

Serum anti-Mullerian hormone throughout the human menstrual cycle

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BACKGROUND: The anti-Mullerian hormone (AMH) is a member of the transforming growth factor (TGF) superfamily. In women, AMH serum levels can be almost undetectable at birth, with a subtle increase noted after puberty. Data are lacking with regard to menstrual cycle day-to-day fluctuations. This longitudinal study was designed to investigate the pattern of secretion of AMH throughout the menstrual cycle in regularly cycling women. **METHODS:** Twelve healthy female subjects aged 18–24 years participated in this study. Blood samples were taken every other day throughout one menstrual cycle. Serum FSH, LH, estradiol (E₂), progesterone, inhibin B and AMH levels were assayed by double-antibody radioimmunoassay using commercial kits. **RESULTS:** Serum AMH in the first days of the menstrual cycle (days –14 to –12) was 3.8 ± 1.2 ng/ml (mean \pm SD). No significant changes were observed in serum AMH levels throughout the menstrual cycle. The highest value was 3.9 ± 1.3 ng/ml at day –12 and the lowest value was 3.4 ± 1.1 ng/ml at day 14, and the difference was not significant. **CONCLUSION:** In this study, we demonstrated that serum AMH levels do not change significantly throughout the menstrual cycle. Hence, AMH exhibits a relatively stable expression during the menstrual cycle, making it an attractive determinant of ovarian activity.

Key words: anti-Mullerian hormone/gonadotrophins/inhibin B/physiological menstrual cycle/sex steroids

Introduction

Anti-Mullerian hormone (AMH) is a member of the transforming growth factor (TGF) superfamily (Massague, 1998). AMH is strongly expressed in Sertoli cell from testicular differentiation up to puberty and to a much lesser degree in granulosa cells from birth up to menopause (Behringer *et al.*, 1994; Josso *et al.*, 2001; Grujters *et al.*, 2003).

AMH seems to act only in the reproductive organs (Lee and Donahoe, 1993). The most striking effect of AMH is its capacity to induce regression of the Mullerian ducts, the anlage of the female internal reproductive organs. In the absence of AMH, Mullerian ducts of both sexes develop into uterus, Fallopian tubes and the upper part of the vagina (Munsterberg and Lovell-Badge, 1991).

During fetal life, only the male testes express the hormone (Munsterberg and Lovell-Badge, 1991). In the female, AMH expression begins post-natally and acts by modulating follicular growth (Durlinger *et al.*, 2001; Ikeda *et al.*, 2002) and preventing recruitment of non-dominant follicles (Josso *et al.*, 1998; Durlinger *et al.*, 1999; Durlinger *et al.*, 2002). AMH seems to have a dual role in folliculogenesis. Firstly, it inhibits the FSH-independent recruitment of follicles from the primordial follicle pool. Secondly, it seems to have, at least in rodents, a role in modifying the FSH sensitivity of pre-ovulatory follicles (Visser *et al.*, 2006).

In the human, AMH is expressed only in the ovarian granulosa cells. Granulosa cells of primary follicles show homogeneous AMH expression; in larger follicles, AMH is mainly produced in cells near the oocyte and in few cells surrounding the antrum. AMH continues to be expressed in the growing follicles in the ovary until they have reached the size and differentiation state at which they are to be selected for dominance by the action of pituitary FSH (Weenen *et al.*, 2004).

In women, AMH serum levels can be almost undetectable at birth (Rajpert-De Meyts *et al.*, 1999), with a subtle increase noted after puberty (Hudson *et al.*, 1990), then AMH seems to be stable until adulthood, when it decreases as a sign of follicular reserve exhaustion (Lee *et al.*, 1996).

To assess an individual's ovarian reserve, early follicular phase serum levels of FSH, estradiol (E₂) and inhibin B have been measured. However, all these hormones are part of the pituitary-ovarian feedback system; hence, their serum levels are not independent of each other. A serum marker that is not controlled by gonadotrophins would benefit both patients and clinicians.

Serum AMH levels have been measured at three different times during the menstrual cycle (follicular, ovulatory and luteal phases) suggesting minimal fluctuation (Cook *et al.*, 2000). The peak value (not significant) seems to be reached in the late follicular phase (Cook *et al.*, 2000). Minimal fluctuations

in serum AMH levels may be consistent with continuous non-cyclic growth of small follicles.

