The number of antral follicles in normal women with proven fertility is the best reflection of reproductive age

G.J. Scheffer1,6, F.J.M. Broekmans1, C.W.N. Looman2, M. Blankenstein3, B.C.J.M. Fauser4, F.H. de Jong5 and E.R. te Velde1

1Department of Reproductive Medicine, Division of Perinatology and Gynecology, University Medical Center Utrecht, 2Department of Public Health, Faculty of Medicine, Erasmus University, Rotterdam, 3Department of Clinical Chemistry, Free University Medical Center, Amsterdam, Division of Reproductive Medicine, 4Department of Obstetrics and Gynecology and 5Department of Internal Medicine III, Erasmus University Medical Center, Rotterdam, The Netherlands

6To whom correspondence should be addressed at: Department of Reproductive Medicine, Division of Perinatology and Gynecology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. E-mail: gabriellescheffer@hotmail.com

BACKGROUND: The purpose of this study was to compare the predictive capacity of several markers of reproductive age in normal women. METHODS: Healthy female volunteers (n = 162) aged 25–46 years with proven, normal fertility and regular menstrual cycles were recruited. In this selected group, chronological age was assumed to approximate reproductive age and, therefore, was taken as the proxy-variable for reproductive age. The number of antral follicles with 2–10 mm diameter, total ovarian volume, total follicular volume, mean follicular volume, and volume of either the smallest or largest ovary were estimated by transvaginal sonography of the ovaries. Serum levels of early follicular FSH, estradiol and inhibin B, as well as the response of estradiol and inhibin B to exogenous GnRH agonist administration (GAST), were also evaluated. RESULTS: Regression analysis revealed that the antral follicle number showed the highest correlation with age (r = -0.68, P = 0.001), and explained 46% of its variance. All other variables, except inhibin B, were moderately correlated with age. Responses of estradiol and inhibin B to the GnRH agonist were moderately correlated with age, but highly correlated with the number of antral follicles. CONCLUSIONS: It is concluded that the number of antral follicles has the closest association with chronological age in normal women with proven fertility. As stimulated estradiol and inhibin B clearly reflect the size of the antral follicle cohort, the GAST may be considered the second best single test to predict reproductive age.

Key words: antral follicles/GnRH agonist stimulation test/inhibin B/reproductive ageing/transvaginal sonography

Introduction

Delaying the period in life to have children considerably contributes to the proportion of couples with involuntary childlessness (Mosher et al., 1991). Demographic (Wood, 1989) and clinical (Noord-Zaadstra et al., 1991) studies have shown that a woman experiences her optimal fertility before the age of 30–31 years. Thereafter, fertility gradually decreases, with an acceleration towards the age of 40 years. Already at an age of 40–41 years half of the women will have completely lost their capacity for reproduction. It is generally accepted that reproductive ageing is in fact ovarian ageing and is related to the decreasing quantity and quality of the pool of follicles preserved in the ovary (Seifer et al., 1996; te Velde and Pearson, 2002).

The number of antral follicles and the total ovarian volume as measured by transvaginal ultrasound (Lass et al., 1997; Tomas et al., 1997; Chang et al., 1998; Ng et al., 2000; Bancsi et al., 2002), basal FSH (Muasher et al., 1988; Scott et al., 1990; Bancsi et al., 2000), inhibin B (Seifer et al., 1997; Corson et al., 1999; Hall et al., 1999; Creus et al., 2000), estradiol (E2) (Evers et al., 1998; Mikkelsen et al., 2001) and the E2 and inhibin B response to exogenous GnRH agonist (GAST) (Winslow et al., 1991; Avrech et al., 1996; Galtier-Dereure et al., 1996; Ranieri et al., 1998; Ravhon et al., 2000) or FSH stimulation (EFORT) (Fanchin et al., 1994; Dzik et al., 2000; Fabregues et al., 2000; Elting et al., 2001) have all been mentioned in the literature to predict declining fertility related to reproductive ageing. Most of these studies were performed in infertility populations, using pregnancy rates or response to ovarian hyperstimulation in IVF as outcome measures. However, the establishment of a pregnancy is influenced by many more, partly unknown, factors. To what extent ovarian ageing per se contributes to the reproductive failure of an individual couple remains a matter of speculation.

