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Serum anti-Mullerian hormone levels during controlled ovarian hyperstimulation in women with polycystic ovaries with and without hyperandrogenism

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BACKGROUND: Anti-Mullerian hormone (AMH) is expressed in pre- and small-antral follicles. High serum levels are found in women with polycystic ovaries (PCO), accordant with their increased content of small follicles. To evaluate the relationship between AMH, folliculogenesis and hyperandrogenism, we compared serum AMH levels between women with PCO with and without hyperandrogenism and normal controls during controlled ovarian hyperstimulation (COH). METHODS: Nineteen women with PCO and hyperandrogenism (group A), 10 women with PCO but no hyperandrogenism (group B) and 23 ovulatory women with normal ovarian morphology (group C, controls) underwent COH with the long protocol. Serum levels of AMH, estradiol, androstenedione and follicular tracking were determined before gonadotropins treatment (day 0) and every 2–4 days up to the day of HCG administration. RESULTS: AMH levels declined gradually throughout COH in the three groups, but remained higher in groups A and B compared with the controls. Significantly higher levels were found in group A compared with group B, despite comparable numbers of small follicles. Multiple regression analysis revealed that both the number of small follicles and serum androgens were correlated to AMH. CONCLUSIONS: Women with PCO have higher serum AMH levels during COH than controls. Hyperandrogenism is associated with an additional increase in AMH. It is conceivable that hyperandrogenism may reflect more severe disruption of folliculogenesis in women with PCO or may affect AMH secretion.

Key words: anti-Mullerian hormone/controlled ovarian hyperstimulation/hyperandrogenism/polycystic ovaries

Introduction

The dimeric glycoprotein anti-Mullerian hormone (AMH), also termed Mullerian inhibiting substance, is a member of the transforming growth factor-β superfamily. In the male, it is produced during fetal sex differentiation by Sertoli cells, in which it induces Mullerian duct degeneration (Cate *et al.*, 1986). In females, AMH is produced only postnatally by granulosa cells from preantral and small antral follicles (Durlinger *et al.*, 2002; Weenen *et al.*, 2004).

Serum AMH level is strongly correlated with the number of antral follicles and is more strongly related to ovarian reserve than other known markers such as day 3 FSH, inhibin B or estradiol (de Vet *et al.*, 2002; Seifer *et al.*, 2002; van Rooij *et al.*, 2002; Fanchin *et al.*, 2003a). Women with polycystic ovary syndrome (PCOS) have higher serum AMH levels than normal controls (Fallat *et al.*, 1997; Cook *et al.*, 2002; Pigny *et al.*, 2003; La Marca *et al.*, 2004a; Laven *et al.*, 2004). This is presumably related to the increased number of small antral follicles in PCOS. However, the diagnosis of PCOS in these studies was usually based on the Rotterdam consensus (Rotterdam ESHRE/ASRM Sponsored PCOS Consensus Workshop Group, 2004), where only two out of three criteria—hyperandrogenism, typical ultrasonic ovarian morphology and oligo- or amenorrhoea—were needed for the diagnosis. Therefore, they did not differentiate between women with or without hyperandrogenism. Women with PCOS were reported to have higher AMH serum levels than other WHO 2 infertile women (Laven *et al.*, 2004). PCOS women, however, have both increased serum androgens and higher number of small antral follicles. Moreover, although serum AMH levels were positively correlated with the number of small antral follicles (Pigny *et al.*, 2003; Laven *et al.*, 2004), controversies existed as to the correlation with serum androgens.

Serum AMH levels decline gradually during controlled ovarian hyperstimulation (COH), whereas other hormones such as estradiol, inhibin A, inhibin B and progesterone increase (Fanchin *et al.*, 2003b; La Marca *et al.*, 2004b).

