

Serum anti-Müllerian hormone is more strongly related to ovarian follicular status than serum inhibin B, estradiol, FSH and LH on day 3

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BACKGROUND: The study aim was to compare the relationship between serum anti-Müllerian hormone (AMH) levels and other markers of ovarian function with early antral follicle count on day 3. **METHODS:** A total of 75 infertile women was studied prospectively. On cycle day 3, serum levels of AMH, inhibin B, estradiol (E₂), FSH and LH levels were measured, and the number of early antral follicles (2–10 mm in diameter) estimated at ultrasound scanning to compare the strengths of hormonal–follicular correlations. **RESULTS:** Median (range) serum levels of AMH, inhibin B, E₂, FSH and LH were 1.39 ng/ml (0.24–6.40), 90 (16–182) pg/ml, 31 (15–111) pg/ml, 7.0 (2.9–19.3) mIU/ml and 4.7 (1.2–11.7) mIU/ml respectively, and follicular count was 12 (1–35). Serum AMH levels were more strongly correlated ($P < 0.001$) with follicular count ($r = 0.74$, $P < 0.0001$) than were serum levels of inhibin B ($r = 0.29$, $P < 0.001$), E₂ ($r = -0.08$, $P = \text{NS}$), FSH ($r = -0.29$, $P < 0.001$) and LH ($r = 0.05$, $P = \text{NS}$). **CONCLUSIONS:** Serum AMH levels were more robustly correlated with the number of early antral follicles than inhibin B, E₂, FSH and LH on cycle day 3. This suggests that AMH may reflect ovarian follicular status better than the usual hormone markers.

Key words: anti-Müllerian hormone/antral follicles/endocrine profile/Müllerian-inhibiting substance (MIS)/ovarian reserve

Introduction

Anti-Müllerian hormone (AMH) is a glycoprotein hormone that belongs to the transforming growth factor β superfamily (Pepinsky *et al.*, 1988) and is chiefly expressed in the fetal testis to drive differentiation of the mammalian reproductive tract (Lee and Donahoe, 1993). In women, granulosa cell production of AMH (Vigier *et al.*, 1984) is barely detectable at birth (Rajpert-De Meyts *et al.*, 1999) and reaches the highest values after puberty (Hudson *et al.*, 1990). During adulthood, although AMH continues to be expressed at basal and similar levels by both the Sertoli and granulosa cells (Hudson *et al.*, 1990), its biological role is poorly understood.

Basic research data obtained from the adult ovary indicate that AMH is likely to be involved in the regulation of follicular steroidogenesis. Experiments conducted in animals suggest that AMH reduces aromatase activity and the number of LH receptors in FSH-stimulated granulosa cells (Josso *et al.*, 1998), and also influences testosterone production by theca cells (Ingraham *et al.*, 2000). In addition, growing evidence indicates that AMH mainly is expressed in pre-antral and early antral follicles (Baarends *et al.*, 1995) and has either direct or

indirect roles in various phases of folliculogenesis, from the primordial (Durlinger *et al.*, 2002) to FSH-sensitive (Durlinger *et al.*, 2001; McGee *et al.*, 2001) follicular stages, probably via specific AMH type II receptors that are expressed in granulosa and theca cells. This suggests that, in contrast to other hormonal markers of ovarian function, AMH secretion might concurrently reflect the activity of pre-antral and early antral follicles, which makes it a promising parameter in the evaluation of ovarian follicular reserve.

Recent clinical evidence is in agreement with this hypothesis. One group (de Vet *et al.*, 2002) demonstrated that serum AMH levels on cycle day 3 decrease progressively along with age and become undetectable after menopause. This suggests that peripheral AMH levels are a valuable parameter to monitor the relative follicular exhaustion due to ovarian ageing. Consistently, others (Seifer *et al.*, 2002) showed that day 3 AMH levels are positively related with the number of oocytes retrieved after controlled ovarian hyperstimulation (COH). Taken together, these results indicate that circulating AMH levels reflect the number of selectable follicles during the early follicular phase. Indeed, the early antral follicle count has been

shown to reliably predict the fertility potential of women (Reuss *et al.*, 1996) and their responsiveness to COS (Chang *et al.*, 1998; László *et al.*, 2002).