Studies concerning physiological ovarian ageing in women without fertility problems have only been performed in a limited number of relatively young women (Schipper et al., 1998) or have compared small groups of relatively aged
Antral follicle number reflects reproductive age

Table I. Median values and ranges of endocrine and sonographic characteristics in the three age categories

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Young (n = 49)</th>
<th>Middle (n = 53)</th>
<th>Old (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25–34 years</td>
<td>35–40 years</td>
<td>41–46 years</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>6.7 (2.0–21.2)</td>
<td>6.8 (2.7–21.4)</td>
<td>8.1 (3.2–35.9)a</td>
</tr>
<tr>
<td>Estradiol (pmol/l)</td>
<td>193 (51–290)</td>
<td>206 (77–528)</td>
<td>278 (51–816)b</td>
</tr>
<tr>
<td>Inhibin B (pg/ml)</td>
<td>101 (18–180)</td>
<td>104 (0–177)</td>
<td>97 (0–210)</td>
</tr>
<tr>
<td>No. of follicles (2–10 mm)</td>
<td>15 (3–30)</td>
<td>9 (1–25)b</td>
<td>4 (1–17)c</td>
</tr>
<tr>
<td>Total ovarian volume (ml)</td>
<td>11.8 (4.7–40.3)</td>
<td>11.4 (4.9–32.2)</td>
<td>8.3 (4.5–19.7)a</td>
</tr>
<tr>
<td>Smallest ovary volume (ml)</td>
<td>5.0 (1.8–16.1)</td>
<td>4.6 (1.7–14.6)</td>
<td>3.4 (1.2–7.9)c</td>
</tr>
<tr>
<td>Largest ovary volume (ml)</td>
<td>7.4 (2.4–24.2)</td>
<td>7.0 (3.3–19.7)</td>
<td>5.2 (2.7–13.4)a</td>
</tr>
<tr>
<td>Total follicular volume (ml)</td>
<td>0.71 (0.16–2.04)</td>
<td>0.58 (0.4–1.83)</td>
<td>0.39 (0–1.51)c</td>
</tr>
<tr>
<td>Mean follicular volume (ml)</td>
<td>0.05 (0.02–0.13)</td>
<td>0.06 (0.02–0.20)</td>
<td>0.09 (0.002–0.4)c</td>
</tr>
</tbody>
</table>

aMedian values of the old age group differed significantly from the young age group.
bMedian values of the middle age group differed significantly from the young age group.
cMedian values of the old age group differed significantly from the middle age group.

Range values are given in parentheses.

Materials and methods

This study was approved by the local Ethics Committee and written informed consent was obtained from all participants. Healthy female volunteers (n = 162, age range 25–46 years) were recruited by advertisement in the local newspapers. Women were enrolled in the study protocol if they met all of the following criteria: (i) regular menstrual cycles varying from 21 to 35 days, (ii) a biphasic body temperature chart, (iii) proven natural fertility by having had at least one pregnancy carried to term, (iv) each of their pregnancies arisen spontaneously within 1 year after the start of unprotected intercourse, (v) no evidence of endocrinological disease, (vi) no history of ovarian surgery, (vii) no ovarian abnormalities as assessed by transvaginal ultrasound and (viii) hormonal contraception stopped ≥2 months before entering the study protocol. For study participation the volunteers received monetary compensation.

