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Anti-Müllerian Hormone Levels in the Spontaneous Menstrual Cycle Do Not Show Substantial Fluctuation

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Context: Anti-Müllerian hormone (AMH), a quantitative marker for ovarian reserve, has been suggested to be independent of the classical endocrine fluctuations of the menstrual cycle.

Objective: The objective of the study was to determine whether AMH levels are constant throughout the menstrual cycle, compared with those of FSH, LH, and estradiol.

Design/Patients: Frequent blood sampling was performed in 44 fertile, regularly cycling, female volunteers during one full menstrual cycle.

Setting: The study was conducted at a university hospital.

Main Outcome Measures: AMH, FSH, LH, and estradiol measurements were allocated to one of seven cycle phases, and a multilevel analysis was performed. Consistent fluctuation patterns were tested by fitting sine patterns to the data. Finally, the frequency in which randomly selected individual samples would remain in one of five preset level categories (quintiles) for each of the variables was studied.

Results: A sine pattern fitted to the AMH data was not statistically significant ($P = 0.40$). In contrast, sine patterns for FSH, LH, and estradiol were highly significant. Comparing the seven cycle phases, no significant differences could be observed between phase-specific AMH levels ($P = 0.06$). Repeated selection of AMH samples for each individual showed that in 71.5% of selections, AMH values remained in the same quintile, whereas in 27.9% values fell in an adjacent quintile.

Conclusions: AMH levels measured through a full menstrual cycle did not show consistent fluctuation patterns in contrast to levels of FSH, LH, and estradiol. Furthermore, random fluctuations were small, indicating that AMH can be relied on as a cycle-independent marker for ovarian reserve. **(*J Clin Endocrinol Metab* 91: 4057–4063, 2006)**

ANTI-MÜLLERIAN HORMONE (AMH, also called Müllerian-inhibiting substance) is produced by granulosa cells of the follicles in the ovary (1). AMH plays a role in the regulation of ovarian function during both early and late follicle development. From studies in mice, it has emerged that AMH impedes the transition from primordial follicles into growing, primary follicles (2), although a recent study on human ovarian tissue has suggested a more facilitating role for AMH (3). Surely AMH can be considered a factor in the depletion rate of the primordial follicle pool and the maintenance of the pool of growing follicles. In small antral follicles that start to grow under the influence of rising FSH levels at the luteofollicular transition of the menstrual cycle, AMH limits the sensitivity of these follicles to FSH (2, 4). When human follicles in the growing cohort have reached a diameter of 6 mm, AMH production decreases and is absent in the dominant follicle (5), rendering this follicle more FSH sensitive, compared with the nondominant ones. Therefore, it is believed that AMH is one of the autocrine factors that regulate dominant follicle selection (6).

The production of AMH by small antral follicles is believed to determine the measurable levels of AMH in premenopausal women (7, 8). In prepubertal girls AMH values appear rather low, with a slight tendency to rise toward the onset of puberty (9–11). This may be consistent with the finding that in girls from birth to puberty, ovarian size and antral follicle presence gradually increase (12, 13). After puberty AMH seems to reach maximum levels, subsequently show a gradual decrease over many years, and become undetectable in women after menopause (11, 14). AMH levels correlate well with the number of antral follicles measured by ultrasound (14–16) and are believed to be the best representation of the gradual decline in reproductive capacity among proven fertile women (17, 18). Finally, AMH has been shown to be an accurate marker for the occurrence of poor response to ovarian hyperstimulation with gonadotropins in *in vitro* fertilization (IVF) (16, 19, 20). On the basis of these findings, AMH may well become a frequently applied marker in reproductive medicine.

