

Biological variability in serum anti-Müllerian hormone throughout the menstrual cycle in ovulatory and sporadic anovulatory cycles in eumenorrheic women

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STUDY QUESTION: Does serum anti-Müllerian hormone (AMH) vary significantly throughout both ovulatory and sporadic anovulatory menstrual cycles in healthy premenopausal women?

SUMMARY ANSWER: Serum AMH levels vary statistically significantly across the menstrual cycle in both ovulatory and sporadic anovulatory cycles of healthy eumenorrheic women.

WHAT IS KNOWN ALREADY: Studies to date evaluating serum AMH levels throughout the menstrual cycle have conflicting results regarding intra-woman cyclicity. No previous studies have evaluated an association between AMH and sporadic anovulation.

STUDY DESIGN, SIZE, DURATION: We conducted a prospective cohort study of 259 regularly menstruating women recruited between 2005 and 2007.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Women aged 18–44 years were followed for one ($n = 9$) or two ($n = 250$) menstrual cycles. Anovulatory cycles were defined as any cycle with peak progesterone concentration ≤ 5 ng/ml and no serum LH peak on the mid or late luteal visits. Serum AMH was measured at up to eight-time points throughout each cycle.

MAIN RESULTS AND THE ROLE OF CHANCE: Geometric mean AMH levels were observed to vary across the menstrual cycle ($P < 0.01$) with the highest levels observed during the mid-follicular phase at 2.06 ng/ml, decreasing around the time of ovulation to 1.79 ng/ml and increasing thereafter to 1.93 (mid-follicular versus ovulation, $P < 0.01$; ovulation versus late luteal, $P = 0.01$; mid-follicular versus late luteal, $P = 0.05$). Patterns were similar across all age groups and during ovulatory and anovulatory cycles, with higher levels of AMH observed among women with one or more anovulatory cycles ($P = 0.03$).

LIMITATIONS, REASONS FOR CAUTION: Ovulatory status was not verified by direct visualization. AMH was analyzed using the original Generation II enzymatically amplified two-site immunoassay, which has been shown to be susceptible to assay interference. Thus, absolute levels should be interpreted with caution, however, patterns and associations remain consistent and any potential bias would be non-differential.

WIDER IMPLICATIONS OF THE FINDINGS: This study demonstrates a significant variation in serum AMH levels across the menstrual cycle regardless of ovulatory status. This variability, although statistically significant, is not large enough to warrant a change in current clinical practice to time AMH measurements to cycle day/phase.

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Key words: menstrual cycle / anovulation / anti-Müllerian hormone

Introduction

Anti-Müllerian hormone (AMH) has been widely studied as a predictor of the quantity and quality of the ovarian follicle pool (Nardo *et al.*, 2009; Nelson *et al.*, 2009; Domingues *et al.*, 2010; Jayaprakasan *et al.*, 2010; Ledger, 2010; Dólleman *et al.*, 2013; Broer *et al.*, 2013). Recently, AMH has also been recognized as a surrogate marker for the traditional ovarian ultrasound markers of the common ovulatory disorder, polycystic ovary syndrome (PCOS) (Pigny *et al.*, 2006; Pawelczak *et al.*, 2012), as well as a clinically useful tool in diagnosing and monitoring ovarian granulosa cell tumors (La Marca *et al.*, 2007; Chong *et al.*, 2012), further highlighting the extensive use of AMH in the clinical management of female reproductive health.

The use of AMH as an ideal marker for ovarian reserve is appealing not only because of its clinically non-invasive ease of detection, but also more importantly based on the premise that serum AMH does not vary throughout the menstrual cycle. However, findings from previous studies in regards to AMH variability across the menstrual cycle in eumenorrhic women have been conflicting (Cook *et al.*, 2000; La Marca *et al.*, 2004, 2006; Hehenkamp *et al.*, 2006; Tsepelidis *et al.*, 2007; Wunder *et al.*, 2008; Streuli *et al.*, 2009; Sowers *et al.*, 2010; Zec *et al.*, 2010; Robertson *et al.*, 2011; Overbeek *et al.*, 2012; Hadlow *et al.*, 2010; Randolph *et al.*, 2014). Existing studies evaluating AMH across the menstrual cycle are limited by number of participants ($n \leq 44$), lack of adequate number of measurements across more than a single menstrual cycle, determinations of cycle phase based on time alone or other means besides utilizing validated markers of ovulation and inherent differences in the AMH assay used or in the pre- and post-assay procedures, including collection, storage and freeze/thaw process. Notably, no studies to our knowledge examined the association between sporadic anovulation and serum AMH levels among regularly menstruating women without clinically identified gynecologic or ovarian disorders.

