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Assessment of reproductive ageing patterns by hormonal and ultrasonographic ovarian reserve tests

E.Tufan[1], K.Elter[1,2] and F.Durmusoglu[1]

[1]Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Marmara University School of Medicine, Istanbul, Turkey

[2]To whom correspondence should be addressed at: Kuyubasi S.Fenik Apt. No: 20/17, 34724 Feneryolu, Istanbul, Turkey. E-mail: korayelter@marmara.edu.tr

BACKGROUND: Tests to assess the change in ovarian reserve (OR) with age have been analysed only in monophasic or biphasic linear patterns. Our aim was to analyse an optimum curve that might define the relationship between different OR tests and age. METHODS: A total of 81 regularly menstruating women without a history of infertility were included in this prospective study. On cycle day 3, antral follicle (AF) counts, ovarian volume (OV), and serum FSH and estradiol levels were determined. Curve estimation was performed to determine the optimal relationship between age and OR tests. Optimum curves were also compared with monophasic and biphasic linear relationships. RESULTS: A quadratic model ($y = a \times x^2 + b \times x + c$) had the highest coefficient of determination for the AF count, OV and serum FSH level. The predictive power of this quadratic model was comparable with biphasic linear models for the OV and serum FSH level, but was better than that of the AF count. CONCLUSIONS: The pattern of reproductive ageing as assessed by hormonal and ultrasonographic OR tests does not appear to show an abrupt change at a certain age, but follows a continuously increasing rate of decline in the third decade of life. The changes in serum FSH levels and ultrasonographic OR tests follow a quadratic model in regularly menstruating women.

Key words: age/antral follicle/FSH/ovarian reserve/ovarian volume

Introduction

It is known that the decline in fecundity with female age occurs long before menopause (O'Connor *et al.*, 1998). Fertility is remarkably reduced with increasing age of women in both spontaneous conceptions (Tietze, 1957) and assisted reproductive methods (Templeton *et al.*, 1996). The decrease in fertility with ageing is due to decreases in both oocyte quality and quantity. Direct data on oocyte quality or number of follicles present in the ovaries in *in vivo* situations are limited for obvious reasons. Parameters such as early follicular serum FSH and estradiol (E_2) levels and various stimulation tests, as well as ultrasound-based measurements, i.e. antral follicle (AF) count and ovarian volume (OV), have been used to predict advanced ageing of the ovary and the probability of treatment success in infertility populations. Therefore, the so-called ovarian reserve (OR) tests are frequently used to evaluate the probability of pregnancy in infertile women (Bukman and Heineman, 2001).

Many women in developed countries delay childbearing. Since age is not a good predictor of reproductive potential (Bukman and Heineman, 2001), use of OR tests for an accurate assessment of the reproductive potential in these older women may help clinicians during counselling. The determination of OR may help clinicians in making the decision regarding prophylactic oophorectomy in selected women (Barnes-Kedar and Plon, 2002). Younger women can be screened for early ovarian ageing, especially those in high-risk groups, as has been proposed recently for the prevention of subfertility (Nikolaou and Templeton, 2003). Detection of early ovarian ageing is not only important for infertility, but is also of interest in relation to general somatic ageing (Kirkwood, 1998). Therefore, the establishment of normal curves for OR tests is important. A normal curve for OV may also help to define abnormal values at different ages in screening for early ovarian cancer (DePriest *et al.*, 1997).

It has been suggested that the normal rate of oocyte depletion follows a biphasic pattern, accelerating below a number of ~25 000 at a mean age of 37–38 years based on histological analyses of ovaries (Faddy *et al.*, 1992). This suggested midlife acceleration in oocyte loss is of great interest. Although the OR tests have been studied in infertile women, there are few studies addressing the effects of age on OR tests in women without infertility (Ruess *et al.*, 1996; Scheffer *et al.*, 1999; Ng *et al.*, 2003). Furthermore, these studies have not analysed an optimum curve for the relationship between OR and age. Some authors have suggested a monophasic linear relationship, and others have suggested

a biphasic pattern without analysing a comparison between different models (Ruess *et al.*, 1996; Ng *et al.*, 2003).