However, the question of whether serum AMH measurements on cycle day 3 reflect ovarian follicular status better than the usual hormonal parameters remains unanswered. Hence, the present investigation was designed to weigh the relationship between early antral follicles and AMH levels against that between early antral follicles and other putative markers of ovarian function and fertility potential, including serum levels of inhibin B, estradiol (E₂), FSH and LH on cycle day 3.

Materials and methods

Subjects

A total of 75 infertile women (age range 25–40 years) was studied prospectively. In order to be included, the women had to meet the following criteria: (i) regular, ovulatory menstrual cycles every 25–35 days; (ii) both ovaries present; (iii) no current or past diseases affecting ovaries or gonadotrophin or sex steroid secretion, clearance or excretion; (iv) a body mass index (BMI) of 18–27 kg/m²; (v) no current hormone therapy; and (vi) adequate visualization of ovaries at transvaginal ultrasound scanning. All patients were undergoing routine explorations before IVF–embryo transfer. Informed consent was obtained from all women, and the investigation was approved by the authors' internal Institutional Review Board.

Study protocol

On day 3 of the menstrual cycle, each woman underwent blood sampling by venipuncture for measurement of serum levels of AMH, inhibin B, E₂, FSH and LH at approximately 09:00 h. Later in the morning, ovarian ultrasound scanning (Figure 1) was performed using a 4.5–7.2 MHz multi-frequency transvaginal probe (Siemens Elegra; Siemens S.A.S., Saint-Denis, France) by one operator (R.F.), who was blinded to the results of the hormone assays. The objective of the ultrasound examination was to evaluate the number and sizes of early antral follicles and to calculate the mean ovarian volume. The sum of all follicles measuring 2–10 mm in mean diameter (mean of two orthogonal diameters) in both ovaries was considered. In an attempt to optimise the reliability of ovarian follicular assessment, the ultrasound scanner used was equipped with a tissue harmonic imaging system (Thomas and Rubin, 1998); this allowed improved image resolution and adequate recognition of follicular borders. Ovarian volumes, calculated according to the formula for an ellipsoid (0.526 × length × height × width) (Sharara and McClamrock, 1999), corresponded to the mean volume for both ovaries. Intra-analysis coefficients of variation (CV) for follicular and ovarian measurements were <5%, and their lower limit of detection was 0.1 mm.

Hormone measurements

Serum was separated from all blood samples and frozen in aliquots at –20°C until used for subsequent centralized analysis. Serum AMH levels were determined using an ultrasensitive enzyme-linked immunosorbent assay (ELISA) (Beckman-Coulter, Villepinte, France) as described previously (Long *et al.*, 2000). For AMH, functional sensitivity was 0.24 ng/ml and intra-assay and inter-assay CV were <5 and <8% respectively. Serum inhibin B levels were determined using a double antibody ELISA (Serotec, Varilhès, France) as described previously (Groome *et al.*, 1996). For inhibin B, functional sensitivity was 15 pg/ml and intra-assay and inter-assay CV were <6 and <9% respectively. Serum levels of E₂, FSH and LH

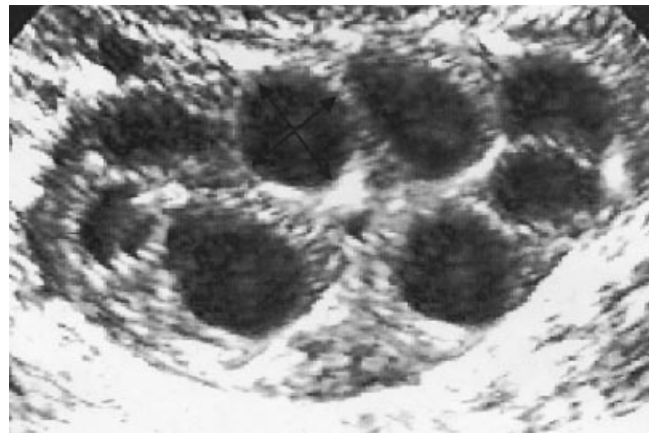


Figure 1. Ultrasound scan of the ovary on cycle day 3. A tissue harmonic imaging system was used to improve image resolution and enable adequate recognition of follicular borders. Note that the number and mean diameter of early antral follicles can be easily assessed.

