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Elevated Serum Level of Anti-Mullerian Hormone in Patients with Polycystic Ovary Syndrome: Relationship to the Ovarian Follicle Excess and to the Follicular Arrest

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The serum level of anti-Mullerian hormone (AMH), a product from granulosa cells involved in follicle growth, has been shown to correlate tightly with the small antral follicle number (FN) at ultrasonography (U/S) in women who do not have polycystic ovary syndrome (PCOS). Because PCOS is associated with a 2- to 3-fold increase in growing FN, we investigated whether an increased AMH serum level correlates to other hormonal and/or U/S features of PCOS. Serum AMH has been assayed in 104 women (59 symptomatic PCOS, 45 controls) between d 2 and 7 after the last either spontaneous or progestin-induced (in PCOS) menstrual period. Mean serum AMH level was markedly increased in the PCOS group (47.1 ± 22.9 *vs.* 20.8 ± 11.6 pmol/liter in controls; $P < 0.0001$), an increase in the same order of magnitude as the one of the FN in the 2- to 5-mm range at U/S (12.8 ± 8.3 *vs.* 4.8 ± 1.9; $P < 0.0001$, respectively). The ratio AMH/FN was similar between the two groups (4.8 ± 3.4 *vs.* 4.8 ± 2.9; $P = 0.55$). By simple regression, both in PCOS and controls, the AMH level was positively related to the 2- to 5-mm FN at U/S ($P < 0.0001$ and $P < 0.03$, respectively), but not to the 6- to 9-mm FN, and was negatively correlated to the serum FSH level ($P < 0.02$ and $P < 0.04$, respectively). AMH was also positively related to the serum testosterone and androstenedione levels, in PCOS exclusively ($P < 0.0005$ and <0.002, respectively). No relationship was found between AMH and age, serum estradiol, inhibin B, and LH levels in both groups. After multiple regression only the 2- to 5-mm FN remained significantly related to AMH in PCOS whereas testosterone, androstenedione, and FSH were no longer. In conclusion, the assay of the serum AMH may represent an important breakthrough in the diagnosis and in the understanding of PCOS. Our data suggest that the increase of AMH serum level in PCOS is the consequence of the androgen-induced excess in small antral FN and that each follicle produces a normal amount of AMH. We hypothesize that an increased AMH tone within the cohort could be involved in the follicular arrest of PCOS, by interacting negatively with FSH at the time of selection. (*J Clin Endocrinol Metab* 88: 5957–5962, 2003)

POLYCYSTIC OVARY SYNDROME (PCOS) is the most frequent cause of anovulatory infertility and hyperandrogenism in young women (1). The mechanism(s) leading to anovulation is (are) still poorly understood. For many years the excess in intraovarian androgens has been suspected to disturb folliculogenesis, through a proatretic effect on growing follicles (2). However, more recent experimental data in rhesus monkeys strongly suggest that in fact, intraovarian androgens promote granulosa cell (GC) proliferation and inhibit apoptosis, especially in small follicles whose GCs are the richest in androgen receptors (3). Accordingly, polycystic ovaries (PCOs) are characterized by an excessive number of growing follicles (2- to 3-fold that of normal ovaries), up to the stage of 2–5 mm in size (small antral follicles) (4). We recently demonstrated in patients with PCOS that the excess of follicles detected by ultrasonography (U/S) applies to this range (5). We also showed that the follicle number (FN) in the 2- to 5-mm range was tightly related to the androgen serum level (5), thus reflecting the promoting effect of intraovarian androgens on follicle growth (3). On the other hand, follicles in the 6- to 9-mm range (selected follicles) were not in excess, and their number was negatively related to the body mass index (BMI) and to the fasting insulin serum level (5). The discrepancy between the 2- to 5-mm and 6- to 9-mm FN substantiates the theory of follicular arrest in PCOS, which assumes that the progression of small antral follicles to selected follicles and then to the dominant follicle is altered. This phenomenon is thought to result from an impaired action of FSH on the follicle cohort, whose mechanism(s) is (are) unclear.

