

# Circadian variation in concentration of anti-Müllerian hormone in regularly menstruating females: relation to age, gonadotrophin and sex steroid levels

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**BACKGROUND:** Anti-Müllerian hormone (AMH) is a promising marker of ovarian reserve. The aim of the study is to assess the circadian variation in AMH, and to evaluate its clinical relevance and biological aspects as an effect of age and other endocrine mechanisms involved in the regulation of AMH secretion.

**METHODS:** Nineteen healthy non-smoking, regularly menstruating female volunteers with body mass index below 30 kg/m<sup>2</sup>, 10 aged 20–30 years (Group A) and 9 aged 35–45 (Group B) were included. Blood sampling, initiated at 8:00 a.m. on Days 2–6 of the menstrual cycle, was continued every second hour until 8:00 a.m. the following day. Serum levels of AMH, FSH, LH, progesterone and estradiol were measured.

**RESULTS:** With 8:00 a.m. values at the first day of investigation as a reference, the mean concentrations in the pooled data revealed a significantly lower level at 4:00 a.m. ( $P = 0.021$ ) and 6:00 a.m. ( $P = 0.005$ ) with a maximum mean difference of 1.9 pmol/l (10.6%). The same pattern was seen in both the age groups. Including both the age groups, the overall circadian variation of the AMH levels did not reach statistical significance ( $P = 0.059$ ). A significant positive correlation between AMH and LH concentration was seen over the 24-h period ( $P < 0.001$ ).

**CONCLUSIONS:** A slight decrease in serum AMH levels during the late night appears not clinically relevant. Co-variation in the levels of LH and AMH might indicate joint regulatory mechanisms for the latter hormone and gonadotrophins.

**Key words:** Müllerian inhibitory substance / endocrinology / circadian variation

## Introduction

Anti-Müllerian hormone (AMH), originally named for role in regression of the Müllerian ducts in the male fetus, is a dimeric glycoprotein classified as a member of the transforming growth factor- $\beta$  superfamily (Jost, 1946; Cate *et al.*, 1986). In females, the hormone is produced in ovarian granulosa cells, by the primary follicles, secondary follicles and small antral follicles. In prepubertal girls, AMH concentrations are quite low, whereas they seem to reach their highest levels after puberty. Subsequently, a gradual decrease over many years occurs and finally AMH becomes undetectable after menopause. AMH is secreted into the circulation where the concentration of the hormone can be regarded as a reliable quantitative measure of the

ovarian pool of primordial follicles (Visser *et al.*, 2006). The significance of AMH as an endocrine marker of the ovarian function is supported by its stable age-dependent decline in serum, which mirrors the reduction in the pool of primordial follicles over time (van Rooij *et al.*, 2005; Broekmans *et al.*, 2008). In a clinical perspective, AMH is a promising marker for predicting the onset of menopause as well as predicting the ovarian response to hormonal stimulation prior to assisted reproduction treatments (Sowers *et al.*, 2008).

As the follicle transits into the early antral stage, it becomes responsive to gonadotrophin and further development renders the follicle completely dependent on the presence of gonadotrophins. Animal studies and experiments on human ovarian tissue have identified an inhibitory role of AMH in primordial follicle recruitment (Themmen,

2005; Visser and Themmen, 2005; Nilsson *et al.*, 2007) and a regulatory role in the FSH sensitivity of large antral follicles reaching the stage of sensitivity to pituitary gonadotrophins (Durlinger *et al.*, 2001). However, it is not known whether the regulation of FSH sensitivity by AMH represents its entire intra-ovarian function. Knowledge regarding the extra-ovarian control and action of this hormone in females is also lacking.

The number of granulosa cells increases slowly over several menstrual cycles and therefore rapid fluctuations in AMH should not be expected (Gougeon, 1996). However, knowledge of the diurnal rhythms in the secretion of ovarian hormones is limited, in contrast to the well-established profiles of adrenal hormones cortisol and dehydroepiandrosterone that exhibit a marked circadian rhythm (Aedo *et al.*, 1977). A single study reported that LH revealed a diurnal variation during the luteal phase but not in the follicular phase (Aedo *et al.*, 1981). In some studies, AMH measured during daytime has been reported to be relatively stable in the course of a normal menstrual cycle (Hehenkamp *et al.*, 2006; La Marca *et al.*, 2006), whereas others papers describe significant variation (Wunder *et al.*, 2008; Sowers *et al.*, 2010). Under conditions characterized by a significantly diminished gonadotrophin release, such as during pregnancy (La Marca *et al.*, 2005), during GnRH agonist therapy (Mohamed *et al.*, 2006) and during oral contraceptive administration (Arbo *et al.*, 2007), a potential circadian variation of the hormone would be of clinical as well as biological interest and recently the need of such type of studies has been stressed (Al-Qahtani and Groome, 2006).