Therefore, in this prospective study, we aimed to find an optimum curve that might define the relationship between different OR tests and age, by comparing the predictive power of monophasic and biphasic regression lines with different cut-off values of age.

Materials and methods

Subjects

Eighty-one women presenting to our out-patient clinic for acute minor gynaecological problems such as vaginitis were included in this prospective study. To qualify for participation, subjects were required to (i) have regular 21–35 day cycles; (ii) be on no medications or exogenous hormones for 6 months before participation; (iii) have no evidence of endocrinological disease; and (iv) have no history of infertility. Seventy-three women had proven fertility, and the remaining eight (9.9%), all aged ≤26 years, were nulliparous with no desire for fertility. Each woman had both of her ovaries, and women with previous ovarian surgery, endometriomas or follicles measuring at least 10 mm on baseline ultrasound were excluded from the study. The study was approved by the institutional review board at Marmara University, and written informed consent was obtained from each subject.

On cycle day 3 of spontaneous bleeding, baseline vaginal ultrasonography was performed for bilateral AF count and OV determinations, and venous blood samples were withdrawn for serum FSH and E_2 determinations.

Assays and ultrasonographic measurements

All blood samples were centrifuged within 2 h after withdrawal and stored at −20°C until assayed. Serum FSH and E_2 concentrations were determined using the Immulite immunoassay system (Diagnostic Products Corporation, Los Angeles, CA). This assay is standardized to the World Health Organization Second International Reference Preparation 78/549. The inter- and intra-assay coefficients of variation were 6.6 and 5.4% for FSH, and 5.4 and 4.4% for E_2, respectively.

Transvaginal ultrasound was performed by the same physician (E.T.), using a GE Logiq 200 Pro (GE Medical Systems, Milwaukee, WI) 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 cycle day 3. Ovarian volume subsequently was computed using the ellipsoid formula: $OV = D1 \times D2 \times D3 \times \pi/6$, where D1, D2 and D3 are the maximal perpendicular diameters of each ovary. The volumes and AF counts of both ovaries were added, and the total number of follicles and total OV per patient were used for calculations.

Statistical analysis

Correlations between OR tests and age were analysed by using the Pearson's correlation test. For the significantly correlated parameters, curve estimation was performed to determine the optimal relationship between age and OR tests. For the curve estimation procedure, curve estimation regression statistics were performed for 11 different regression models, including linear, logarithmic, inverse, quadratic, cubic, power, compound, S-curve, logistic, growth, and exponential models. The model with the highest coefficient of determination (r^2) was accepted as the optimal model for the relationship. The coefficient of determination is the amount of the scatter in one variable that can be explained by another. In the present study, it is the rate (e.g. 35% when $r^2 = 0.35$) of variation in the values of the OR test which can be accounted for by knowing the age through using the relevant model.

Standard deviations for the OR test in different age groups, i.e. 21–25, 26–30, 31–35, 36–40 and 41–45 years, were determined. The predictive power of the model with the highest r^2 value was analysed by determining the number of observed values in 'the mean as determined by the regression equation ± 2 SDs' range. Any observed value below or above the ± 2 SDs of the predicted value was accepted as the 'outlying value'. The number of outlying values was determined and the ratio of outlying values to the total number of values ($n = 81$) was used for the statistical analysis.

Linear regression equations were also determined. The regression coefficient β and the y-intercept were determined for the regression formula: OR test $= \beta \times$ age + y-intercept. The number of observed values below and above the ± 2 SDs of the predicted values was determined. Following determination of the monophasic line for the significantly correlated OR tests, the predictive power of biphasic lines was analysed. Each of the cut-offs between 30 and 38 for the age was analysed separately, and the number and ratio of outlying values to the total number of values ($n = 81$) were determined for each biphasic line. The ratio of outlying values was compared by using the χ^2 or Fisher's exact tests, where appropriate. SPSS, Release 11.5 (SPSS, Inc, Chicago, IL) was used for the statistical analysis, and a P-value of <0.05 was considered significant.