Anti-Mullerian hormone (AMH), also termed Mullerian inhibiting substance, is a member of the TGF-β superfamily that also includes the GC and theca cell-derived inhibins and activins as well as the oocyte-derived growth differentiation factor 9. Although the roles of inhibins, activins, and growth differentiation factor 9 on ovarian folliculogenesis have been extensively described (6), data about the role of AMH are still scarce. In the ovary, AMH is produced by the GC from preantral and small antral follicles (7). From experimental data, mainly obtained in rodents, the proposed functions of AMH are 1) inhibition of the initial recruitment of primordial follicles, through a paracrine effect (GC-oocyte cross-talk) (7,

Abbreviations: AMH, Anti-Mullerian hormone; BMI, body mass index; E2, estradiol; FN, follicle number; GC, granulosa cell; PCO, polycystic ovary; PCOS, PCO syndrome; U/S, ultrasonography.

8) and 2) inhibition of aromatase activity in GC, thus reducing the production of estradiol (E2) (9). This last effect combined with the fact that AMH could reduce the follicle sensitivity to FSH in the mouse both *in vitro* and *in vivo* (10) raises the possibility that an excessive production of AMH could be involved in the follicular arrest of PCOS.

Although the effects of AMH on the ovarian functions are not fully elucidated and even though it is still questioned whether AMH is a marker of primordial follicles or later stages of follicle development or both (7), its serum level appears as a reliable marker of the ovarian follicle pool. It has been shown recently that AMH levels decreased in situations of ovarian aging, along with follicle depletion (11). In normoovulatory infertile women undergoing *in vitro* fertilization, Van Rooij *et al.* (12) demonstrated that the serum AMH level was strongly and positively related to the number of antral follicles assessed by U/S at baseline. It was also related to the number of oocytes retrieved after ovarian stimulation, in line with a previous report from Seifer *et al.* (13). The serum AMH status has been less investigated in situations of follicle excess. High levels of AMH were reported in the follicular fluid (14) and recently in the serum (15) of patients with PCOS, but these authors did not examine the relationship between AMH and the FN. To determine whether AMH could play a role in the ovulation disorder of PCOS, the aims of the present study were as follows: 1) to confirm the increase of serum AMH in a larger series of patients with PCOS, 2) to relate the AMH level to the follicle status at U/S, and 3) to search for any relationship of AMH to the serum level of the main hormones that are involved in the late stages of follicle maturation.

Patients and Methods

Patient population

This study was approved by the Institutional Review Board of the Lille University Hospital, and informed consent was obtained from all patients and controls before entry into the study. The main clinical and ultrasound data in controls and in patients with PCOS are presented in Table 1.

Controls. The control population consisted of 45 healthy women (mean age, 28.3 yr; 10–90th percentiles, 24–33 yr). These women were recruited by the Department of Assisted Reproductive Medicine in our institution. They were referred for *in vitro* fertilization because of tubal and/or male infertility. Exclusion criteria were a history of menstrual disturbances (*i.e.* cycle length either <25 days or >35 days), hirsutism, abnormal serum level of prolactin or androgens (*i.e.* serum testosterone and/or androstenedione > 0.7 or 2.2 ng/ml, respectively), PCOs at U/S (see below), and hormonal treatment during the 3 months before the study. Their mean BMI was 23.1 kg/m^2 (10–90th percentile range, 19.2–33.2 kg/m^2).

Women with PCOS. Fifty-nine women were recruited for this study. Mean patients' age was 27.4 yr (10–90th percentiles, 21.3–33.1 yr). Mean BMI was 26.7 kg/m^2 (18.7–37.7 kg/m^2). The diagnosis of PCOS was based on the association of at least two of the three following criteria: 1) hyperandrogenism (in 71% of patients), as defined either by hirsutism (modified Ferriman and Gallwey score > 8), or minor signs such as acne or seborrhea, and/or testosterone > 0.7 ng/ml and/or androstenedione > 2.2 ng/ml; 2) menstrual and/or ovulatory disturbances, mainly oligomenorrhea (in 61% of patients) and amenorrhea (in 19% of patients); and 3) U/S criterion of PCO (*i.e.* an ovarian area more than 5.5 cm^2 unilaterally or bilaterally) (16) (in 60% patients).