Hence, AMH seems relatively convenient to measure, especially as it seems to exhibit a relatively stable expression during the menstrual cycle. However, published data (Cook *et al.*, 2000) are lacking with regard to day-to-day fluctuations.

This longitudinal study was designed to investigate the pattern of secretion of AMH throughout the menstrual cycle in regularly cycling women. The relationship between AMH, inhibin B, ovarian steroids and gonadotrophins was also investigated during the menstrual cycle.

Materials and methods

Twelve healthy female subjects participated in this study after providing written consent. The women were aged 22 ± 3 years (mean ± SD) (range 18–24), and the mean BMI was 23 ± 1.8 kg/m² (mean ± SD). None of the subjects took any medication for at least 3 months before they entered the study. The duration of the last three menstrual cycles in these subjects was of normal length (28–34 days). In this longitudinal study, blood samples (5 ml) were taken every other day throughout one menstrual cycle starting from day 1–2 until day 3–4 of the next cycle. The day of the mid-cycle gonadotrophin surge was identified by the occurrence of the day of LH peak, day of FSH peak and day of (or day after) E₂ peak.

After a 12-h fast, blood samples were taken between 8:00 a.m. and 9:00 a.m. from the cubital vein. Blood was centrifuged and serum was separated and stored at –20°C for hormone measurements.

Hormone measurements

LH, FSH, E₂ and progesterone

Serum FSH, LH and E₂ levels were assayed by double-antibody radioimmunoassay using commercial kits from Radim (Rome, Italy) for FSH and LH, from Sorin (Saluggia-VC, Italy) for E₂ and progesterone. Samples were assayed in duplicate at two dilutions. Samples from subjects were analysed for each hormone in the same assay to avoid inter-assay variation. Quality control pools at low, normal and high LH, FSH, E₂ and progesterone concentrations were present in each assay. The detection limit of the assay was 0.20 IU/l for LH, 0.18 IU/l for FSH, 5 pg/ml for E₂ and 0.16 nmol/l for progesterone. Intra- and inter-assay variations were 7.8 and 8.2% for LH, 6.2 and 6.5% for FSH, 4.2 and 4.9% for E₂ and 8.5 and 10.8% for progesterone.

AMH

Serum AMH was measured by enzyme-linked immunosorbent assay (ELISA) using the AMH/MIS ELISA kit (Immunotech-Beckman, Marseilles, France). The detection limit of the assay was 0.7 pmol/l (0.1 ng/ml); intra- and inter-assay coefficients of variation (CV) were 5.3 and 8.7%, respectively, for a serum AMH concentration of 35 pmol/l and 4.9 and 7.8% for a serum AMH concentration of 1100 pmol/l. No cross-reaction was observed with TGF-beta.

Inhibin B

Inhibin B concentrations were measured in all serum samples using specific two-site ELISAs purchased from Serotec (Oxford, UK). The assay detection limit for inhibin B was <5 pg/ml. Within-plate and between-plate CVs were 6 and 8%, respectively.

Statistical analysis

Data are expressed as mean ± SD. Menstrual cycle profiles for the women were aligned relative to the day of the mid-cycle gonadotrophin

surge (day 0). First, the distribution of the variables was analysed. Outcomes for hormones were natural-log transformed to satisfy assumptions of normality and equal variance. Because the values were normally distributed, parametric tests were used for statistical analysis. Comparisons between the groups were performed using one-way analysis of variance followed by Bonferroni’s multiple comparison test. Student’s paired *t*-test was used to compare differences between the means of variables within the same group. In addition, to verify the correctness of statistical analysis for hormones, their median values on the original scale were recalculated using non-parametric tests. Because the results of this analysis are consistent with the ones obtained after using parametric tests, they are not reported. Linear regression or Pearson correlations were used to evaluate the between- and within-subject relations of different variables. Statistical analyses were performed with the Statsoft software. *P* < 0.05 was considered significant.

