

# Serum anti-Müllerian hormone levels remain high until late reproductive age and decrease during metformin therapy in women with polycystic ovary syndrome

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**BACKGROUND:** Anti-Müllerian hormone (AMH) is secreted by granulosa cells of ovarian early developing follicles and its serum levels have been shown to correlate with small antral follicle number. Since the pronounced androgen secretion from follicles/stroma in women with polycystic ovary syndrome (PCOS) remains until late reproductive age, and since AMH reflects the number of antral follicles, it was of interest to study the possible age-related relationship between AMH, androgens and follicle number in women with PCOS and in control women. Moreover, the possible effect of metformin on serum AMH levels and the relationship to follicle count and volume were studied. **METHODS:** Forty-four healthy women (aged 21–44 years) and 65 women with previously diagnosed PCOS (aged 16–44 years) participated in the study. Serum basal AMH levels were correlated with those of serum androstenedione, testosterone, estradiol (E<sub>2</sub>), LH, FSH and inhibin B, and with follicle number. The effect of metformin on serum AMH concentrations, follicle number and ovarian volume was studied in 26 women (aged 20–41 years) with PCOS after 6 months of treatment. **RESULTS:** Serum AMH levels were 2- to 3- fold higher in PCOS women than in healthy women. In control women, serum AMH levels correlated positively with those of serum androstenedione ( $r = 0.564$ ,  $P < 0.001$ ) and testosterone ( $r = 0.328$ ,  $P = 0.036$ ) and negatively with serum FSH concentrations ( $r = -0.374$ ,  $P = 0.012$ ) and age ( $r = -0.691$ ,  $P < 0.001$ ). In women with PCOS, serum AMH levels correlated positively with those of androstenedione ( $r = 0.311$ ,  $P = 0.011$ ) and testosterone ( $r = 0.310$ ,  $P = 0.011$ ) and with follicle count ( $r = 0.352$ ,  $P = 0.012$ ), and negatively with age ( $r = -0.300$ ,  $P = 0.014$ ). Serum AMH levels, the number of antral follicles and ovarian volume decreased significantly during metformin treatment. **CONCLUSIONS:** Serum AMH levels decreased with age both in healthy women and in women with PCOS, although they were always 2- to 3-fold higher and remained elevated until 40 years of age in PCOS subjects. Thus, since serum AMH levels correlate well with antral follicle count and serum androgen levels, the measurement of AMH could be used as a tool to assess ovarian ageing, to diagnose polycystic ovaries/PCOS and to evaluate treatment efficacy.

*Key words:* anti-Müllerian hormone/follicle pool/metformin/ovarian ageing/PCOS

## Introduction

Anti-Müllerian hormone (AMH), also known as Müllerian-inhibiting substance (MIS), is a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) family. In men, AMH is produced by Sertoli cells and it causes regression of the Müllerian ducts, which is a requirement for normal male reproductive tract development (Allard *et al.*, 2000). In females, AMH is mainly secreted by the granulosa cells of ovarian early developing follicles. Until puberty, serum levels of AMH are negligible, but they increase thereafter to levels comparable with those in men, probably as a result of follicular growth, and they remain detectable until the end of ovarian activity

(Vigier *et al.*, 1984; Hudson *et al.*, 1990; Josso *et al.*, 1993; Young *et al.*, 1999). The expression of AMH has been demonstrated in granulosa cells, and its receptors have been found both in granulosa and in theca cells by *in situ* hybridization in animal studies (Baarends *et al.*, 1995). Similarly, in the human, the expression of AMH is localized in granulosa cells of primary, pre-antral and small antral follicles, suggesting an important role for AMH in human folliculogenesis (Weenen *et al.*, 2004). Since AMH is secreted exclusively in the gonads, its serum concentrations in females are thought to reflect the size of the ovarian follicle pool (van Rooij *et al.*, 2002). In fact, several investigators have

reported a correlation between serum AMH concentrations and antral follicle count measured by transvaginal ultrasonography (de Vet *et al.*, 2002; van Rooij *et al.*, 2002; Fanchin *et al.*, 2003; Laven *et al.*, 2004). Furthermore, the results of earlier studies suggest that AMH may play an important role in follicle recruitment, possibly by decreasing the sensitivity of ovarian follicles to FSH and by inhibiting the initiation of FSH-induced follicle growth and selection of the dominant follicle, thereby slowing down follicle pool depletion (Durlinger *et al.*, 1999, Durlinger *et al.*, 2002; Seifer *et al.*, 2002; Gruijters *et al.*, 2003). This may indicate that a lack or excess of AMH may be associated with abnormal follicular development, and thus disturbances in reproductive functions.