Results

Means (± SD) for the age and body mass index (BMI) among women in the present study were 34.4 (± 6.4) years (range 21–43) and 24.3 (± 4.5) kg/m^2. When the correlations between the OR tests and age were evaluated, OV, AF count and serum FSH level were found to be significantly correlated with age (Table I).

The changes in OV and AF count with age are shown in Figure 1. The quadratic model ($y = a \times x^2 + b \times x + c$) had the highest coefficient of determination for both of these ultrasonographic OR tests (Table II). The regression equations for this quadratic model were as follows: AF count $= -0.0165 \times \text{age}^2 + 0.7643 \times \text{age} + 0.2793$, and $OV = -0.0196 \times \text{age}^2 + 1.0193 \times \text{age} - 1.9988$ (Figure 1). Standard deviations in different age groups are shown in Table III. 'The mean as determined by the regression equation ± 2 SD' range for the AF count included, and therefore predicted, 97.5% (79 out of 81) of the observed values. 'The mean as determined by the regression equation ± 2 SD' range for the OV included, and therefore predicted, 96.3% (78 out of 81) of the observed values.

The change in serum FSH level with age is shown in Figure 2. An exponential model had the highest coefficient of determination (Table II). Assessment of the 11 models after

Table I. Correlations between age and ovarian reserve tests

	Mean ± SD	r	P
Antral follicle count (n)	6.4 ± 3.1	−0.64	<0.001
Ovarian volume (ml)	9.0 ± 3.3	−0.51	<0.001
Serum FSH level (mIU/ml)	9.7 ± 4.0	0.55	<0.001
Serum E_2 level (pg/ml)	56.9 ± 30.1	0.13	NS

NS = not significant.

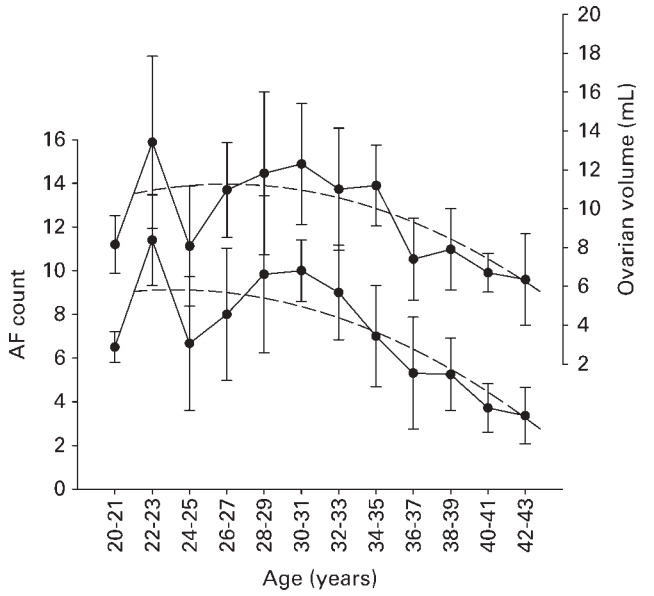

Figure 1. The change in OV and AF count with age. Dashed lines indicate the regression equations for the ovarian volume and AF count [AF count $= -0.0165 \times \text{age}^2 + 0.7643 \times \text{age} + 0.2793$; OV (ml) $= -0.0196 \times \text{age}^2 + 1.0193 \times \text{age} - 1.9988$].

