Changes in anti-Müllerian hormone serum concentrations over time suggest delayed ovarian ageing in normogonadotrophic anovulatory infertility

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BACKGROUND: Anti-Müllerian hormone (AMH), produced by growing pre-antral and early antral ovarian follicles, has been shown to be a useful marker for ovarian ageing. Serum AMH concentrations are elevated during reproductive life in anovulatory women, especially in those patients exhibiting polycystic ovaries (PCO). The current study was designed to investigate whether the decrease in AMH serum concentrations over time is different comparing women with normogonadotrophic anovulation [World Health Organization (WHO) group 2 (including polycystic ovary syndrome (PCOS)] and normo-ovulatory controls. METHODS and RESULTS: AMH serum levels were assessed on two occasions in 98 patients suffering from WHO 2 anovulatory infertility as well as in 41 normo-ovulatory premenopausal women. Median time interval between both visits was 2.6 years (range 0.3–9.0) for WHO 2 patients compared with 1.6 years (range 1.0–7.3) in controls. Serum AMH concentrations were significantly ($P<0.0001$) elevated on both occasions in WHO 2 patients (AMH₁, median = 7.5 μg/l, range 0.1–35.8; and AMH₂, median = 6.7 μg/l, range 0.0–30.6) compared with controls (AMH₁, median = 2.1 μg/l, range 0.1–7.4; and AMH₂, median = 1.3 μg/l, range 0.0–5.0). Regression analysis, corrected for age, indicated a significant relative decrease in serum AMH concentrations over time for both groups ($P<0.001$). However, the decline in serum AMH in WHO 2 patients was significantly less compared with controls ($P=0.03$). CONCLUSION: The present longitudinal study shows that serum AMH concentrations decrease over time both in women presenting with WHO 2 anovulatory infertility and in normo-ovulatory controls. The decrease in WHO 2 patients is less pronounced despite distinctly elevated concentrations. This observation may suggest retarded ovarian ageing and hence a sustained reproductive life span in these patients.

Key words: anti-Müllerian hormone (AMH)/anovulation/infertility/ovarian ageing/PCOS

Introduction

The dimeric glycoprotein anti-Müllerian hormone (AMH), a member of the transforming growth factor-β (TGF-β) superfamily, is produced exclusively in the gonads (Lee and Donahoe, 1993) and is involved in the regulation of growth and development (Cate et al., 1986). During male fetal sexual differentiation, AMH [also known as Müllerian-inhibiting substance (MIS)] is synthesized by testicular Sertoli cells and induces degeneration of the Müllerian ducts (Jost, 1947; Josso et al., 1993; Lee and Donahoe, 1993). AMH expression in the ovary starts at the end of the third trimester of pregnancy (Rajpert-De Meyts et al., 1999), where it is produced in the granulosa cells of early developing follicles (Baarends et al., 1995). Ovaries of AMH knock-out mice as well as female mice heterozygous for the AMH deletion showed an accelerated exhaustion of the primordial follicle stock (Durlinger et al., 1999), suggesting important roles for AMH in depletion of the primordial follicle pool. Moreover, AMH was able to inhibit the initiation of primordial follicle growth in cultured neonatal mouse ovaries (Durlinger et al., 2002), and AMH has been shown to inhibit FSH-induced follicle growth in female mice (Durlinger et al., 2001). Recent data suggest that AMH expression in the human ovary is similar to that observed in mouse and rat (Weenen et al., 2004), suggesting important roles for AMH in the regulation of human early follicle growth as well.
AMH serum levels decline with increasing age in normo-ovulatory women (de Vet et al., 2002) and are strongly correlated with the number of antral follicles. Hence AMH may be used as a marker for ovarian ageing (de Vet et al., 2002; van Rooij et al., 2002; Fanchin et al., 2003a). In fact, poor response during ovarian stimulation for IVF [indicative of ovarian ageing (Beckers et al., 2002)] has been shown to be associated with reduced early follicular phase AMH serum concentrations (van Rooij et al., 2002; Seifer et al., 2002; Fanchin et al., 2003b).