One of the possible advantages of the use of AMH as ovarian reserve test over established markers like basal FSH, the clomiphene citrate challenge test, and the antral follicle count is its presumed menstrual cycle independence (8, 21). In the study by Cook *et al.* (21), AMH levels at ovulation were

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Abbreviations: AMH, Anti-Müllerian hormone; CV, coefficient of variation; IVF, *in vitro* fertilization; WHO, World Health Organization.

only slightly higher than in the early follicular and midluteal phases, suggesting that AMH levels are not cycle dependent. Also in the study by La Marca *et al.* (8), AMH levels did not show any change over the course of the follicular phase. Apparently, AMH release into the systemic circulation does not follow the same pattern as for the classical hormones that regulate the pituitary-ovarian interplay. Finally, the lack of acute response of AMH combined with the presence of a substantial rise in estradiol and inhibin B after a massive exposure to endogenous FSH and LH indicates that AMH release is not FSH mediated (16).

To verify the contention that AMH is a cycle-independent marker for ovarian reserve, we analyzed AMH levels throughout a full menstrual cycle in an adequately sized group of female volunteers. To evaluate our method of analysis, we compared cycle AMH patterns with those of FSH, LH, and estradiol.

Subjects and Methods

Subjects

The study was conducted at the Department of Reproductive Endocrinology and Fertility of the University Medical Centre, Utrecht. Approval was obtained from the local ethics committee, and written informed consent was obtained from all participants. Healthy women were recruited by advertisement in local newspapers. Volunteers were enrolled in the study protocol if they met all of the following criteria: 1) regular menstrual cycles varying from 21 to 35 d, 2) a biphasic body temperature chart, 3) proven natural fertility by having had at least one pregnancy carried to term, 4) spontaneously arising pregnancy within 1 yr after the start of unprotected intercourse, 5) no evidence of endocrine disease, 6) no history of ovarian surgery, 7) no ovarian abnormalities as assessed by transvaginal ultrasound, and 8) hormonal contraception stopped at least 2 months before entering the study protocol. For study participation the volunteers received monetary compensation (22).

Experimental design

The design of the study has been described earlier (22, 23). In brief, investigations were started in the midluteal phase of the first study cycle. The luteal phase was assumed to have started when a temperature rise on the basal body temperature (BBT) chart, based on classical criteria [World Health Organization (WHO), 1967], had been observed. From the seventh day after the temperature shift onward, the volunteers visited the clinic every 2 or 3 d for blood sampling until the occurrence of menstruation. After onset of the menstrual bleeding of the second study cycle, volunteers returned on cycle d 2, 3, or 4 and every 2–3 d thereafter for ultrasound scanning to observe the emergence and development of the dominant follicle until this follicle had reached a mean diameter of at least 14 mm. From that point onward, ultrasound scans were performed daily until 4 d after ovulation. Ovulation day was defined as the day at which a complete disappearance of the follicle or a reduction of its mean diameter by at least 5 mm was observed (24, 25). In addition to performing serial ultrasound, blood samples were taken at each visit in the second cycle. In all blood samples, levels of AMH, FSH, LH, and estradiol were measured.

Hormone assays

AMH concentrations were measured in serum, whereas FSH, LH, and estradiol concentrations were measured in plasma. Specimens were stored at −20 C until processed. In all samples, AMH levels were estimated using an enzyme-immunometric assay (Diagnostic Systems Laboratories, Webster, TX). Inter- and intraassay coefficients of variation (CVs) were less than 5% at the level of 3 μg/liter and less than 11% at the level of 13 μg/liter. The detection limit of the assay was 0.026 μg/liter. Repeated freezing and thawing of the samples or storage at 37 C for 1 h did not affect results of the assay (16).