The present study describes the circadian variation in AMH levels in 19 healthy regularly menstruating women, with the intention of elucidating (i) possible age-related differences and (ii) endocrine associations with AMH secretion. For the first purpose, two age groups were studied; one group included women below the age of 30 years, whereas the age of the other group consists of women over 35 years of age. For the second aim, variations in the levels of AMH were compared with the circadian changes in levels of FSH, LH, progesterone and estradiol.

## Materials and Methods

The study was conducted at the Reproductive Medicine Centre at Skåne University Hospital Malmö, Lund University, Sweden. Healthy women, enrolled by recruitment posters or advertisement in the local newspapers, received oral and written information before signing a consent form. The local Ethical Committee at Lund University approved the study.

### Subjects

Twenty healthy non-smoking female volunteers with body mass index below 30 kg/m<sup>2</sup>, 10 women aged 20–30 years (Group A) and 10 aged 35–45 years (Group B), were enrolled in the study.

A regular menstrual period with a cycle length of 21 and 35 days was required for inclusion. Exclusion criteria were as follows: use of hormonal medication, history of infertility and/or other gynaecological or chronic diseases.

### Blood sampling

Each study subject called the research team on the first day of the menstrual bleeding. On the basis of results from previous studies, where no major variation in AMH serums levels was observed during the early follicular phase, blood sampling was initiated on Days 2–6 of the menstrual cycle. The circadian profile was performed during a 24-h period by

drawing blood samples every second hour, starting at 8:00 a.m. and continuing until 8:00 a.m. the following day. From three subjects, one sample was not obtained, whereas in the remaining 16 women all 13 samples were obtained.

Through a heparinized catheter inserted into a forearm vein, each blood sample consisted of 10 ml blood drawn into vacuumed vials containing gel. Within 2 h, the samples were centrifuged at 2000g for 10 min, and serum was isolated and stored at –20 °C and assayed within in the period of 2 months.

### Assays

Serum AMH was analysed using the Immunotech EIA AMH/MIS assay from Beckman–Coulter Inc., Marseille, France (Long *et al.*, 2000). The lowest detectable level distinguishable from zero with 95% confidence is 0.7 pmol/l. The total coefficient of variations (CVs) obtained were 25% at 5.7 pmol/l and 12% at 52 pmol/l.

For FSH, LH, progesterone and estradiol, all samples from one participant were analysed within the same assay run at a Beckman Access<sup>®</sup> Immunoassay System on a UniCel<sup>™</sup>Dxl800 from Beckman–Coulter Inc., Brea, CA, USA. The lowest detectable level distinguishable from zero with 95% confidence and total CVs are 0.2 IU/l and <9% for FSH and LH, 0.25 nmol/l and <14% for progesterone and 73 pmol/l and <13% for estradiol.

All measurements were performed according to the instructions from the manufacturers.

### Statistical analysis

We performed mixed model analyses for the repeated measurements of AMH, LH, FSH, estradiol and progesterone that were considered to be independent continuous variables (continuous) modelled with Group (A/B) and time (all time points: 8:00 a.m., 10:00 a.m., 12:00 p.m., 2:00 p.m., 4:00 p.m., 6:00 p.m., 8:00 p.m., 10:00 p.m., 12:00 a.m., 2:00 a.m., 4:00 a.m., 6:00 a.m. and 8:00 a.m.) as categorical variables. For AMH, LH, FSH, estradiol and progesterone, the analysis was performed with and without the other hormones as continuous covariates.

Mixed model analysis allows for evaluation of differences in repeated measurement between patient groups. Compared with more simple statistical methods, mixed model analysis focuses on the overall mean difference between the groups and the overall time pattern of the variance, and thereby avoids multiple testing at individual time points. Another advantage of this statistical method is that clinically important differences between patient groups under investigation can be adjusted for. Repeated measurements at different time points imply that measurements for the same patient are more similar than those for different patients, i.e. the residuals of the mixed model for repeated measurements within a patient will be correlated. This correlation was assumed to follow an autoregressive structure with one time lag. A random coefficient was kept in the model only if its estimated variance was non-zero. Group-specific circadian variations were estimated as marginal means. The mixed model analysis does also allow for comparison of each single time point with the first value (8:00 a.m. on the first day).