Given the widespread clinical use of serum AMH as a marker of ovarian reserve regardless of the cycle day in which the measurement is taken, further evaluation of AMH variability across the menstrual cycle is needed. Moreover, elucidation of an association between serum AMH levels and sporadic anovulation has the potential to provide insight into the mechanism of pathologic ovarian processes involving anovulation. We, therefore, assessed the variability of AMH across the menstrual cycle as well as its association with sporadic anovulation in a cohort of healthy, premenopausal women in the BioCycle Study.

Materials and Methods

Study population and design

Conducted in 2005–2007, the BioCycle Study followed 259 healthy premenopausal women, aged 18–44 years from Buffalo, NY, USA, for one ($n = 9$) or two ($n = 250$) menstrual cycles. The study population, materials and

methods have been previously described in detail (Wactawski-Wende *et al.*, 2009). Following informed consent, women were provided a questionnaire to determine study eligibility based on inclusion and exclusion criteria. Detailed questions on menstrual and reproductive history were administered to all potential subjects for eligibility purposes. Only women with a self-reported menstrual cycle length between 21 and 35 days for each menstrual cycle in the preceding 6 months without evidence of oligo, poly, or amenorrhea or metromenorrhagia as identified by screening questionnaire were included. Exclusion criteria included use of depo-provera, norplant or intrauterine device in the preceding 12 months, and oral contraceptive or other hormone supplement in the preceding 3 months prior to study enrollment. Women with conditions known to affect menstrual cycle function such as laparoscopic confirmed endometriosis of any stage, PCOS, uterine fibroids, a self-reported BMI < 18 or > 35 kg/m² at time of screening (based on standard cut-points for underweight and class II obesity), a recent history of alcohol abuse or illicit drug use, a soy-based diet and future plans to restrict diet were also excluded. Pregnancy, breast feeding or report of actively trying to conceive in the previous 6 months or plans to attempt to conceive in the next 3 months were also cause for exclusion. Lastly, women with a history of chronic diseases, such as heart disease, inflammatory or autoimmune diseases, diabetes mellitus, thyroid disease or any other endocrine dysfunction, were excluded.

Study visits and data collection

Participants provided fasting blood specimens on up to eight visits per cycle for two menstrual cycles, with visits timed using fertility monitors (FMs) to correspond to menstruation (visit 1), mid-follicular (visit 2), late follicular (visit 3), LH surge (visit 4), ovulation (visit 5), and early (visit 6), mid (visit 7) and late (visit 8) luteal phase. Study visits were scheduled to occur during specific phases of the menstrual cycle using an algorithm accounting for each woman's self-reported cycle length, with the day of the mid-cycle visits adjusted based on FM data (Mumford *et al.*, 2011). FMs (Clearblue® Easy Fertility Monitor, Inverness Medical, Waltham, MA, USA) measured both urinary estrone-3-glucuronide and LH, and have been shown previously to improve menstrual cycle standardization (Howards *et al.*, 2009).

Covariate assessment

At study enrollment, height and weight were measured using standardized protocols by trained study staff to obtain BMI, while age, race, smoking, physical activity, past medical and reproductive history were obtained using validated questionnaires (Craig *et al.*, 2002; Gaskins *et al.*, 2009). No covariate was missing for $> 5\%$ of women. Cycle length was defined as the number of days from the first day of bleeding (menstruating by 1600 h) until the day before the next onset of bleeding after confirming two consecutive days of bleeding (Howards *et al.*, 2009; Mumford *et al.*, 2011). Anovulatory cycles were defined as any cycle with peak progesterone concentration ≤ 5 ng/ml and no serum LH peak on the mid or late luteal visits (Gaskins *et al.*, 2009; Howards *et al.*, 2009).

Laboratory assays

Fasting morning blood draws were scheduled between 0700 and 0830 with immediate processing and freezing at -80°C within 90 min of collection.

Samples were shipped on dry ice to analytical laboratories as a complete cycle batch (up to eight samples) from each participant. Samples were measured consecutively, within a single run, to limit analytical variability. Estradiol (E_2), progesterone, LH and FSH were measured at the Kaleida Health Center for Laboratory Medicine (Buffalo, NY, USA) using solid-phase competitive chemiluminescent enzymatic immunoassay by Specialty Laboratories, Inc. (Valencia, CA, USA) on the Diagnostics Product Corporation Immulite 2000 Analyzer (Siemens Medical Solutions Diagnostics, Deerfield, IL, USA). Inter-assay coefficients of variation (CV) were $<10\%$ for E_2 , $<14\%$ for progesterone and $<4\%$ for LH and FSH.