Transvaginal sonography measurements

Transvaginal sonography of the ovaries was carried out on cycle day 1, 2, 3 or 4. All sonography measurements were performed by the same observer (G.S.) using the 7.5 MHz transvaginal probe on a Toshiba Capasee SSA-220A (Toshiba Medical Systems Europe BV, Zoetermeer, The Netherlands). Examination of the ovary was established by scanning from the outer to the inner margin (Pache et al., 1990; van Santbrink et al., 1995). All follicles 2–10 mm in size were measured and counted in each ovary. The sum of both counts was the antral follicle count. Follicle size was calculated from two or three perpendicular measurements depending on the diameter (≤6 or >6 mm). The volume of each follicle was calculated by applying the equation of the volume of an ellipsoid (L × W × D × π/6). By adding all volumes of follicles up to 10 mm in size in both ovaries, the total follicular volume was obtained (Haning et al., 1982). Mean follicular volume was calculated by dividing the total follicular volume by the number of follicles counted. The volume of the left and right ovary was assessed by measuring the diameter of the ovarian contour in three perpendicular directions and applying the equation for the volume of an ellipsoid (V = 4/3 × π × D1 × D2 × D3 × π) to calculate ovarian volume. Total ovarian volume was then obtained by summing the volume of the left and right ovary. Intra- and inter-observer variations of the antral follicle count and ovarian volume assessment have been published elsewhere (Scheffer et al., 2002).

Endocrine testing

Blood sampling was performed on the same day as the sonography examination. Hormone concentrations were measured in plasma (E2 and FSH) and serum (inhibin B). Specimens were stored at −20°C until processing. A random subgroup of 40 women underwent a GnRH agonist stimulation test in the cycle subsequent to the one in which the basal endocrine and ultrasound characteristics were assessed. A single s.c. injection of 100 μg of triptorelin (Decapeptyl; Ferring, Hoofddorp, The Netherlands) was administered at day 3 of the cycle (Ranieri et al., 1998). Blood samples were taken immediately before and 24 h after GnRH agonist administration. The administered dose is assumed to provide maximal stimulation of the pituitary with mean peak levels of 52 IU/l for LH and 25 IU/l for FSH at 4 h after the injection and LH levels of 12.0 IU/l and FSH levels of 10.3 IU/l after 24 h (Broekmans et al., 1993). E2 concentrations were assayed with a microparticle enzyme immunoassay (MEIA) purchased from Abbott Laboratories (Abbott Park, IL, USA) and using a semi-automated IMx analyser. Between-run coefficients of variation (CV) for E2 were 10.1, 7.0 and 6.9% at 533, 1354 and 4197 pmol/l respectively (n = 49, 49 and 30). Concentrations of FSH were measured with the use of the MEIA technology on a fully automated AxSYM immunoanalyser (Abbott Laboratories) according to the manufacturer’s instructions. All specimens of each volunteer were analysed in the same run. The standard of the FSH assay was referenced against the World Health Organization Second International Reference Preparation for human FSH (78/549). The between-run CV of the FSH assay was 6.0, 6.6 and 8% at levels of 5.0, 25 and 75 IU/l (n = 46). Inhibin B levels were measured using an immuno-enzymometric assay (Serotec, Oxford, UK).
Results

Median values and ranges of the various endocrine and sonographic parameters for the three age groups are given in Table I. Values of all variables in the old age group differ significantly from those of the young group (except inhibin B) and from those of the middle group (except $E_2$, inhibin B and FSH). The number of follicles is the only variable for which values in the middle age group also significantly differ from those of young women. Both the numbers of antral follicles and the total follicular volume decrease with increasing age. However, the decrease of total follicular volume is less steep than that of the follicle number, while the mean volume per follicle increases as a woman grows older and almost doubles in the old age group as compared with the young age group.

The correlation matrix (Table II) should be interpreted in conjunction with Figure 1. Volumes of the smallest, the largest and both ovaries are highly correlated, indicating that they will provide almost the same information. Therefore, in subsequent analyses only one of them—total ovarian volume—is taken into account. The correlation between follicle number and total follicular volume is also strong ($r = 0.69$), while the mean volume per antral follicle increases with age ($r = 0.37$). The sonographic variables, except mean follicular volume, together form one cluster, in which correlation between the ovarian volume cluster and the follicle cluster is between 0.4 and 0.5 (Figure 1). The interrelationship between the endocrinological parameters is less clear-cut. One cluster is formed by inhibin B and FSH, confirming their rather strong and inverse correlation ($r = -0.40$). $E_2$ and mean follicular volume also form one, positively correlated cluster, which is in line with the positive correlation of both $E_2$ and mean follicular volume with age (Table II).
The results of the univariate and partial correlations of the endocrine and sonographic variables with age are shown in Table III. Number of follicles shows the best correlation with age ($r = -0.68$). In contrast, the correlation of inhibin B with age was weak and did not reach statistical significance. The correlations of the remaining variables were all highly significant ($P < 0.001$).