were determined using an automated multi-analysis system with chemiluminescence detection (ACS-180; Bayer Diagnostics, Puteaux, France). For E₂, functional sensitivity was 15 pg/ml and intra-assay and inter-assay CV were 8 and 9% respectively. For FSH and LH, functional sensitivity was 0.1 mIU/ml and intra-assay and inter-assay CV were 3 and 5% respectively.

Statistical analysis

The measure of central tendency used was the median, and data variability was expressed by the ranges. Relationship between two different continuous variables was assessed by correlation. The Fisher *r* to *z*-test was used to determine if the coefficient of correlation (*r*) was significantly different from zero. Comparison of strength of correlations was performed using the Hotelling's *t*-test (Hotelling, 1940), which assesses the difference between coefficients of correlation for correlated samples that share a common variable. A *P*-value < 0.05 was considered statistically significant.

Results

The median age of participants was 34 (range 25–40) years, BMI was 21.3 (range 16–27) kg/m², and menstrual cycle length 28 (range 25–35) days. Data distribution of serum hormone levels and of the number of early antral follicles on cycle day 3 is shown in Figure 2. Median serum levels of AMH, inhibin B, E₂, FSH and LH were 1.39 (range 0.24–6.40) ng/ml, 90 (range 16–182) pg/ml, 31 (range 15–111) pg/ml, 7.0 (range 2.9–19.3) mIU/ml and 4.7 (range 1.2–11.7) mIU/ml respectively. Ultrasound scans revealed a median number of early antral follicles of 12 (range 1–35), and an ovarian volume of 6 (range 4.4–8.0) cm³. In addition, serum levels of AMH were slightly and negatively correlated with age (*r* = –0.22, *P* < 0.04), but this did not apply to serum levels of either inhibin B, E₂, FSH or LH (*r* = 0.06, *r* = 0.09, *r* = –0.13 and *r* = –0.02 respectively).

Relationships between the number of antral follicles and serum levels of AMH, inhibin B, E₂, FSH and LH on cycle day 3 are shown in Figure 3. Unlike serum levels of E₂ and LH (*r* = –0.08 and *r* = 0.05), those of AMH, inhibin B and FSH were significantly correlated with the number of early antral follicles