Blood sampling was performed in the early follicular phase (*i.e.* between days 2 and 7 after the last menstrual period) both in PCOS patients and control women, as previously described (17). In PCOS patients, the last menstrual period was either spontaneous or induced by the administration of didrogesterone (10 mg/d for 7 d). Any patient with at least one follicle with a diameter greater than 9 mm at U/S or a serum E2 level >80 pg/ml was excluded from the study so as not to confound the data with the presence of a dominant follicle.

Hormonal immunoassays

Serum AMH levels were measured in duplicate using an ultrasensitive ELISA (AMH-EIA, Beckman Coulter, Villepinte, France) according to the supplier's instructions. Results are expressed in picomoles per liter using human recombinant AMH as a standard. The detection limit of this assay using the ultrasensitive protocol is 0.7 pmol/liter. E2, inhibin B, androstenedione, testosterone, dehydroepiandrosterone, LH, and FSH were measured by immunoassays as described previously (17). Fasting serum insulin levels were measured in duplicate by an immunoradiometric assay (Bi-Insulin IRMA Pasteur, Bio-Rad, Marnes la Coquette, France) that uses two monoclonal antiinsulin antibodies. Intra- and interassay coefficient of variation were <3.8 and 7.5%, respectively. Results are expressed as milliinternational units per liter in terms of the World Health Organization 66/304 reference preparation.

Statistical methods

A P value <0.05 was considered significant. Comparisons of two independent groups were made using the Student t test or the χ^2 test. Significant relationships between AMH and the various parameters were evaluated by the nonparametric Spearman correlation coefficient. Multiple regression analysis was used to control for potential confounding variables. All statistic procedures were run on Statview 4.5 (Abacus Concepts Inc., Berkeley, CA).

Results

The main hormonal findings in each group are presented and compared in Table 2. As shown by Fig. 1, the mean serum

TABLE 1. Clinical and ultrasound data in controls and in patients with PCOS

	Controls n = 45	PCOS n = 59	P
Age (yr)	28.3 (24–33)	27.4 (21.3–33.1)	0.3[a]
BMI (kg/m^2)	23.1 (19.2–31.2)	26.7 (18.7–37.7)	0.03[a]
% BMI > 25	37.5%	54%	0.18[b]
% with hyperandrogenism	0%	71%	0.0001[b]
% with menstrual disorder	0%	80%	0.0001[b]
Ovarian area[c] (cm^2)	8.1 (5.6–9.5)	12.6 (8.9–17.0)	0.0001[a]
% with ovarian area > 5.5 cm^2	0%	60%	0.0001[b]
2- to 9-mm FN[d]	7.3 (4.5–11.3)	16.6 (10.8–28.5)	0.0001[a]
2- to 5-mm FN[d]	4.8 (2.5–7)	12.8 (4.0–25.7)	0.0001[a]
6- to 9-mm FN[d]	2.6 (0–5.5)	3.7 (0–8.8)	0.12[a]

Values are expressed as mean with 10–90th percentiles in *parentheses.*
[a] P value by Student t test; [b] P value by χ^2 test.
[c] Sum of both ovaries; [d] mean of both ovaries.