Stored serum (at -80°C) was later analyzed using Generation II enzymatically amplified two-site immunoassay (Beckman Coulter, Brea, CA, USA) by the Advanced Research and Diagnostic Laboratory of Minnesota, Minneapolis, MN, USA, to determine AMH and inhibin B levels. Specifically for AMH assay determination, controls, calibrators and assays were incubated in anti-AMH antibody coated wells, after which biotin labeled anti-AMH antibody was added for a second incubation followed by washing and a third incubation with streptavidin–horseradish peroxidase. Lastly, tetramethylbenzidine substrate was added to the wells followed by an acidic stopping solution. The degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 nm and between 600 and 630 nm. The reported concentration of AMH was directly proportional to the absorbance measured. To reduce measurement error, select batches of measurements were then recalibrated post-assay by a calibration curve estimated from all the calibration data (Whitcomb et al., 2010). Using recalibrated data, the inter-assay CVs of AMH and inhibin B were <12.0 and $<9.1\%$, respectively, while intra-assay CVs were 1.6 and 1.8%, respectively. The original lower limit of detection for AMH prior to recalibration was 0.103 ng/ml and after recalibration and utilizing machine observed values reduced to 0.001 ng/ml (Whitcomb et al., 2010).

Statistical analysis

Descriptive statistics were calculated for demographic characteristics. Fisher's exact test and the Kruskal–Wallis test were used to test for associations between demographic variables and tertiles of mean serum AMH (each averaged across up to 16 visits per woman). Percentage of anovulatory cycles, and mean hormone levels across the cycle, and by cycle phase, were compared between tertiles of mean AMH per cycle, accounting for multiple cycles per woman. Linear mixed effect models were used to explore the variation of AMH over the menstrual cycle to account for both between-women variation and within-woman cycle correlation. Geometric mean (GM) AMH levels were assessed across phases of the menstrual cycle overall and by age category (≤ 20 , 21–25, 26–30, 31–35, >35 years). GMs were compared between the late follicular phase (visit 3) and expected ovulation (visit 5) visits and P -values calculated to assess statistical significance during the peri-ovulatory period. The mean of the maximum observed difference in AMH across the cycle was calculated by averaging the difference between the maximum and minimum AMH level observed during each cycle, and was compared across age categories. All AMH related results were adjusted for age. Additional covariates, including years since menarche, marital status, nulliparity and past hormonal contraceptive use were considered but did not appreciably alter the estimates. Based on common serum AMH levels used to clinically identify low ovarian reserve, the percentage of women with AMH levels ≤ 0.3 or <1.0 ng/ml were calculated for each cycle phase and compared across the cycle (Tremellen et al., 2005; Nardo et al., 2009; Hadlow et al., 2010; Blazar et al., 2011; Fridén et al., 2011; Celik et al., 2013). AMH levels were also categorized by ovulatory status (ovulatory cycles of women with only ovulatory cycles observed ($n = 443$ cycles), ovulatory cycles of women with 1 ovulatory and 1 anovulatory cycle ($n = 24$ cycles) and all anovulatory cycles ($n = 42$ cycles; 28 women had 1 observed anovulatory cycle and 7 women had 2 anovulatory cycles

during the study)). Statistical analyses were conducted using SAS 9.3 (SAS Institute, Cary, NC, USA).

Ethical approval

The University at Buffalo Health Sciences Institutional Review Board (IRB) approved the study, and served as the IRB designated by the National Institutes of Health for this study under a reliance agreement. All participants provided written informed consent (Wactawski-Wende et al., 2009).

Results

Women in the BioCycle Study cohort were on average 27.3 years of age (SD 8.2), with an average BMI of 24.1 kg/m^2 (SD 3.9), predominantly Caucasian (60%), non-married (75%), nulliparous (72%) and with history of past use of hormonal contraception (54%) (Table I). Women in the highest AMH tertile were younger on average. Factors positively associated with AMH included years since menarche, non-married marital status and nulliparity. AMH levels were not associated with BMI, smoking status or educational status. Past hormonal contraceptive use was negatively associated with AMH.

Mean overall and luteal phase progesterone levels as well as mean overall, follicular and luteal phase FSH levels were lower with increasing AMH tertile (Table II). The mean LH/FSH ratio and menstrual cycle length were positively associated with AMH tertile. Mean overall, follicular phase and luteal phase inhibin B were positively associated with AMH. We observed the highest mean E_2 levels in the middle AMH tertile, while follicular phase E_2 was negatively associated with increasing AMH tertile. A higher percentage of anovulatory cycles were observed in the highest AMH tertile.

AMH was observed to vary significantly across the menstrual cycle ($P < 0.01$) with the highest levels observed during the mid-follicular phase at 2.06 ng/ml (95% confidence interval (CI): 1.83, 2.34), decreasing immediately prior to expected time of ovulation to 1.79 ng/ml (95% CI: 1.58, 2.02); and increasing throughout the rest of the luteal phase to 1.93 (95% CI: 1.72, 2.17) (mid-follicular versus ovulation, $P < 0.01$; ovulation versus late luteal, $P = 0.01$; mid-follicular versus late luteal, $P = 0.05$) (Fig. 1). A similar pattern was observed within each age group (Fig. 2).

