dosed in the very early days of the cycle, the AMH remains constant during all the phases of the menstrual cycle. Its decrease to minimal levels might correlate with a reduced number of ovarian follicles, while its abnormal increase correlates to an excess of follicles in precocious maturation stage, as in PCOS.

There are lots of scientific evidences supporting the thesis that in the PCOS, in addition to intrinsic thecal dysregulation leading to hyperandrogenism, a granulosa cell (GC) dysregulation may occur: AMH expression is suspected to be overexpressed in PCOS granulosa cells [7].

Follicular fluid (FF) is a mixture of chemical constituents, comprising a variety of different proteins as well as growth factors, reactive oxygen species, anti-apoptotic factors, fatty acids, sugars, and hormones [8]. Among them, AMH seems to be the most prominent targets for reproductive health research [9,10].

Intrafollicular and serum AMH levels are elevated in patients with PCOS due to increased number of small follicles and an increased secretion within each of these small follicles. This excess of AMH is strongly suspected to play a role in the characteristic follicular arrest of PCOS, through a negative action on aromatase expression and on FSH action. Value above 5 ng/ml or 35 pmol/l might be considered as a diagnostic criterion for PCOS [11,12].
The positive correlation between high AMH values and PCOS was also put in evidence in a work of Tomova, that showed how AMH value decreases after treatment with metformin, the most used insulin-sensitizing agent administered to correct the metabolic aspect of the syndrome [13].

Thus, AMH serum levels could be also used as a prognostic factor of metformin therapy in clinical practice [14].

Indeed, insulin is one of the causes of increased AMH in PCOS: hyperinsulinemia may stimulate the development of antral follicles and improve the sensitivity of granulosa cells to FSH, thus increasing the number of follicles and ovarian volume [15].

AMH positively correlates with ovarian volume and peripheral follicle distribution, especially in young PCOS adolescents [16].

Particularly, AMH serum levels were significantly higher in PCOS patients with hyperandrogenism. Li et al. assert that the diagnostic power of AMH is limited when used to predict patients without hyperandrogenism [17].

Intraovarian hyperandrogenism may cause follicular arrest and follicles’ excess with a consequent increased intraovarian AMH level; thus, elevated AMH in PCOS may be due to increased follicle numbers rather than increased production by each follicle [18].

This research point is controversial: in fact, other authors demonstrated that an ovulatory PCOS groups had lower concentrations of AMH than those who were equally hyperandrogenic but anovulatory, which supports the hypothesis that increased AMH is due to increased production of AMH by each follicle [19].

On the other hand, Rosenfield noticed that AMH levels are independently related to ovarian androgenic function and polycystic ovaries: very high AMH levels are specific but not sensitive for PCOS. In the absence of hyperandrogenism, moderate AMH elevation in women with normal-variant polycystic ovaries seems to indicate just an enlarged oocyte pool [20].

A serum AMH ≥35 pmol/l (or ≥5 ng/mL) appears to be more sensitive and specific than a USS follicle count >19 in PCOS women [21], but there is not yet an international consensus that validates the threshold for AMH. In a recent study, Lin et al. have divided patients in reproductive age into three groups: high AMH (>11 ng/mL), moderate AMH (4–11 ng/mL), and low AMH (<4 ng/mL). As the AMH level increased, the prevalence of PCOS increased significantly from 21% in the low-AMH group to 37% in the moderate-AMH group, up to 80% in the high-AMH group [22].

As shown above, values of serum AMH have been deeply investigated [23,24], but there is less data regarding the behavior of AMH concentrations in FF, since FF has usually been designated as waste product during oocyte collection for IVF treatment [10]. Recent studies revealed the importance of FF in oocyte development [25], providing information on FF, oocyte quality and fertilization. Information on individual follicular AMH concentrations within one stimulated IVF cycles is still not available, although it is tempting to speculate that individual follicular AMH could be a potential predictor of fertilization success in IVF treatments [10,26].

Mean follicular AMH showed to be negatively correlated with age, which is in line with the overall AMH decrease in patients with increasing age [10].

A very recent study demonstrated that individual follicular AMH concentrations reflected serum AMH values of a stimulated cycle during IVF treatment and that follicular AMH concentrations did not significantly differ within one patient [10].

The aim of our study is to demonstrate the presence of higher AMH serum levels and higher AMH intrafollicular fluid level of PCOS patients, undergone to IVF cycles, compared to normovulatory patients.

Materials and methods

All the patients enrolled in this study attended the ‘Reproductive Diseases Outpatient Clinic’ at the Institute of Obstetric and Gynecological Pathology (‘Santo Bambino’ Hospital, Catania) from January 2016 to February 2017.

In the 12-month enrollment phase a total of 110 women with infertility history were selected: 55 PCOS patients (Group A) and 55 normovulatory women with tubaric disease (such as PID outcomes) or male infertility factors, which constituted the ‘Control Group B’.