Table II. Coefficients of determination (r^2) of different models for the relationship between age and OR tests

Models	Serum FSH	AF count	Ovarian volume
Linear	0.301	0.403	0.257
Exponential	0.333	0.426	0.260
Logarithmic	0.287	0.375	0.233
Quadratic	0.314	0.442	0.308
Inverse	0.268	0.342	0.205
Cubic	0.313	0.440	0.303
Power	0.323	0.390	0.233
Compound	0.333	0.426	0.260
S-curve	0.307	0.349	0.203
Logistic	0.333	0.426	0.260
Growth	0.333	0.426	0.260

Table III. Standard deviations of OR tests in different age groups

Age (years)	Serum FSH (mIU/ml)	AF count	Ovarian volume (ml)
21–25	1.4	3.2	4.4
26–30	1.5	3.3	3.3
31–35	1.6	2.4	2.3
36–40	4.7	2.1	2.0
41–45	4.1	1.3	2.1

this logarithmic transformation revealed that the quadratic model improved the r^2 value from 33 to 37%. The regression equation for this combined model was as follows: serum FSH level (mIU/ml) $= e^{(0.0006 \times \text{age}^2 - 0.0075 \times \text{age} + 1.6793)}$ (Figure 2). Standard deviations for the serum FSH level in different age groups are shown in Table III. 'The mean as predicted by the regression equation ± 2 SD' range for the serum FSH level included, and therefore predicted, 96.3% (78 out of 81) of the observed values.

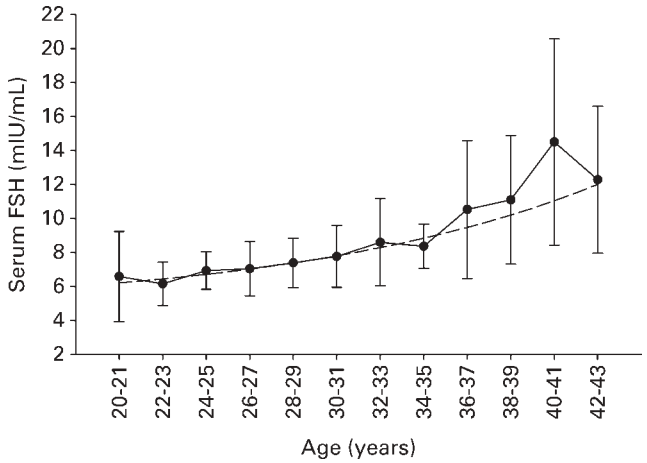

Figure 2. The change in serum FSH level with age. The dashed line indicates the regression equation for the serum FSH level [serum FSH level (mIU/ml) $= e^{(0.0006 \times \text{age}^2 - 0.0075 \times \text{age} + 1.6793)}$].

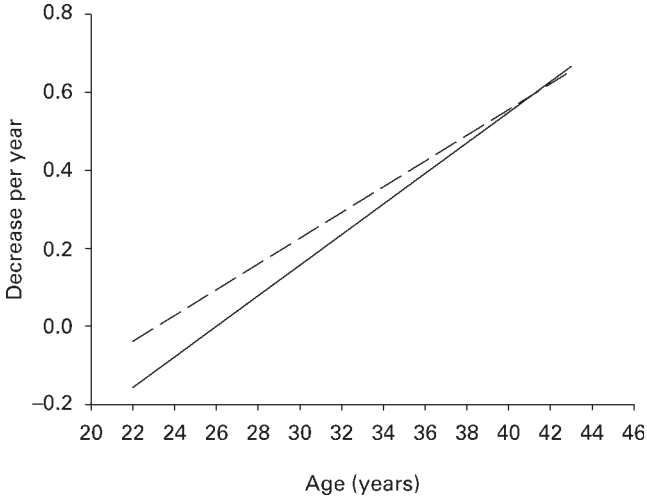

Figure 3. Decline rate in the OV (ml per year) and AF count (*n* per year) with age. The dashed line indicates the decline rate for the AF count.

The amount of decrease in the AF count and OV per year increased linearly with age, but it was <1 (*n* for the AF count and ml for the OV) per year for all ages between 21 and 43 years for both of these ultrasonographic variables (Figure 3). The amount of increase in the serum FSH level per year also increased with age (Figure 4). The percentage change in the serum FSH level per year increased linearly with age and it was between 2 and 5%/year for ages between 21 and 43 years (Figure 4).

