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Intercycle variabilities of basal antral follicle count and ovarian volume in subfertile women and their relationship to reproductive aging: A prospective study

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Abstract

Background. Reproducibility is an important issue when using tests for estimating ovarian reserve and counseling patients. However, little is known about the intercycle variabilities of basal antral follicle count and ovarian volume. In this prospective study, we analysed the intercycle variabilities of the antral follicle count and ovarian volume, and compared them with those of other basal ovarian reserve tests in subfertile patients.

Method. Fifty-two ovulatory and infertile women were followed for two consecutive spontaneous cycles. The antral follicle count, ovarian volume, serum follicle stimulating hormone and estradiol levels were determined on day 3 of both cycles. Limits of agreement between two measurements were determined.

Results. Limits of agreement were –6.9 and 6.5 for the antral follicle count, and –8.3 and 8.6 for the ovarian volume. These degrees of variation corresponded to a range of 1.30 and 1.45 times their means for the ovarian volume and antral follicle count, respectively. The variability in the antral follicle count was greater in women who were younger than 24.5 years than in those who were older.

Conclusions. Intercycle variabilities of the antral follicle count and ovarian volume were clinically significant. More variation was observed in the antral follicle count of young infertile patients. Therefore, a low antral follicle count in young, infertile, but ovulatory women should be cautiously interpreted. This may not reflect a low ovarian reserve, and these women may have a high antral follicle count in the next cycle.

Keywords: *Antral follicle, infertility, intercycle variability, ovarian reserve, ovarian volume, reproducibility*

Introduction

To identify patients at risk for a poor response to ovarian stimulation and cycle cancellation as a result of decreased ovarian reserve, multiple basal and provocative endocrine tests have been described. These include early follicular phase follicle stimulating hormone (FSH) and estradiol levels, gonadotropin releasing hormone (GnRH) stimulation and the clomiphene citrate challenge test [1]. More recently, the ovarian antral follicle count and ovarian volume have emerged as useful indicators of functional ovarian age and a predictor of stimulation quality in *in vitro* fertilization (IVF) cycles [1].

Reproducibility is an important issue when using these tests for estimating ovarian reserve and counseling patients. Variability is important for any new methods to be validated. The intercycle variability is well known for basal serum FSH values [2]. However, little is known about the intercycle variabilities of basal antral follicle count and ovarian volume.

It has been reported that the antral follicle count is one of the better predictors of response to ovarian stimulation [3]. As a result, many practices have incorporated the antral follicle count into the evaluation of infertility patients for counseling purposes. If antral follicle counts are going to be used to counsel subfertile women regarding their chances for conception, a number of questions regarding the reproducibility of the test have to be addressed. One of these is defining the magnitude of the intercycle variation. Intercycle variability in the antral follicle count has been investigated only in women with proven fertility in a prospective study, and in those undergoing IVF [4–6]. To our knowledge, the intercycle variability in the antral follicle count has not been previously investigated prospectively in general subfertile women, and that in the

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ovarian volume has not been previously investigated, in either a retrospective or a prospective design.

In this prospective study, we analysed the intercycle variabilities of the antral follicle count and ovarian volume, and compared them with those of other basal ovarian reserve tests in a subfertile group of women.

Materials and methods

Fifty-two subfertile women were included in this prospective study. To qualify for participation, subjects were required to have regular 21–35-day cycles; be ovulatory, as suggested by a luteal-phase progesterone level of > 3 ng/ml in a recent cycle; be on no medications or exogenous hormones for 6 months before participation; have no history of endocrinological disease (e.g., hirsutism or galactorrhea); and have a history of infertility. Each woman had both of her ovaries, and women with previous ovarian surgery, endometriomas, or follicles measuring at least 10 mm on baseline ultrasound examination were excluded from the study. The study was approved by the institutional review board at the Marmara University, and written informed consent was obtained from each subject.

Baseline vaginal ultrasonography was performed for bilateral antral follicle count and ovarian volume determinations, and venous blood samples were withdrawn for serum FSH and estradiol determinations on day 3 of a spontaneous cycle. Ovulation was confirmed during the first cycle by ultrasonographic folliculometry. Transvaginal ultrasound confirmed the presence of a developing follicle in the midfollicular phase, a follicle of at least 16 mm in diameter in the periovulatory phase, and a corpus luteum in the midluteal phase. The work-up at baseline was repeated on day 3 of the following spontaneous cycle.