Concentrations of FSH and LH were measured using a fully automated AxSYM immunoanalyzer (Abbott Laboratories, Abbott Park, IL) according to the manufacturer's instructions. The standard of the LH assay was calibrated against the WHO First International Reference Preparation for human LH (68/40), whereas that of the FSH assay was referenced against the WHO Second International Reference Preparation for human FSH (78/549). For LH, the between-run CVs were 5.5, 7.2, and 7.9% at 4.8, 39, and 83 IU/liter, respectively (n = 48). For FSH, the between-run CV was 6.0, 6.6, and 8% at levels of 5.0, 25, and 75 IU/liter, respectively (n = 46). Estradiol concentrations were assayed using a microparticle enzyme immunoassay kit (Abbott Laboratories) on a semi-automated IMx analyzer. Between-run CVs for estradiol were 10.1, 7.0, and 6.9% at 533, 1354, and 4197 pmol/liter, respectively (n = 49, 49, and 30).

Statistical analysis

Statistical analysis was performed by using the linear mixed-effects model in SPlus (version 6.0; Mathsoft Inc., Seattle, WA). Furthermore, SPSS 12.1 (SPSS Inc., Chicago, IL) was used to construct bar graphs. Cycle phases were defined as a range of days counted either from menstruation (M) or from the ultrasound assessed ovulation (O) day in cycle 2. The seven cycle phases were defined as follows: midluteal: M-9 to M-5; late luteal: M-4 to M-1; early follicular: M to M+4; midfollicular: O-9 to O-6; late follicular: O-5 to O-2; periovulation: O-1 to O+1; and early luteal O+2 to O+4. To visualize the values for all four variables per cycle phase, all available values per cycle phase were averaged, and box plots were constructed using these data. By doing so, the data in the bar graph may become biased by the fact that not every woman contributed equal numbers of observations per cycle phase. To compensate for this, a multilevel regression analysis was applied to the data for comparison of the hormone levels across the cycle phases.

For analysis of fluctuations, the logarithm of the AMH, FSH, LH, and estradiol concentration was first modeled by a trigonometric function as fixed factor and woman as random factor, using a linear mixed-effects model. Individual sine functions were calculated to obtain periodicity according to the woman's cycle length. By entering both sine and cosine as fixed factors in the model, the phase (relative moment of the maximum value) of the function could be estimated (26). The pattern, however, may be more intricate than a simple sine. Therefore, in a second model, the trigonometric function was replaced by a categorical variable with the seven cycle phases described above. In this model the pattern will consist of a sequence of seven different levels, one for each phase of the cycle. In both models an ANOVA yielded the significance levels for differences in log (AMH, FSH, LH, or estradiol) level during the cycle.

To evaluate the size of the effect of intraindividual fluctuations, we classified values of all four variables in five quintiles and registered how often two different measurements of an individual were located in the same quintile, adjacent quintiles, or nonadjacent quintiles. The cutoff levels used for the analysis of quintile categories were 0.22, 0.50, 1.14, and 2.02 μg/liter for AMH; 3.32, 4.80, 6.70, and 9.10 IU/liter for FSH; 2.80, 4.54, 6.56, and 12.70 IU/liter for LH; and 228, 350, 621, and 1008 pmol/liter for estradiol.

Finally, only for AMH, we evaluated clinically relevant fluctuations by analyzing how many patients crossed an earlier reported cutoff level for the prediction of the reproductive outcome pregnancy in IVF: 1.0 μg/liter (20). In this publication an assay from Immunotech-Coulter (Miami, FL) was used. Comparison of the Diagnostic Systems Laboratories and Immunotech-Coulter assays can be done by multiplying the Diagnostic Systems Laboratories results by a factor 2.02. This was based on a comparison of 82 samples across all concentration ranges, yielding a correlation coefficient of 0.85. The formula of the regression line was: AMH (Diagnostic Systems Laboratories) = 0.495 * AMH (Immunotech-Coulter) − 0.03. Therefore, the AMH cut-off level for our series was set at 0.5 μg/liter.

Results

A total of 44 women were recruited for this study [median age 38.3 yr (range 25.6–46.2)]. A total of 396 blood samples were collected (median eight per volunteer; range 6–14). The mean overall AMH level was 1.05 μg/liter (SD 0.92; range 0.01–4.60).