It has been suggested that this reflected the reduction in the number of small antral follicles in parallel to the increase in the number of larger ones. However, serum levels of androgens, such as androstenedione and testosterone, also increase during COH (Hamori *et al.*, 1992; Martin *et al.*, 1997; Fanchin *et al.*, 2003b). To gain further insight into the relationship between ovarian androgens, folliculogenesis and AMH secretion, we investigated AMH levels during COH in women having PCO with or without hyperandrogenism, and compared them with normal controls.

Materials and methods

The Shaare-Zedek Medical Center Research and Ethics Committee approved the study. A total of 52 IVF patients were prospectively enrolled in this study. The inclusion criteria were age between 20–39 years, both ovaries present, no previous ovarian operation, adequate visualization of ovaries on transvaginal ultrasound and no current hormone therapy. The indications for IVF were sperm abnormality (17 patients), mechanical factor (seven patients), ovulatory dysfunction after failure of superovulation and intrauterine inseminations (seven patients), unexplained infertility (five patients) and combined indications (16 patients).

Twenty-nine women were diagnosed with PCOS according to the Rotterdam consensus (Rotterdam ESHRE/ASRM Sponsored PCOS Consensus Workshop Group, 2004). They had oligo- or amenorrhoea and at least 12 follicles 2–9 mm in diameter per ovary. Nineteen of the women had hyperandrogenism (testosterone >3 nmol/l, free androgen index >4.5 and/or androstenedione >12 nmol/l) (group A, PCO with hyperandrogenism) and 10 women had normal serum androgens levels and no clinical hyperandrogenism (group B, PCO without hyperandrogenism). Twenty-three women had normal ovulatory cycles, no endocrine abnormalities (normal TSH, prolactin, day 3 FSH and estradiol, and no hyperandrogenism) and normal ultrasonic ovarian morphology (group C, controls).

The women were treated with the long down-regulation protocol consisting of Decapeptyl Depot 3.75 mg (Ferring Ltd, Herzliya, Israel) intramuscularly starting in the midluteal phase or on the 16th day of using contraceptive pills. Ovarian stimulation was commenced after at least 2 weeks, when estradiol levels were <150 pmol/l and ovarian cysts were excluded by vaginal ultrasound. The standard protocol consisted of one ampoule of HMG (Menogon; Ferring Ltd) and 150 IU recombinant FSH (Gonal F; Serono, Herzliya, Israel). The standard protocol was modified when there was a previous history of poor response or a risk of hyperstimulation. Ultrasound for follicular tracking and blood sampling for estradiol levels were performed every 2–3 days. After the first 3–5 treatment days, the daily dose could be adjusted based on the follicular development and estradiol levels, and 10 000 IU HCG (Pregnyl; Organon, Petach Tiqva, Israel) was administered when at least three follicles with a diameter of 17 mm were detected. Oocyte retrieval was performed 36 h later. Up to three embryos were transferred 48–72 h later.

Basal hormone levels were determined on day 3–4 of spontaneous or induced menses. Insulin levels were measured after fasting for at least 8 h. Blood samples were obtained on the day in which pituitary desensitization was confirmed, before starting gonadotropins treatment (day 0) and every 2–3 days from the third day of gonadotropins treatment up to the day of HCG administration. Serum was stored at -70 °C until assayed for AMH and androstenedione concentrations. Serum androstenedione was measured on day 0 and day of HCG.

Serum levels of estradiol, androstenedione, testosterone, FSH, LH and sex hormone binding globulin (SHBG) levels were measured using Immulite 2000 (Diagnostic Products Corp, Los Angeles, CA, USA). Insulin was measured using ADVIA Centaur (Bayer Corporation, Tarrytown, NY, USA). Free androgen index was calculated as: 100 × total testosterone (nmol/l)/SHBG (nmol/l). AMH was measured using a commercially available ultrasensitive two-site ELISA (Immunotech-Coulter, Marseilles, France). The assay sensitivity was 0.7 pmol/l. Inter- and intra-assay coefficients of variation were 8.7% and 5.3%, respectively.

