Which follicles make the most anti-Müllerian hormone in humans? Evidence for an abrupt decline in AMH production at the time of follicle selection

J.V. Jeppesen¹, R.A. Anderson², T.W. Kelsey³, S.L. Christiansen¹, S.G. Kristensen¹, K. Jayaprakasan⁴, N. Raine-Fenning⁴, B.K. Campbell⁴, and C. Yding Andersen¹,*

¹Laboratory of Reproductive Biology, Section 5712, The Juliane Marie Centre for Women, Children and Reproduction, University Hospital of Copenhagen, University of Copenhagen, Blegdamsvej 9, Rigshospitalet, DK-2100 Copenhagen, Denmark ²MRC Centre for Reproductive Health, Queen’s Medical Research Institute, University of Edinburgh, Edinburgh, UK ³School of Computer Science, University of St Andrews, St Andrews, Scotland, UK ⁴Nottingham University Research and Treatment Unit in Reproduction (NURTURE), Division of Human Development, School of Clinical Sciences, University of Nottingham, Nottingham, UK

*Correspondence address. Tel: +45-35455822; Fax: +45-35455824; E-mail: yding@rh.dk

Submitted on December 18, 2012; resubmitted on March 4, 2013; accepted on March 24, 2013

ABSTRACT: Anti-Müllerian hormone (AMH) is exclusively produced by granulosa cells (GC) of the developing pre-antral and antral follicles, and AMH is increasingly used to assess ovarian function. It is unclear which size follicles make the most AMH (total content) and are the main contributors to circulating AMH concentrations. To determine AMH gene expression in GC (q-RT–PCR) and follicular AMH production (Elisa and RIA) in relation to follicular development, 87 follicles (3–13 mm diameter) including both GC and the corresponding follicular fluid (FF) were collected in connection with fertility preservation of human ovaries. Further, follicle number and diameter, graded in 1 mm increments, were determined by 3D ultrasound in 113 women in their natural menstrual cycle to determine follicle number and diameter in relation to circulating AMH levels. This study demonstrates for the first time a positive association between AMH gene expression in human and both total follicular fluid AMH (P = 0.02) and follicular fluid AMH concentration (P = 0.01). AMH gene expression and total AMH protein increased until a follicular diameter of 8 mm, after which a sharp decline occurred. In vivo modelling confirmed that 5–8 mm follicles make the greatest contribution to serum AMH, estimated for the first time in human to be 60% of the circulating concentration. Significant positive associations between gene expression of AMH and FSHR, AR and AMHR2 expression (P < 0.00001 for all three) and significant negative association between follicular fluid AMH concentration and CYP19a1 expression were found (P < 0.0001). Both AMH gene expression (P < 0.02) and follicular fluid concentration of AMH (P < 0.00001) correlated negatively with estradiol concentration.

Key words: AMH / human small antral follicles / granulosa cells / follicular fluid / modelling

Introduction

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor β-family (Pepinsky, 1988; Visser and Themmen, 2005) exclusively produced by granulosa cells (GC) of developing pre-antral and small antral follicles (Vigier et al., 1984; Donahoe et al., 2003; Weenen et al., 2004; Knight and Glister, 2006; La Marca et al., 2009). Although many publications in recent years have demonstrated the clinical use of AMH in assessing the ovarian reserve and the likely response to controlled ovarian stimulation (La Marca et al., 2009; Broer et al., 2010; Anderson et al., 2012; Nelson et al., 2012), the relative contributions of follicles of different sizes to serum AMH and the functional implications of changing AMH production during folliculogenesis are unknown.

AMH expression has been shown to be present in a window of follicular development from the secondary stage until a diameter of around 8–10 mm (Weenen et al., 2004; Anderssen et al., 2010; Grandahl et al., 2011), corresponding with follicular selection for dominance. Using immunohistochemistry, Weenen et al. (2004) showed that AMH in human GC showed maximal expression in antral follicles...
<4 mm in diameter and a gradual disappearance of AMH expression in larger follicles (Weenen et al., 2004). Likewise, the intrafollicular concentrations of AMH in normal human antral follicles show a gradual reduction as the diameter of the follicle increases, and a sharp decline is observed at 8–10 mm (Andersen et al., 2010), but whether the higher expression in smaller follicles (4 mm and less) means that they are the major contributors to serum AMH is unclear. Consistent with this, AMH gene expression is undetectable in GC from pre-ovulatory follicles, although it continues to be present in cumulus cells (Grandsåhl et al., 2011). The abrupt decline in AMH expression coincides with the selection of follicles for dominance. Functionally, this involves a transition from a low-estrogen producing state, when pituitary FSH production is not regulated by follicular activity, to one of rapidly increasing estrogen production which underlies the ovarian/pituitary dialogue that ensures that only one dominant follicle emerges.