The mean of the maximum observed change in serum AMH levels overall was 1.30 ng/ml, and varied by age category, with a value of 1.73 ng/ml (95% CI: 0.99, 1.55) and 0.61 ng/ml (95% CI: 0.43, 0.52) in the ≤ 20 year old age group and the ≥ 35 year old group ($P < 0.01$), respectively. For every year increase in age, the average change in AMH across the menstrual cycle decreased by ~ 0.05 ng/ml (95% CI: 0.04, 0.07).

Overall, 25 (9.65%) women had serum AMH levels ≤ 0.3 ng/ml during at least one visit. Eighteen women (6.95%) had consistently low AMH levels across the cycle, while seven (2.7%) crossed this cut-off at least once throughout the menstrual cycle. Forty-four (17%) women had values consistently below a higher range cut-off of 1.0 ng/ml, while 24 (9.2%) crossed this level at least once during their menstrual cycle.

A similar pattern of variability in AMH across the menstrual cycle was observed during both ovulatory and anovulatory cycles (Fig. 3). After adjustment for age, the GM AMH levels of anovulatory cycles were significantly higher than ovulatory cycles. Moreover, GM AMH levels were lowest during the ovulatory cycles of women with only ovulatory

Table 1 Baseline characteristics of 259 healthy, premenopausal women from the BioCycle Study per tertile of AMH.

	Total cohort	Mean AMH (tertiles) ^a			P-value ^b
		≤1.87 ng/ml	1.88–3.61 ng/ml	>3.61 ng/ml	
n (number of women)	259	86	86	87	
Demographics mean ± SD					
Age, years	27.3 ± 8.2	33.1 ± 1.2	26.1 ± 7.5	22.7 ± 8.2	<0.001
Age at menarche, years	12.5 ± 1.2	12.5 ± 1.2	12.1 ± 1.3	12.7 ± 1.2	0.01
Years since menarche	14.9 ± 8.3	20.5 ± 8.6	14.1 ± 7.5	10.0 ± 4.3	<0.001
BMI, kg/m ²	24.1 ± 3.9	24.5 ± 4.0	24.2 ± 3.4	23.5 ± 4.1	0.11
n (%)					
Race					
White	154 (59.5)	58 (67.4)	46 (53.5)	50 (57.5)	0.34
Black	51 (19.7)	12 (14.0)	19 (22.1)	20 (23.0)	
Other	54 (20.8)	16 (18.6)	21 (24.4)	17 (19.5)	
≤High school education	33 (12.7)	14 (16.3)	8 (9.3)	11 (12.6)	0.39
Married	66 (25.5)	43 (50.0)	15 (17.4)	8 (9.2)	<0.001
Nulliparous	187 (72.2)	44 (51.2)	66 (76.7)	77 (88.5)	<0.001
Current smoker	10 (3.9)	2 (2.3)	4 (4.7)	4 (4.6)	0.78
Past HC use	140 (54.1)	62 (72.1)	42 (48.8)	36 (41.4)	<0.001

HC, hormonal contraceptive.

^aWomen were categorized according to tertile of mean AMH and across all visits for up to two menstrual cycles.

^bThe Kruskal–Wallis test (a non-parametric version of analysis of variance) is used for the continuous variables, and Fisher's exact test for categorical variables.

cycles observed ($P = 0.02$) (Fig. 3). Mean serum AMH levels between anovulatory cycles of women with at least one anovulatory cycle and ovulatory cycles of women with at least one anovulatory cycle were not significantly different.

Discussion

Our findings demonstrate that AMH concentrations significantly vary throughout the menstrual cycle in healthy eumenorrhic women. The fluctuations in AMH over the menstrual cycle were observed during both ovulatory and anovulatory cycles and were consistent across all age groups, though the variability in AMH declined with increasing age. Moreover, higher AMH levels were observed during anovulatory cycles, as well as during the ovulatory cycles of women who experienced an anovulatory cycle during the study period. The variation in serum AMH we observed was not large enough to warrant a change in current clinical practice to time AMH measurement to menstrual cycle phase as a measure of ovarian reserve. However, the statistically significant change across the menstrual cycle supports the complex endocrine and paracrine actions of AMH involved in female reproductive physiology, while highlighting a need for greater understanding of AMH in normal follicle development and selection.

Variability of AMH across the menstrual cycle in premenopausal healthy women has been evaluated in previous epidemiological and case studies. Although the timing of peak and nadir serum AMH across the menstrual cycle, pattern of variation, as well as statistical significance was inconsistent between them, nine previous studies have demonstrated some degree of variation in AMH across the menstrual cycle in regularly menstruating women (Cook *et al.*, 2000; Wunder *et al.*,