Results

Cycle lengths varied from 25 to 35 days. All cycles were ovulatory. LH, FSH, E₂ and progesterone showed the expected changes (Figures 1 and 2). There was a significant increase (*P* < 0.05) in LH levels at the days –2, 0 and +2 of the menstrual cycle with respect to the other time-points. FSH levels showed a significant increase (*P* < 0.05) at the days –2, 0, +2 and +14 of the cycle. Progesterone levels were significantly higher in

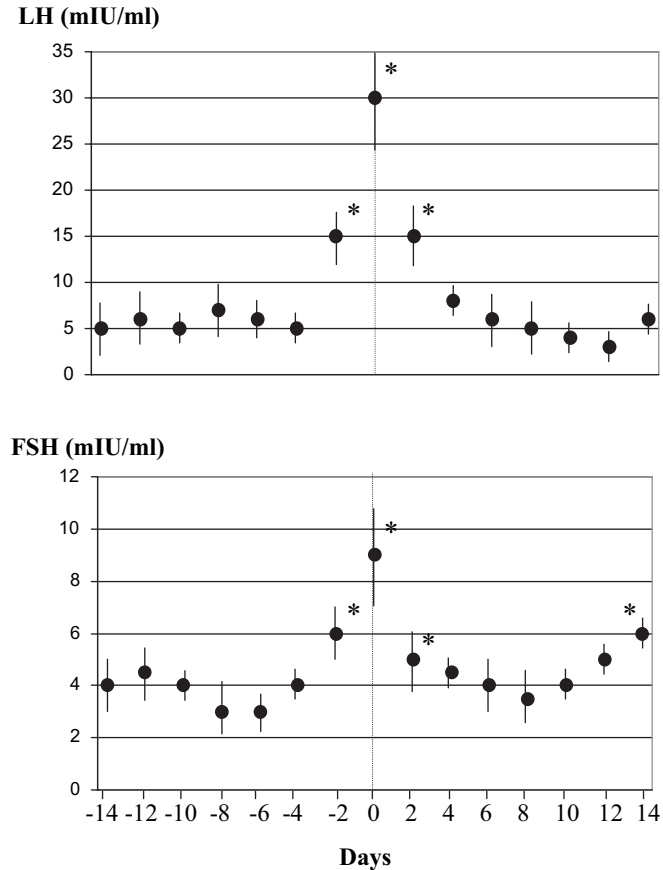


Figure 1. Circulatory patterns of FSH and LH during the menstrual cycle of young healthy women aged 18–24 years. Day 0 = day of LH surge. Data are mean ± SD. *, *P* < 0.05 versus –14 time-point.

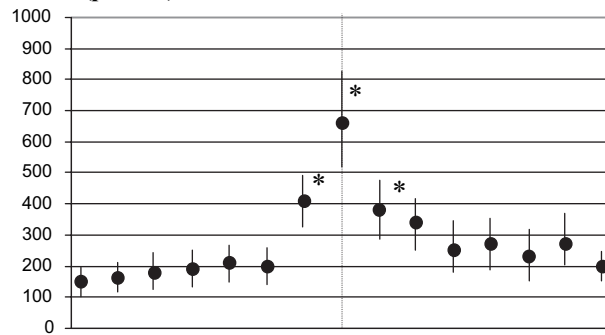
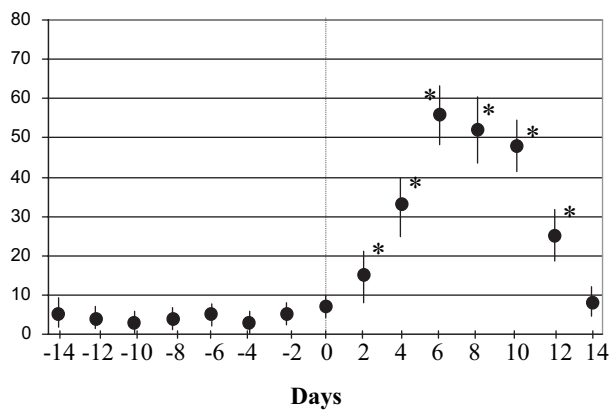
Estradiol (pmol/L)**Progesterone (nmol/L)**

Figure 2. Circulatory patterns of estradiol and progesterone during the menstrual cycle of young healthy women. Day 0 = day of LH surge. Data are mean \pm SD. *, $P < 0.05$ versus -14 time-point.

the luteal phase than in the follicular phase of the cycle (days +2 to +12 versus -14 to 0; $P < 0.05$). Gonadotrophins surge led to a significant increase ($P < 0.05$) in E_2 levels at the days -2 to +2.