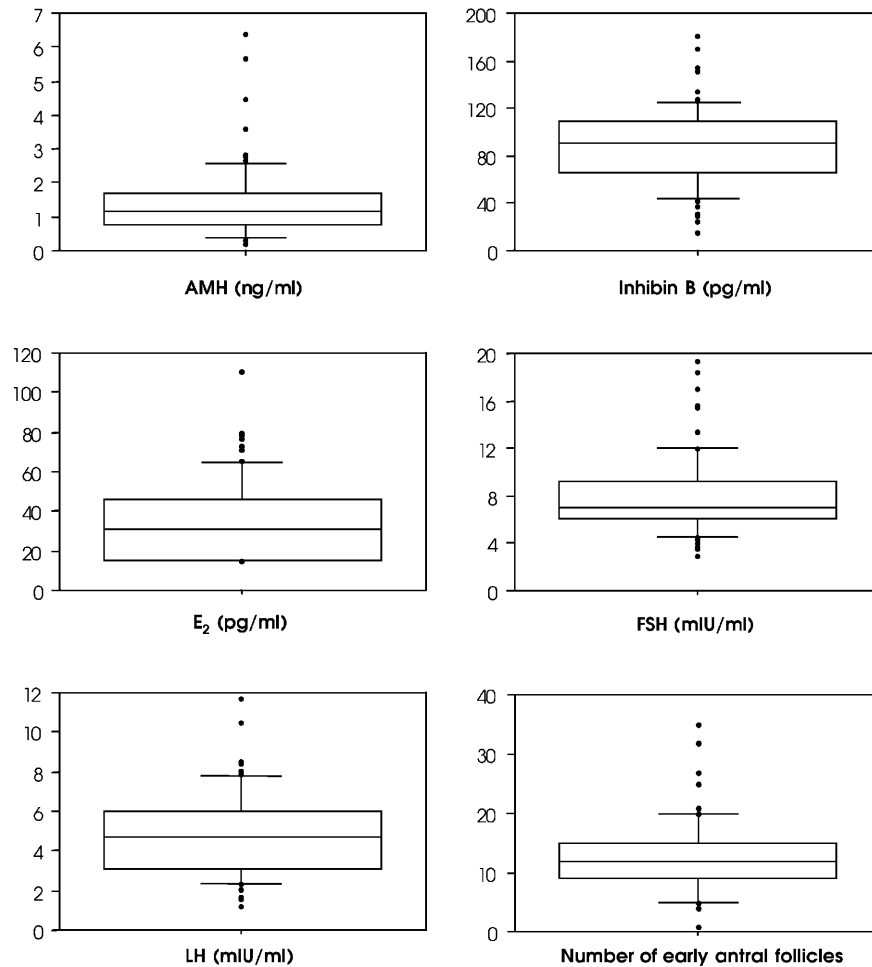


Figure 2. Data distribution of serum levels of AMH, inhibin B, E₂, FSH and LH and early antral follicle count on cycle day 3. Central horizontal lines are medians; additional horizontal lines indicate the 10th, 25th, 75th and 90th centiles. Each value outside the 10th and 90th centiles is represented by a dot. Extreme lower and upper dots represent ranges.

on cycle day 3. It is noteworthy that the correlation between serum AMH levels and the number of early antral follicles ($r = 0.74$, $P < 0.0001$) was significantly stronger ($P < 0.001$) than correlations between serum levels of inhibin B and FSH and the same parameter ($r = 0.29$, $P < 0.001$; $r = 0.29$, $P < 0.001$ respectively). In agreement with this, serum AMH levels showed a stronger correlation ($P < 0.001$) with ovarian volume ($r = 0.43$, $P < 0.0001$) than did those of inhibin B ($r = 0.11$, $P = \text{NS}$), E₂ ($r = 0.13$, $P = \text{NS}$), FSH ($r = -0.27$, $P < 0.02$) and LH ($r = 0.02$, $P = \text{NS}$). Incidentally, serum AMH levels were correlated with those of inhibin B ($r = 0.26$, $P < 0.02$) and FSH ($r = -0.27$, $P < 0.02$), but not with those of E₂ and LH.

Discussion

The present investigation was designed to evaluate the direct relationship between peripheral AMH levels, measured by an ultrasensitive ELISA technique, and the ovarian follicular status on cycle day 3, and to compare the strength of correlations between the number of early antral follicles and some hormonal parameters implicated directly or indirectly in the eventual stages of folliculogenesis. It was observed that

serum AMH levels are tightly related to early antral follicle count, with a relationship that was remarkably more intense than those obtained with serum levels of inhibin B, E₂, FSH and LH.