TABLE 2. Main hormonal findings in controls and patients with PCOS

	Controls n = 45	PCOS n = 59	P^a	Conversion factor for SI units
AMH (pmol/liter)	20.8 (3.6–37.2)	47.1 (24.4–85.0)	0.0001	1 ng/ml = 7.14 pmol/liter
Testosterone (ng/ml)	0.3 (0.17–0.45)	0.53 (0.25–0.83)	0.0001	1 ng/ml = 3.467 nmol/liter
Androstenedione (ng/ml)	1.6 (0.99–2.14)	2.6 (1.61–4.2)	0.0001	1 ng/ml = 3.492 nmol/liter
E2 (pg/ml)	30 (20–52)	32 (20–47)	0.36	1 pg/ml = 3.671 pmol/liter
LH (IU/liter)	3.7 (2.1–5.9)	8.0 (3.3–14.9)	0.0001	NA
FSH (IU/liter)	5.8 (4.5–7.3)	5.6 (4.0–7.4)	0.35	NA
Inhibin B (pg/ml)	84.6 (50–119)	90.2 (33–142)	0.49	NA
Insulin (mIU/liter)	4.5 (1.0–8.5)	7.9 (1.4–18.3)	0.0001	NA

Values are expressed as mean with 10–90th percentiles in *parentheses.* NA, Not applicable.
[a] *P* level by Student *t* test.

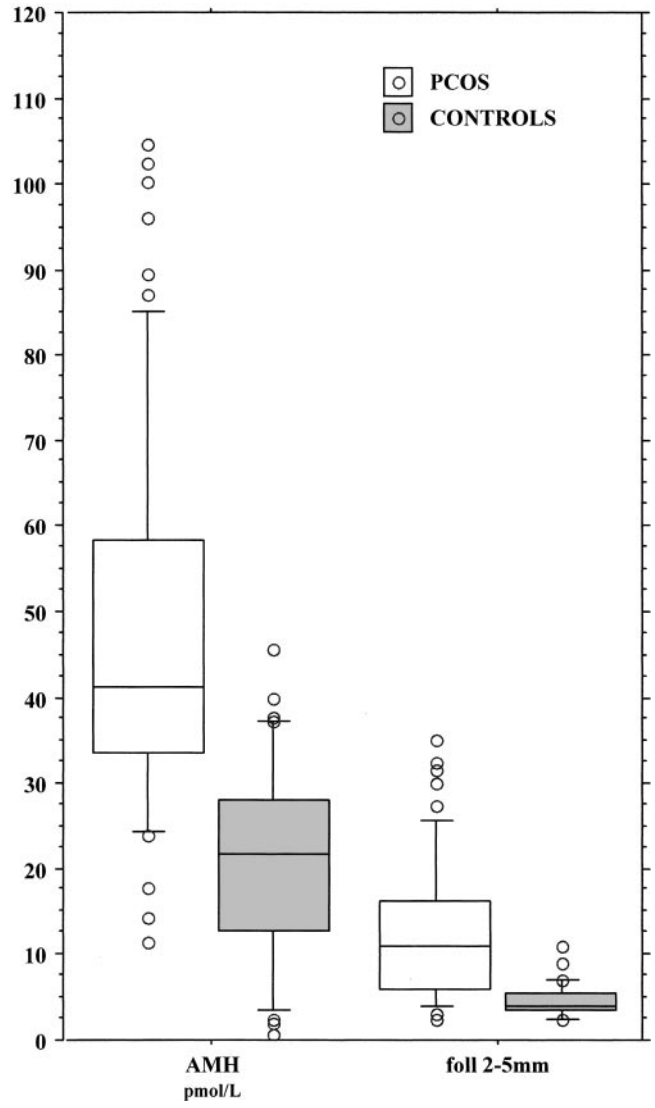

FIG. 1. Box-and-whisker plots showing the values of serum AMH (1 ng/ml = 7.14 pmol/liter) and 2- to 5-mm follicle number in patients with PCOS (n = 59) and in controls (n = 45). *Horizontal small bars* represent the 10–90th percentile range, and the *boxes* indicate the 25th-75th percentile range. The *horizontal line* in each box corresponds to the median.

AMH level was 2- to 3-fold higher in PCOS than in controls, an increase of the same order of magnitude as the one of FN in the 2- to 5-mm range (Table 1). Therefore, the ratio AMH/2- to 5-mm FN was similar between the two groups (4.8 ± 3.4 *vs.* 5.3 ± 3.4; $P = 0.55$).