The maximum relative intra-individual variations in AMH levels found in subjects of Groups A and B were compared using the Mann–Whitney test. Statistical analysis was done using statistical software (SPSS 17.0 for Windows; SPSS Inc., Chicago, IL, USA). A *P*-value of <0.05 was considered statistically significant.

## Results

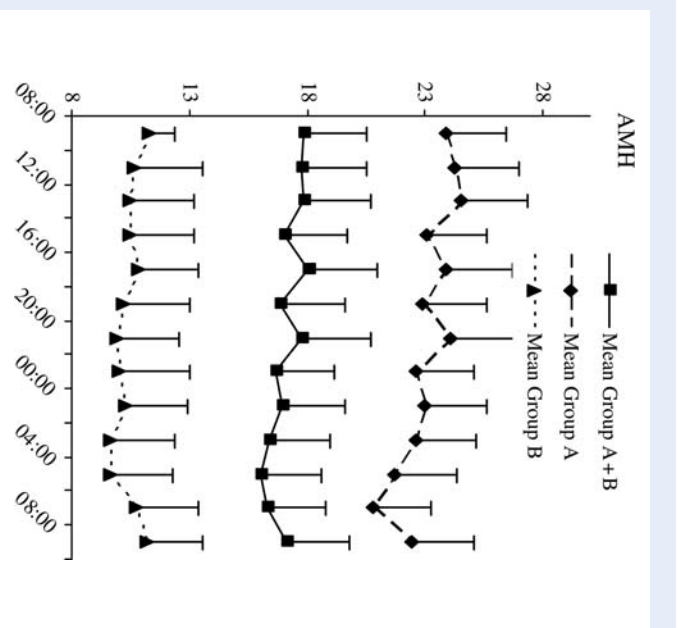
One study participant in Group B was excluded during the course of the study due to orthopaedic surgery performed requiring anaesthesia

and pain-relieving medication. The median age in Group A was 26 years (range 22–45) and the median menstrual cycle length 30 days (range 25–35). The corresponding subject characteristics in Group B were a median age of 39 years (range 35–45) and a cycle length of 28 days (range 22–30).

### Circadian variation in AMH

The circadian variation in AMH in both groups is shown in Fig. 1 and Table 1. With 8:00 a.m. values at the first day of investigation as the reference, the mean concentrations in the pooled data revealed a statistically significantly lower level at 4:00 a.m. ( $P = 0.021$ ) and 6:00 a.m. ( $P = 0.005$ ) with a maximum mean difference of 1.9 pmol/l (10.6%). Group A demonstrated significantly lower values at 6:00 a.m. when compared with 8:00 a.m., the maximum mean difference being 3.1 pmol/l (13%). In the older age group, significant lower values compared with 8:00 a.m. were found at 2:00, 4:00 and 6:00 a.m., the maximum mean difference being 1.7 pmol/l (15.0%). Including both age groups, the overall circadian variation of the AMH levels was not statistically significant ( $P = 0.059$ ). The median (range) maximum intra-individual variation in AMH concentration was 23% (10–230%) in Group A and 68% (17–147%) in Group B; this difference being statistically significant ( $P = 0.045$ ). For the whole group of subjects, the lowest concentration was reduced with 11% when compared with the value at 8:00 a.m. The relative variation was approximately at the same level in the older age group (15%) when compared with the youngest group (13%).

A significant difference in mean AMH levels between the groups was observed, the highest values being found in the younger age group ( $P = 0.011$ ).