Serum concentrations of inhibin B rose in the early follicular phase, reaching a peak (212 ± 46.4 pg/ml) around day -12. Inhibin B concentrations fell after day -12 and had a small peak after the LH surge and declined gradually during the luteal phase (Figure 3). Overall, inhibin B levels were significantly lower during the luteal phase ($P < 0.05$) than during the follicular phase.

Serum AMH in the first days of the menstrual cycle (days -14 to -12) was 3.8 ± 1.2 ng/ml. No significant modifications were observed in serum AMH levels throughout the menstrual cycle. The highest value was 3.9 ± 1.3 ng/ml at day -12 and the lowest value was 3.4 ± 1.1 ng/ml at day 14, and the difference was not significant (Figure 4).

Classically, E_2 and inhibin B have an endocrine effect on pituitary FSH secretion. E_2 and inhibin B inhibit pituitary FSH secretion. We found an inverse correlation between E_2 and FSH ($r = -0.56$) and inhibin B and FSH ($r = -0.37$); however, statistical significance was not reached.

Early follicular (day -14 to -12) serum levels of AMH was significantly inversely correlated to FSH ($r = -0.45$; $P < 0.05$). No significant correlations were found between AMH and inhibin B, LH, E_2 or progesterone.

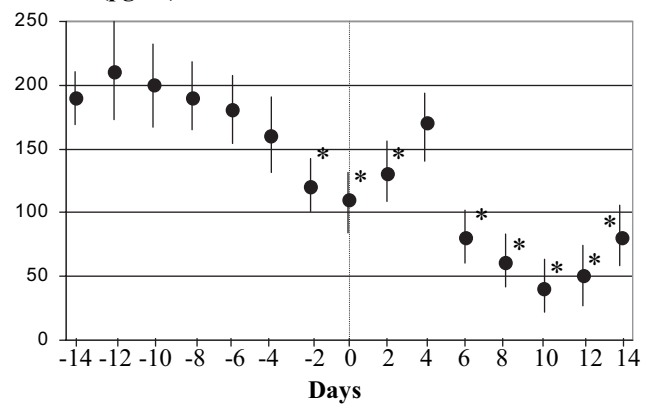
Inhibin B (pg/ml)

Figure 3. Circulatory pattern of inhibin B during the menstrual cycle of young healthy women. Day 0 = day of LH surge. Data are mean \pm SD. *, $P < 0.05$ versus -14 time-point.

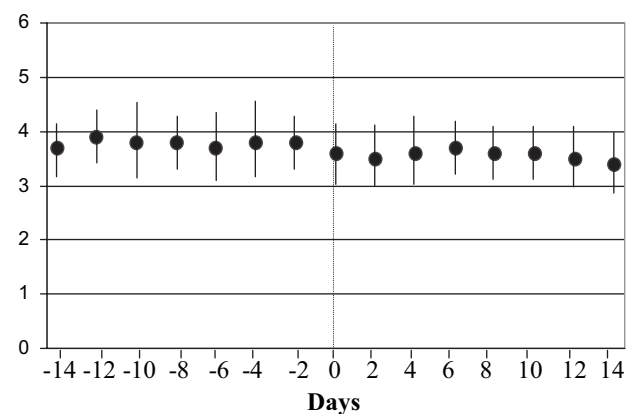
AMH (ng/ml)

Figure 4. Circulatory pattern of anti-Mullerian hormone (AMH) during the menstrual cycle of young healthy women. Day 0 = day of LH surge. Data are Mean \pm SD.

Discussion

This study reports that serum AMH levels do not change significantly throughout the menstrual cycle. Whereas, all other steroids and peptide (inhibin B) secreted by the ovary show significant variations during the cycle. This observation is not surprising as serum AMH has been thought to reflect the continuous, non-cyclic growth of small follicles in the ovary (Visser *et al.*, 2006).

Activation of primordial follicles and the pace of follicular development are regulated by both positive and negative factors. AMH is considered as a negative regulator of the early stages of follicular development (Visser *et al.*, 2006). Indeed, female homozygous AMH knockout mice have more growing pre-antral and small antral follicles compared with wild type when they are pre-pubertal and as young adults (Durlinger *et al.*, 1999). However, their stock of primordial follicles becomes depleted earlier in life. Hence, it has been suggested that AMH acts as a brake on the activation of primordial

follicles and the growth of pre-antral follicles (Durlinger *et al.*, 2002).