Figure 1A shows the box plot graph for AMH levels per cycle phase, using all available measurements per volunteer per cycle phase. As in the midluteal and early luteal phase, several volunteers did not contribute to the data, and observations tended to become less reliable for these phases. By coincidence, younger cases with higher AMH were overrepresented in these two cycle periods, creating relatively high median levels. Figure 1, B–D, shows the box plot graphs for the FSH, LH, and estradiol levels per cycle phase. Patterns across the cycle phases were highly compatible with current knowledge on the fluctuations of these hormones in the normal cycle (27).

Figure 2A shows all observed AMH values for each volunteer on a logarithmic scale, plotted with the day of ovulation in the second cycle as reference. No consistent fluctuation related to the classical cycle events menstruation and ovulation becomes apparent from the graph, except for a possible periovulatory rise in some of the younger patients with high AMH levels. The modeling of sine patterns in which cycle length was taken into account resulted in Fig. 2B, in which the fitted values for a sine model for every individual woman are shown. The mean amplitude for the sinus was 7.6%, *i.e.* the top was 3.8% higher and the bottom 3.8% lower than a woman's specific mean. Mean sinus amplitudes for FSH, LH, and estradiol were 80, 232, and 207%, respectively. Data for FSH are graphically represented in Fig. 2, C and D.

The goodness of fit of the sine patterns for AMH showed to be statistically nonsignificant ($P = 0.40$). This was in contrast to the sine patterns for FSH, LH, and estradiol, of which the goodness of fit statistics were consistently highly significant ($P < 0.0001$).