Antral follicle number reflects reproductive age

<table>
<thead>
<tr>
<th>Variables</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH</td>
<td>0.25*</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>Estradiol</td>
<td>0.29*</td>
<td>0.22*</td>
<td>0.21*</td>
</tr>
<tr>
<td>Inhibin B</td>
<td>-0.12</td>
<td>0.11</td>
<td>0.06</td>
</tr>
<tr>
<td>No. of follicles</td>
<td>-0.68*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Total ovarian volume</td>
<td>-0.30*</td>
<td>0.05</td>
<td>0.001</td>
</tr>
<tr>
<td>Total follicular volume</td>
<td>-0.34*</td>
<td>0.24*</td>
<td>—</td>
</tr>
<tr>
<td>Mean follicular volume</td>
<td>0.37*</td>
<td>0.23*</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Step 1: univariate correlations; step 2: partial correlations controlling for the number of follicles; step 3: partial correlations controlling for the number of follicles and total follicular volume.

Values are medians and ranges.

The number of antral follicles correlated much better with the age of the women evaluated in this study, than other presumed basal markers for reproductive age, including FSH, inhibin B, E2 and ovarian volume. As expected, total follicular volume appeared to be a simple derivative from number of follicles, evidenced by a strong and positive correlation with numbers of follicles ($r = 0.69$) and a negative correlation with reproductive age ($r = -0.34$). We were surprised, however, to find that the mean follicular volume considerably increased with age and had almost doubled in the old age group in comparison with the young ones. The correlation of mean follicular volume, a derivative of the total follicular volume

<table>
<thead>
<tr>
<th>Variables</th>
<th>Baseline</th>
<th>Stimulated</th>
<th>Response</th>
<th>$P$-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol (pmol/l)</td>
<td>187 (51–462)</td>
<td>536 (9–1163)</td>
<td>276 (–11–994)</td>
<td>0.001</td>
</tr>
<tr>
<td>Inhibin B (pg/ml)</td>
<td>89.5 (5–170)</td>
<td>150 (11–351)</td>
<td>62 (46–246)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values of age and the basal endocrine and ultrasound variables studied in the GAST group did not differ from those in the remaining group (data not shown). Baseline levels, levels after GnRH agonist stimulation and the corresponding responses of inhibin B and E2 in the GAST group are shown in Table IV. The rise in hormone levels after the GnRH agonist stimulation was statistically significant for both hormones. Correlations of age and the investigated hormones and hormonal responses as well as the ultrasound variables are shown in the correlation matrix (Table V). Note that the correlations among age and the endocrine and ultrasound baseline variables are in the same order as for the total group (Table II), with the exception that the correlation for inhibin B with antral follicle count seems more favourable, presumably due to chance variation. The weak or statistically non-significant correlations for baseline levels of inhibin B and E2 with age are changed into more robust and significant correlations when the response in the GnRH agonist test is considered (Tables II and IV). Baseline levels of inhibin B show a statistically significant correlation with antral follicle numbers. However, the correlations with the number of antral follicles of the E2 and inhibin B responses after GnRH agonist stimulation become highly significant and clearly stronger than for basal inhibin B. Moreover, E2 and inhibin B responses are clearly better correlated with antral follicle number ($r = 0.75$ and 0.73 respectively) than with age ($r = -0.42$ and -0.44 respectively). Finally, E2 and inhibin B responses are correlated as expected.