TABLE 3. Relationships between the AMH plasma level and BMI or other biological data (serum levels) in patients with PCOS and in controls

	PCOS (n = 59)		Controls (n = 45)	
	Correlation coefficient[a]	*P*	Correlation coefficient[a]	*P*
AMH, 2- to 5-mm FN	0.670	0.0001	0.370	0.022
AMH, 6- to 9-mm FN	0.042	0.73	0.137	0.37
AMH, FSH	−0.313	0.018	−0.311	0.037
AMH, testosterone	0.454	0.0004	0.163	0.288
AMH, androstenedione	0.430	0.0013	0.217	0.153
AMH, E2	−0.083	0.54	0.072	0.64
AMH, BMI	−0.061	0.66	−0.280	0.048
AMH, insulin	−0.024	0.87	−0.258	0.091

[a] Spearman correlation coefficient.

As shown in Table 3, the AMH level was positively and significantly related to the 2- to 5-mm FN at U/S (Fig. 2), but not to the 6- to 9-mm FN, both in PCOS and controls. It was positively related to the serum testosterone and androstenedione levels, in PCOS exclusively (Fig. 2), whereas it was negatively correlated to the serum FSH level, in both groups (Fig. 2). Multiple regression was performed in the PCOS group, including AMH as the dependent variable and the other above mentioned parameters as independent variables. Only the 2- to 5-mm FN remained significantly related to the AMH level ($r = 0.52$; $P < 0.0001$), whereas testosterone, androstenedione, and FSH were no longer significantly related ($r = 0.183$, 0.173, and −0.123, respectively).

The mean serum AMH level tended to be lower in obese than in nonobese controls (15.0 ± 9.3 *vs.* 22.0 ± 12.7 pmol/liter, respectively; $P = 0.07$), whereas no difference was observed between obese and nonobese women with PCOS (44.5 ± 16.6 *vs.* 50.8 ± 27.6 pmol/liter, respectively; $P = 0.32$). In addition, in controls exclusively, the BMI was negatively and significantly related to the serum AMH level, whereas the tendency with the fasting insulin serum level did not reach the level of significance (Table 3). After controlling for BMI in this group, the correlation coefficient between the AMH level and the 2- to 5-mm FN was slightly below the level of significance ($r = +0.25$; $P = 0.08$), whereas the relationship between the AMH and FSH levels remained significant ($r = -0.31$; $P < 0.02$).

No relationship was found in both groups between AMH and age, serum E2, inhibin B, and LH levels (data not shown).