Chronic anovulation is a common cause of infertility and it is diagnosed in ~20–25% of couples with fertility problems (ESHRE Capri Workshop Group, 1995; Laven et al., 2002). Most of these women present with irregular menstrual cycles and normal serum FSH concentrations [World Health Organization (WHO) group 2] (Rowe et al., 2000). Recent data have shown that serum levels of AMH are elevated in WHO 2 and polycystic ovary syndrome (PCOS) patients (Cook et al., 2002; Pigny et al., 2003; Laven et al., 2004). Moreover, it seems that AMH levels correlate well with the extent of ovarian dysfunction in anovulatory women (Laven et al., 2004). Finally, the decline in AMH serum levels with increasing age in this cross-sectional data set differs when comparing anovulatory women and normo-ovulatory controls (Laven et al., 2004).

Since AMH constitutes an important regulator of primordial follicle pool depletion (Durlinger et al., 1999), an increased intra-ovarian AMH production may slow down the process of depletion of the primordial follicle pool. Due to retarded exhaustion of the primordial stock of follicles, the age of menopause might be delayed in these anovulatory women. The current longitudinal cohort study was designed to investigate whether the decrease in AMH serum concentrations over time is different comparing women with WHO 2 anovulation (including PCOS) and ovulatory controls.

Materials and methods
The local Medical Ethics Review Committee approved this study and informed consent was obtained from all participants. Ninety-eight patients attending our fertility clinic between 1993 and 2003 with: (i) infertility; (ii) oligomenorrhea (interval between periods >35 days) or amenorrhea (absence of vaginal bleeding for at least 6 months); (iii) serum FSH concentrations within normal limits (1–10 IU/l) (van Sanbrink et al., 1997); and (iv) between 16 and 41 years of age were included in the present study. A subgroup of these patients was diagnosed as having PCOS due to hyperandrogenism and/or polycystic ovaries (PCO) on ultrasound (Rotterdam ESHRE/ASRM-sponsored PCOS Consensus Workshop Group, 2004). PCO were diagnosed in the case of an increased follicle count (>11 follicles in one or both ovaries) and/or an increased ovarian volume (>10.0 ml) of at least one ovary (Balen et al., 2003). All WHO 2 women participated in previously published studies (Imani et al., 1998, 1999; Mulders et al., 2003a,b).

The control group consisted of 41 normo-ovulatory women, as described before (de Vet et al., 2002). All control women participated in previous studies between 1993 and 1999 (van Sanbrink et al., 1995; Schipper et al., 1998; Hohmann et al., 2001). Inclusion criteria were regular menstrual cycle (26–30 days), 20–36 years of age, body mass index (BMI) (19–26 kg/m²), absence of endocrine disorders or any other relevant disease, and no use of medications or oral contraceptives during the 3 months prior to the start of the study.

For the anovulatory patients, repetitive standardized screening [clinical investigation, fasting blood withdrawal and transvaginal sonography (TVS)] was performed on a random day between 9 and 11 a.m., as previously described (Imani et al., 1998). For each individual anovulatory patient, the length of the interval between visits is dependent on the time between each step of the treatment regimen (Imani et al., 1998). For the normo-ovulatory controls, repetitive TVS and blood sampling were performed during the early follicular phase (cycle day 3, 4 or 5) (de Vet et al., 2002). For each control, the interval length between visits is dependent on the time between participation in both studies (de Vet et al., 2002).

Blood samples were obtained by venepuncture and processed within 2 h after withdrawal, as described previously. Serum was stored at −20°C until assayed. The hormone assays used have all been described elsewhere (Imani et al., 1998; de Vet et al., 2002). Serum AMH levels were measured by using an ultrasensitive enzyme-linked immunosorbent assay (Immunotech-Coulter, Marseilles, France) as described elsewhere (Long et al., 2000). This assay uses the same components as the normal assay, but some procedural adaptations result in increased sensitivity, making it possible to determine lower serum concentrations of AMH as they exist in women. Intra- and inter-assay coefficients of variation were <5 and <15% for LH, <3% and <8% for FSH, <8% and <11% for androstenedione (AD), <3% and <5% for testosterone, <5% and <7% for estradiol (E2), <4% and <5% for sex hormone-binding globulin (SHBG), <9% and <15% for inhibin B, <5% and <8% for AMH, respectively (Imani et al., 1998; de Vet et al., 2002).