2008; Streuli *et al.*, 2009; Hadlow *et al.*, 2010; Sowers *et al.*, 2010; Zec *et al.*, 2010; Robertson *et al.*, 2011; Overbeek *et al.*, 2012; Randolph *et al.*, 2014). Previously demonstrated fluctuations of serum AMH have ranged from highest levels in mid-follicular phase with a start of decline prior to serum E₂ rise and lowest levels in early luteal phase (Wunder *et al.*, 2008) to serum elevations seen only at time of ovulation (Cook *et al.*, 2000). Recently, Randolph *et al.* (2014) demonstrated a biphasic pattern in a group of 20 healthy premenopausal women, with an elevation and depression in both follicular and luteal phases. Others have found serum AMH to both steadily rise and decline throughout the entire luteal phase in premenopausal women (Robertson *et al.*, 2011). After categorizing daily serum AMH levels into four quartiles based on area under the curve, Sowers *et al.* (2010) described a pattern of ovarian aging not consistent with chronological age in 20 regularly menstruating women aged 30–40 years. The younger ovarian pattern described with a mid-follicular phase peak in serum AMH is in agreement with our findings. The aging ovarian pattern is also seemingly consistent with the chronologically older women in our study; however, after adjusting for age in our large cohort, we were able to determine that the pattern of variation is both consistent with those of younger age categories and significant. We observed serum AMH to peak during the mid-follicular phase and reach nadir peri-ovulation, with a subsequent incline throughout the remaining luteal phase. The discrepancy between the aforementioned studies and ours is not only the size of cohort studied, but also the result of less frequent monitoring of AMH across the cycle, and the use of other modalities to time menstrual cycle phase/day. Moreover, as outlined in a recent critical review of AMH assays, existing studies varied in serum sample collection, processing and storage prior to AMH analysis as well as the AMH assay utilized, not only in the manufacturer of the

Table II Mean reproductive hormones and menstrual cycle characteristics in healthy, premenopausal women by tertile of mean AMH by cycle.

	Total cohort	Mean AMH (tertiles) ^a			P-value ^b
		≤1.87 ng/ml	1.88–3.61 ng/ml	>3.61 ng/ml	
n (number of cycles) ^b	509	178	170	161	
Mean ± SD					
Cycle length, days	28.8 ± 4.1	27.1 ± 3.2	29.2 ± 4.0	30.4 ± 4.5	<0.001
Inhibin B (pg/ml) ^c	69.3 ± 40.6	52.2 ± 34.9	70.2 ± 27.7	87.2 ± 49.2	<0.001
Follicular phase	86.6 ± 46.0	70.0 ± 39.0	86.6 ± 29.3	105.0 ± 58.9	<0.001
Luteal phase	50.2 ± 46.8	32.9 ± 36.3	51.4 ± 41.7	68.2 ± 54.7	<0.001
LH (ng/ml) ^c	9.5 ± 3.6	8.9 ± 3.4	9.9 ± 3.8	9.7 ± 3.6	0.032
Follicular phase	10.1 ± 5.5	10.0 ± 5.4	10.3 ± 5.2	10.0 ± 6.1	0.50
Luteal phase	8.8 ± 5.8	7.6 ± 5.3	9.5 ± 6.9	9.4 ± 5.0	<0.001
FSH (mIU/ml) ^c	6.4 ± 2.4	7.6 ± 3.1	5.8 ± 1.4	5.6 ± 1.7	<0.001
Follicular phase	7.4 ± 3.1	8.9 ± 4.2	6.7 ± 1.7	6.5 ± 2.1	<0.001
Luteal phase	8.8 ± 5.8	6.2 ± 2.8	4.8 ± 2.1	4.7 ± 2.0	<0.001
Estradiol (pg/ml) ^c	112.0 ± 39.6	113.5 ± 39.6	116.8 ± 45.4	105.3 ± 44.6	0.044
Follicular phase	102.5 ± 51.7	109.8 ± 46.7	106.5 ± 55.3	90.2 ± 51.2	<0.001
Luteal phase	122.1 ± 54.7	117.3 ± 54.2	128.3 ± 54.0	120.8 ± 55.5	0.081
Progesterone (ng/ml) ^c	3.4 ± 1.8	3.7 ± 1.5	3.5 ± 1.7	3.0 ± 1.9	<0.001
Follicular phase	0.9 ± 1.0	1.0 ± 1.1	1.0 ± 0.9	0.9 ± 0.8	0.36
Luteal phase	6.1 ± 3.2	6.7 ± 2.7	6.4 ± 3.3	5.1 ± 3.5	<0.001
Other					
LH/FSH ratio ^d	1.5 ± 0.6	1.2 ± 0.5	1.6 ± 0.6	1.7 ± 0.5	<0.001
Peak LH ^e	31.5 ± 18.4	31.2 ± 17.0	33.4 ± 18.8	29.7 ± 19.4	0.08
n (%)					
Anovulatory ^f	42 (8.3)	5 (2.8)	10 (5.9)	27 (16.8)	<0.001

^aAll available cycles (n = 509) from the cohort of 259 women were categorized according to tertile of mean AMH per cycle.

^bTwo hundred fifty women in the BioCycle study were followed for 2 cycles, 9 women were followed for 1 cycle, for a total of 509 cycles.