Table 1 displays the results of the multilevel analysis, in

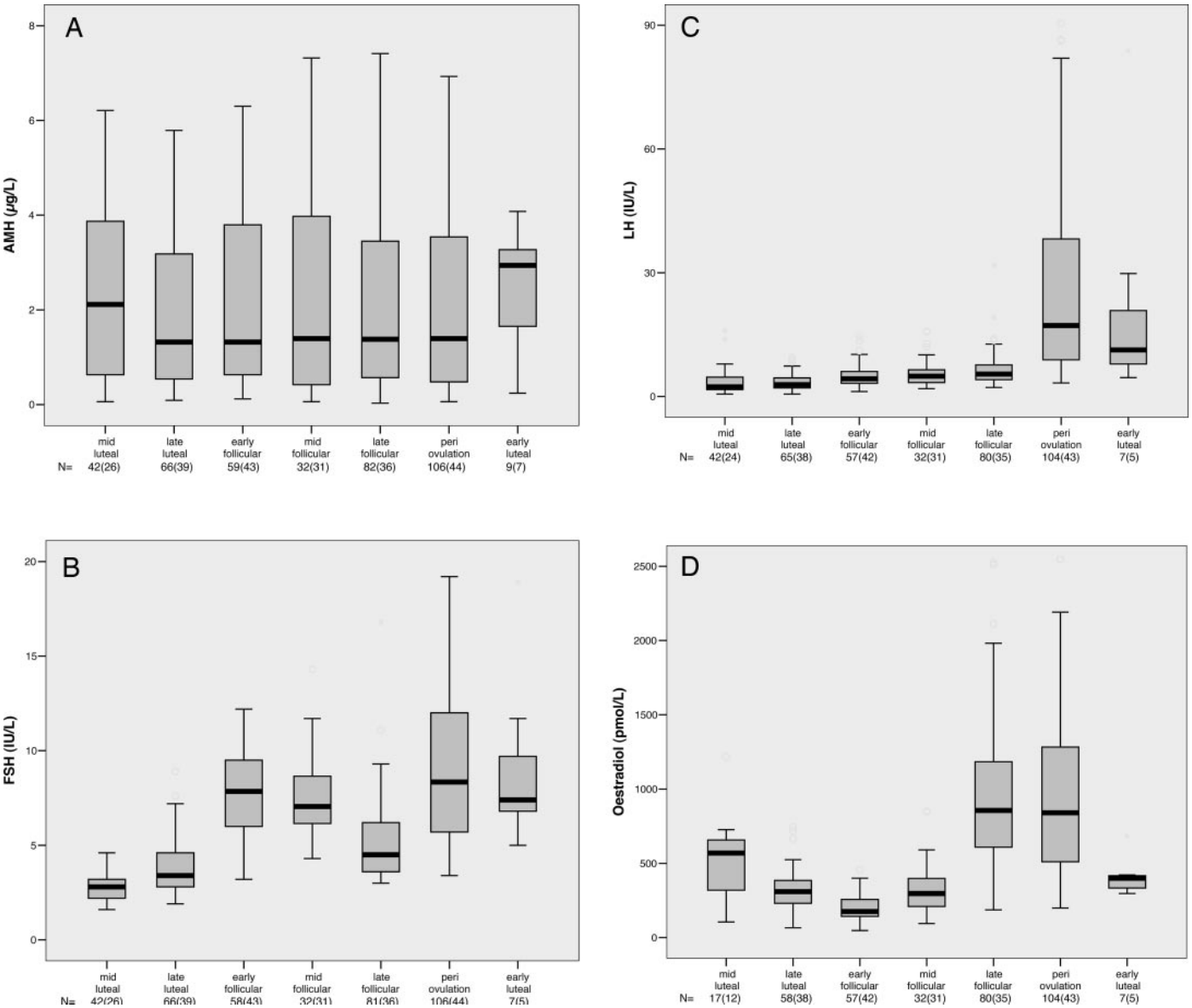

FIG. 1. AMH (A), FSH (B), LH (C), and estradiol (D) levels for each phase of the menstrual cycle. Data are given as box and whisker plots indicating the median (*bold line*), interquartile range (box), and 95% limits of all observations (whiskers). On the x-axis per phase the number of measurements and number of cases contributing (between brackets) are indicated.