Moreover, it has been hypothesized that AMH could be one of the factors involved in determining the responsiveness of ovarian follicles to FSH during cyclic recruitment (Durlinger *et al.*, 2001).

Recent studies indicate that AMH may be a novel measure of ovarian reserve. Serum AMH levels show a reduction through reproductive life (de Vet *et al.*, 2002). Undetectable AMH levels after spontaneous menopause have been reported (Lee *et al.*, 1996; Van Rooij *et al.*, 2004). Ovariectomy in regularly cycling women is associated with disappearance of AMH in 3–5 days, demonstrating that circulating AMH is exclusively of ovarian origin (La Marca *et al.*, 2005a). Similarly, premature ovarian failure has been associated with undetectable serum AMH levels (La Marca *et al.*, 2006).

Serum AMH levels on day 3 of the menstrual cycle show a progressive decrease with age, which correlates with antral follicle counts (AFC) (de Vet *et al.*, 2002). Serum AMH levels have been shown to decrease over time in young normovulatory women (de Vet *et al.*, 2002) and to correlate with age, FSH and the number of antral follicles. In a recent study, a group of women were studied twice, and the interval between the two visits ranged from 1.1 to 7.3 years. A reduction in mean AMH levels of about 38% was observed, whereas the number of antral follicles and the levels of FSH and inhibin B did not change (de Vet *et al.*, 2002).

More recently, 81 women were prospectively studied for 4 years (mean age 39.6 and 43.6 at the beginning and at the end of the study, respectively). It was found that AFC did not change over time, whereas AMH, FSH and inhibin B significantly changed. However, AMH was the only marker of ovarian reserve showing a mean longitudinal decline over time both in younger women (<35 years) and in women >40 years (Van Rooij *et al.*, 2005).

With respect to other known markers, AMH seems to better reflect the continuous decline of the oocyte/follicle pool with age (Van Rooij *et al.*, 2005). The decrease in AMH with advancing age may be present before changes in currently known ageing-related variables, and this indicates that serum AMH levels may be the best marker for ovarian ageing and menopausal transition.

To date, no data have been published on the modifications of AMH levels in serum during the menstrual cycle. In the only published study, it has been showed that AMH exhibits minimal fluctuation throughout the menstrual cycle. However, the study was based on only three samples obtained in the follicular, ovulatory and luteal phase of the cycle (Cook *et al.*, 2000).

In the present article, we demonstrate that AMH levels do not significantly change in serum throughout the menstrual cycle. Whereas, all other steroids and peptide (inhibin B) secreted by the ovary show significant variations during the cycle.

AMH serum levels were significantly, inversely correlated to FSH serum levels. No significant correlations were found between FSH and E₂ or inhibin B. The inverse relationship between FSH and AMH could be secondary to a direct FSH effect on granulosa cell AMH secretion. Indeed, it has been proposed that FSH may down-regulate the AMH expression in

adult rat ovaries (Baarends *et al.*, 1995). Moreover, the relationship between FSH and AMH could be secondary to a mutual relationship between each hormone and the follicle pool (La Marca and Volpe, 2006).

This study included a very young group of women aged 22 ± 3 years (range 18–24). However, AMH as a test will be often used in older women to assess ovarian reserve. Hence, we cannot exclude the possibility that in women of advanced reproductive age, AMH serum levels could show significant fluctuation.

In the human, follicle development to the antral stage continues throughout life until depletion of follicles at the menopause, even in the presence of conditions under which endogenous gonadotrophin release is substantially diminished (Richardson and Nelson, 1990). Such conditions include contraception, pregnancy and hypogonadism (La Marca *et al.*, 2005a,b). Hence, ovarian AMH secretion seems to be independent of endogenous gonadotrophins. This may explain our finding of no modifications in AMH serum levels throughout the human menstrual cycle. Moreover, AMH is produced by the growing antral follicles up to the selection stage (4–6 mm), hence reflecting the continuous non-cycling growth of small follicles.

In conclusion, AMH seems relatively convenient to study, making it an attractive determinant of ovarian activity. The relative stability and consistency of AMH serum levels indicate that AMH could be used as a marker for ovarian ageing and for ovarian response to controlled ovarian stimulation. Compared with the other ovarian tests, AMH seems to be the best marker for reflecting the oocyte/follicle pool.

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Submitted on May 17, 2006; resubmitted on June 16, 2006; accepted on June 27, 2006