Statistical analysis was performed using Student's *t*-test and the Mann–Whitney *U*-test as appropriate. Longitudinal changes between the three groups were assayed using Kruskal–Wallis one-way analysis of variance by ranks, Cuzick test for trend and Pee-Freedman test for all strata trend. Relationship between day 0 AMH and other data was assayed using univariate and multivariate linear regression analyses. A *P*-value <0.05 was considered statistically significant.

Results

Clinical, endocrine and ultrasonic data of the women are presented in Table I. The groups were similar in their mean age, weight and body mass index (BMI). The women in group A (PCO and hyperandrogenism) were significantly different from the remaining two groups in their hormonal profile. On the other hand, groups B (PCO but no hyperandrogenism) and C (controls) were similar. The number of small follicles (<10 mm) was similar between groups A and B, but significantly higher than group C (Table II).

Table I. Clinical, basal endocrine and ultrasound data

	Group A ($n = 19$)	Group B ($n = 10$)	Group C ($n = 23$)	*P*-value		
				A versus C	B versus C	A versus B
Age (years)	27.9 ± 4.1	29.8 ± 2.1	30.7 ± 4.2	NS	NS	NS
BMI (kg/m^2)	27.7 ± 6.1	27.2 ± 7.5	25.1 ± 4.5	NS	NS	NS
FSH (IU/l)	5.0 ± 1.4	6.2 ± 1.9	6.1 ± 1.7	<0.05	NS	NS
LH (IU/L)	9.3 ± 4.8	5.4 ± 3.5	5.2 ± 2.4	<0.005	NS	<0.05
LH/FSH	2.0 ± 1.1	0.9 ± 0.5	0.9 ± 0.5	<0.001	NS	<0.005
Testosterone (nmol/l)	2.9 ± 1.1	1.6 ± 0.4	1.4 ± 0.7	<0.0001	NS	<0.0005
Free androgen index	8.9 ± 4.7	5.4 ± 2.8	5.0 ± 2.9	<0.05	NS	<0.05
Androstenedione (nmol/l)	11.8 ± 5.2	7.5 ± 3.9	7.7 ± 3.1	<0.05	NS	<0.01

Values are presented as mean ± SD.
Group A = PCO with hyperandrogenism; group B = PCO without hyperandrogenism; group C = normal controls; NS = non significant.

Table II. Treatment outcome

	Group A	Group B	Group C	*P* value		
				A versus C	B versus C	A versus B
Day 0						
AMH (pmol/l)	51.7 ± 30.8	29.1 ± 16.8	11.2 ± 5.5	<0.0002	<0.01	<0.05
Number of follicles <10 mm	29.2 ± 12.7	26.4 ± 9.2	10.5 ± 5.2	<0.001	<0.001	NS
Androstenedione (nmol/l)	11.5 ± 5.2	5.6 ± 2.7	5.5 ± 2.0	<0.01	NS	<0.05
Day of HCG administration						
AMH (pmol/l)	30.4 ± 21.7	9.6 ± 5.4	3.0 ± 2.7	<0.0001	<0.005	<0.001
Number of follicles <10 mm	23.8 ± 10	21.5 ± 8.7	8.3 ± 6.0	<0.001	<0.05	NS
Estradiol (pmol/l)	8064 ± 3281	9118 ± 4473	9330 ± 4028	NS	NS	NS
Androstenedione (nmol/l)	20.3 ± 8.3	15.1 ± 5.9	16.6 ± 8.0	NS	NS	NS
Androstenedione/estradiol ratio	2.75 ± 1.39	1.68 ± 0.33	1.73 ± 0.81	<0.05	NS	<0.05
Oocytes number	19.2 ± 8.5	21.0 ± 2.3	15.3 ± 6.6	NS	<0.001	NS
Total FSH dose (IU)	2196 ± 819	2704 ± 626	3277 ± 904	<0.001	<0.05	NS
Oocyte number/total FSH dose (kIU)	9.5 ± 6.6	8.2 ± 2.5	5.1 ± 2.5	<0.01	<0.05	NS
Clinical pregnancy/ET	5/15	4/10	12/22	NS[a]		

Values are presented as mean ± SD. Day 0, after pituitary suppression with GnRH agonist, before gonadotropin administration.
NS[a] = non-signifiant; ET = embryo transfer; NS between the 3 groups.