Results are presented as the mean ± SD if distributed normally, or otherwise as the median and range. To assess differences between groups, Mann–Whitney or Kruskal–Wallis tests were used. Associations between different parameters were assessed by Spearman’s rank correlation coefficient. To establish whether variables changed over time, the Wilcoxon matched pairs signed rank sum test was used. To determine the rate of change over time, regression analysis was used. After log transformation, the ratio (value visit 2:visit 1) of variables was plotted against the time interval between visit 1 and 2. The relative decline per year was introduced in the present analysis since ovarian follicle depletion occurs at a constant rate of proportional decline for women under 38 years of age (Faddy et al., 1992). A possible difference in rate of decline between WHO 2 and controls was tested for in the analysis. All regression analyses were corrected for age. Data were analysed using the commercially available software package SPSS (Chicago, IL). A P-value of 0.05 was chosen as the threshold level for statistical significance.

Results
Clinical, endocrine and ultrasound characteristics during the first assessment in the WHO 2 and normo-ovulatory control group are summarized in Table I. During the second assessment, BMI (P < 0.001), cycle duration (P < 0.001), serum E2 concentrations (P < 0.001) and mean number of follicles (P < 0.001) were significantly elevated in WHO 2 patients when compared with the controls. Age (P = 0.03) and serum FSH concentrations (P < 0.001) were significantly elevated during the second assessment for the normo-ovulatory controls. Serum testosterone concentrations decreased significantly (P < 0.001) over time for the WHO 2 patients.
Serum AMH concentrations were significantly ($P < 0.001$) elevated on both occasions in WHO 2 patients (AMH$_1$, median = 7.5 μg/l, range 0.1–35.8; and AMH$_2$, median = 6.8 μg/l, range 0.0–30.6) compared with controls (AMH$_1$, median = 2.1 μg/l, range 0.1–7.4; and AMH$_2$, median = 1.3 μg/l, range 0.0–5.0) (Figure 1). Levels of AMH were negatively correlated with age at visit 1 (WHO 2, $r = -0.30$, $P = 0.003$; controls, $r = -0.47$, $P = 0.002$) and visit 2 (WHO 2, $r = -0.25$, $P = 0.01$; controls, $r = -0.57$, $P < 0.001$) (Figure 2). Additionally, associations between AMH and mean follicle number are shown for both groups at visit 1 and visit 2 (Figure 2). A statistical analysis on the serum levels of AMH from participants in whom levels at both visits were assessed within 2 years after withdrawal was performed to exclude bias due to long-term storage (Lee et al., 1996). A one-sample $t$-test showed a significant decrease in AMH levels. Storage time did not significantly influence the slopes of the regression lines of follicle number compared with AMH level. The slope of the regression line for follicle number versus AMH serum levels was 0.36, 0.30 and 0.29 in groups with a storage time of 2 years, 2–3 years and >4 years, respectively ($P = 0.8$).

AMH serum levels declined in both groups (Figure 3). The rate of decline, as shown by the regression lines, in WHO

**Table 1.** Clinical, endocrine and ultrasound characteristics [medians with (range)] during the first assessment in 98 patients with WHO 2 anovulatory infertility compared with 41 normo-ovulatory controls