The predictive power of monophasic trend lines and biphasic trend lines created by placing the cut-off point at various ages from 30 to 38 years is shown in Tables IV, V and VI. The lowest number of outlying values was observed with biphasic lines with the cut-off values of 30 and 31 in both of the ultrasonographic OR tests. The rate of outlying values was comparable between the quadratic and biphasic linear models for OV and serum FSH level (three out of 81 versus

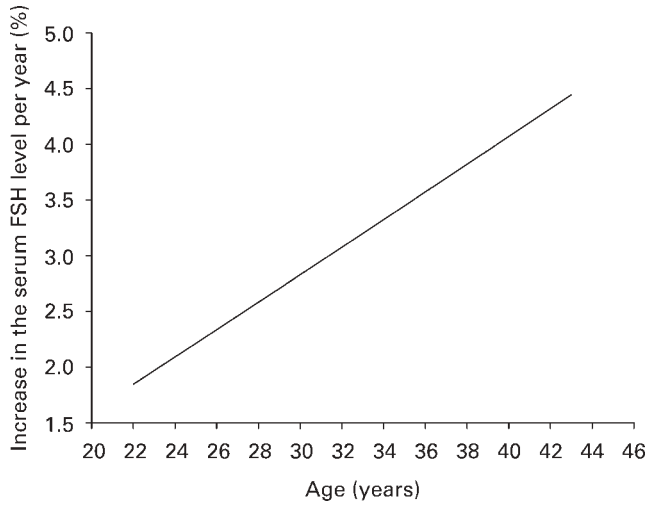

Figure 4. Increase in the serum FSH level (% per year).

three out of 81 in both of these variables). The rate of outlying values was higher in the biphasic linear model when compared with the quadratic model for the AF count (three out of 81 versus two out of 81; $P = 0.001$).

Discussion

The present study showed that markers of OR change in a quadratic model with increasing age. This model is optimal after logarithmic transformation for the serum FSH level. The present study also showed that the rate of change increases with age. To our knowledge, a quadratic model has not been used previously as a means of testing the relationship between age and OR.

It has been suggested that oocyte depletion accelerates toward the onset of menopause, and this acceleration is characterized by a bend in the scatter of points on a log-linear plot of follicle number by age (Richardson *et al.*, 1987; Faddy *et al.*, 1992; Gougeon *et al.*, 1994). This abrupt change in the rate of follicular loss has been suggested to occur at the age of 37.5 years (Faddy *et al.*, 1992). Thus, follicular atresia has been interpreted as biphasic. A linear relationship after logarithmic transformation indicates that the rate of percentage change is constant. However, what happens at some critical age to make the rate of follicular depletion increase abruptly? Recently, this biphasic pattern has been questioned by Leidy *et al.* (1998). They have tested four models with the use of data drawn from published studies and histological analyses of ovaries (Leidy *et al.*,

Table IV. Regression equations for monophasic and different biphasic linear relationships between the AF count and age

	n_1/n_2	β_1	*y*-intercept	β_2	*y*-intercept$_2$	Outlying values (*n*)
Monophasic	81/NA	−0.312	17.126	NA	NA	6
Biphasic relationships with different ages (in years) as cut-offs						
30	22/59	0.062	7.399	−0.507	24.581	3
31	24/57	0.099	6.493	−0.479	23.466	3
32	26/55	0.005	8.779	−0.495	24.129	3
33	28/53	0.061	7.419	−0.427	21.392	5
34	32/49	−0.021	9.466	−0.379	19.459	3
35	38/43	−0.123	12.044	−0.348	18.208	4
36	43/38	−0.197	13.949	−0.397	20.209	3
37	51/30	−0.242	15.148	−0.437	21.870	4
38	58/23	−0.244	15.204	−0.194	11.706	4

The predictive power of these relationships was expressed as the number of outlying values.
n_1 and n_2 indicate the number of subjects, who were younger than or equal to the relevant cut-off and those older than the cut-off, respectively.
NA = non applicable.