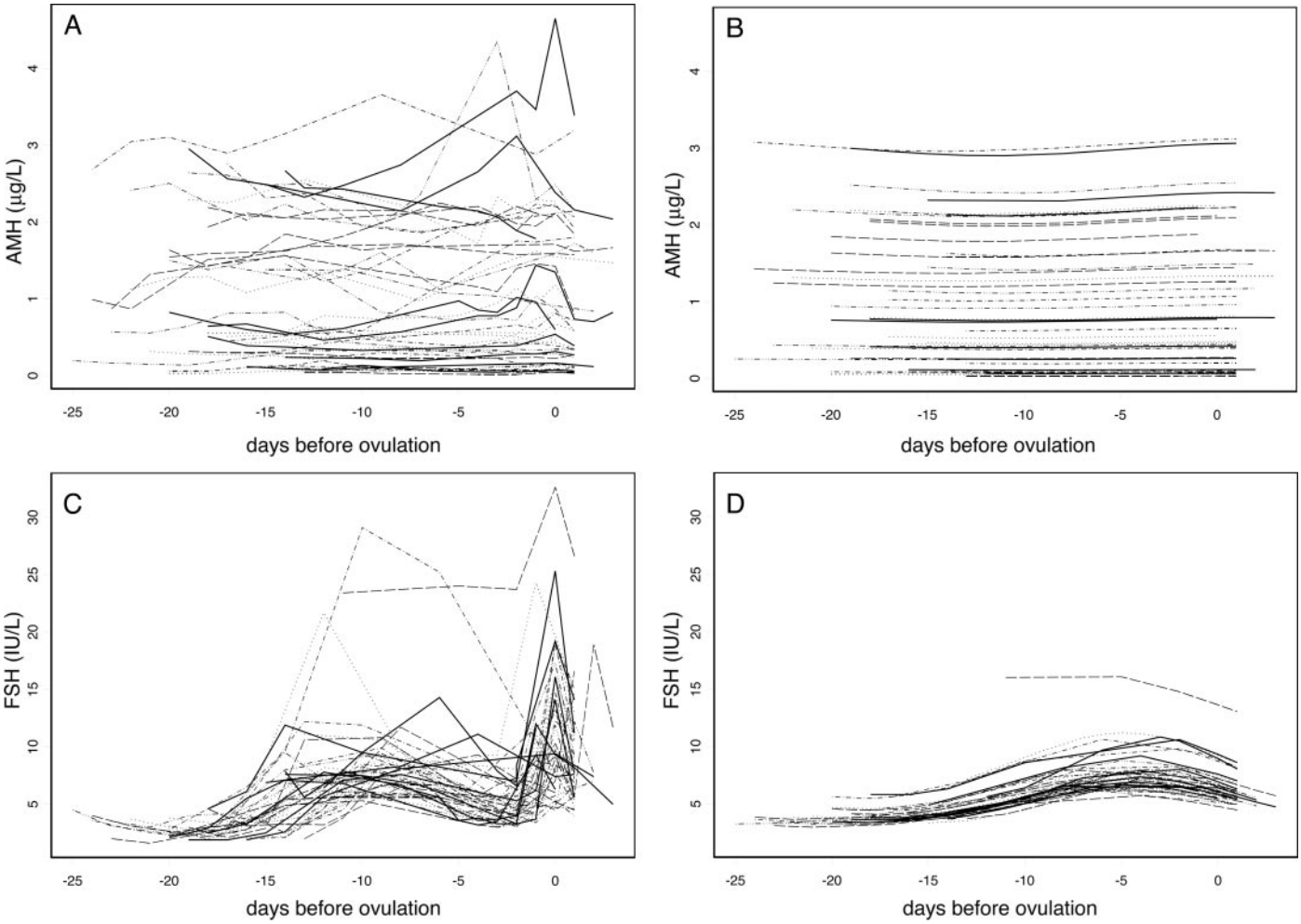

FIG. 2. A and C, Observed AMH and FSH levels per individual case. B and D, fitted values of AMH and FSH based on the trigonometric regression model. Each line represents one case. AMH and FSH values are represented on a logarithmic scale (y-axis). Zero represents the ovulation of the second cycle. The sine pattern is relative to the start of the cycle.

which AMH levels within the seven cycle phases relative to the overall mean AMH level per case are shown. ANOVA revealed that the differences between the cycle phase-specific AMH levels were of borderline significance ($P = 0.064$). This is reflected in the fact that the confidence interval for the difference from the mean in the periovulation phase and the early follicular phase did not include zero, whereas it did in the other five phases. The overall P value of 0.064 was corrected for the effects of multiple testing for all possible differences between cycle phases. For the early follicular phase, the mean AMH value was on average 9.2% (95% confidence interval, −16.6 to −1.1%) lower than the case-specific mean AMH level. For FSH, LH, and estradiol, the differences between the cycle phases showed to be highly significant (all < 0.0001). ANOVA showed that 95.5% of the variation of log (AMH) was due to the variance between woman-specific levels; 0.1% was due to a systematic pattern with the phase of the cycle, and 4.4% was left as random error (*i.e.* unexplained variance).

TABLE 1. Differences (%) across cycle phase-specific AMH levels relative to the mean case specific AMH (linear mixed-effect model) in comparison to FSH, LH, and estradiol (E2)