Polycystic ovary syndrome (PCOS) is the most common endocrinological disorder in women of fertile age. It is characterized by menstrual irregularity, androgen excess, polycystic ovaries (PCO) and disturbances in glucose metabolism. Since women with PCOS are known to have an excessive amount of small antral follicles in the ovaries and at the same time increased serum AMH levels (Pigny *et al.*, 2003; Laven *et al.*, 2004; Mulders *et al.*, 2004), it is possible that AMH may indeed play a role in PCOS, being one of the factors that cause/reflect functional or morphological features typical of the syndrome.

Insulin-lowering agents, such as metformin, have been shown to improve hyperinsulinaemia, hyperandrogenism, menstrual pattern and ovulatory function in women with PCOS (Velazquez *et al.*, 1994; Nestler and Jakubowicz, 1997; Morin-Papunen *et al.*, 1998; Elter *et al.*, 2002), although the exact mechanism of action of this drug remains controversial. Up to now, there are only few data on the possible effects of metformin on ovarian morphology (ovarian follicle number and volume) (De Leo *et al.*, 1999; Stadtmauer *et al.*, 2001; Elter *et al.*, 2002) and no data on its potential effect on serum AMH levels in women with PCOS. Thus, it was of interest to study whether the improvement of hyperandrogenism induced by metformin treatment (Velazquez *et al.*, 1994; Morin-Papunen *et al.*, 2003) would be associated with changes in serum AMH levels and ovarian morphology in women with PCOS. Furthermore, our recent studies have shown that ovarian steroid production capacity differs significantly between healthy subjects and women with PCOS (Piltonen *et al.*, 2003, 2004). The pronounced androgen production in PCOS lasts the whole of a woman's reproductive life, while in healthy women ovarian androgen secretion capacity decreases after the age of 30 years. This difference may partly be a result of a higher number of antral follicles in women with PCOS. Since serum AMH concentrations have been shown to correlate with the number of remaining follicles, it was also of interest to analyse serum AMH levels in relation to ovarian ageing and endocrine function in women with PCOS.

### Patients and methods

Forty-four healthy women [aged 21–44 years, body mass index (BMI), 19–31.77 kg/m<sup>2</sup>] and 65 women with previously diagnosed

PCOS (aged 16–44 years, BMI, 18–44 kg/m<sup>2</sup>) participated in the study. The majority of the subjects had participated in our earlier studies (Morin-Papunen *et al.*, 2000, Morin-Papunen *et al.*, 2003; Piltonen *et al.*, 2003, 2004). All healthy women had regular cycles (28–35 days) and normal appearing ovaries on transvaginal ultrasonography. All women with PCOS had oligomenorrhea (intermenstrual interval  $\geq 36$  days) or irregular menstruation (menstrual interval  $> 7$  days from one period to another), hyperandrogenism (hirsutism score  $> 7$  according to Ferriman and Gallwey, acne or serum testosterone  $\geq 2.7$  nmol/l) and PCO (at least eight follicles of 3–8 mm diameter in one plane in one ovary) confirmed by transvaginal ultrasonography. These criteria also fulfil the Rotterdam consensus meeting definition of PCOS (ESHRE/ASRM, 2004). The women with PCOS were otherwise healthy and were under no medication (including oral contraceptive pills). Progesterin (dydrogesterone, 10 mg/day for 10 days) was used to induce menstrual bleeding in PCOS cases with oligomenorrhoea/amenorrhoea. In both study groups, a break/wash out period of at least 2 months in the use of oral contraceptive pills was required before the study. In clinical practice, a break longer than 2 months in oral contraceptive pills is not convenient for the patients and we have used a 2 month break in all our previous studies (Koivunen *et al.*, 2001; Piltonen *et al.*, 2002, 2003, 2004; Morin-Papunen *et al.*, 2003). Informed written consent was obtained from each subject and the study was approved by the ethics committee of Oulu University Hospital (Oulu, Finland).