<table>
<thead>
<tr>
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<th>WHO 2 anovulation</th>
<th>Normo-ovulatory controls</th>
<th>$P$-value</th>
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<tbody>
<tr>
<td><strong>Clinical</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age (years)</td>
<td>27.4 (16.1–41.3)</td>
<td>29.9 (19.6–35.6)</td>
<td>0.04</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>27.9 (17.7–50.6)</td>
<td>22.3 (18.8–27.3)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Cycle duration (days)</td>
<td>92.8 (13–199)</td>
<td>28.3 (25.0–31.0)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td><strong>Endocrine</strong></td>
<td></td>
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<tr>
<td>FSH (IU/l)</td>
<td>4.4 (0.1–15.7)</td>
<td>6.2 (3.3–13.5)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>6.8 (0.1–24.4)</td>
<td>NA</td>
<td></td>
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<tr>
<td>E$_2$ (pmol/l)</td>
<td>207 (44–1137)</td>
<td>153 (64–404)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Inhibin B (ng/l)</td>
<td>102 (1–621)</td>
<td>113 (12–213)</td>
<td>0.9</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>2.4 (0.3–6.8)</td>
<td>NA</td>
<td></td>
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<tr>
<td>FAI (100 × testosterone/SHBG)</td>
<td>5.5 (0.5–27.8)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>AMH (μg/l)</td>
<td>7.5 (0.1–35.8)</td>
<td>2.1 (0.1–7.4)</td>
<td>$&lt;0.001$</td>
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<tr>
<td><strong>Ultrasound</strong></td>
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<td>Mean number of follicles (both ovaries)</td>
<td>29.0 (5.0–84.0)</td>
<td>14.9 (6.0–28.0)</td>
<td>$&lt;0.001$</td>
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*Indicating amenorrhea; NA = not available.
2 patients differed significantly from controls \((P = 0.03)\) (Figure 3). The regression coefficient \((\beta)\) (representing the logarithm of 1 minus the relative decrease per year) for the normo-ovulatory controls was \(-0.16\), compared with only \(-0.08\) for women with WHO 2 anovulation. This implies a yearly decrease in AMH concentrations of 15% of the level of the year before in controls, compared with only 8% in WHO 2 women. Since there was a significant difference in age between the WHO 2 women and the controls, age was corrected for in the regression model. Correction for possible differences in age did not change either the data or the outcome. Figure 4 shows the fitted curves for the decline of AMH serum levels [mean (95% confidence interval)] over time for both groups.

There were no significant differences in the rate of change of AMH between the WHO 2 women with and without PCOS (data not shown).

Figure 2. Scatter plots depicting the correlations between the individual AMH serum concentrations versus age and number of follicles, respectively, in 98 women with WHO 2 anovulation and 41 normo-ovulatory women at visit 1 (filled circles) and visit 2 (open circles). Spearman’s correlation coefficients and corresponding \(P\)-values are depicted.

Figure 3. Scatter plot depicting the change of the ratio of AMH (value visit 2: visit 1) in relation to the time interval between visit 1 and visit 2 in 98 women with WHO 2 anovulation (filled circles, solid line) and 41 normo-ovulatory women (open circles, dotted line). The decline in serum AMH over time was significantly less in WHO 2 patients \((P = 0.03)\).

Figure 4. Serum levels of AMH in relation to age in 98 women with WHO 2 anovulation and 41 normo-ovulatory women. Solid lines indicate the fitted regression line. Shaded areas indicate the 95% confidence intervals around the fitted lines. Note the difference in the decline of the regression lines between WHO 2 women and normo-ovulatory controls. The intersection with the cut-off value of AMH (arbitrarily defined as 0.2 \(\mu g/l\)) of WHO 2 women and normo-ovulatory controls was 74 and 42 years, respectively.
Discussion

The present data once more show that AMH serum concentrations are increased in women with normogonadotrophic normo-estrogenic anovulatory infertility compared with normal controls with regular menstrual cycles. Similar results in a previous publication from our group were based on a cross-sectional set of data (Laven et al., 2004). However, the current longitudinal analysis indicates for the first time that serum AMH concentrations decline less rapidly over time in women with WHO 2 anovulatory infertility compared with normo-ovulatory controls. This may indicate a sustained reproductive life span in these anovulatory patients.

PCO differ from normal ovaries in that follicle development is arrested at the stage where dominant follicle selection would have taken place under normal conditions (Pache et al., 1992a; van Santbrink et al., 1995; Fauser and Van Heusden, 1997). Upon histological examination, it has been shown that the number of developing and subsequently atretic follicles was doubled compared with normo-ovulatory controls (Hughesdon, 1982; Webber et al., 2003). Moreover, the number of primordial follicles per section did not differ between women with and without PCO (Hughesdon, 1982; Webber et al., 2003). However, since the total ovarian volume is increased in PCO, it might be speculated that the primordial follicle pool is enlarged in these women. Indeed, recent historical studies using more sophisticated morphometric techniques suggest that the increased density of small pre-antral follicles in PCO possibly could result from a higher initial population of primordial follicles (Webber et al., 2003). Alternatively, the rate of follicle depletion in women with PCO may also vary. At present, evidence regarding dissimilarities involved in regulation of ovarian ageing in women with and without PCO is lacking. However, data currently available suggest that the intrinsic ovarian abnormality associated with aberrant follicular dynamics in the PCO might cause a reduced rate of atresia (Webber et al., 2003).