Group A patients characteristics were:

- Patient’s age >20 but <35 years old (in order that AMH value would not be affected by a high maternal age);
- BMI <30 (it is an internal criterion for all the patients of our ART center, because it is related to better outcomes);
- Ethnicity: all the patients were Caucasian females of Sicilian ancestry. Thus, patients from Bangladesh, Japan, China, India, USA and Northern Europe were excluded.
- Patients not on any medications for menstrual disorders (oral contraceptives, metformin, inositol) or other pathologies (hypertension, dyslipidemia or any other medical or psychiatric illness) for at least 3 months before study enrollment;
- Diagnosis of PCOS, according to Rotterdam Criteria [27];
- Exclusion of other endocrine diseases (hypoprolactinemia, thyroid dysfunctions, Cushing’s syndrome, congenital adrenal hyperplasia or androgen-secreting tumors).

Inclusion criteria for Group B patients were:

- Patient’s age >20 but <35 years old;
- BMI <30;
- Ethnicity: all the patients were Caucasian females of Sicilian ancestry. Thus, patients from Bangladesh, Japan, China, India, USA and Northern Europe were excluded.
- Patients not on any medications for menstrual disorders (oral contraceptives, metformin, inositol) or other pathologies (hypertension, dyslipidemia or any other medical or psychiatric illness) for at least 3 months before study enrollment;
- Presence of both ovaries;
- Exclusion of PCOS, hyperprolactinemia, thyroid dysfunctions, endometriosis, POI (premature ovarian insufficiency) and other menstrual irregularities (hypothalamic amenorrhea, idiopathic oligomenorrhea or polycystic ovaries).

AMH serum levels were determined by AMH Gen II Elisa (enzymatically amplified two-site immunoassay) during the third day of menstrual bleeding: the analytical sensitivity of this assay is 0.14 ng/mL.

All the patients followed the long-down regulation protocol: the women were injected leuprolein acetate 0.2 ml every day from the 21st day of their menstrual cycle. Controlled ovarian hyperstimulation (COH) was performed by administration of recombinant FSH after pituitary suppression was reached, with an initial dose of 125 IU/daily. Follicle development was monitored with serial ultrasonographic examinations and serum estradiol measurements. Afterwards, 5000–1000 IU of hCG were administered when the follicles were 18 mm in diameter.
Table 1. Patients’ characteristics and AMH values in Group A and B.

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women’s age (years)</td>
<td>28±4</td>
<td>27±1</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27±2</td>
<td>27±2</td>
<td>NS</td>
</tr>
<tr>
<td>AMH (blood levels, ng/mL)</td>
<td>9.4±3.3</td>
<td>3.25±2.62</td>
<td>.00001</td>
</tr>
<tr>
<td>AMH (intrafollicular, ng/g protein)</td>
<td>8.2±1.6</td>
<td>2.8±1.8</td>
<td>.001</td>
</tr>
</tbody>
</table>

Transvaginal ultrasound-guided follicular aspiration (ooocytes’ pick-up) was performed 34–36 h later.

The FF from more than one follicle was gently aspirated and subsequently frozen at −20°C for AMH measurement using an ultrasensitive enzyme-linked immunosorbent assay. The lower detection limit was estimated at 0.1 ng/mL. To avoid possible bias due to FF volume variability, AMH concentrations in the FF were adjusted to its protein content by the Bradford assay.

The differences between the groups regarding age and BMI were determined through the Mann–Whitney U test; to compare serum and intrafollicular AMH levels of the two groups the T-Test for two independent means was used.

Discussion

The serum levels of AMH reflect the size of the ovarian follicular pool [28]. The main function of AMH seems to be the inhibition of the early stages of follicular development and of the FSH-dependent selection progress. In ovaries, in fact, AMH inhibits primordial follicle recruitment [29], meiosis II [30], granulosa cell division and progesterone production [31]. AMH concentrations in FF is inversely correlated with granulosa cell proliferation, although normal physiology is disrupted with advancing age and in PCOS [32,33].

Serum AMH assessment has demonstrated its utility in the treatment of infertility; but the absence of an international standard for serum AMH assay and the inability to define clear thresholds makes application of serum AMH valuation more difficult [34].

AMH could be used as a menstrual cycle-independent marker of ovarian response to controlled ovarian stimulation [35]. AMH appears to correspond well with antral follicle counts and ovarian response to hyperstimulation in in vitro fertilization (IVF) [36,37] and to be useful for predicting ovarian response in women undergoing IVF treatment [38–40].

One of the main advantages of AMH measurement in IVF treatment, comparing to other markers of ovarian reserve, might be its low inter- and intra-cycle variability [41–43]. Moreover, serum AMH level is independent from the hypothalamic pituitary axis and it is not modified in pathologies such as hyperprolactinemia and functional hypothalamic amenorrhea [44].

Indeed, the increased serum basal AMH concentrations in PCOS have been explained by the increased number of small ovarian follicles responsible for AMH secretion [45]. Additionally, these high AMH levels are probably related to the follicular arrest, during the selection process of the dominant follicle, through a negative interaction between AMH and FSH [46].

Moreover, the demonstration that intrafollicular AMH levels are greater in women with PCOS compared to the controls, may suggest increased ‘per follicle’ AMH secretion in PCOS [47].