Fasting blood samples for basal serum AMH, LH, FSH, inhibin B, androstenedione, testosterone and estradiol (E<sub>2</sub>) assays were taken on days 1–5 of the menstrual cycle or on days 1–5 of progesterin-induced menstrual bleeding. In healthy women, serum progesterone levels were  $\leq 7.1$  nmol/l, confirming the follicular phase of the menstrual cycle.

Twenty-six subjects with PCOS (aged 20–41 years, BMI 18–41 kg/m<sup>2</sup>) were treated with metformin. Fourteen women were treated with metformin (metformin hydrochloride, Diformin; Bristol-Myers Squibb Co., Leiras, Finland) at 1500 mg/day for 6 months (Morin-Papunen *et al.*, 1998). The other 12 women were treated with metformin at 1000 mg/day for 3 months and thereafter the dose was doubled to 2000 mg/day for the next 3 months (Morin-Papunen *et al.*, 2000, 2003).

Serum AMH concentrations were determined by enzyme immunoassay (Immunotech-Beckman Coulter, Marseille, France). Serum concentrations of testosterone and progesterone were analysed by using an automated chemiluminescence system (Advia Centaur, Bayer Corporation, New York). Inhibin B concentrations were analysed by enzyme-linked immunosorbent assay (ELISA) (Serotec Ltd., Oxford, UK). Serum concentrations of FSH and LH were analysed by fluoroimmunoassays (Perkin Elmer, Wallac Ltd., Turku, Finland) and radioimmunoassays were used for androstenedione (Diagnostic Products Corporation, Los Angeles, CA) and E<sub>2</sub> (Orion Diagnostica, Oulunsalo, Finland), following the instructions of the manufacturers. The intra- and interassay coefficients of variation were 5.1 and 6.6%, respectively, for AMH, 3.8 and 4.3% for FSH, 4.9 and 6.5% for LH, 5.2 and 6.4% for inhibin B, 5.0 and 8.6% for androstenedione, 4.0 and 5.6% for testosterone, 5.7 and 6.4% for E<sub>2</sub> and 3.7 and 5.4% for progesterone.

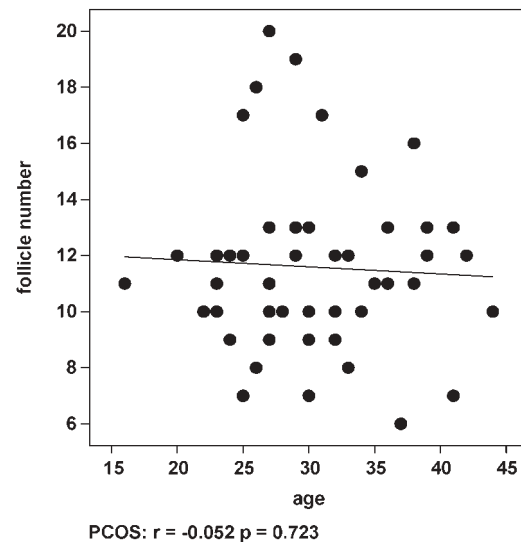
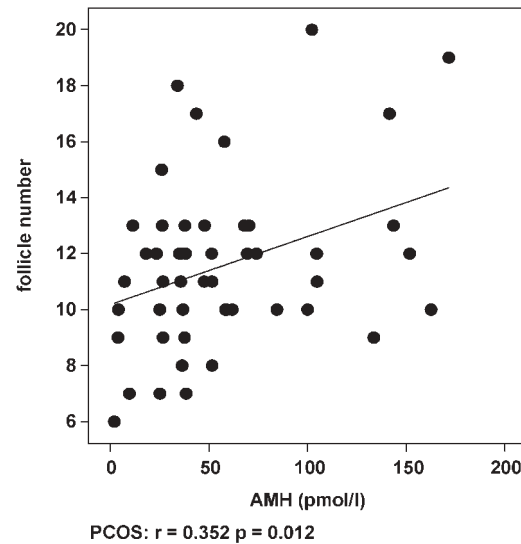
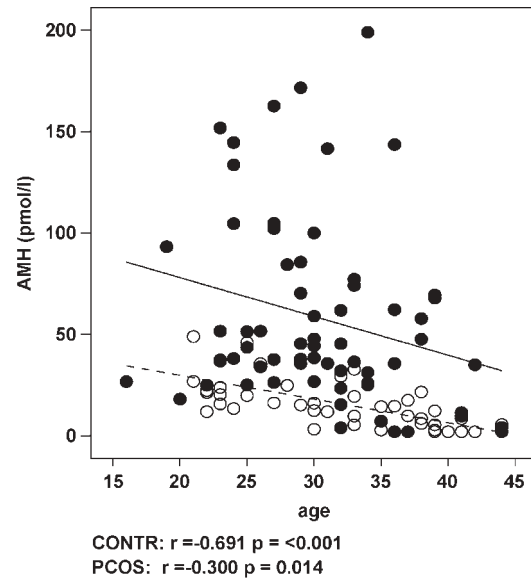
Data were analysed by means of the Statistical Program for Social Science (SPSS Inc., Chicago, IL). To compare basal serum hormone levels between control women and women with PCOS, and inside the PCOS group when setting the age divisions at 25, 30 and 35 years, the independent samples *t*-test was used for normally distributed variables and the Mann–Whitney test for variables with skewed distribution. Pearson's (normal distributions) and Spearman's