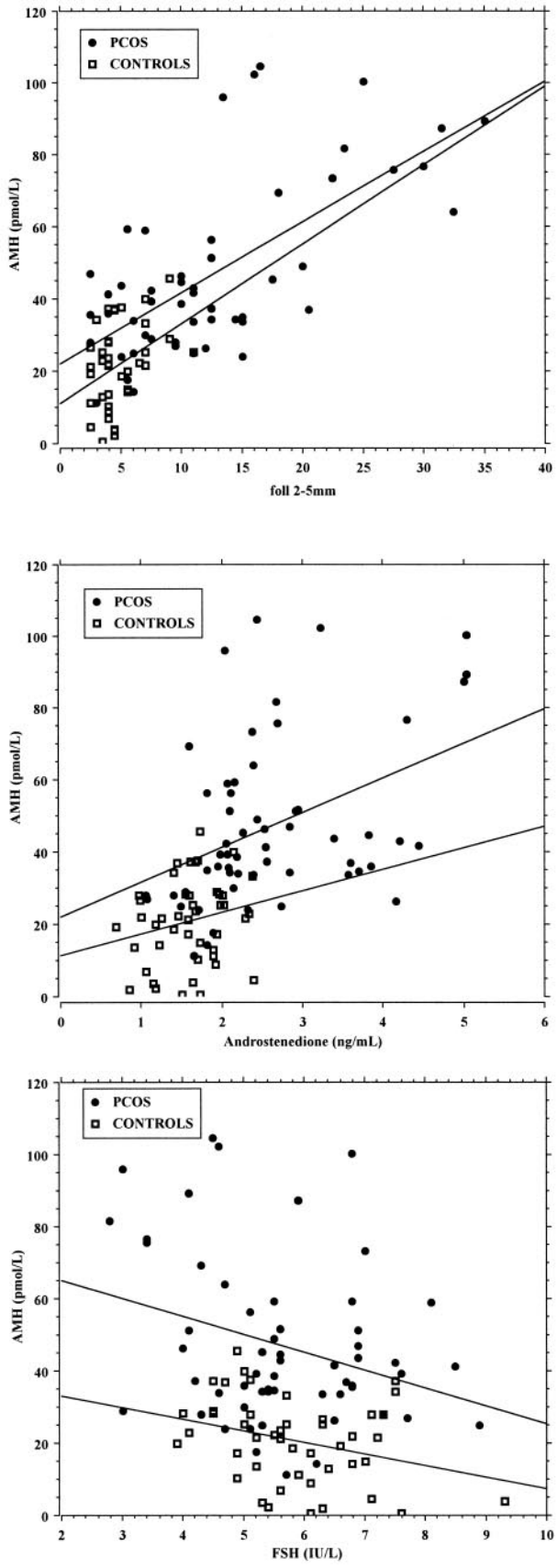

FIG. 2. Relationship between serum AMH level (1 ng/ml = 7.14 pmol/liter) and the 2- to 5-mm FN (*top*), serum androstenedione (*middle*), and serum FSH (*bottom*) in controls (n = 45) and in patients with PCOS (n = 59). See Table 2 for the values of the Spearman coefficient of correlation. For each panel, the upper and the lower regression lines apply to PCOS and control groups, respectively.

Discussion

Data about our control group agree with previous reports from Van Rooij *et al.* (12) and Fanchin *et al.* (18) who found a tight relationship between the AMH serum level and the FN at U/S in regularly menstruating infertile women studied at baseline d 3, before undergoing *in vitro* fertilization. Van Rooij *et al.* (12) also showed that the AMH level was a good predictor of the number of retrieved oocytes, as previously reported by Seifer *et al.* (13). However, our data have produced new information by showing that the 6- to 9-mm follicles do not contribute to the serum AMH level in normoovulatory women, whereas being overweight has a mild, although significant, negative influence on this parameter.

We also confirm the data from Cook *et al.* (15) indicating a marked elevation of the serum AMH level in PCOS. However, our data bring further information by showing a tight relationship between AMH serum level and the 2- to 5-mm FN at U/S, with the same slope of regression as in controls. Therefore, the marked elevation of AMH is not surprising in such a situation of follicle excess, which is a salient feature of the syndrome. In a previous series of women with PCOS, we showed that the 2- to 5-mm FN was positively related to the serum androstenedione or testosterone level (5). We hypothesized that this reflected the promoting effect of intraovarian androgens on follicle growth (3). In this study we expand our previous data by showing a significant relationship between AMH and androgens that seems specific to PCOS because it has not been found in our controls. However, this relationship was no longer significant after controlling for the 2- to 5-mm FN. Therefore it must be viewed as the consequence of the androgen-induced excess in FN rather than an indicator of any positive effect of androgens on ovarian AMH secretion, which has not been reported so far. In contrast to controls, BMI did not influence the serum AMH level in the PCOS group, although it displayed a higher mean BMI. Even if it can be speculated that the mild effect of BMI was masked by the strong positive relationship between AMH and androgens in women with PCOS, the reason for this discrepancy remains unclear.