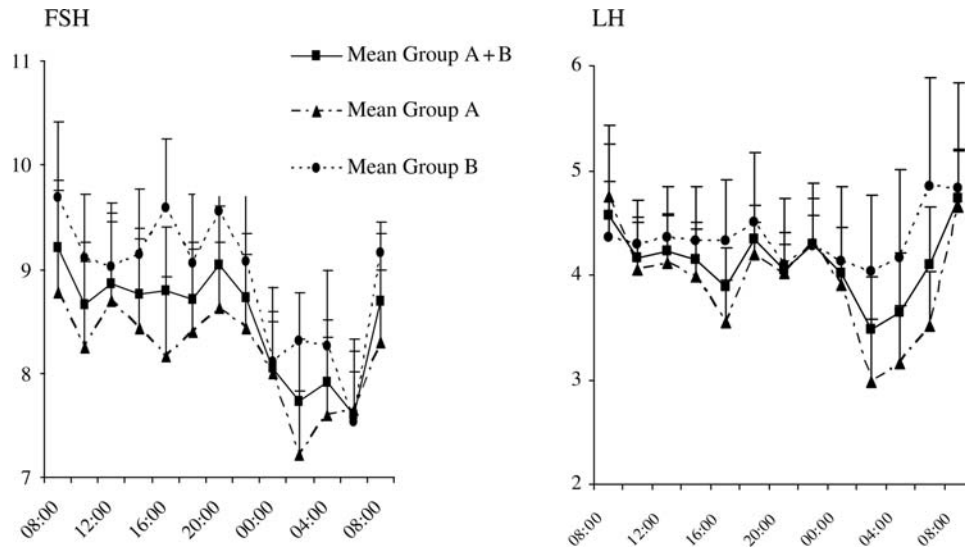


**Figure 1** Circadian variation in AMH (pmol/l). Figure illustrates mean values  $\pm$  standard error of the mean (SEM) in Group A + B, Group A and Group B.

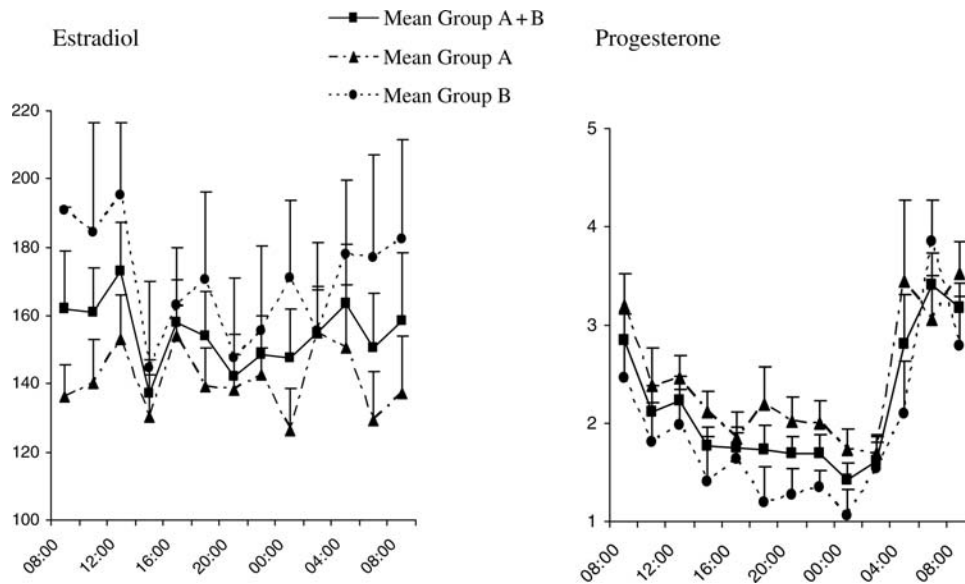
**Table 1** Serum concentration of AMH, FSH and LH in relation to the time of the day and age groups.