(skewed distributions) correlation coefficients (*r*) were calculated to correlate age, follicle number, BMI, testosterone, androstenedione, E<sub>2</sub>, inhibin B, LH and FSH data with serum AMH levels. Multiple linear regression analysis and analysis of variance (ANOVA) were used to adjust for the impact of BMI. Paired samples *t*-test (normally distributed variables) and Wilcoxon test (variables with skewed distribution) were used to compare the changes in serum AMH levels, ovarian follicle count and ovarian volume before and after 6 months of metformin treatment. The limit of statistical significance was set at  $P \leq 0.05$ .

**Results**

Serum AMH levels were 2- to 3-fold higher in women with PCOS than in control women in all age groups (Tables I and II). Serum AMH concentrations decreased with age in control women, and a similar tendency was also observed in women with PCOS (Table II). Our previous studies on control and PCOS subjects have shown that 25, 30 and 35 years are important cut-off points as regards the changes in female gonadotrophin and androgen secretion (Piltonen *et al.*, 2003). Therefore, serum AMH levels were also analysed according to these age divisions. Serum AMH concentrations were significantly higher in younger PCOS subjects when the age division was set at 35 years, whereas in control women the same difference was already observed when the age division was set at 25 years (Table II). Interestingly, in control women beyond the age of 38 years, serum AMH concentrations were very low and in most subjects undetectable (<5 pmol/l), while most women with PCOS at the same age still had high AMH levels (10–70 pmol/l) (Figure 1).

Serum AMH levels correlated positively with those of androstenedione and testosterone (androstenedione,



**Table I.** Basal serum AMH levels in control women and in women with PCOS

	Controls	PCOS	<i>P</i> -value
<i>n</i>	44	65	
Age (years)	31.6 ± 1.1	30.6 ± 0.7	NS
Follicle number	<8	11.6 ± 0.4	NA
BMI (kg/m <sup>2</sup> )	23.3 ± 0.4	30.1 ± 0.9	<0.001
AMH (pmol/l)	16.7 ± 1.8	57.8 ± 5.7	<0.001

Data are shown as means ± SE.  
NS = non-significant; NA = not analysed.

**Table II.** Basal serum AMH levels in different age groups in control women and in women with PCOS

Age (years)	Controls		PCOS		<i>P</i> -value
	AMH (pmol/l)	<i>n</i>	AMH (pmol/l)	<i>n</i>	
25	26.5 ± 3.3	13	65.4 ± 18.1	15	0.045
>25	12.0 ± 1.7 <sup>a</sup>	31	49.6 ± 7.7	50	<0.001
≤30	23.4 ± 2.7	20	63.5 ± 10.5	32	0.001
>30	10.3 ± 1.8 <sup>a</sup>	24	43.9 ± 9.4	33	0.002
≤35	21.2 ± 2.3	28	62.0 ± 8.9	50	<0.001
>35	7.7 ± 1.5 <sup>a</sup>	16	28.1 ± 7.5 <sup>a</sup>	15	0.02

Data are shown as means ± SE.  
<sup>a</sup>Statistically significant between the age groups when the age division was set at 25, 30 or 35 years.  
 $P < 0.001$ .