^cMean inhibin B, LH, FSH, estradiol and progesterone were taken across all visits per cycle for up to two menstrual cycles.

^dLH/FSH ratio is taken as mean LH across all visits per cycle for up to two menstrual cycles and divided by the mean FSH across all visits per cycle for up to two menstrual cycles.

^ePeak LH is the mean peak LH across the entire menstrual cycle.

^fTwenty-eight women in the study had only 1 anovulatory cycle, and 7 women in the study had 2 anovulatory cycles for a total of 35 women with at least 1 anovulatory cycle. There were a total of 42 anovulatory cycles out of a total 509 in the BioCycle Study.

assay, but also in generation of assay from the same manufacturer (Rustamov et al., 2014). Ultimately, previous studies evaluating healthy regularly women were small (n < 44) and lacked adequate power to detect statistically and biologically significant variability in serum AMH throughout a menstrual cycle (Cook et al., 2000; La Marca et al., 2004, 2006; Hehenkamp et al., 2006; Tsepelidis et al., 2007; Wunder et al., 2008; Streuli et al., 2009; Hadlow et al., 2010; Sowers et al., 2010; Zec et al., 2010; Robertson et al., 2011; Overbeek et al., 2012; Randolph et al., 2014).

The fluctuations in serum AMH we observed across the cycle, with AMH levels beginning to decline during the peri-ovulatory period prior to E₂ peak, are consistent with the known inhibitory effect of AMH on the sensitivity of growing follicles to FSH. Sensitivity to FSH in normal follicles lowers the expression of AMH from granulosa cells, allowing E₂ secretion from selected follicles that have escaped AMH inhibition (Son et al., 2011; Dewailly et al. 2014). After serum AMH starts to decline, serum E₂ rises. As previously demonstrated, the nadir of AMH

expression coincides with the greatest follicular growth (>8 mm in size), enabling selection of a dominant follicle prior to ovulation (Jeppesen et al., 2013). Not only did the lowest serum AMH occur mid-cycle, but the greatest decline in serum AMH, as expressed by the mean of the maximum observed difference, corresponded to study visits correlating with dominant follicle selection and ovulation, respectively. Throughout the remaining luteal phase, serum AMH levels rose sequentially, likely representing gonadotrophin independent growth of pre-antral and small antral follicles (Son et al., 2011; Jeppesen et al., 2013).

The mean of the maximum observed difference in AMH across the cycle was higher than previous studies demonstrating variation. In existing studies, the mean of the maximum observed change ranged from 0.29 ng/ml in a population of 35 women aged 18–45 years, using a diagnostic systems laboratories (DSLs) AMH enzyme-linked immunoassay (ELISA) on serum stored at –20°C (Zec et al., 2010) to 0.65 ng/ml in a population of 36 women aged 20–32 years, using a DSL ELISA to analyze serum stored at –80°C (Wunder et al., 2008), in comparison

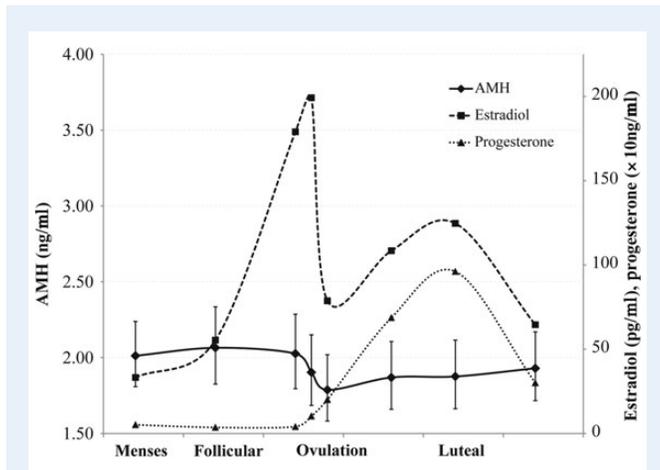


Figure 1 Geometric mean AMH levels across the menstrual cycle by ovulatory category, adjusted for age. AMH levels from ovulatory cycles of women with only ovulatory cycles were significantly different from both of the other groups with anovulatory cycles ($P = 0.02$). No significant difference between anovulatory cycles from women with both observed cycles being anovulatory and anovulatory cycles from women with one observed anovulatory cycle and one observed ovulatory cycle were seen. There were 443 ovulatory cycles from 224 women with ovulatory cycles only, 24 ovulatory cycles from 24 women with 1 ovulatory and 1 anovulatory cycle and 42 anovulatory cycles from 35 women with any anovulatory cycle (total of 509 cycles from 259 women).