Assays and ultrasonographic measurements

All samples were centrifuged within 2 h after withdrawal and stored at -20°C until assayed. Serum FSH and estradiol concentrations were determined by using the Immulite immunoassay system (Diagnostic Products Corporation, Los Angeles, CA, USA). This assay is standardized to the World Health Organization Second International Reference Preparation 78/549. The interassay and intra-assay coefficients of variation were 6.6% and 5.4% for FSH, and 5.4% and 4.4% for estradiol, respectively.

Transvaginal ultrasound examination was performed by the same physician (A.S.) by using a GE Logiq 200 Pro machine (GE Medical Systems, Milwaukee, WI, USA) with a 6.5-MHz vaginal transducer. Round or oval echo-free structures were regarded as follicles, and all ovarian follicles measuring 2–10 mm on both ovaries were counted on day 3 of the cycle. Ovarian volume was subsequently computed using the ellipsoid formula: Ovarian volume = D1 X D2 X D3 X $\pi/6$, where D1, D2 and D3 are the maximal perpendicular diameters of each ovary. The volumes and antral follicle counts of both ovaries were added, and the total number of follicles and total ovarian volume per patient were used for calculations.

Statistical analysis

To evaluate the intercycle variability, limits of agreement were determined as described by Bland and Altman in 1986 [7] and 1995 [8]. The objective of this specific method is to describe the degree of agreement of measurements. The limits-of-agreement method is based on calculating the mean difference between two measurements as well as the SD of the differences. The limit of agreement is defined as 1.96 times the SD above or below the mean difference, and indicates to what extent two measurements can vary. These limits indicate the range, in which 95% of differences will lie. Since the scales and units of ovarian reserve tests are different, it is not possible to compare the variation without any modification. To compare the magnitudes of variation, we eliminated the effect of scale, and standardized the range between the limits of agreement by dividing the range by its mean. Therefore, the range was expressed as multiples of the mean (MoM).

The absolute value for the difference between the ovarian reserve tests in the two cycles was calculated for each woman. Correlations of these absolute differences with both age and their mean values (mean of the values in the two cycles) were analysed by using the Pearson's correlation test.

For the tests, whose variability was significantly correlated with both age and their mean value, stepwise multiple regression analysis was performed to determine whether these were independently related to the variability. The absolute difference was used as the dependent variable during the regression analysis.

For the tests whose variability was significantly correlated with age, women were divided into two groups according to the absolute difference between the two cycles: those in the upper 25th centile for the absolute difference were accepted as women with a highly variable result, and others were included in the group of women with a relatively constant cycle-to-cycle variation.

Subsequently, receiver operating characteristic (ROC) analysis was performed to determine the optimum cut-off value for the age to predict women with high variability between cycles. Diagnostic sensitivity and specificity were calculated, and the ROC curve was constructed by plotting the sensitivity against the false-positive rate (1 – specificity) of various cut-off values. The value with the optimal

Table 1. Mean values and upper and lower limits of agreement between the two measurements of ovarian reserve tests.

Variable	mean (± SD)	95% CI for the mean	mean (± SD) difference	95% CI for the mean differenc	LLA	95% CI for the LLA	ULA	95% CI for the ULA	Range between UPL and LLA	MoM
Serum FSH level (IU/L)	7.26 ± 1.93	6.73 - 7.79	0.27 ± 1.83	−0.24 - 0.78	−3.32	−4.20 - − 2.44	3.86	2.98 - 4.74	7.18	0.99
Serum estradiol level (pg/mL)	40.66 ± 20.55	34.91 - 46.41	2.43 ± 29.30	−5.77 - 10.64	−54.99	−69.20 - − 40.78	59.85	45.65 - 74.06	114.84	2.82
Ovarian volume (mL)	13.00 ± 4.26	11.84 - 14.16	0.19 ± 4.31	−0.98 - 1.36	−8.25	−10.28 - − 6.22	8.63	6.60 - 10.65	16.88	1.30
Antral follicle count (n)	9.22 ± 3.98	8.14 - 10.30	−0.17 ± 3.41	−1.10 - 0.75	−6.85	−8.45 - − 5.24	6.50	4.90 - 8.10	13.35	1.45

CI = confidence interval, LLA = lower limit of agreement, ULA = upper limit of agreement, MoM = multiples of mean.

combination of sensitivity and specificity was chosen as the optimum cut-off value. The absolute intercycle differences in subjects who were younger than the optimum cut-off value were compared with those in older subjects by using the Mann–Whitney U test. SPSS, Release 11.5 (SPSS, Inc, Chicago, IL, USA) was used for the statistical analysis, and a p value of < 0.05 was considered significant.