**Figure 1.** Correlation between AMH and age in control women 21–44 years (open circles, *n* = 44) and in women with PCOS aged 16–44 years (filled circles, *n* = 65) and correlations between follicle number, AMH and age in women with PCOS.

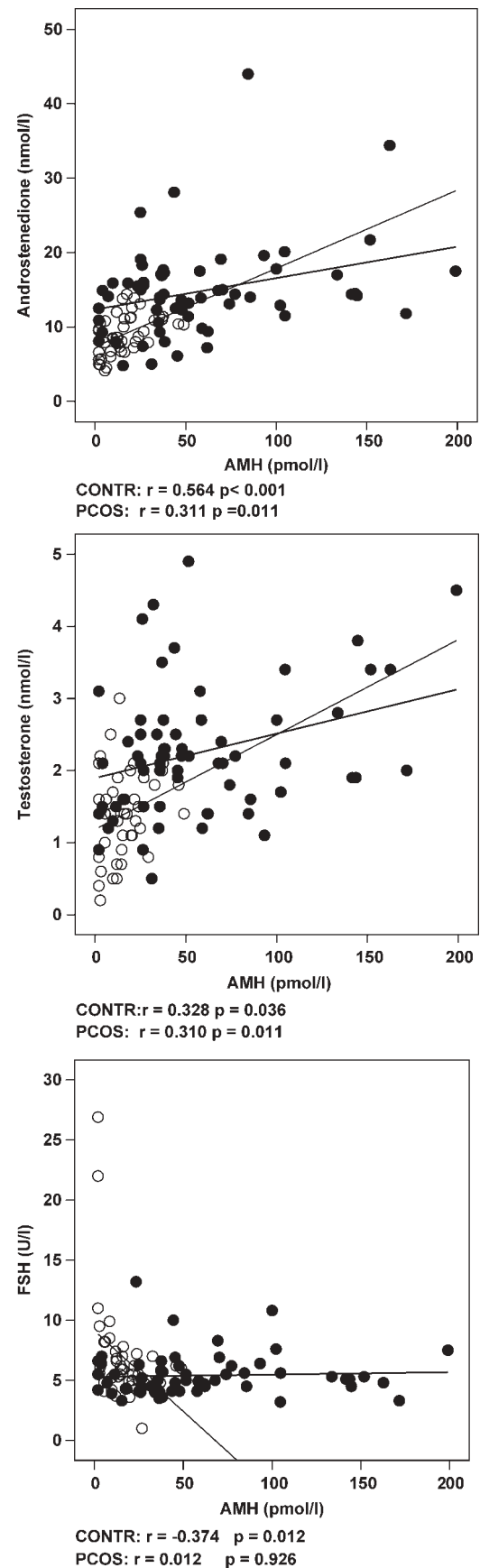
$r = 0.311$ ,  $P = 0.011$ ; testosterone,  $r = 0.310$ ,  $P = 0.011$ ) in PCOS subjects, and a similar correlation was observed between AMH and androstenedione and AMH and testosterone in control women (androstenedione,  $r = 0.564$ ,  $P < 0.001$ ; testosterone,  $r = 0.328$ ,  $P = 0.036$ , Figure 2). Serum AMH levels correlated negatively with those of FSH ( $r = -0.374$ ,  $P = 0.012$ , Figure 2) in control women but no correlation between AMH and FSH was observed in the PCOS group ( $r = 0.012$ ). However, when the two extreme values observed in control women were excluded from the statistical analyses, the  $P$ -value decreased to  $P = 0.053$ . AMH levels correlated negatively with age in women with PCOS ( $r = -0.300$ ,  $P = 0.014$ , Figure 1) and in control women ( $r = -0.691$ ,  $P < 0.001$ , Figure 1). Follicle number correlated positively with AMH concentrations in PCOS subjects ( $r = 0.352$ ,  $P = 0.012$ , Figure 1) and a negative but non-significant correlation was observed between follicle number and age ( $r = -0.052$ ,  $P = 0.723$ , Figure 1). However, in women with PCOS, the correlation between follicle number and age became statistically significant after adjustment for BMI (from  $P = 0.723$  to  $P = 0.039$ ). No correlation was found in the control or PCOS groups between AMH versus  $E_2$ , LH, inhibin B or BMI (data not shown).