Discussion

In this study we showed that the number of antral follicles 2–10 mm in diameter, as measured by vaginal sonography during the early follicular phase, appeared to have the best correlation with chronological age in a carefully selected group of women who had regular cycles and proven normal fertility. In such women the age-dependent changes of ovarian function are likely to reflect the physiological decline of female fecundity. Hence, their chronological age can be assumed to be a better approximation of reproductive age than that of women who suffer from infertility which may be associated with accelerated reproductive ageing. We realize, however, that such an approximation is not perfect, because also in normal fertile women of the same chronological age, variations in reproductive status may be present. However, we are not aware of a better ‘gold standard’ for the process of reproductive ageing.

It is generally accepted that reproductive ageing is directly related to the remains of the stock of primordial follicles, which is established during fetal life. This pool progressively empties as a woman grows older and is (almost) completely exhausted when menopause is reached (Faddy et al., 1992). In a previous study we showed that the pattern of age-dependent loss of antral follicle numbers is strikingly similar to that of the primordial follicle pool (Scheffer et al., 1999). It seems plausible, therefore, that the number of antral follicles, as assessed by sonography, reflects what is left of the primordial follicle pool and, thus, the reproductive age of an individual woman.

The number of antral follicles correlated much better with the age of the women evaluated in this study, than other presumed basal markers for reproductive age, including FSH, inhibin B, E2 and ovarian volume. As expected, total follicular volume appeared to be a simple derivative from number of follicles, evidenced by a strong and positive correlation with numbers of follicles ($r = 0.69$) and a negative correlation with reproductive age ($r = -0.34$). We were surprised, however, to find that the mean follicular volume considerably increased with age and had almost doubled in the old age group in comparison with the young ones. The correlation of mean follicular volume, a derivative of the total follicular volume
and the number of follicles, with age, therefore, is positive ($r = 0.37$). 

E$_2$ levels are also positively correlated with age. Apparently, the few antral follicles which are still present in the early follicular phase in older aged women, are not only larger but also produce more E$_2$ than the many, but smaller, antral follicles present at a younger age. Several studies have shown that the follicular phase of the menstrual cycle in older aged women becomes considerably shorter (Lenton et al., 1984; Klein et al., 1996a; van Zonneveld et al., 2003). This phenomenon has been postulated as suggesting accelerated growth of antral follicles in older women (Klein et al., 1996a). Since the diameter on the day of ovulation and the mean follicular growth per day were almost the same in older and younger women (van Zonneveld et al., 2003), we suggest that follicular development in older women is not accelerated but advanced. Dominant follicle growth in older women is likely to have started already in the luteal phase of the previous cycle before menstruation starts. This earlier start of development is in accordance with data from the literature, indicating that the intercycle FSH rise is not only higher, but also starts earlier in older women (Klein et al., 1996a; van Zonneveld et al., 2003). Advanced development of follicular growth fully explains why at the onset of the menstrual cycle, antral follicles are larger and, though lower in number, produce higher E$_2$ levels in older compared with younger women.

While numbers of follicles already have clearly decreased in the middle-aged, hormone levels of FSH, E$_2$ and inhibin B only become notably changed in women aged >40 years. Apparently, age-dependent hormonal changes are a relatively late phenomenon and only occur when follicle numbers are greatly reduced (te Velde and Pearson, 2002). This conclusion is in line with the results of several studies in normal volunteers. Neither maximum FSH and inhibin B concentrations in the follicular phase nor cycle day 3 FSH levels were correlated with age in normally cycling, female volunteers aged 20–35 years (Schipper et al., 1998). A study in young controls (aged 20–25 years) and reproductively aged women (aged 40–45 years) showed that inhibin B serum levels were only significantly lower in the reproductively aged women at the day of maximal FSH (Klein et al., 1996a). Finally, lower early follicular inhibin B serum levels were only found in older cycling women compared with young controls (Welt et al., 1999). All this explains why the correlations of basal FSH, E$_2$ and inhibin B with chronological age are only weak to moderate.