Experimental data carried out on cultured GCs demonstrated that AMH inhibits the conversion rate of androgens to E2 by down-regulating the aromatase gene expression (19). This supports the physiological relevance of the inverse relationship between AMH and E2, which has been found in PCOS women (15) or in non-PCOS patients (13). In contrast to these studies, we did not find this negative relationship. Likewise, we did not find a relationship between AMH and inhibin B, in contrast to Fanchin *et al.* (18). Because AMH is an earlier product from the follicle cohort than E2 or inhibin B (20), these discrepancies might simply reflect differences in the sampling day. Indeed, in the study of Cook *et al.* (15), blood was collected at random in their oligomenorrheic women, whereas our patients were sampled in the early

follicular phase after either spontaneous or progestin-induced menstruations. Not surprisingly therefore, the range of E2 values was higher in the other series (13, 15), thus allowing the authors to unravel the inverse relationship between AMH and E2.

As already reported by others in non-PCOS patients (12, 13, 18), we observed a negative correlation between AMH and FSH serum levels, both in our PCOS patients and controls, with a similar regression slope. Whether this correlation, now observed in four independent studies, reflects a physiological link between these two parameters cannot be established by these studies. Yet it supports the hypothesis that FSH may behave as a negative regulator of AMH synthesis in the human adult ovary, whereas it is well established that it is a positive regulator of testicular AMH gene expression in adults (21). Although few experimental data are available so far in the literature to support a regulatory role of FSH on the ovarian AMH, Baarends *et al.* (22) previously reported that FSH may down-regulate the AMH and AMH type II receptor expression in adult rat ovaries. Conversely, follicles from AMH knockout mice have been shown to be more sensitive to FSH than those from the wild-type (10), suggesting that the above mentioned inhibiting effect of AMH on aromatase activity acts through a decrease in GC sensitivity to FSH. This balance between the opposite effects of AMH and FSH on aromatase activity might be crucial for the cohort, at the time of the selection process. The acquirement by the small antral follicles of an exquisite sensitivity to FSH would lower AMH expression. This would allow aromatase to escape from AMH inhibition, thus conferring to the selected follicles the ability to secrete E2.

Such a phenomenon could be altered in PCOS. Indeed, higher levels of AMH in our study and that of Cook *et al.* (15) were associated with lower values of FSH levels (in both studies) and with lower E2 levels (in the latter). From the aforementioned experimental data, it is therefore tempting to speculate that the excess of AMH is involved in the lack of FSH-induced aromatase activity, which characterizes the follicular arrest of PCOS. This could be explained by an excessive AMH tone operating at the cohort level rather than within the GCs. Indeed, the ratio AMH/FN was not increased in our patients, thus suggesting that each follicle produces a normal amount of AMH. Conversely, the robust and independent positive correlation that we found between the FN and the serum AMH level argues in favor of the hypothesis that the excess in the 2- to 5-mm FN is *per se* responsible for the excess of AMH. This hypothesis could reconcile the contradiction between the *in vivo* findings in women with PCOS showing a reduced responsiveness to FSH (23) and the *in vitro* observation that GCs from PCOs are highly sensitive to FSH in terms of E2 production (24). As recently emphasized (23), a putative aromatase inhibitor within the ovarian microenvironment of PCOs could be responsible for this.

In conclusion, our data about AMH provides new insights into the comprehension of follicular arrest in PCOS. Notwithstanding the role of a premature LH action (25) and the negative effect of obesity on the late stages of follicle maturation (5), the available data about AMH make it a good candidate as the putative interfollicular surrounding factor that inhibits aromatase within the cohort and thus refrains the selection process. Because of the increased FN leading to an exaggerated AMH tone in PCOs, the negative effect exerted by FSH on AMH would not be sufficient, despite the normal serum level of this hormone. This fits with the rationale for ovulation induction in PCOS, which implies that the FSH serum level needs to be increased at the time of selection. In practice, as shown in states of follicle depletion and/or in aging women (12, 13), our study suggests that the AMH serum level could also be a useful follicle marker in states of multifollicularity, such as PCOS. More particularly, additional studies are warranted to evaluate whether serum AMH could be a good predictor of the ovarian stimulation outcome in PCOS, as are the FN or ovarian volume (26, 27) and the basal serum FSH level (28). More particularly, its usefulness to predict ovarian hyperstimulation has to be checked.

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