	AMH		FSH		LH		E2	
	Est. (%)	95% CI	Est. (%)	95% CI	Est. (%)	95% CI	Est. (%)	95% CI
Midluteal	−9.2	−16.6 to −1.1	−51	−55 to −45	−57	−64 to −49	4	−16 to 30
Late luteal	2.6	−4.3 to 10.0	−37	−42 to −31	−48	−56 to −40	−27	−36 to −17
Early follicular	5.3	−2.0 to 13.2	33	21 to 45	−27	−38 to −15	−54	−59 to −47
Midfollicular	−3.7	−12.1 to 5.5	29	16 to 45	−18	−33 to −0.4	−30	−40 to −17
Late follicular	−0.2	−6.6 to 6.7	−17	−23 to −9	2	−12 to 19	97	75 to 122
Periovulation	7.0	0.8 to 13.6	43	32 to 54	203	165 to 245	95	74 to 117
Early luteal	−0.9	−16.4 to 17.4	57	24 to 99	146	64 to 270	6	−24 to 47

The mean is taken as the mean of the seven estimates (on the log-scale), disregarding the length of each phase. Est., Estimate; CI, confidence interval.

Investigation of all possible combinations of two AMH samples per woman in this data set showed that in 71.5% of selections of AMH pairs, values remained in the same quintile category, whereas in 27.9% of pairs, one of the two values fell in an adjacent quintile category. In 0.6% of cases, one of the two sample values fell in a quintile category, which was two quintiles away from the original quintile category. For FSH only 23% of measurements remained in the same level quintile category, and 32% fell in the adjacent category, whereas 6% of measurements were even four quintiles apart. For LH this was 23, 33, and 7%, respectively, and for estradiol 21, 30, and 7%.

Analysis of all combinations of two AMH samples based on a clinically relevant cutoff level revealed that for the cutoff of 0.5 μg/liter, the percentage of values that remained in the original cutoff category was 92.0%.

Discussion

This study shows that AMH levels across the normal menstrual cycle do not follow consistent patterns of variation, indicating that the fluctuations observed are merely due to chance. The fact that AMH levels seem higher in the early luteal and midluteal phase (Fig. 1A) may be explained by the fact that these two phases represent the head and tail of the data acquisition, and several volunteers did not contribute to the build-up of data in these cycle phases. From Fig. 2 it becomes apparent that especially cases with lower AMH levels have not been adequately represented in these two phases. Furthermore, if within each case the deviation of the early luteal and midluteal AMH levels from the individual's mean level across the whole cycle is calculated, the mean change was nonsignificant for both cycle phases (Table 1). This indicates that AMH levels do not rise in the luteal phase.

For every cycle phase, the average deviation from the individual cycle mean is very modest and may reach a maximal value of 17.4% (Table 1). The multilevel analysis showed individual means of AMH to range from 0.05 to 2.92 (95% confidence interval). This implies that a certain woman can have a mean AMH level that is 50-fold higher than that of another woman. In the context of these differences between individuals, an intracycle deviation of maximally 17.4% can be considered to be relatively small. For two-cycle phases, the limits of the confidence intervals were just below or above zero (periovulatory and early follicular). Because the overall multilevel ANOVA appeared not to be significant, these deviations should be regarded as nonrelevant.

The data in this study show that AMH levels fail to follow the endocrine fluctuations that are so typical for the menstrual cycle. FSH, LH, and estradiol levels across the cycle showed fluctuations following consistent patterns that are known from the literature (27), showing that our methods of analysis of AMH patterns can be considered valid. One may wonder whether there is a biological basis for this contrast. In the human ovary, AMH is mainly produced by granulosa cells of small antral follicles sized 2–6 mm, but immunoreactive AMH can be detected already in the stage of early follicle development when follicles have left the primordial pool (5, 28). AMH levels therefore may well represent the size of the FSH-sensitive cohort of antral follicles. In contrast, the follicle selected to become dominant will show a reduced AMH production as it grows beyond 6–8 mm (5, 7). The early follicular rise of endogenous FSH that initiates the selection of the dominant follicle is not paralleled by a change in AMH. This is probably because the majority of the cohort will not reach the stage in which synthesis and production of AMH become halted (28). If the majority of follicles are driven into dominant growth, as in ovarian hyperstimulation for IVF, a clear reduction in AMH levels can be elicited, whereas the remaining numbers of small antral follicles continue to be correlated with AMH levels (7). In the normal cycle, as the development of the dominant follicle progresses, the remaining cohort will sustain the AMH production at a level that is proportional to its size.