| Time                        | 8:00 a.m.   | 10:00 a.m.  | 12:00 p.m.  | 2:00 p.m.   | 4:00 p.m.   | 6:00 p.m.   | 8:00 p.m.   | 10:00 p.m.  | 12:00 a.m.  | 2:00 a.m.   | 4:00 a.m.    | 6:00 a.m.    | 8:00 a.m.   |
|-----------------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|--------------|--------------|-------------|
| <b>AMH</b>                  |             |             |             |             |             |             |             |             |             |             |              |              |             |
| Group A (pmol/l), mean (SD) | 23.8 (10.)  | 24.2 (11.3) | 24.5 (11.8) | 23.0 (10.9) | 23.9 (12.6) | 22.9 (11.8) | 24.0 (11.7) | 22.6 (10.0) | 23.0 (10.7) | 22.6 (10.2) | 21.7 (10.9)  | 20.8* (11.3) | 22.4 (12.0) |
| Group B (pmol/l), mean (SD) | 11.3 (3.2)  | 10.7 (8.8)  | 10.5 (8.2)  | 10.4 (8.2)  | 10.8 (7.9)  | 10.2 (8.6)  | 9.9 (7.9)   | 10.1 (9.1)  | 10.2 (8.0)  | 9.6* (8.3)  | 9.6* (8.0)   | 10.8* (8.0)  | 11.2 (7.0)  |
| All (pmol/l), mean (SD)     | 17.9 (11.5) | 17.8 (11.9) | 17.9 (12.3) | 17.1 (11.3) | 18.1 (12.6) | 16.9 (11.8) | 17.8 (12.6) | 16.6 (10.9) | 16.9 (11.4) | 16.4 (11.2) | 16.0* (11.2) | 16.3* (10.7) | 1.71 (11.5) |
| <b>FSH</b>                  |             |             |             |             |             |             |             |             |             |             |              |              |             |
| Group A (IU/l), mean (SD)   | 8.8 (3.1)   | 8.2 (2.6)   | 8.7 (2.6)   | 8.4 (2.7)   | 8.5 (2.3)   | 8.4 (2.5)   | 8.6 (2.0)   | 8.4 (2.3)   | 8.0 (1.9)   | 7.2* (1.9)  | 7.6* (2.4)   | 7.7* (2.4)   | 8.3 (2.2)   |
| Group B (IU/l), mean (SD)   | 9.7 (2.6)   | 9.1 (2.7)   | 9.0 (2.8)   | 9.1 (2.9)   | 9.6 (2.9)   | 9.1 (2.3)   | 9.6 (3.1)   | 9.1 (3.1)   | 8.1* (2.0)  | 8.3* (3.1)  | 8.3* (3.0)   | 7.5* (1.3)   | 9.2 (3.4)   |
| All (IU/l), mean (SD)       | 9.2 (2.8)   | 8.7 (2.6)   | 8.9 (2.6)   | 8.8 (2.7)   | 9.0 (2.5)   | 8.7 (2.4)   | 9.0 (2.5)   | 8.7 (2.7)   | 8.0* (1.9)  | 7.7* (2.6)  | 7.9* (2.6)   | 7.6* (1.8)   | 8.7 (2.8)   |
| <b>LH</b>                   |             |             |             |             |             |             |             |             |             |             |              |              |             |
| Group A (IU/l), mean (SD)   | 4.8 (1.6)   | 4.1 (1.6)   | 4.1 (1.5)   | 4.0 (1.4)   | 3.8 (1.2)   | 4.2 (0.9)   | 4.0 (1.2)   | 4.3 (1.8)   | 3.9 (1.7)   | 3.0* (1.9)  | 3.2* (1.7)   | 3.5* (1.7)   | 4.7 (1.7)   |
| Group B (IU/l), mean (SD)   | 4.4 (1.3)   | 4.3 (1.5)   | 4.4 (1.6)   | 4.3 (1.7)   | 4.3 (2.0)   | 4.5 (1.9)   | 4.1 (0.9)   | 4.3 (2.2)   | 4.1 (2.2)   | 4.0 (2.5)   | 4.2 (3.1)    | 4.9 (3.0)    | 4.8 (2.3)   |
| All (IU/l), mean (SD)       | 4.6 (1.4)   | 4.2 (1.5)   | 4.2 (1.5)   | 4.2 (1.5)   | 4.0 (1.6)   | 4.3 (1.4)   | 4.1 (1.1)   | 4.3 (1.9)   | 4.0 (1.9)   | 3.5* (2.2)  | 3.6* (2.5)   | 4.1 (2.4)    | 4.7 (2.0)   |

\* $P < 0.05$  in comparison to 8 a.m. levels.



**Figure 2** Circadian variation in FSH (IU/l) and LH (IU/l). Figures illustrate the mean values + SEM in Group A + B, Group A and Group B.



**Figure 3** Circadian variation in estradiol (pmol/l) and progesterone (nmol/l). Figures illustrate mean values + SEM in Group A + B, Group A and Group B.

### Circadian variation in gonadotrophins

The circadian variation in gonadotrophins in both groups is shown in Fig. 2 and Table I. For the combined groups, FSH concentration showed a significant variation over the 24-h period ( $P = 0.001$ ). The variation did not reach significance within the individual groups, Group A ( $P = 0.075$ ) and Group B ( $P = 0.095$ ). No difference was seen between the groups ( $P = 0.524$ ).