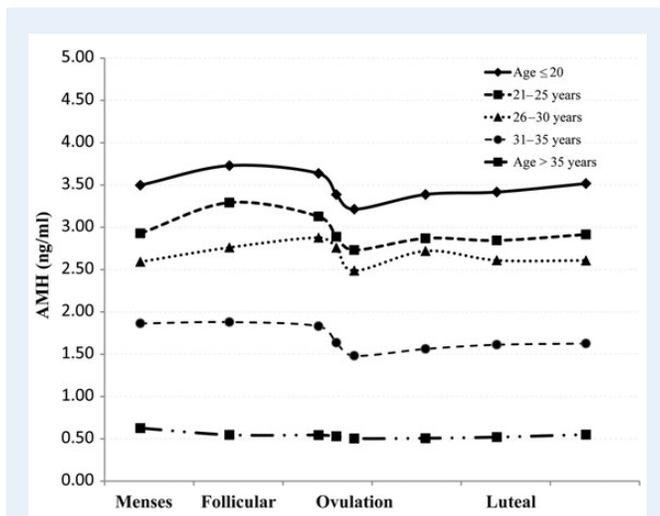


Figure 2 Geometric mean AMH across the menstrual cycle in eumenorrhic women by age category. $P < 0.05$ for mid-cycle comparisons (visit 3 versus visit 5) for AMH levels for all age groups (years). Age ≤ 20 years: $n = 73$ women, 20–25 years: $n = 71$ women, 26–30 years: $n = 33$ women, 31–35 years: $n = 20$ women, > 35 years: $n = 62$ women. The n values reflect women in each age category; each woman contributes up to 2 cycles at each of the 8-cycle visits for a total of up to 16 possible measurements of serum AMH across the 2 menstrual cycles.

with an average of 1.30 ng/ml in our study. The mean of the maximum observed difference was found to be greater in younger women, which is consistent with a re-analysis by Overbeek *et al.* (2012). A limitation

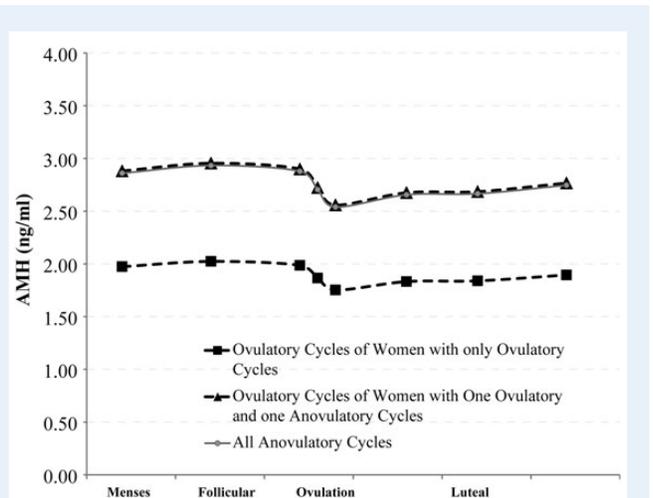


Figure 3 Geometric mean AMH levels across the menstrual cycle by ovulatory category, adjusted for age. AMH: ovulatory cycles of women with only ovulatory cycles were significantly different from both of the other groups ($P = 0.02$). No significant differences between ovulatory cycles of women with anovulatory cycles and anovulatory cycles. There were 443 ovulatory cycles from 224 women with ovulatory cycles only, 24 ovulatory cycles from 24 women with 1 ovulatory and 1 anovulatory cycle and 42 anovulatory cycles from 35 women with any anovulatory cycle (total of 509 cycles from 259 women).

in these comparisons is inherent discrepancies in the assays used to analyze AMH (Rustamov *et al.*, 2012; Rustamov *et al.*, 2014) including potential complement interference in the Beckman Coulter assay (Han *et al.*, 2014, Clark *et al.*, 2014, Dewailly *et al.*, 2014).

While the variability in serum AMH throughout the menstrual cycle we demonstrated is statistically significant and has biological implications, it is not likely large enough to warrant a clinically significant change in ovarian reserve classification for several reasons. Our study included 25 women who would have been classified as having diminished ovarian reserve on at least one of their cycle visits based on an AMH cut-off of 0.3 ng/ml, which has been shown to have a sensitivity and specificity of 71.4 and 66.67%, respectively, for the diagnosis (Celik *et al.*, 2013). Only 7 (2.7%) women would have been classified differently depending on phase of cycle measurement. Given the established literature on the range of cut-offs around 1.0 ng/ml indicating poor ovarian reserve and the lack of significant variation in the number of women crossing the cut-off between 0.896 and 1.3 ng/ml (Tremellen *et al.*, 2005, Nardo *et al.*, 2009; Nelson *et al.*, 2009; Blazar *et al.*, 2011; Fridén *et al.*, 2011), we also evaluated the change in characterization of diminished ovarian reserve based on a cut-off of 1.0 ng/ml. Using this higher cut-off, only 24 (9.2%) women would have been misclassified based on day of measurement. This speaks to the clinical insignificance in the degree of AMH variability and lends greater credence to its current clinical measurements irrespective of cycle day or phase.