The aim of the present study was to examine the production of AMH in human antral follicles, using both in vitro molecular methods and in vivo modelling to obtain more insights into the production of AMH at different stages of folliculogenesis. These data give new insights into the transition in follicle function at the time of follicle selection and emerging estrogenic activity.

Materials and Methods

Patients and collection of follicular fluid and GC from small antral follicles

Granulosa cells and follicular fluid (FF) samples were isolated from aspirates of individual small antral follicles from ovaries surgically removed for fertility preservation. Fertility preservation of the ovarian cortex was offered to women with a disease where the appropriate treatment was gonadotoxic and posed a risk of rendering her sterile. A total of 87 follicles (one to eight per ovary) from which mRNA was purified were obtained from 38 women aged 7–38 years (median 26 years) at various times during their menstrual cycle. None of the women had any reproductive disorder and all ovaries appeared normal.

All antral follicles exposed on the surface of the ovary or visible during the isolation of ovarian cortex were collected with a 1 ml syringe with a 26-gauge needle (Becton Dickinson, Brøndby, Denmark). To isolate the GC each aspirated follicle was centrifuged at 2000 × g for 2 min. The follicle volume ranged from 15 to 1080 μl, corresponding to ~3–13 mm in diameter. Granulosa cells and follicular fluid were snap-frozen in liquid nitrogen and stored at −80 °C until RNA purification or hormone measurement.

In a cohort of 395 small antral follicles, only the volume and the intrafollicular concentration of AMH were determined. These follicles were collected and treated as stated above. All antral follicles exposed on the surface of the ovary or visible during the isolation of ovarian cortex were collected with a 1 ml syringe with a 26-gauge needle (Becton Dickinson, Brøndby, Denmark). To isolate the GC each aspirated follicle was centrifuged at 2000 × g for 2 min. The follicle volume ranged from 15 to 1080 μl, corresponding to ~3–13 mm in diameter. Granulosa cells and follicular fluid were snap-frozen in liquid nitrogen and stored at −80 °C until RNA purification or hormone measurement.

In a cohort of 395 small antral follicles, only the volume and the intrafollicular concentration of AMH were determined. These follicles were collected and treated as stated above.

The ethical committee of the municipalities of Copenhagen and Frederiksberg approved the project (KF)01-170/99 and H-2-2011-044), and the patients or their parents signed an informed consent form. The concentration of AMH in some of the follicular fluid from the small antral follicles and small part of the mRNA data has previously been published (Andersen et al., 2010; Jeppesen et al., 2012a, b).

RNA purification

Total RNA was purified for each GC sample using a combination of TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) and the RNeasy Mini Kit (Qiagen, Hilden, Germany) to optimize the quality of the mRNA. The quality of the RNA samples were analysed using an Agilent 2100 Bioanalyzer and an RNA 6000 Pico LabChip (RNA 6000 Pico assay kit, Agilent Technologies, Waldbronn, Germany). Only samples with an RNA integrity number (RIN) higher than the internal threshold value were included in the study. Total RNA content in each sample was measured using a Beckman Coulter Du730 life science UV/vis spectrophotometer.

cDNA synthesis and qPCR analysis

First-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Briefly, a master mix containing 2.0 μl 10× RT Buffer, 0.8 μl 25× dNTP Mix (100 mM), 2.0 μl 10X RT Random Primers, 1.0 μl Multi-Scribe™ Reverse Transcriptase (50 U/μl), 0.5 μl RNase inhibitor and 4.7 μl nuclease-free (DEPC) water was prepared for each 20 μl reaction. Eleven microliters of master-mix was added to 9 μl of total RNA. Samples were centrifuged briefly at 12,000 × g and then incubated at room temperature for 10 min, followed by 37 °C for 2 h and finally at 85 °C for 5 s.