Serum AMH levels (mean ± SEM) in groups A, B and C were 51.7 ± 7.8, 29.1 ± 5.3 and 11.2 ± 1.2 pmol/l, respectively, on day 0 (Figure 1). Throughout gonadotropins treatment, serum AMH levels were significantly different between the three groups, being highest in group A and lowest in group C ($P = 0.002$, Kruskal–Wallis one-way analysis of variance). AMH levels in all three groups decreased during gonadotropins treatment ($P < 0.001$, Cuzick test for trend). The decrease in AMH from day 0 to the last day of gonadotropins treatment (the day of HCG administration) was 34 ± 24%, 60 ± 25% and 71 ± 18% in groups A, B and C, respectively.

IVF cycle treatment outcome in the three groups is presented in Table II. On day 0, the number of small follicles was similar between groups A and B, but significantly higher from group C. On the other hand, serum androstenedione level was similar between groups B and C, but significantly higher in group A. The number of small follicles seemed to decrease during gonadotropin ovarian stimulation, but the differences between day 0 and the day of HCG administration were not statistically significant. Although on the day of HCG administration the estradiol and androstenedione levels were similar in the three groups, the ratio androstenedione to estradiol was higher in group A compared with either group B or C. Since we aimed to examine changes in serum androgen levels during gonadotropins treatment, we chose androstenedione, because it was shown in previous studies that only its increase was significantly higher in PCOS compared with controls (Hamori *et al.*, 1992). HCG administration and oocyte retrieval were cancelled in one patient in group A owing to high risk for ovarian hyperstimulation syndrome (OHSS). No moderate or severe OHSS developed in any patient.

Highly significant ($P < 0.001$) positive correlations were found between day 0 AMH and androgens (testosterone and free androgen index), the number of small follicles, the number of oocytes per total FSH dose and AMH on the day of

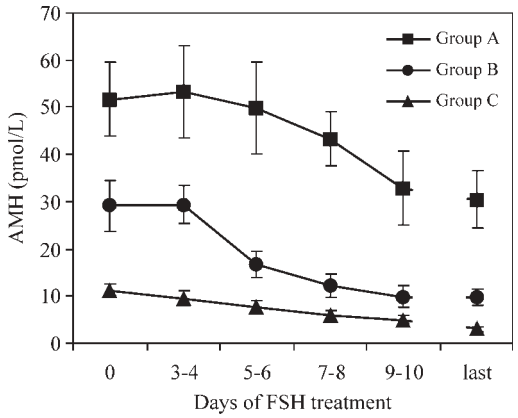

Figure 1. Serum AMH levels (mean ± standard error of the mean) during gonadotropins treatment.

Table III. Association (Pearson's correlation) between serum AMH and other parameters comprising all three groups together

	Correlation	No.[a]	*P*-value
Day 0			
Age	−0.38	52	<0.01
BMI	0.11	48	NS
LH	0.30	47	<0.05
FSH	−0.38	47	<0.01
LH/FSH ratio	0.46	47	<0.001
Testosterone	0.66	46	<0.001
Free androgen index	0.66	33	<0.001
Androstenedione	0.45	39	<0.01
Fasting insulin	−0.10	18	NS
Number of follicles <10 mm	0.78	52	<0.001
Number of oocytes	0.08	51	NS
Control group only	0.38	23	NS
Total FSH dose	−0.59	52	<0.001
Number of oocyte/total FSH dose	0.55	51	<0.001
AMH on day of HCG administration	0.89	48	<0.001
Maximal estradiol	0.21	52	NS
Control group only	0.68	23	<0.001
Day of HCG administration			
Number of follicles <10 mm	0.79	48	<0.001
Androstenedione/estradiol ratio	0.71	39	<0.001

[a]Number of women tested.