Our study is the first to demonstrate an association between systemic AMH levels and sporadic anovulation in healthy women with no underlying gynecologic or clinically recognized ovarian disorders. Like previous studies demonstrating higher serum AMH concentrations from women with known anovulatory PCOS (Pigny *et al.*, 2003), we observed women with sporadic anovulatory cycles to have elevated AMH levels compared

with ovulatory women. The mechanism by which premenopausal women with PCOS have elevated systemic AMH remains elusive. However, the inhibitory effect of AMH, and perhaps more so, the altered opposing actions of FSH and AMH on E₂ expression and dominant follicle development is thought to play a substantial role in the pathophysiology of anovulation (Jonard et al., 2004). Healthy premenopausal women with elevated systemic basal levels of AMH may be at risk for an inhibitory endocrine effect from systemic AMH elevation or a disruption in the available intra-follicular AMH resulting in impaired ovulation.

The pattern of menstrual cycle AMH variability observed was similar between ovulatory and anovulatory cycles. The peri-ovulatory decline in serum AMH was unexpected in anovulatory cycles given the mechanism expanded upon above (Son et al., 2011; Jeppesen et al., 2013; Dewailly et al. 2014). The consistency of the mid-follicular phase to the mid-cycle decline in serum AMH despite anovulatory status could be a reflection of the difference between systemic and local ovarian milieu during any given menstrual cycle or an indication of a potential benign phenotype of anovulation in this cohort of healthy women.

The many strengths of this study included a large sample size, frequent sampling of AMH and other biomarkers across two menstrual cycles using standardized procedures to time menstrual phase accurately. This is the largest study to date to demonstrate a significant biological variation in AMH throughout the menstrual cycle regardless of ovulatory status. We recognized the potential for error in the absolute values of AMH based on possible complement interference and performed a sensitivity analysis taking into consideration the maximum possible assay interference in the study samples (the 'worst case scenario'). The statistical significance of all associations made herein either remained the same or were strengthened, including the pattern of AMH variability observed across the cycle in both ovulatory and anovulatory cycles. However, we are limited in evaluating and comparing absolute values. Despite a recent study showing comparable serum AMH values between assays available through different manufacturers and an assay available through Beckman Coulter (Randolph et al., 2014) and more data reinforcing the reproducibility of serum AMH levels (Fleming and Nelson 2012), the mean of the maximum observed difference and percentage of values below previously established cut-offs reported herein and its relationship to values reported in previous studies should be interpreted with caution as they are dependent on absolute values.

We also recognize that many experts consider radiological-assisted direct visualization of ovulation and follicular count to be the gold standard and therefore a lack thereof is a possible limitation to this study. However, studies suggest that ultrasound assisted determination of ovulation is comparable with determination with FMs and phase-specific biomarkers (Behre et al., 2000; Li et al., 2002). Regardless, this study was not designed to test the diagnostic accuracy of AMH as a marker of follicular quantity or quality. In addition, our findings are limited to a select group of healthy eumenorrheic women without gynecologic or endocrine disorders limiting reproductive capacity, who were not seeking pregnancy. Results reported here cannot be generalized to all women regardless of age and fertility status, or medical, geographic, or socioeconomic background.

Overall, we observed that serum AMH is cyclically dependent across both ovulatory and sporadic anovulatory cycles of regularly menstruating women, with higher levels of serum AMH associated with sporadic anovulation. The serum AMH variability throughout the menstrual cycle is in keeping with the cyclicity of granulosa cell production and an underlying

intricate endocrine related mechanism responsible for an optimal range of serum AMH regardless of cycle day or phase. As shown here, these fluctuations across the cycle are not of clinical relevance and do not support a change in clinical practice to time AMH to menstrual cycle phase or day. Regardless, in premenopausal and otherwise healthy women, serum AMH levels designated as too low have poor prognostic implications in regards to ovarian reserve (Nardo et al., 2009; Domingues et al., 2010; Jayaprakasan et al., 2010; Ledger, 2010; Dólleman et al., 2013; Nelson et al., 2014). Likewise, AMH levels allocated as too high also may have an equally poor prognosis, suggestive of poor follicular quality associated with ovarian malignancy and anovulatory infertility, or adverse outcome with assisted reproduction technologies (Pigny et al., 2006; La Marca et al., 2007; Chong et al., 2012; Pawelczak et al., 2012; Broer et al., 2013). Further research into the variability of serum AMH as measured with a standardized reliable universal AMH assay throughout the menstrual cycle regardless of ovulatory status in healthy premenopausal women has profound implications in understanding the optimal age-dependent range of serum AMH, the role of serum AMH in normal follicle development and selection, as well as its role in anovulatory disorders, and ultimately the advancement of female reproductive health.

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Authors' roles

All authors were involved in study design, execution, analysis, manuscript drafting and critical discussion.

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Conflict of interest

None declared.

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