Circadian variation in LH was not statistically significant in the pooled data set (both groups;  $P = 0.11$ ); however, statistical significance was found in Group A ( $P = 0.045$ ), but not in the older

women ( $P = 0.98$ ). No difference was seen between the groups ( $P = 0.57$ ).

For both hormones, the levels were lower during late night and early morning hours when compared with the 8:00 a.m. concentration.

### Circadian variation in ovarian-derived hormones

The circadian variation in ovarian-derived is shown in Fig. 3 and Table II. For estradiol, pooled data did not show statistically significant

**Table II** Serum concentrations of progesterone and estradiol in relation to the time of the day and age groups.

| Time                        | 8:00 a.m.    | 10:00 a.m.   | 12:00 p.m.   | 2:00 p.m.     | 4:00 p.m.     | 6:00 p.m.    | 8:00 p.m.     | 10:00 p.m.   | 12:00 a.m.   | 2:00 a.m.    | 4:00 a.m.    | 6:00 a.m.    | 8:00 a.m.     |
|-----------------------------|--------------|--------------|--------------|---------------|---------------|--------------|---------------|--------------|--------------|--------------|--------------|--------------|---------------|
| <b>Progesterone</b>         |              |              |              |               |               |              |               |              |              |              |              |              |               |
| Group A (nmol/l), mean (SD) | 3.15 (1.0)   | 2.34 (1.2)   | 2.39 (0.8)   | 2.23* (0.7)   | 2.01* (0.8)   | 2.27 (1.2)   | 2.0* (0.8)    | 1.9* (0.8)   | 1.6* (0.7)   | 1.61* (0.6)  | 3.27 (2.6)   | 3.08 (1.4)   | 3.59 (1.0)    |
| Group B (nmol/l), mean (SD) | 2.5 (1.2)    | 1.8 (1.1)    | 2.0 (1.4)    | 1.4* (0.8)    | 1.61* (1.0)   | 1.2* (0.8)   | 1.3* (0.5)    | 1.3* (0.8)   | 1.1* (0.8)   | 1.5* (1.6)   | 2.1 (1.3)    | 3.8* (1.5)   | 2.80 (1.0)    |
| All (nmol/l), mean (SD)     | 2.8 (1.1)    | 2.1* (1.2)   | 2.2* (1.1)   | 1.8* (0.8)    | 1.80* (0.9)   | 1.8* (1.1)   | 1.7* (0.8)    | 1.6* (0.8)   | 1.3* (0.8)   | 1.6* (1.1)   | 2.7 (2.1)    | 3.4* (1.5)   | 3.20 (1.0)    |
| <b>Estradiol</b>            |              |              |              |               |               |              |               |              |              |              |              |              |               |
| Group A (pmol/l), mean (SD) | 136.4 (29.8) | 140.0 (41.1) | 153.1 (40.9) | 130.1 (39.6)  | 157.9 (27.6)  | 139.1 (37.1) | 138.4 (32.6)  | 142.6 (24.9) | 126.3 (39.2) | 155.2 (41.5) | 150.8 (57.5) | 129.4 (44.5) | 137.2 (53.0)  |
| Group B (pmol/l), mean (SD) | 190.7 (96.2) | 184.2 (63.5) | 195.3 (75.6) | 144.8 (50.8)  | 163.0 (76.77) | 170.4 (70.1) | 147.4* (74.6) | 155.6 (67.6) | 171.1 (77.5) | 155.3 (65.0) | 178.0 (91.0) | 177.0 (87.2) | 182.3 (109.9) |
| All (pmol/l), mean (SD)     | 162.1 (73.0) | 160.9 (56.1) | 173.1 (62.0) | 137.1* (44.6) | 160.3 (54.4)  | 153.9 (55.9) | 142.4 (53.6)  | 148.7 (48.8) | 147.5 (63.0) | 155.3 (52.3) | 163.7 (74.3) | 150.6 (69.1) | 158.6 (85.5)  |

\* $P < 0.05$  in comparison to 8 a.m. levels.

diurnal variation ( $P = 0.059$ ), and no significant diurnal variation was seen when the two groups were analysed separately (Group A:  $P = 0.21$  and Group B:  $P = 0.17$ ). In contrast, a significant difference in mean levels was seen between the two groups with highest concentrations in the older group ( $P = 0.025$ ).