Gene expression levels were evaluated by RQ (relative quantification)-PCR analysis using TaqMan® technology (Applied Biosystems) and the LightCycler 480 qPCR instrument (Roche, Copenhagen, Denmark). Pre-designed AMH, AMHR2, FSHR, LHR, AR and CYP19a1 TaqMan® Gene Expression Assays, as well as the Endogenous Control Assays for human B-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were purchased from Applied Biosystems (Assay id-no.: B-actin: #4326315E, GAPDH: #4333764F AMH: #Hs00174915_m1, AMH-R2: #Hs00179718_m1, FSH-R: #Hs00178463_m1, A-R: #Hs00171172_m1, CYP19a1 (aromatase): #Hs00903413). Data were quantified and compared to the Comparative CT Method (LightCycler®480 Software, Roche); expression levels are presented as 2 −ΔΔCT normalized to GAPDH (Schmittgen and Livak, 2008).

Hormone measurements

AMH was measured using a specific ELISA-kit according to the manufacturer’s instructions (DSL-10-14400, Diagnostic System Laboratories). follicular fluid samples were diluted either 1:500 or 1:3000 in the zero-standard and dilution curves proved parallel to the standard curve (Yding Andersen et al., 2008). Inter-assay variation was 4.4% (7.6 ng AMH/ml) (n = 12) and intra-assay variation was 3.3% (0.45 ng/ml) (n = 5).

Inhibin-B was measured using a specific ELISA-kit according to the manufacturer’s instructions (The Oxford Bio-innovation kit; Biotech-lgG, Copenhagen, Denmark) with samples diluted 1:100 or 1:500 in serum obtained from a pool of five post-menopausal women who did not show any inhibin-B activity (CV < 7%).

Estradiol, progesterone, androstenedione and testosterone were initially measured using commercially available RIA kits (DSL-43100, DSL-3400, DSL-3800, DSL-4000; DSL, Webster, TX) using in-house prepared steroid-free serum for dilution. However, during the course of sample collection, the RIA assays became unavailable and it was necessary to use an alternative assay in around half the samples. Commercially available ELISA assays (NovaTec Immundiagnostica, Dietzenbach, Germany; DNOV 002, 003, 006 and 008, respectively) were used and performed according to the manufacturer’s instructions. To compare ELISA and RIA results, 39 follicular fluid samples were used where enough material was present to conduct both assays. Based on highly significant linear correlations between the two types of assay (correlation coefficients for estradiol, progesterone, testosterone and androstenedione r = 0.99; r = 0.93; r = 0.91; respectively), results were expressed in concentrations relating to the RIA assays.

The limited volume of most of these samples did not allow re-measurement when the first result was below the detection limit of the
Ultrasound data

A total of 113 women about to have their first IVF cycle were recruited as previously described (Jayaprakasan et al., 2010). They were aged <41 years, had regular menstrual cycles, had no history of ovarian surgery and did not have ovarian cysts. They had a variety of causative factors for their subfertility, including tubal disease (27 subjects; 23.9%), endometriosis (seven subjects; 6.2%), male factor (43 subjects; 38.1%), combined factors (three subjects; 2.7%) and unexplained subfertility (33 subjects; 29.1%). The participants underwent venepuncture and a pretreatment 3D transvaginal ultrasound examination in the early follicular phase (days 2–4) of a spontaneous menstrual cycle. All ultrasound scans were performed by a single investigator (KJ) using a Voluson Expert-730 (GE Medical Systems, Zipf, Austria) and a three-dimensional, 5–9 MHz transvaginal probe. Follicles were initially identified automatically by sono-AVC (GE Medical Systems) and a three-dimensional, 5–9 MHz transvaginal probe. Follicles were initially identified automatically by sono-AVC (GE Medical Systems), which provides an automated mean diameter calculated from the actual volume of the follicle by using the formula for the volume of a sphere. Post-processing was then done manually by scrolling through the 3D ovarian volume to ensure that all follicles were included and then subdivided into cohorts according to their mean diameter: ≤2 mm, 2.1–3 mm, 3.1–4 mm, 4.1–5 mm, 5.1–6 mm, 6.1–7 mm, 7.1–8 mm, 8.1–9 mm and 9.1–10 mm. Serum AMH was measured using the DSL ELISA, as described above. The study received Ethical committee approval and written informed consent was obtained from all women.