Results

Means (± SD) for the age and duration of infertility among women in the present study were 28.5 (± 5.8) years (range 18–42 years) and 3.6 (± 3.1) years, respectively. Mean (± SD) for the body mass index (BMI) was 23.6 (± 4.4) kg/m^2. The main indications for treatment were tuboperitoneal factor ($n = 10$), male factor ($n = 14$) and unexplained infertility ($n = 28$). Fourteen subjects had secondary infertility. No pregnancies or drop-outs occurred during the study.

Intercycle variability as assessed by calculating limits of agreement between two day-3 measurements is shown in Table I and Figure 1. The mean differences for the antral follicle count and ovarian volume between repeated measurements were −0.17 and 0.19 ml, respectively. The upper and lower limits of agreement for the antral follicle count were −6.9 and 6.5, respectively. The corresponding values for the ovarian volume were −8.3 and 8.6, respectively (Table I). These results show that the antral follicle count in the subsequent cycle may be in the range of approximately ± 6 of the measured value in the index cycle with a probability of 95%. This implies a weak or no agreement between the measurements for a variable with a mean of 9 (the mean for the antral follicle count). This range of variation is 1.45 MoM, and this is clinically significant. The lowest variation was observed in the serum FSH level with a MoM value of 0.99 (Table I). A ± 3 change in serum FSH level also implies a lack of agreement. The largest variation was observed in serum estradiol level (Table I). The variations of ultrasonographic parameters were between those of hormonal parameters (Table I).

When correlations between the absolute differences in ovarian reserve tests and age were evaluated, only variability in the antral follicle count had a significant, but moderate, correlation with age ($r = -0.3$, $p = 0.03$; Table II; Figure 2). When correlations between the absolute difference and the mean of the two measurements were evaluated, significant positive correlations were observed for all of the ovarian reserve tests, i.e., the variability increased as the ovarian reserve test value increased (Table II).

Among the ovarian reserve tests, only the difference in the antral follicle count was significantly correlated with both age and its mean value. Regression analysis revealed that age was the only