These results not only corroborate but also expand clinical data reported previously by other investigators (de Vet *et al.*, 2002; Seifer *et al.*, 2002). First, the age-related decrease in AMH levels (de Vet *et al.*, 2002) probably results from the relative follicular attrition that characterizes the decline of ovarian function, with a noticeable reduction in the number of early antral follicles (Reuss *et al.*, 1996). The negative relationship between day 3 serum AMH levels and the women's age seen in the present study supports this hypothesis. Second, the reported correlation between serum AMH levels on cycle day 3 and the number of oocytes retrieved after COS (Seifer *et al.*, 2002) is in keeping with the relationship between AMH and the number of early antral follicles shown in the present study. Indeed, the number of early antral follicles is a putative predictor of COS outcome (Chang *et al.*, 1998; László *et al.*, 2002). Third, the correlation between serum AMH levels and early antral follicle count confirms preliminary data reported by others (de Vet *et al.*, 2002), though the latter

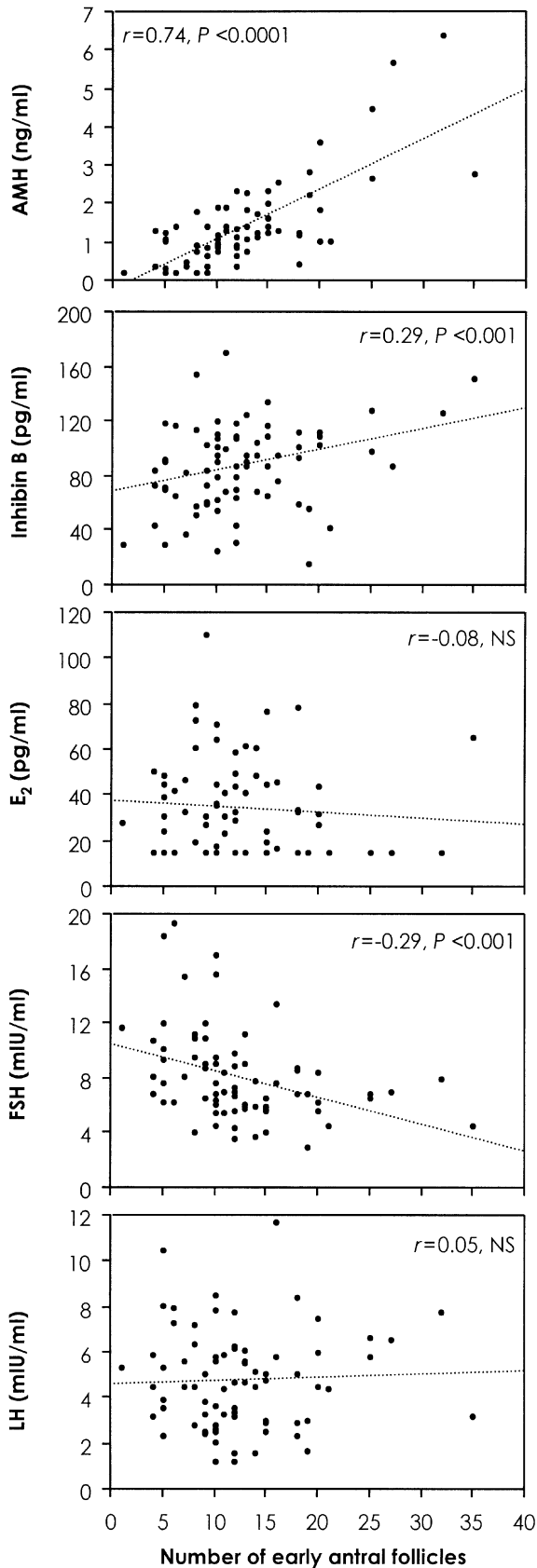


Figure 3. Relationships between the number of antral follicles and serum levels of AMH, inhibin B, E₂, FSH and LH on cycle day 3. The serum AMH level was more strongly correlated with follicular count ($P < 0.001$) than levels of inhibin B, E₂, FSH and LH.

authors observed similar coefficients of correlation for this relationship (0.71) as in the present study (0.74).