Statistics and modelling

For evaluation of a possible correlation between the levels of the measured substances, the Spearman correlation coefficient test (SC) was used. One-way analysis of variance (ANOVA) was used when the number of groups exceeded two and student’s t-test was used as a post-hoc test when the ANOVA proved statistically significant. A P-value of <0.05 was accepted as statistically significant. To calculate the relative contribution to serum AMH by each follicle cohort (graded in single mm increments), we related serum AMH for each woman to the number of follicles in each cohort and a factor for the AMH contribution from each follicle of that cohort. A general linear regression model was used for analysis of AMH concentrations by follicle cohorts within each woman:

$$AMH = a_2AFC_2 + a_3AFC_3 + \ldots + a_{10}AFC_{10}$$

which relates AMH levels to a weighted linear combination of antral follicle counts (AFC). AFC denotes the number of AFCs of size i millimetres (e.g. i = 3 indicates follicles measuring 2.1–3 mm diameter) and ai denotes the computed coefficient value that minimizes the predictive error when applied to every subject. The general linear regression model using the R open-source statistical analysis package was initially determined using individual follicle cohorts. A subsequent analysis was performed after grouping into 3 contiguous sizes (<5 mm, 5.1–8 mm, >8 mm).

Since a large and positive coefficient for a generalized linear regression model need not correspond to a small P-value and since a small P-value can be obtained for coefficients that have an insignificant effect on the model, our results were inspected for collections of follicle cohorts that (i) had the largest positive coefficient, thereby having the largest relative effect on the model and (ii) had the smallest P-value indicating that the large effect is unlikely to be due to chance. Estimates for the relative contributions to AMH levels of antral follicles of different sizes were obtained by positing that the majority of AMH is produced by antral rather than pre-antral follicles and using the sizes of the coefficients in a generalized linear regression model that only considers the contribution of antral follicles.

Results

In vitro data: AMH gene expression

There was a significant positive association between the mRNA expression of AMH in GC and the concentration of AMH in the corresponding follicular fluid (P < 0.01) (Table I). The total content of AMH in each individual follicle (ng) also showed a significant positive association with the corresponding mRNA expression of AMH in the corresponding GC (P < 0.02) (data not shown).

AMH gene expression in GC from 87 follicles was readily detectable in follicles of 7 mm or less in diameter, but dropped to almost undetectable levels in GC from follicles exceeding 8 mm in diameter (Fig. 1). Due to a very limited number of antral follicles exceeding 8 mm in diameter, there are no significant differences between the group exceeding 8 mm in diameter and the other groups, but a strong tendency is seen.

AMH gene expression showed a highly significant positive correlation with gene expression of AMHR2 (Table I and Fig. 2) and with AR and FSHR gene expression (Table I and Fig. 2) (P < 0.00001 for all three), while there was no significant correlation with the gene expression of CYP19a1 (Table I). AMH gene expression showed a significant inverse correlation with corresponding follicular fluid concentration of estradiol and positive correlations with follicular fluid concentrations of testosterone and progesterone, and no relationship with follicular fluid androstenedione (Table I).

In vitro data: AMH protein expression

The content of AMH in follicular fluid of 395 follicles in relation to follicular diameter showed a distinct peak at 8 mm with a steady rise leading up to that size and a more abrupt decline in larger follicles, while the intrafollicular concentrations are concomitantly reduced with a sharp decline at 8 mm (Fig. 3). The average concentration of AMH in follicular fluid from 87 human small antral follicles was 820 ± 62 ng/ml (mean ± SEM). Highly significant negative correlations between the protein level of AMH (FF) and the corresponding gene expression of CYP19a1 and the follicular fluid concentration of estradiol were found (both P < 0.0001) (Table II). Further significant negative associations were found with follicular fluid concentrations of progesterone and inhibin-B (Table II). No significant correlations were found, however, between the protein levels of AMH (FF) and FSHR, AR or AMHR2 expression (Table II).