Taking into account the interassay coefficient of variation in 15 patients out of 26, serum AMH levels decreased during metformin therapy, in four patients the levels increased and in seven patients they remained unchanged. The mean serum concentrations of AMH decreased significantly in both metformin protocol groups and did not differ between the two different protocols. Therefore, the data were combined. The mean serum AMH levels ( $P < 0.01$ ), mean number of ovarian follicles ( $P < 0.001$ ) and the ovarian volumes ( $P < 0.01$ ) decreased significantly after 6 months of metformin treatment (Table III).

## Discussion

The present results confirm those of previous studies (Fallat *et al.*, 1997; Cook *et al.*, 2002; Pigny *et al.*, 2003; Laven *et al.*, 2004; Mulders *et al.*, 2004) showing that AMH levels are 2- to 3-fold higher in women with PCOS compared with healthy women. We also demonstrated that this difference remains until late reproductive age. Increased serum AMH concentrations in PCOS have been explained by the increased number of small ovarian follicles responsible for AMH secretion (Pigny *et al.*, 2003). The positive correlation between AMH concentrations and follicle number in women with PCOS in the present study supports this concept, but whether AMH has a regulatory role in follicle development or whether it is a consequence of increased antral follicle number in PCOS subjects is not clear. Although AMH has been shown to inhibit initial follicle recruitment (Durlinger *et al.*, 1999) and FSH-stimulated follicle growth (Durlinger *et al.*, 2001) in animal studies and cell culture conditions, the definitive role of AMH in the regulation of human follicle development remains to be investigated.

There are several factors that have been reported to be associated with AMH secretion. For example, FSH has been



**Figure 2.** Correlation between AMH and androstenedione, testosterone and FSH in control and PCOS subjects. Statistical significance was reached, when  $P \leq 0.05$ .

**Table III.** The effect of metformin treatment on serum AMH levels, follicle number and ovarian volume in women with PCOS

	0 months ( <i>n</i> = 26)	6 months ( <i>n</i> = 26)	<i>P</i> -value
AMH (pmol/l)	87.5 ± 15	81.4 ± 16.0	<0.01
Follicle number	11.9 ± 0.5	8.3 ± 0.6	<0.001
Ovarian volume	8.5 ± 0.6	7.0 ± 0.5	<0.01

Data are shown as means ± SE.

shown to decrease the expression of AMH and its type II receptors in granulosa cells (Baarends *et al.*, 1995), and a negative correlation between FSH and AMH levels has been observed in several studies (Seifer *et al.*, 2002; van Rooij *et al.*, 2002; Fanchin *et al.*, 2003; Pigny *et al.*, 2003). A negative association between serum AMH and FSH was also observed in the present study in healthy women but not in women with PCOS. The difference between healthy women and those with PCOS can be explained by unchanged serum FSH levels until late reproductive age (Piltonen *et al.*, 2004) and high although variable AMH levels in women with PCOS. In contrast to the study by Laven *et al.* (2004), no correlation was observed between serum LH and AMH levels. This difference could be a result of different age distributions in the studies. In our study, the age range was wider, and especially in women close to 40 years of age the individual variation of both LH and AMH levels was larger. Furthermore, it is also possible that the induction of menstrual bleeding by progestin in some subjects could have affected the results since it has been shown to decrease follicular phase LH concentrations (Anttila *et al.*, 1992), which may have weakened the correlation of LH and AMH.

Since pronounced androgen secretion is one of the typical features of PCOS, it was interesting to study whether AMH levels, in addition to their correlation to follicular count, also correlate to serum androgen levels, thus reflecting the degree of hyperandrogenism. Serum AMH concentration correlated significantly with serum androstenedione and testosterone levels in women with PCOS, and a similar correlation was observed between AMH and androstenedione concentrations in control subjects. These findings strengthen the results of previous studies (Pigny *et al.*, 2003; Laven *et al.*, 2004) and confirm the importance of small ovarian follicles in the production of both AMH and androgens.