Table V. Regression equations for monophasic and different biphasic linear relationships between the ovarian volume and age

	n_1/n_2	β_1	*y*-intercept$_1$	β_2	*y*-intercept$_2$	Outlying values (*n*)
Monophasic	81/NA	−0.262	18.055	NA	NA	4
Biphasic relationships with different ages (in years) as cut-offs						
30	22/59	0.188	6.351	−0.491	26.799	3
31	24/57	0.194	6.196	−0.473	26.102	3
32	26/55	0.016	10.556	−0.541	28.814	4
33	28/53	0.109	8.251	−0.472	26.044	4
34	32/49	0.120	7.981	−0.350	21.136	5
35	38/43	0.043	9.927	−0.180	14.223	5
36	43/38	−0.076	13.016	−0.197	14.924	5
37	51/30	−0.179	15.724	−0.286	18.621	5
38	58/23	−0.210	16.552	−0.297	19.089	5

The predictive power of these relationships was expressed as the number of outlying values.
n_1 and n_2 indicate the number of subjects, who were younger than or equal to the relevant cut-off and those older than the cut-off respectively.
NA = non applicable.

Table VI. Regression equations for monophasic and different biphasic linear relationships between the serum FSH level and age

	n_1/n_2	β_1	y-intercept$_1$	β_2	y-intercept$_2$	Outlying values (n)
Monophasic	81/NA	0.347	−2.164	NA	NA	5
Biphasic relationships with different ages (in years) as cut-offs						
30	22/59	0.158	2.885	0.498	−7.957	4
31	24/57	0.157	2.913	0.505	−8.234	4
32	26/55	0.272	0.088	0.574	−10.950	3
33	28/53	0.183	2.288	0.539	−9.549	3
34	32/49	0.171	2.609	0.510	−8.418	3
35	38/43	0.164	2.773	0.405	−4.139	3
36	43/38	0.206	1.701	0.364	−2.458	3
37	51/30	0.260	0.278	0.320	−0.607	4
38	58/23	0.234	0.975	−0.683	41.442	3

The predictive power of these relationships was expressed as the number of outlying values.
n_1 and n_2 indicate the number of subjects, who were younger than or equal to the relevant cut-off and those older than the cut-off respectively.
NA = non applicable.

1998). These models did not include a quadratic model. Leidy *et al.* (1998) concluded that follicular atresia was not linear on either original measurement or log-linear scales. They also suggested that the data on follicular atresia did not support the notion that an abrupt change in the exponential rate of decline exists, and concluded that a biphasic pattern was most likely to be an artefact (Leidy *et al.*, 1998). It is more acceptable to hypothesize the existence of an increase in the decline rate of follicles through the years instead of an abrupt change at a certain age.

Faddy and Gosden also revised their original model, and suggested a gradual change in follicle depletion (Faddy and Gosden, 1996, 2000; Faddy *et al.*, 1992). In their model, the follicles were classified into three stages of growth: stage I, primordial follicles; stage II, early growing forms; and stage III, more advanced stages. Following re-analysis of ovaries in their initial study (Faddy *et al.*, 1992), they suggested a model that shows a gradual increase in follicle depletion based on either growth of follicles between these three compartments or atresia. Their model suggested that a proportional number of primordial follicles enter the growth phase. This model suggests that the AF count can reliably reflect the primordial follicle pool, which is the actual OR. This is consistent with the results of clinical studies, which showed that AF count is a reliable OR test. Since their model was based on histological findings and a three compartment model, we avoided detailed comparison between their model and that in the present study, which was based on OR tests. However, the similarity of the graph in the present study to that in their model supports the proportional relationship between the primordial follicle cohort and growing follicles (Faddy and Gosden, 2000).