In vivo modelling

Data from a total of 1463 follicles in 113 women were analysed with further statistical analysis (Jayaprakasan et al., 2010). Initial analysis using single-millimetre follicle diameter increments demonstrated a significant relationship between the number of follicles 5.1–6 mm (P = 0.003) in diameter and a non-significant relation between the number of follicles 7.1–8 mm in diameter (P = 0.086) and serum AMH concentration (Table III), whereas other follicle groups failed to show significant relationships with AMH. This might reflect the power of the analysis (many women had no follicles within several cohorts when analysed at such a high resolution), follicle size ranges
were also grouped into ≤5 mm; 5.1–8 mm and >8 mm cohorts. This analysis demonstrated that the medium-sized cohort of antral follicles (5.1–8 mm) showed a highly significant relationship with serum AMH ($P < 0.0001$), whereas the cohort of small follicles (<5 mm) showed a modest relationship that approached statistical significance ($P = 0.056$), and there was a negative but non-significant association between the large follicle cohort and AMH ($P = 0.3$) (Table III).

Relative contribution analysis indicated that about 60% of circulating AMH is produced by medium-sized antral follicles. Accurate estimates of the proportion of AMH produced by small and large antral follicles could not be produced from our data, but estimates of 15–20% for large and 20–25% for small sized follicles are likely ranges.

**Discussion**

To our knowledge, this is the first study to integrate information of AMH gene expression by granulosa cells from normal human antral follicles, with the total follicular content of AMH protein in these follicles and in vivo modelling studies to determine that 5–8 mm human follicles contribute the most to the concentrations of AMH measured in circulation. A significant association was found between the intrafollicular mRNA expression of AMH in GC and AMH protein level in the corresponding follicular fluid in a large number of individual antral follicles from normal human ovaries. Clear relationships with increasing estrogenic activity, reflecting maturational progression towards selection for dominance and ultimately ovulation, are demonstrated. The significant association between gene expression on one side and both the follicular protein concentration and the total protein content of AMH suggests that protein concentration indeed reflects gene expression. Therefore, the follicular fluid concentration of AMH is likely to reflect synthesis at that particular stage of follicular development, rather than an accumulation of previously synthesized protein. There were no effect of the age on the gene expression and follicular fluid measurements (Spearman).

Given that the follicular fluid AMH concentration reflects AMH gene activity in the GC, the present study provides several lines of evidence to suggest that follicles 5–8 mm in diameter contribute the most to the concentration of AMH found in circulation. The total AMH content per follicle clearly peaks at a diameter of 8 mm, despite the
intrafollicular concentration of AMH drop compared with earlier stages (Andersen et al., 2010). The number of granulosa cells per follicle and the volume of each follicle increase as the diameter increases and the collective output of AMH from each follicle then surprisingly turns out to peak at 8 mm despite intrafollicular AMH concentration being considerably lower than earlier on. Since AMH undergoes a tremendous dilution in circulation, the important parameter is the total content of AMH and not the intrafollicular concentration.

This peak in total follicular AMH content could also reflect an accumulation of AMH from earlier follicular developmental stages where AMH production was higher, but the present study demonstrated that the AMH gene expression in GC correlates positively with the intrafollicular AMH concentration and drops abruptly at a follicular diameter of 8 mm, which suggests that the peak of total follicular AMH content at 8 mm does not reflect an accumulation of AMH from earlier stages and in vivo modelling of three groups of follicles (i.e. <5 mm, 5.1–8 mm and >8 mm) closely supports these direct observations clearly demonstrating that follicles 5.1–8 mm contribute the most to the AMH concentration found in circulation. While the 5.1–6 mm cohort appeared the most important contributor, this may in part reflect the lower power of the analysis at the single-mm level of resolution and the relative abundance of follicles of this size as shown in the present study. Taken together, the present study strongly suggests that the circulating concentration of AMH is mostly influenced by the presence of follicles with diameters of 5–8 mm, which contribute ~60% of serum AMH. Although smaller follicles have been reported to show the most intense immunoexpression of AMH (Weenen et al., 2004), this analysis suggests that the cohort of follicles of 5 mm diameter contribute no more than 25% of serum AMH probably due to a reduced number of granulosa cells.