Mean levels of progesterone revealed a rapid fall during the daytime, when compared with the initial 8:00 a.m. measurement and increased again in the early morning. These variations were highly significant in the pooled data ( $P < 0.001$ ), as well as in both individual groups ( $P < 0.001$ ). There was no difference between groups ( $P = 0.134$ ).

### Co-variation between serum levels of AMH and other reproductive hormones

Statistically significant co-variation was found between AMH and LH, which was the case for both groups, Group A ( $P = 0.035$ ) Group B ( $P = 0.002$ ) and Group A + B ( $P < 0.001$ ). No such association was found between the variation in AMH and any other of the hormones measured.

### Co-variation between serum levels of gonadotrophins and ovarian-derived hormones

Statistically significant co-variation was found between LH and progesterone, which was the case for both groups, Group A ( $P = 0.02$ ), Group B ( $P = 0.049$ ) and the total cohort ( $P < 0.001$ ). The same was also true for the co-variation between FSH and progesterone, Group A ( $P = 0.047$ ), Group B ( $P = 0.010$ ) and Groups A + B ( $P = 0.002$ ). There was no co-variation between LH and estradiol or between FSH and estradiol.

## Discussion

To our knowledge, this is the first study to describe circadian variation in circulating levels of AMH in regularly menstruating women who have no signs of polycystic ovarian syndrome. The overall circadian variation in AMH was quantitatively small and did not reach statistical significance. However, when comparing different time points with the 8:00 a.m. value, we found significantly lower AMH levels earlier in the morning, at 4:00 and 6:00 a.m. For the whole group of women, AMH was 11% lower when compared with the value at 8:00 a.m. The relative variation was approximately at the same level in the older age group (15%) when compared with the younger group (13%). However, although more subtle variability might be discovered if more subjects were included, due to a relatively limited magnitude of the observed diurnal fluctuations and stable levels during daytime, our findings do not abrogate the usefulness of AMH as a clinical marker for ovarian reserve. The CV for the AMH assay is 5.7 pmol/l, indicating that the functional sensitivity of the assay is much higher than 0.7 pmol/l given as the limit of detection. With very low levels of AMH that could be expected in the older group, this lack of precision could mask circadian AMH variation or contributed to high intra-individual variation in AMH. However, no measurements, either in the older or the younger group, displayed such low levels; the lowest value recorded was 1.2 pmol/l. Furthermore, the intra-individual variation seen in some subjects was much higher

than the intra-assay CV and could therefore not be ascribed to limitations in the laboratory analysis.

Notably, in this study, a significant positive co-variation between serum levels of AMH and LH was observed. Furthermore, concentrations of FSH and LH displayed significant circadian variation. With respect to LH, these fluctuations were more pronounced in the youngest group. Thus, the association between LH and AMH levels might mirror the presence of a joint regulatory mechanism for AMH and LH or FSH. Estradiol revealed no significant diurnal variation, but the serum levels of this hormone were higher in the oldest group ( $P = 0.025$ ). This finding correlates well with earlier observations of an accelerated follicular phase in women of more advanced reproductive age, where estrogen excretion was reportedly higher compared with younger women, indicating an accelerated growth of the dominant follicle (Santoro *et al.*, 1996).

Progesterone was significantly and profoundly decreased in the evening and night and, when measured in the early follicular phase, serum concentrations were found to be highest in the morning. The circadian variation of this hormone in the early phase of the menstrual cycle has not been much studied and the relative contribution of the ovary and the adrenal gland to the overall production of progesterone during the follicular phase has not been defined. By catheterization of the ovarian veins, a significant higher level of progesterone was seen at the side of the growing follicle, proving that progesterone is also produced by the ovary in the follicular phase (Coutts, 1981). The statistically significant correlation between LH and progesterone levels in this study suggests the ovarian production of the latter hormone, but proof of such mechanism still needs to be provided.

In conclusion, we found a slight decrease in serum AMH levels during the early morning hours. An interesting co-finding was the significant positive correlation between AMH and LH concentration over a 24-h period, which should encourage further studies on possible association between LH or FSH action and the secretion of AMH from the granulosa cells.

## Authors' roles

L.B.: first author, substantial contribution to conception and design; A.-K.J.: acquisition of data; F.R.: acquisition of data; C.B.: analysis and interpretation of data; C.Y.A.: revising the article critically for important intellectual content; N.G.: analysis and interpretation of data; A.G.: substantial contribution to conception and design, revising the article critically for important intellectual content.

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