Clinical experience and demographic data obtained from natural populations suggest that the period of optimal fertility only lasts until age 30–31 and decreases thereafter (van Noord-Zaadstra *et al.*, 1991; te Velde *et al.*, 1998). The suggested model for the AF count and OV in the present study indicates almost a constant OR between ages 21 and 30. The OV decreases by 1.7% and the AF count decreases by 7.7% during that time. Thereafter, although the amount of decrease per year is constant according to the model, the percentage change increases with age. Therefore, the apparent acceleration in decline is merely due to a constant depletion and a decreasing denominator; percentage change = amount of difference (which is constant)/previous amount (which decreases with age). The percentage change in the serum FSH level increases with age through all ages between 21 and 43. However, this increase is slight for ages between 21 and 30, i.e. 23% in 9 years.

These models may also help to analyse the mechanism for ovarian ageing. It has been suggested previously that the accelerated increase of oocyte depletion might be due to a rising serum FSH level (Gosden and Faddy, 1994; Gougeon, 1996). In the present study, we observed that the serum FSH level increases slightly at the third decade of age, but AF count and OV appear to decline slightly in the same period. The increase in serum FSH level was slightly faster than the decline in AF count. In the model, serum FSH level increased 60% between ages 25 and 40. The AF count decreased 51% during the same period.. These findings suggest that increasing serum FSH level may initiate the accelerated loss. However, it should be mentioned that the evidence that the FSH receptor is not expressed in follicles until they have reached a certain stage argues against this FSH-dependent hypothesis (Oktay *et al.*, 1997). The underlying mechanism for the accelerated loss of oocytes remains unclear. However, the results of egg donation programmes suggest that the oocyte itself is responsible for the declining fertility in humans. It appears that ageing has some negative effects on the quiescent oocytes. This may be a result of either damage to DNA, decreasing density of oocytes or changes in growth factor tone (Faddy and Gosden, 2000).

In the present study, the quadratic model for the AF count was superior to any biphasic linear relationship for predicting the observed values. This model indicates a slower increase in the decline rate instead of an abrupt change. This relationship between age and AF count is similar to fertility change by age in different populations (Menken *et al.*, 1986). Compared with women aged 20–24, fecundity is reduced on average by 6% for women aged 25–29, 14% for those aged

30–34, and 31% for women aged 35–39 (Menken *et al.*, 1986). The corresponding values were 3, 14 and 34%, respectively, when the model in the present study for the AF count was used.

The model for the AF count in the present study means that the existence of an FSH-sensitive cohort will have ended at the age of 46.7 years. This seems biologically unlikely in view of the fact that the vast majority of women will still have cycles at this age, albeit irregular. Although this value is consistent with mean age at menopause in Turkey (47 years) (Neslihan Carda *et al.*, 1998), it should also be mentioned that models in the present study are based on the data of women between the ages of 20 and 43 years, and therefore extrapolating these results to older ages may not be correct.

The AF count had the highest correlation with age among the OR tests in the present study. Scheffer *et al.* (2003) also showed that the number of AFs correlated much better with the age of the women evaluated in their study than other presumed basal markers for reproductive age, including FSH, inhibin B, E_2 and OV. Therefore, results in the present study also support the hypothesis that the AF count is superior to other static measures of reproductive ageing.

Although nomograms for OV in post-menopausal women have been studied (Goswamy *et al.*, 1983, 1988), data in premenopausal women are limited (van Nagell *et al.*, 1990). Surprisingly, there are reports that used a constant cut-off value for the OV while studying the effectiveness of transvaginal sonography in screening for ovarian cancer in premenopausal women (DePriest *et al.*, 1997). The present study showed that OV decreases with age, especially after the age of 30 years. Therefore, using a constant cut-off value for the OV in premenopausal women may not be appropriate for screening purposes.

In conclusion, the pattern of reproductive ageing as evidenced by hormonal and ultrasonographic OR tests does not show an abrupt change at a certain age, but follows a continuously increasing rate of decline in the third decade of life. The change in serum FSH level and ultrasonographic OR tests with age follows a quadratic model in regularly menstruating premenopausal women.

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