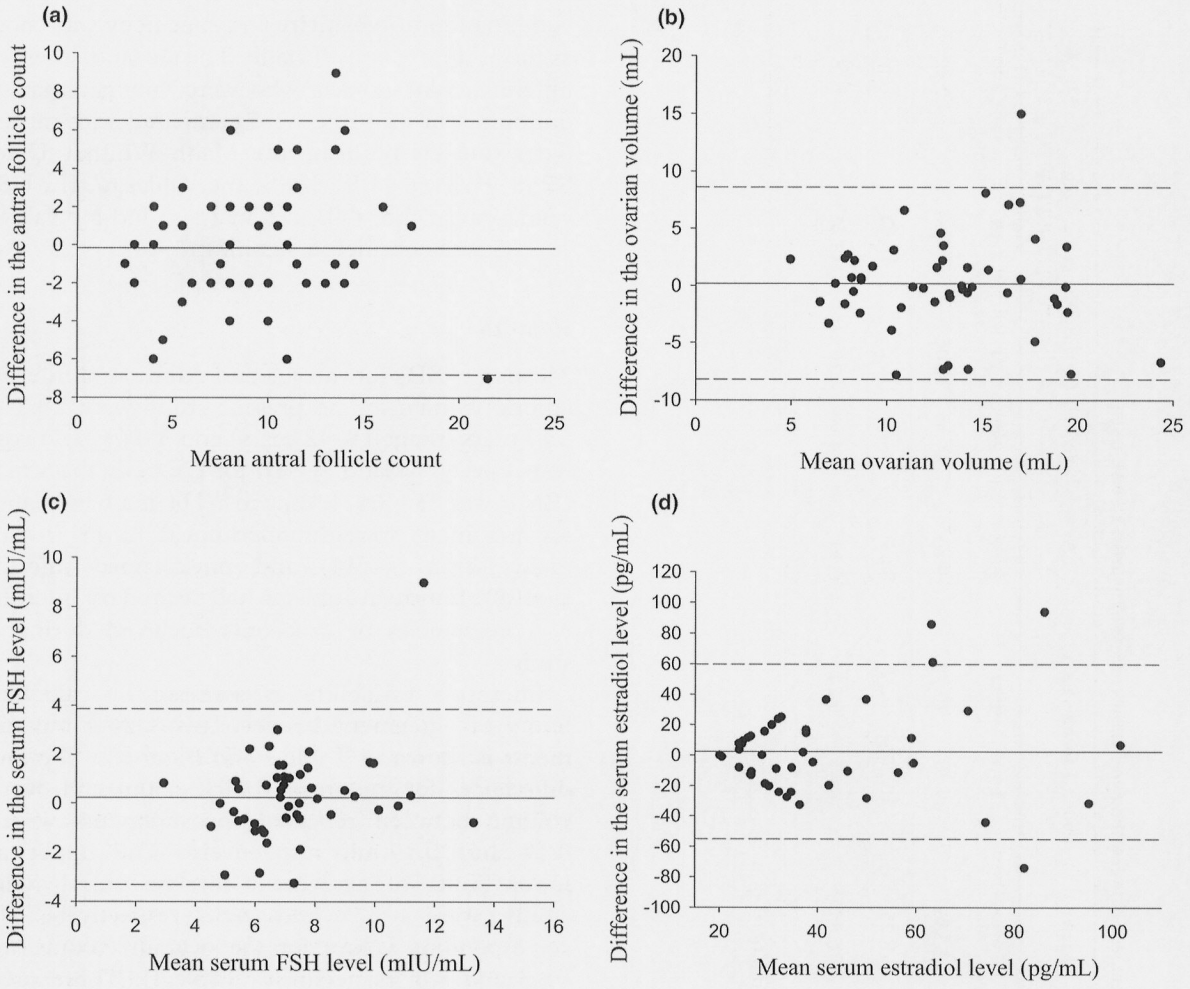

Figure 1. Limits-of-agreement plots for the antral follicle count (a), ovarian volume (b), serum follicle stimulating hormone level (c) and serum estradiol level (d). The solid line indicates the mean difference between the two measurements. Dashed lines represent the upper and lower limits of agreement, which are the ± 1.96 SD values for the difference. The *X*-axis is the mean of the two measurements

independent predictor of its intercycle variability ($r^2 = 0.10$, $\beta = -0.11$, $p = 0.03$).

The absolute difference in the antral follicle counts of the two cycles ranged from zero to nine, and the upper 25th centile corresponded to a difference of > 4. To predict women with a high variability in the antral follicle count (absolute difference > 4), the optimum cut-off value for the age was 24.5 years. The variability was greater in women who were younger than 24.5 years ($n = 16$) than those who were older ($n = 36$) (3.6 ± 2.2 versus 2.3 ± 1.9; $p = 0.03$).

To emphasize the clinical importance of the intercycle variability, we analysed the frequency of subjects who were interpreted as having good ovarian reserve in both of the cycles. Although an optimum cut-off value for the antral follicle count has not been validated in the literature, probably owing to the linear relationship between the antral follicle count and the ovarian response [9], we chose to use ten, as suggested previously [10]. Among women with a highly variable antral follicle count ($n = 12$), only three subjects (25%) had more than ten antral follicles suggestive of good ovarian reserve in both of the successive cycles. Only two (17%) had $\leqslant 10$ antral follicles in both of the cycles. Others (58%) were interpreted as having a poor reserve in one cycle and a good reserve in the other cycle.

Discussion

In the present study, we have shown that there is a significant cycle-to-cycle variability in ultrasonographic markers of ovarian reserve in subfertile women. Hormonal tests also had a significant variability between cycles in the present study. Young women had more variability in their antral follicle counts than older women, and approximately 60% of those women with a high variability had antral follicle counts of $\leqslant 10$ in one of the cycles and > 10 in the other cycle. This variability was related to both age and the number of antral follicles, but age was the only independent predictor.

Intercycle variability in the antral follicle count in infertility patients has been evaluated in only two previous studies [4,6]. Hansen et al. retrospectively analysed the extent of intercycle variability in the

Table 2. Correlations between the absolute differences and mean values of the two measurements and age.