Menopause represents the clinical hallmark of follicle pool exhaustion and the definitive end of reproductive life. In addition, the commencement of menopause at an earlier age is associated with an earlier initiation of subfertility, sterility and transition to cycle irregularity, and vice versa (te Velde and Pearson, 2002). For normo-ovulatory women, it has been demonstrated that menstrual cycle irregularities associated with increasing age are dependent on the number of remaining follicles (Richardson et al., 1987). The basis of ovarian ageing in women is depletion of the primordial follicle pool (Richardson et al., 1987; Nikolaou and Templeton, 2003). Critical aspects involved in the process of ovarian ageing are the number of primordial follicles present in the initial stock and the factors that regulate the rate of loss of this stockpile (Wise et al., 1996). It seems likely that the ovary is the predominant pacemaker in reproductive ageing (te Velde et al., 1998; te Velde and Pearson, 2002). Studies in mice suggested an important role for AMH in depletion of the primordial follicle pool (Durlinger et al., 1999, Durlinger et al., 2002). AMH, produced in growing ovarian follicles, has been shown subsequently to be an excellent marker for ovarian ageing (de Vet et al., 2002; Seifer et al., 2002; Fanchin et al., 2003a). Recently, AMH levels were found to be elevated in anovulatory and PCOS patients compared with normal controls (Cook et al., 2002; Pigny et al., 2003; Laven et al., 2004).

In anovulatory women for whom the number of all classes of follicles including the total number of primordial follicles seems to be increased (Pache et al., 1992b; Webber et al., 2003), the age-related menstrual cycle irregularities (Kok et al., 2003) and follicle pool exhaustion might occur later. Moreover, it may be speculated that the process of ovarian ageing is indeed delayed in women with PCO, since levels of AMH, an important inhibitor of primordial follicle pool depletion (Durlinger et al., 1999), are increased. Indeed, cross-sectional data have suggested that women with PCOS may reach menopause at a later age (Dahlgren et al., 1992). Furthermore, it has been reported previously that cycle irregularities improve with increasing age (Dahlgren et al., 1992; Elting et al., 2000; Bili et al., 2001), possibly associated with a decrease in the follicle cohort size (Elting et al., 2003). Although no information is available regarding the age of menopause in women with WHO 2 anovulation, it has been shown in a cross-sectional study that advanced age is associated with lower LH and androgen levels in this group (Bili et al., 2001), as could be confirmed for the androgens in the current longitudinal study. Both oocyte quantity and quality dictate the subsequent reproductive events including decrease of fertility, increased abortion rate, the end of fertility, the beginning of cycle irregularity and, when almost no follicles are left, the occurrence of menopause (te Velde et al., 1998). As a consequence, it might be hypothesized that women with WHO 2 anovulatory infertility when compared with normo-ovulatory controls still might be able to conceive at an advanced age. However, from this point of view, oocyte quality is not taken into account. Finally, the possibility that a deviation in AMH synthesis or receptor is causally related to PCOS cannot be ruled out at this stage.

In summary, the current longitudinal study confirms that AMH serum levels are elevated in anovulatory women presenting with PCO, and demonstrates for the first time that the decline in AMH with age is less pronounced compared with controls. Considering important and well-documented roles of intra-ovarian AMH in the pace of follicle pool depletion and resulting female reproductive ageing, it may be proposed that the reproductive life span is extended in PCOS. Nevertheless, most women included in the present analysis have not yet reached the age of menopause. In order to substantiate further a delayed exhaustion of the primordial stock in women with WHO 2 anovulation, collection of additional follow-up data is required.

References

AMH over time and anovulatory infertility


Submitted on February 23, 2004; accepted on May 25, 2004