Normal (Pigny *et al.*, 2003) or decreased (Cook *et al.*, 2002) serum E<sub>2</sub> levels and a negative correlation between AMH and E<sub>2</sub> concentrations have been shown in women with PCOS. The relationship between E<sub>2</sub> and increased AMH levels could be explained by an inhibitory effect of AMH on aromatase activity (Vigier *et al.*, 1989; Rouiller-Fabre *et al.*, 1998) that could cause the well-known increase in androgen levels and unchanged/decreased levels of E<sub>2</sub> in PCOS subjects, as suggested earlier (Laven *et al.*, 2004). The present and previous (Pigny *et al.*, 2003) results do not, however, support this concept since no correlation between AMH and E<sub>2</sub> concentrations was observed. It is possible, however, that aromatase activity is not maximal at early follicular phase when the measurements were performed, and this could have affected the results.

There was a significant age-related decrease in AMH levels in both control subjects and those with PCOS, although serum AMH levels were always higher in women with PCOS. In fact, serum AMH levels became undetectable in the majority of control women after the age of 38 years, whereas in women with PCOS markedly increased serum AMH levels could still be observed. After dividing the healthy women into two groups, setting the age limit at 25 years, serum AMH levels were significantly lower in the older women, indicating that the decline in AMH secretion begins early. The age-related decrease in AMH levels in control women is also supported by the results of previous studies in which a negative correlation between age and serum AMH levels has been reported (de Vet *et al.*, 2002; Fanchin *et al.*, 2003; Mulders *et al.*, 2004), although this has not been the case in all studies (Pigny *et al.*, 2003). In a previous study, we reported an age-related rise in FSH concentrations in control women which was already evident after 25 years (Piltonen *et al.*, 2003), as was the decline in AMH levels in the present study. It is possible that AMH as a direct marker of ovarian ageing will turn out to be a better marker than FSH, which reflects the changes in the ovarian environment via feedback systems. Inhibin B, another direct marker of ovarian function, has also been used to predict menopausal transition. In our previous studies, no significant correlation between age and inhibin B levels was observed in either healthy women or those with PCOS (Piltonen *et al.*, 2003, 2004), and nor did inhibin B concentrations correlate with those of AMH in the present study.

Earlier studies have shown that the prevalence of PCO decreases with age (Koivunen *et al.*, 1999; Bili *et al.*, 2001). Furthermore, the women with PCOS have been shown to gain regular menstrual cycles when ageing, probably due to age-related follicle loss in the ovaries (Elting *et al.*, 2000). In accordance with this, we observed a decreasing tendency in follicle number with age in PCOS subjects which became significant after adjustment for BMI. The relatively high serum levels of AMH in women with PCOS in the oldest age-group (>35 years), however, indicate that the follicles and thereby fertility may be preserved for longer in women with PCOS. Whether this affects menopausal transition and whether these women enter the menopause later remains open.

Although the positive effect of metformin on menstrual pattern, ovulatory function and hyperandrogenism in women with PCOS is widely documented (Velazquez *et al.*, 1994; Nestler and Jakubowicz, 1997; Morin-Papunen *et al.*, 1998; Elter *et al.*, 2002), studies concerning its effect on ovarian morphology/ultrasonographic appearance are few (Elter *et al.*, 2002), and no data on serum AMH levels during metformin treatment have existed. The present results show for the first time that serum AMH levels decrease during metformin treatment. It is most likely that the decrease is simply related to the decrease of follicle number, but the contribution of the improvement of hyperandrogenism, insulin action or menstrual pattern cannot be excluded. Whether subjects showing lowered serum AMH levels during metformin treatment have special features remains to be studied in a larger number of

subjects. Since the ovarian appearance, i.e. the number of ovarian follicles/volume, is not likely to change in a short period of time, a longer follow-up may be needed to observe even more profound changes in serum AMH levels than in the present study.

In conclusion, serum levels of AMH decreased with age both in women with PCOS and in control subjects, and became undetectable in most of the control women before the age of 38 years. In women with PCOS, AMH levels remained 2- to 3-fold higher throughout the reproductive years but decreased during metformin treatment, as did the follicle number and ovarian volume. This may indicate that metformin has beneficial effects on follicle growth in women with PCOS. The measurement of serum AMH could be a useful tool for estimating follicle number as well as for diagnosing PCO/PCOS and for assessing ovarian ageing.

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