	Mean of the two measurements									
	Age		Serum FSH level		Serum estradiol level		Ovarian volume		Antral follicle count	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
The absolute difference between two measurements										
Serum FSH level	−0.07	0.6	0.3	0.049	NA	NA	NA	NA	NA	NA
Serum estradiol level	−0.01	0.9	NA	NA	0.6	< 0.001	NA	NA	NA	NA
Ovarian volume	−0.02	0.9	NA	NA	NA	NA	0.3	0.04	NA	NA
Antarl follicle count	−0.3	0.03	NA	NA	NA	NA	NA	NA	0.3	0.03

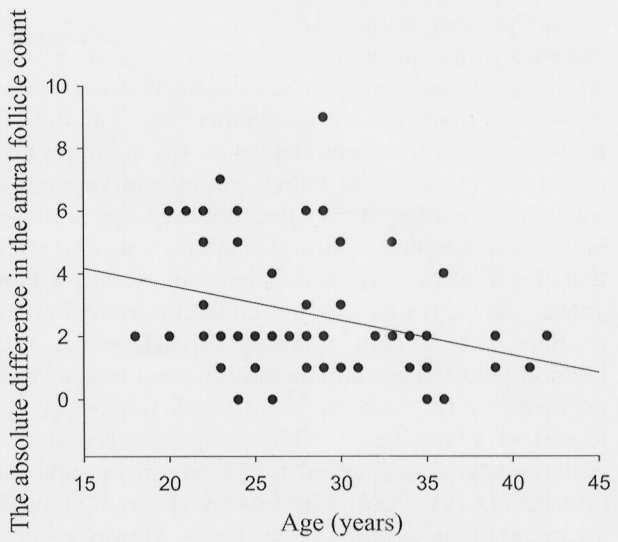

Figure 2. Linear relation between the absolute difference in the antral follicle count and age

antral follicle count and its impact on stimulation outcome in IVF [4]. They observed a variability in antral follicle counts that was correlated with the mean antral follicle count [4]. The mean SD for subjects with a mean antral follicle count of < 15 was 3.0 in their study. This implies a mean absolute difference of 4.2 ($SD^2 = \Sigma(\text{value} - \text{mean})^2$ for $n = 2$) for two values. The mean absolute differences in the present study were 2.3 for subjects who were older than 24.5 years, and 3.6 for subjects who were younger than that age. Although Hansen et al. have not discussed the degree of variation in their study, that variation may mean a lack of agreement for subjects who have < 15 antral follicles [4]. However, they compared cycles within a 1-year duration and, therefore, the duration between cycles was not constant in their study [4]. In addition, ultrasound measurements were not performed by the same physician [4]. Scheffer et al. analysed the intercycle variability of the antral follicle count in healthy female volunteers with proven fertility [5]. They also used the limits-of-agreement method. Although they analysed a different population, they reported similar limits of agreement (–8.9 and 8.4) to those in the present study. This supports the previous suggestion that the antral follicle count does not differ between fertile and infertile women [11].

Bancsi et al. analysed, in a prospective study, the intercycle variability in the antral follicle count in women undergoing IVF [6]. They reported a significant cycle-to-cycle variation with lower and upper limits of agreement of –7.3 and 7.4, respectively. The mean antral follicle count in their study was 9.4. To our knowledge, the present study is the fourth one to analyse the intercycle variability in the antral follicle count, and the first to compare it prospectively to other ovarian reserve tests in a subfertile group of women.

The present study also showed that intercycle variation in the antral follicle count is related to both age and the mean antral follicle count. However, only age could independently predict the variability. Regression analysis revealed that the relation between the mean antral follicle count and variability was dependent on the relation between age and the mean antral follicle count. Scheffer et al. observed that the intercycle variability in women with a low antral follicle count was not different from that in woman with a high number [5]. However, the relation between age and variability was not analysed in their study. Hansen et al. and Bancsi et al. identified a significant relationship between intercycle variability and antral follicle count in subjects undergoing IVF [4,6]. That observed variability was greater at higher antral follicle counts. However, they did not analyse the relation between age and variability [4,6]. Since it is known that the antral follicle count decreases with age [5], it appears logical to expect a higher variability in younger women. However, this has not been previously analysed.