Nonetheless, the central result of the present investigation was that AMH correlated more intensely with early antral follicular counts than did the other hormonal markers of follicular status and development that were monitored. The reasons for this striking phenomenon are unclear, but it may be related to possible different regulation of AMH as compared with inhibin B, E₂ and FSH. During the luteal–follicular transition, the secretion of inhibin B (Welt *et al.*, 1997) and E₂ (Mais *et al.*, 1987) by the early antral follicles modulates their own stimulation by FSH. This implies that inhibin B and E₂ levels depend not only on the bulk of active granulosa cells available, as represented by follicular number and sizes, but also on their stimulation by FSH. Although little is known about FSH effects on AMH expression during the early follicular phase, it can be presumed that this hormone is less FSH-sensitive than inhibin B and E₂. Indeed, AMH is also secreted by follicles that are barely sensitive to FSH, such as pre-antral follicles (Baarends *et al.*, 1995). Therefore, AMH may represent a more independent and reliable marker of early antral follicle activity than inhibin B and E₂, and FSH on cycle day 3. Further studies are required to investigate whether AMH measurements on day 3 reflect not only the activity of early antral follicles but also that of follicles during earlier stages of folliculogenesis.

Nevertheless, a negative relationship was observed between FSH and AMH levels. Whereas these data challenge the hypothesis of a stimulating role of FSH on granulosa cell production of AMH, they do not rule out its potential inhibiting action on AMH production. Indeed, the results of studies conducted in rats treated with GnRH antagonist and FSH have indicated that FSH inhibits AMH and its type II receptor expression in pre-antral and early antral follicles (Baarends *et al.*, 1995). However, since in the present series AMH and FSH levels were both correlated with a third common parameter (early antral follicular count), their mutual correlation might be merely indirect and not causative. Likewise, the correlation observed between AMH and inhibin B levels may not correspond to a direct relationship between both hormones.

Another issue deserving consideration is the observed lack of influence of endogenous LH concentrations on peripheral AMH levels. Indeed, the present data agree with previous results obtained in normo-ovulating and polycystic ovary syndrome (PCOS) patients (Cook *et al.*, 2002). In PCOS, basal AMH levels are significantly higher as compared with normal controls (Cook *et al.*, 2002). Although these results may be attributed to the large number of antral follicles that characterizes PCOS, they do not permit the exclusion of a possible influence of LH on AMH regulation. Experiments conducted in rats and pigs have shown that AMH inhibits the number of LH receptors in FSH-stimulated granulosa cells (Josso *et al.*, 1998, 2001). Further studies involving pituitary-desensitized women treated with controlled FSH and/or LH doses will undoubtedly contribute to clarifying the possible regulation of AMH by pituitary gonadotrophins.

Similarly, conclusive data on AMH secretion patterns during more advanced stages of the follicular phase and during the

luteal phase of the menstrual cycle are still lacking. A preliminary study showed that serum AMH levels are slightly and significantly higher at mid-cycle as compared with early follicular and mid-luteal phase values in women (Cook *et al.*, 2000). However, these data did not detail the possible relationship between AMH production and eventual follicular development and/or corpus luteum activity. Also, they contrast with in-vitro studies that demonstrated a progressive decrease of AMH expression during the final steps of follicular maturation from pre-antral to pre-ovulatory follicles (Baarends *et al.*, 1995). Hence, further characterization of subtle changes in serum AMH levels throughout the menstrual cycle is required. Likewise, investigation of possible effects of COS with exogenous gonadotrophins on peripheral AMH levels may provide further insights into AMH regulation and its pathophysiological role in adult women.

In conclusion, the present results indicate not only that serum AMH levels are strongly correlated with ovarian follicular status during the early follicular phase, but also that this relationship is more strict than that obtained with other hormonal markers of the follicular functioning. This leads to the conclusion that serum AMH measurements on cycle day 3 are a better predictor of the number of early antral follicles than conventional hormone measurements. However, further studies aimed at weighing the predictability of AMH levels against conventional day 3 inhibin B, E₂ and FSH measurements on COS and assisted reproductive technology outcomes are needed to challenge this conclusion. Moreover, uncertainties persist with respect to the control of granulosa cell AMH production and its physiological role during folliculogenesis. The understanding of these key issues will be helpful to refine future clinical applications of AMH measurements in evaluating the fertility potential of women and monitoring infertility treatments.

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