Although the number of antral follicles in both ovaries appeared to have the best correlation with chronological age, basal E$_2$ and total follicular volume slightly improved the prediction already obtained with the antral follicle count. Apparently, the predictive information provided by FSH, inhibin B and total ovarian volume is already covered by the number of follicles. Nevertheless, 90% of the explained variance of age is already obtained with the number of follicles alone, while the additional contributions of total follicular volume and E$_2$ are 6 and 4% respectively. Such an improvement in predictive performance seems almost negligible. Therefore, the use of antral follicle counts as a single test to predict the response to controlled ovarian stimulation and the probability of pregnancy in assisted reproduction seems rational. Several studies have analysed the usefulness of antral follicle counts in this respect. In a study of IVF patients (Tomas et al., 1997) it was shown that the ovarian responsiveness is dependent on the number of small antral follicles (2–5 mm). In another study, patients with an antral follicle (2–8 mm) count of less than four appeared to have a significantly higher rate of cancellation due to poor response and no pregnancies occurred in this group of patients (Chang et al., 1998). In several studies, logistic regression analysis has shown that the number of antral follicles is a significant predictor for the occurrence of poor ovarian response in IVF with an adequate balance between test sensitivity and specificity (Frattarelli et al., 2000; Pohl et al., 2000; Dumiesic et al., 2001; Hsieh et al., 2001; Huang et al., 2001; Bancsi et al., 2002). Most studies, however, also revealed that pregnancy prediction from antral follicle counts, even in combination with other ovarian reserve factors, remains difficult.

It was shown that after the administration of a high dose of GnRH agonist, antral follicles greatly increase their production and release of E$_2$ and inhibin B from the granulosa cells within 24 h. This finding is not new for E$_2$ (Winslow et al., 1991; Ranieri et al., 1998) and confirms results from other studies (Avrech et al., 1996; Galtier-Dereure et al., 1996; Ravhon et al., 2000). As E$_2$ and inhibin B are produced from small antral follicles present in the early follicular phase of the cycle, basal levels would reflect the size of the FSH sensitive cohort of follicles. Although this may be true for inhibin B, E$_2$ release is much more dependent on other sources such as the remnants of the corpus luteum or an advanced growing follicle. Once stimulated by an endogenous FSH (and LH) rise in the GAST, the relation between inhibin B and the cohort size becomes
Moreover, the responses of E2 and inhibin B in the GAST are obtained from other endocrine or ultrasound variables. Additional, though modest, predictive information may be of supremacy that justifies the increased burden put on the literature so far. As yet it cannot be expected that the accuracy with antral follicle count has not been published in the assisted reproduction treatment. Back-to-back comparison in ovarian hyperstimulation for IVF (Fanchin et al., 1994; Dzik et al., 2000; Eldar-Geva et al., 2000; Elting et al., 2001). Moreover, the responses of E2 and inhibin B in the GAST are clearly better related to the chronological age of a woman when compared with baseline levels. This suggests that stimulated E2 and inhibin B may well reflect the quantitative process of reproductive ageing. As the antral follicle count is the best reflection of reproductive age when basal tests are considered, and at the same time is highly correlated with the E2 and inhibin B response in the GAST, the question arises whether the GAST may be a superior test in the prediction of outcome in assisted reproduction treatment. Back-to-back comparison with antral follicle count has not been published in the literature so far. As yet it cannot be expected that the accuracy of the test in the prediction of outcome in IVF will reach a level of supremacy that justifies the increased burden put on the patient by this test.

In summary, in the prediction of chronological age in normal women, the number of antral follicles appeared to be superior to other presumed measures of reproductive ageing. Additional, though modest, predictive information may be obtained from other endocrine or ultrasound variables. Stimulated E2 and inhibin B correlated strongly with the number of antral follicles and therefore may provide the same body of information on reproductive age. Whether these findings will help us to assess the reproductive capacity in individual subfertile women remains to be further elucidated.

References


Submitted on April 27, 2001; resubmitted on September 26, 2002; accepted on November 29, 2002.