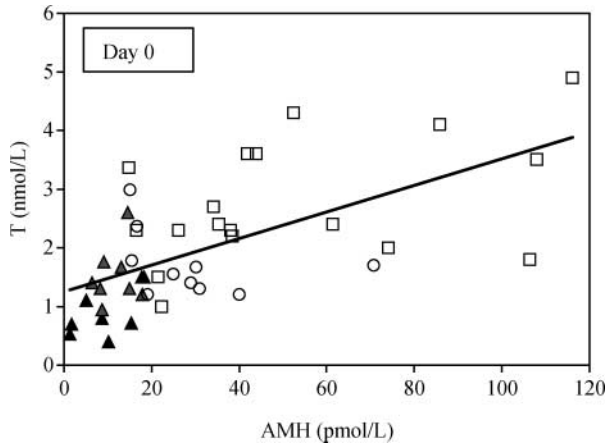

Figure 2. Relationship between serum AMH on day 0 and basal testosterone levels. Group A, squares; group B, circles; group C, triangles.

HCG administration, whilst negative correlation were found with the total FSH dose (Table III; Figures 2 and 3). Day 0 AMH showed significant, but lower ($P < 0.01–0.05$), positive correlations with LH and androstenedione, and negative correlations with patient's age and FSH. When calculated separately, the above correlations in the controls (group C) and the PCO women (groups A & B together) were usually similar, excluding the number of oocytes and maximal

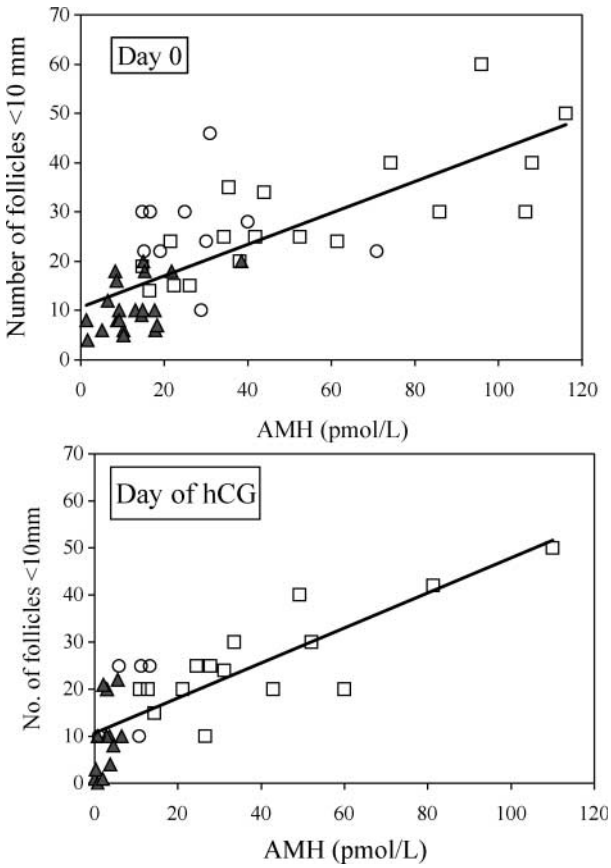

Figure 3. Relationship between serum AMH levels and the number of small antral follicles day 0 and on the last day of FSH treatment. Group A, squares; group B, circles; group C, triangles.