Some studies report higher intra-follicular levels of AMH in PCOS women compared to controls, indicating that AMH-excess could result from overactive follicles [48–50]. However, high intra-follicular levels of AMH do not necessarily imply increased release of AMH from the follicles [48].

Intra-follicular AMH levels were found to be 75-fold higher in anovulatory PCOS-women and intra-follicular AMH decreased with increasing follicle-size [49]: this indicates that follicles of women with PCOM synthetize more AMH than those of women with normal ovary morphology [49].

In PCOS-women referred to routine laparoscopy or laparotomy, intra-follicular AMH-levels were 60-fold higher than serum AMH-levels [50]. Intra-follicular AMH levels in anovulatory
PCOS women were 6-fold higher than in eumenorrhoic women. Serum levels of AMH were highly correlated to the intra-follicular levels in PCOS-women, in contrast to eumenorrhoic controls [50].

The aim of this study was to prove the validity of serum AMH levels as an important marker for PCOS diagnosis, confirming its high levels also in the FF of PCOS women. In summary, the results clearly indicate that blood and intra-follicular AMH levels are significantly higher in PCOS women comparing to the normovulatory population.

Our data confirm those of a recent Chinese study that demonstrated that FF AMH and serum AMH levels in patients with PCOS were significantly higher than those in non-PCOS patients. Additionally, the antral follicular count and the number of oocytes retrieved from patients with PCOS were significantly higher than those obtained from control patients. These results suggested that basal serum AMH values reflect the quantity of oocytes in ovaries [51].

Serum AMH level appears to be a good predictive marker for the risk OHSS, as demonstrated in the meta-analysis of Broer et al. [52,53]. It is well known that the response of PCOS women to gonadotropin stimulation differs significantly from that of normal ovaries: it is defined ‘explosive’ and it is responsible for the higher risk of canceled cycles and/or for OHSS [15,54].

It was shown that during ovarian stimulation, E2 production and E2-to-A ratio are higher in patients with PCOS who have elevated insulin levels than in normo-insulinemic women [15]. Increased insulin levels involve greater ovarian endocrine and morphologic responses to FSH-induced ovulation, which predispose to OHSS.

Therefore, it seems that the typical response of the polycystic ovary to exogenous gonadotropin therapy is related to increased plasma concentrations of insulin [55].

Hyperinsulinaemia is known to be present in anovulatory women more than ovulatory women [56] and a direct correlation was found between serum AMH and insulin insensitivity [57,58]. Hyperinsulinaemia increases androgen production and the raised AMH concentrations may be secondary to an effect of insulin on androgen levels [59].

Several studies had been performed to demonstrate a potential relationship between insulin resistance and hyper-AMH.

Caglar et al. [60] designed a study to evaluate the correlation between AMH levels and insulin-resistance (IR) in normal-weight PCOS patients. Although the PCOS patients were not subdivided according to different phenotypes, no correlation was found between AMH levels and IR. Tian and his Chinese group [61] conducted a more comprehensive study involving more patients, and according to the results, no correlation was observed between AMH and indices of IR among all phenotypes of PCOS cases.

Conversely, an Italian study [57] found a significant positive association between AMH and IR, as well as an association between androgens and AMH in women with PCOS.

Another recent study [62] found a significant relationship between AMH levels and PCOS in patients both with and without IR: it was suggested that women with PCOS, especially those with IR, may have higher serum AMH levels due to a greater release of inflammatory factors into the systemic circulation, thus affecting AMH production in the ovaries. Consistent with these studies, a recent Turkish study found a significant positive correlation between AMH and IR in adolescent patients with PCOS [63].

Therefore, serum AMH levels evaluation should be recommended before starting a controlled ovarian stimulation for IVF not only for confirming the PCOS diagnosis, but also for the right management of patients in order to choose the appropriate dose of gonadotropins and avoid cases of hyperstimulation.

Indeed, according to our experience, PCOS patients with serum AMH levels >11 ng/mL should start COH with a very low dose of gonadotropins (e.g. 75IU daily instead of 125IU per day).

Whether the conventional long protocol can be replaced by the antagonist protocol in IVF for patients with PCOS needs to be confirmed by further studies [51].

However, even if serum AMH determination may be a helpful tool in the prediction of the ovarian response to gonadotropins in PCOS, there is actually no consensus on the threshold for the AMH values [3].

Intrafollicular AMH level might be a good marker for oocyte quantity but not for their quality [41] and this criticism could be an interesting starting point for a future study through the evaluation of oocyte’s quality, embryo’s grade, fertilization and implantation rate in a larger PCOS population.

Follicular AMH concentrations are positively associated with embryo implantation when measuring the AMH concentrations in the dominant follicle [64]. Studies suggested that higher follicular AMH values positively correlated with fertilization [65] and implied higher chances for pregnancy [66].

However, other studies in literature have shown that fertilization rate, high quality grade embryo, and clinical pregnancy rates are unchanged by AMH levels [41,64,67–72].

Disclosure statement
The authors declare that there is no conflict of interest regarding the publication of this paper.

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