During the midcycle gonadotropin surge that will lead to ovulation of the dominant follicle, no change in AMH production was observed, although profound changes in the steroidogenic function of the granulosa cells of the dominant follicle take place in this cycle phase. This seems to be in contrast to data from the study by Cook *et al.* (21) in which a small but significant rise in AMH levels in the periovulatory period was observed. In the present data, obtained in a larger study group applying more frequent blood sampling, the mean deviation from the average AMH level across the cycle per case was estimated to be 7.0% at periovulation and was not significant. When observing the individual AMH series (Fig. 2A), a periovulatory rise seems present in some of the cases with high AMH levels. From a clinical point of view, these minor fluctuations may not be very relevant because quintile analysis revealed that crossovers (*i.e.* more than one quintile away from the original) applied only to 0.6% of cases. Earlier reports have shown that AMH levels do not respond to an acute endogenous rise in LH and FSH produced by exposure to a pharmacological dosage of a GnRH agonist (16), in contrast to estradiol and inhibin B, suggesting that neither LH nor FSH acts as a stimulator of AMH production.

Although median AMH levels in the box plot in Fig. 1A were suggestive of a rising trend after ovulation, AMH levels appeared steady when taking into account that not all individuals contributed observations to all phases. This finding is in line with the observation that the cohort of small antral follicles does not change in size in the days after ovulation. Especially ultrasound data (29) have indicated that the midcycle gonadotropin rise does not alter the size or composition of the antral follicle cohort, although waves of growing follicles over 5 mm have been suggested as a result of peak FSH exposure (30). This wave phenomenon has been well described in other species like cattle (31, 32) but seems to be weakly supported by the human data. In contrast to the natural cycle, in ovarian hyperstimulation for IVF, the decline in AMH levels during stimulation is followed by a further decline after the human chorionic gonadotropin administration, suggesting that smaller follicles are driven into larger stages and decreasing their capacity to produce AMH (7).

From the analysis on repeated, randomly selected AMH values for each individual, it was shown that in 99% of cases in this population, AMH did not change more than one quintile category. This indicates that AMH used as a test in

which, for instance, a cutoff of 0.5 μg/liter is applied will produce cycle-independent values that correctly classify a patient as having or not having a normal quantitative ovarian reserve. Early follicular AMH has shown to be a promising indicator of ovarian reserve status, especially in the field of IVF. Ovarian response correlates well with AMH measurements, and prediction of poor response has shown to be adequate (18, 20). However, prediction of the occurrence of pregnancy from AMH levels has so far failed to be accurate (33), despite a recent promising report by Hazout *et al.* (20). In this study, however, cases with oligoovulation were included, and as such this study group may not represent the average IVF population. At present, therefore, the finding of a low AMH level in infertility patients judged suitable for IVF indicates the need to use other ovarian reserve markers like age, FSH, and antral follicle count to estimate whether indeed severely decreased ovarian reserve is present. If so, counseling on poor prognosis is advocated, but the application of a trial cycle to observe the ovarian response to maximal gonadotropin stimulation may be justified to confirm the poor prospects for the patient. Whether repeat measurements of AMH in separate cycles will provide additional predictive value for the clinical condition of diminished ovarian reserve is currently not known. In a recent study (34), however, it was shown that cycle-to-cycle stability of the AMH level is very high, compared with other ovarian reserve tests. Also, repeat assessments for other tests have shown to be not clearly informative (35–38).

In summary, our data indicate that within the normal menstrual cycle, variability of AMH levels is extremely small. Therefore, a single measurement of AMH obtained at any time in the menstrual cycle may be considered a valid reflection of a woman's ovarian reserve.

Acknowledgments

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