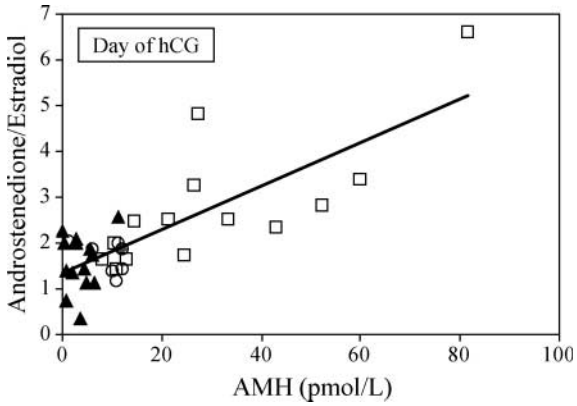

Figure 4. Relationship between serum AMH and androstenedione/estradiol ratio on the day of HCG administration. Group A, squares; group B, circles; group C, triangles.

stradiol, which correlated with AMH only in the control group (Table III). The correlation between day 0 AMH and testosterone in the control group was 0.44 ($P < 0.05$). On the day of HCG administration, serum AMH showed highly significant correlations with both the number of small follicles and androstenedione/estradiol ratio (Figure 4). The correlation between the proportional changes in AMH levels during stimulation and the proportional changes in the number of small follicles was not significant ($r = 0.26$).

Multiple regression analysis was performed including day 0 AMH as the dependant variable and the highly significant parameters mentioned in Table III as independent variables. When we built the univariate linear regression model for testosterone and AMH, the R^2 was 0.429. When we added follicle number, the R^2 increased to 0.653. The added variance was 0.224. The number of small follicles ($P < 0.001$) and testosterone ($P < 0.05$) remained significantly related to AMH level (adjusted $r^2 = 0.636$).

Discussion

Women with PCOS exhibit a broad spectrum of clinical and biochemical characteristics. Not all women have the typical full-blown state, with the stigma of hyperandrogenism, anovulation, obesity and insulin resistance. The ultimate pathogenesis of this syndrome remains obscure, but the distinctive feature is failure of follicular maturation, despite initial recruitment. The role of intraovarian androgens in the disordered folliculogenesis in PCOS is still debated. AMH is an attractive candidate to play a pivotal role in this enigma: it is synthesized in small antral follicles (Durlinger *et al.*, 2002; Weenen *et al.*, 2004), and it inhibits FSH sensitivity (Durlinger *et al.*, 2001; Durlinger *et al.*, 2002) and aromatase activity (di Clemente *et al.*, 1992).

In accordance with previous studies, we found that women with PCO have significantly higher serum AMH levels (Fallat *et al.*, 1997; Cook *et al.*, 2002; Pigny *et al.*, 2003; La Marca *et al.*, 2004a; Laven *et al.*, 2004) and that AMH was associated with the number of small antral follicles (van Rooij *et al.*, 2002; Laven *et al.*, 2004). Our results are in agreement with those of Pigny *et al.* (2003), showing no significant

correlations between AMH and BMI or fasting insulin. However when comparing PCO patients with and without hyperandrogenism, we found that in women with polycystic ovaries, hyperandrogenism was associated with an additional increase in AMH. Furthermore, using multiple regression analysis we showed that both the number of small follicles and serum testosterone levels were independently related to the levels of serum AMH.

Using univariate regression analysis, two previous studies also found significant correlations between serum AMH and androgens in PCOS (Pigny *et al.*, 2003; Laven *et al.*, 2004). However, two small studies found no such correlations in either PCOS or normal women (Cook *et al.*, 2002; La Marca *et al.*, 2004a), while one study found no correlations in normal ovulatory women after GnRH agonist suppression (Fanchin *et al.*, 2003b). The reasons for the differences between the findings are unclear, although the study populations differ in their number, mean age, basal FSH levels, BMI, previous hormonal treatment (pill, GnRH agonist) and possibly other unknown factors. Still, our results are different from those of Pigny and colleagues, who used multiple regression analysis to show that only the number of 2–5 mm follicles, but not androgens, was significantly related to AMH in PCOS women (Pigny *et al.*, 2003). We found that the number of both small follicles and serum androgens were correlated to AMH, in the whole group of patients and in each group (PCO and controls) separately (Table III). However, in the study by Pigny and colleagues, of the 59 PCOS women tested, 29% had no hyperandrogenism and 20% had no menses abnormalities. In addition, the mean number of follicles 2–9 mm in both ovaries was 16.6 (10–90th percentiles 10.8–28.5), compared with 29.2 ± 12.7 and 26.4 ± 9.2 (mean ± SD) in groups A and B, respectively, in our study (Table II).