Intraobserver variability has not been analysed in the present study, since it has been previously reported that intraobserver variability in the antral follicle count and ovarian volume is minimal [12–15]. Scheffer et al. reported a low intraobserver variability in the antral follicle count, which was valid for the whole range of observations, i.e., no change in variance was found when higher numbers (> 12) of antral follicles were compared to lower numbers (< 13) [13]. Therefore, it is unlikely that any more variation with higher numbers of antral follicles, as was observed in the present study, is due to intraobserver variability.

In the present study, age was found as a good predictor for significant variation in the antral follicle count. Intercycle variability in the antral follicle count appears to be more significant in young women. To our knowledge, these are the first data to demonstrate the significant cycle-to-cycle variation in the antral follicle count in young infertile patients. However, this result should be cautiously interpreted, owing to the limited number of young subjects in the present study. It requires to be analysed by further studies. The present study also showed that antral follicle count in older women has a relatively low variability, and that once a patient has a low antral follicle count, this may not differ in another cycle. This result is valuable, since ovarian reserve tests are commonly used in older women.

Approximately 60% of subjects with a high variability had a low antral follicle count ($\leqslant 10$) in one cycle and a high antral follicle count (> 10) in the other cycle. Since high variation is more common in young women, a low antral follicle count in young, infertile, but ovulatory, women should be cautiously interpreted. This may not indicate a low ovarian reserve, and these women may have a high antral follicle count in the next cycle. Hansen et al. evaluated the impact of intercycle variability in the antral follicle count on stimulation outcome in IVF [4]. They compared 'high' and 'low' antral follicle count cycles ($n = 23$ in each group) in individuals who had greater intercycle variability, and observed no significant difference in stimulation outcome [4]. A comparison has also been made between individuals with greater versus less variability in the antral follicle count, and no statistically significant difference was found in stimulation quality or in pregnancy and cycle cancellation rates [4]. However, this may be due to a type II error since numbers of subjects in groups were 23 and 27. Therefore, further studies should be performed to compare the ovarian response of women with a high cycle-to-cycle variation with that of those women with a relatively lower variation independent of age.

Bancsi et al. also reported that the highest count from two cycles gave a statistically significant improvement of the prediction for poor response [6]. Despite this significance, they concluded that the clinical relevance of counting antral follicles in two different cycles instead of only one cycle was limited, and they did not recommend repeating an antral follicle count in a subsequent cycle in women undergoing IVF [6]. However, this may be clinically relevant during counseling subfertile women at initial submission. Repeating an antral follicle count in a subsequent cycle in young subfertile women with a low antral follicle count on initial submission may prevent clinicians leading subjects towards ovulation induction with more aggressive protocols.

To our knowledge, intercycle variability in the ovarian volume has not been previously investigated. The ovarian volume also showed a similar degree of cycle-to-cycle variation as observed in the antral follicle count. Its variation was found to be related to its value. However, in contrast to the antral follicle count, intercycle variability of ovarian volume measurements was not correlated with age in the present study. This shows that age is not a good predictor for cycle-to-cycle variation in ovarian volume. We could not explain the reason for the lack of correlation between this ultrasonographic marker and age.

Intercycle variabilities of basal FSH and estradiol levels are well known [2]. Brown et al. reported that serum FSH level varied significantly less than the serum estradiol level on cycle day 3. The mean coefficient of variation for day-3 serum FSH level was 25.6% as compared to 44.1% for the serum estradiol level [16]. The present study also showed that there was significant cycle-to-cycle variation for both of the hormonal parameters. The serum FSH level showed less variation than the serum estradiol level. It is known that the intercycle variability in serum FSH level is positively correlated with basal FSH level [17]. The results in the present study were consistent with this. Although we observed a similar

correlation in the serum estradiol level, the mean cycle-to-cycle variation, which was highest among the ovarian reserve tests in the present study, weakens its importance, i.e., the serum estradiol level already has a high variation. This has also been previously reported [16].

In conclusion, there is significant cycle-to-cycle variability in ultrasonographic markers of ovarian reserve, i.e., the antral follicle count and ovarian volume, in subfertile women. More variation was observed in the antral follicle count of young infertile patients, when compared to older women. Therefore, a low antral follicle count in young, infertile, but ovulatory women should be cautiously interpreted. This may not indicate a low ovarian reserve, and these women may have a high antral follicle count in the next cycle.

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