We found that serum AMH levels decreased significantly during ovarian stimulation, in accordance with previous studies (Fanchin *et al.*, 2003b; La Marca *et al.*, 2004b). Fanchin and colleagues assumed that the gradual decrease in serum AMH during COH probably reflected the reduction in the number of small antral follicles following gonadotropins treatment. However, in our study, despite similar numbers of small follicles in the two groups of PCO women, AMH levels were significantly higher throughout COH in women with hyperandrogenism. Recently, La Marca and colleagues reported that although AMH plasma levels did not change significantly during the follicular phase in spontaneous cycles, its levels decreased progressively in FSH-treated cycles (La Marca *et al.*, 2004b). They found significant positive correlations between the decrease in AMH and the increase in estradiol plasma levels in FSH-treated cycles and between basal AMH and the peak estradiol during exogenous FSH administration, similar to our findings. They assumed that the decrease in AMH serum levels following exogenous FSH administration was probably secondary to the gonadotropin effect on the process of follicular development. However, Baarends and coworkers showed that treating prepubertal rats with GnRH antagonist and either FSH or estradiol resulted in inhibition of AMH mRNA and AMH-receptor-II mRNA expression in preantral and small antral follicles (Baarends *et al.*, 1995). Cook and coworkers also showed an inverse correlation between serum levels of AMH and estradiol in PCOS, but not in normal women (Cook *et al.*, 2002). In our study, serum estradiol levels during COH were similar between the three groups, but the serum androstenedione/estradiol ratio was significantly higher in the women with hyperandrogenism (Table II), and was positively correlated with AMH ($r = 0.71$; $P < 0.001$; Table III). Furthermore, the decrease in mean serum AMH was lower in PCO patient with hyperandrogenism (group A, 35 ± 24%) compared with PCOS without hyperandrogenism (group B, 60 ± 25%) or to the controls (group C, 71 ± 18%). We speculate that the gradual increase in intraovarian androgen/estradiol ratio during COH may reduce AMH secretion. This ratio is lower in follicular fluid from small follicles in PCOS women compared with size-matched follicles from women with normal ovaries (Eden *et al.*, 1990; Teissier *et al.*, 2000), and may restrain the decrease in AMH.

The role of androgens in preantral follicle development is still unclear. However, it has been hypothesized that intraovarian hyperandrogenism can prompt the increased number of early stages follicles. Hyperandrogenism of extra-ovarian origin, e.g. congenital adrenal hyperplasia, virilizing tumors and especially high-dose exogenous androgen treatment, can cause PCO-like ovarian morphology in women (Pache *et al.*, 1991) and in the androgenized monkey model (Vendola *et al.*, 1998). The trophic effect of androgens predominates in granulosa cells within small follicles due to their richness in androgen receptors (Hillier *et al.* 1997). Our results support the assumption that hyperandrogenism may instigate the increased number of preantral and small antral follicles, leading to increased AMH secretion, which cause refractoriness to FSH-induced follicle differentiation seen in PCOS.

In conclusion, we have demonstrated that AMH levels declined gradually throughout gonadotropins treatment in all women, that women with polycystic ovaries retained significantly higher serum AMH levels during COH than controls, and that hyperandrogenism was associated with an additional increase in AMH. It is conceivable that hyperandrogenism may reflect more severe disruption of folliculogenesis in women with polycystic ovaries, expressed as an increase in the number of preantral (in addition to the small antral) follicles, or may directly affect AMH secretion from ovarian granulosa cells. We recognize that the association between serum AMH and androgens does not necessarily infer a cause and effect. In-vitro studies may help to further understand the causal relationships between androgens and AMH.

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