The functional implications of this include that the biological activity of AMH within and around a follicle is dynamic and is likely to decrease abruptly at around 8 mm of diameter. The present study also found a significant negative association between both AMH gene and protein expression on one side and the intrafollicular estradiol concentration and CYP19a1 expression on the other. These results confirm and extend previous studies showing similar negative effects of AMH on estradiol production and CYP19a1 expression in human GC (Grossman et al., 2008; Nielsen et al., 2010; Pellatt et al., 2011) and comparable data from large animal models (Rico et al., 2009; Monniaux et al., 2011; Campbell et al., 2012). One of the most variable features of human ovaries in relation to age is the number of small antral follicles, which is high in the younger years and declines towards menopause.

Figure 3 The mean total follicular content (diamond; ng) and the mean concentration of AMH (filled square; ng/ml) in 395 individual human antral follicles in relation of the diameter of the follicle. The total follicular content of AMH is calculated based on the assumption that follicles have a spherical form. In brackets is the number of observations in each data point shown.
Table II AMH levels in follicle fluid from 87 human small antral follicles in relation to mRNA expression of FSHR, CYP19a, LHR, AR and AMHR2 in the corresponding granulosa cells and to levels of steroids and inhibin B in (mean ± SEM).

<table>
<thead>
<tr>
<th>AMH in follicle fluid in quintiles</th>
<th>&lt;20%</th>
<th>21–40%</th>
<th>41–60%</th>
<th>61–80%</th>
<th>&gt;81%</th>
<th>Total</th>
<th>Spearman P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of AMH in follicular fluid (ng/ml)</td>
<td>&lt;30</td>
<td>31–150</td>
<td>151–600</td>
<td>601–1000</td>
<td>&gt;1001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>19</td>
<td>16</td>
<td>18</td>
<td>17</td>
<td>17</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>FSHR</td>
<td>110 ± 29</td>
<td>148 ± 28</td>
<td>233 ± 57</td>
<td>210 ± 44</td>
<td>136 ± 25</td>
<td>163 ± 16</td>
<td>NS</td>
</tr>
<tr>
<td>AR</td>
<td>31 ± 6.8</td>
<td>54 ± 9.7</td>
<td>55 ± 8.6</td>
<td>55 ± 8.2</td>
<td>38 ± 3.4</td>
<td>46 ± 3.4</td>
<td>NS</td>
</tr>
<tr>
<td>AMHR2</td>
<td>6.9 ± 1.5</td>
<td>10.3 ± 1.1</td>
<td>18 ± 3.1</td>
<td>16.9 ± 3.2</td>
<td>13.7 ± 2.3</td>
<td>13 ± 1.1</td>
<td>NS</td>
</tr>
<tr>
<td>CYP19a</td>
<td>146 ± 58</td>
<td>111 ± 41</td>
<td>47 ± 20</td>
<td>33 ± 16</td>
<td>29 ± 17</td>
<td>69 ± 15</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Estradiol (nM)</td>
<td>304 ± 84</td>
<td>98 ± 31</td>
<td>76 ± 30</td>
<td>19 ± 3.2</td>
<td>40 ± 15</td>
<td>96 ± 20</td>
<td>P &lt; 0.00001</td>
</tr>
<tr>
<td>Testosterone (nM)</td>
<td>273 ± 32</td>
<td>222 ± 43</td>
<td>394 ± 67</td>
<td>296 ± 37</td>
<td>246 ± 37</td>
<td>275 ± 20</td>
<td>NS</td>
</tr>
<tr>
<td>Progesterone (nM)</td>
<td>442 ± 46</td>
<td>307 ± 32</td>
<td>360 ± 38</td>
<td>330 ± 28</td>
<td>252 ± 30</td>
<td>330 ± 16</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Androstenedione (nM)</td>
<td>2379 ± 495</td>
<td>2075 ± 323</td>
<td>2693 ± 269</td>
<td>2273 ± 274</td>
<td>2198 ± 236</td>
<td>2298 ± 140</td>
<td>NS</td>
</tr>
<tr>
<td>Inhibin B (ng/ml)</td>
<td>133 ± 26</td>
<td>151 ± 27</td>
<td>84 ± 8.3</td>
<td>68 ± 6.7</td>
<td>49 ± 5.1</td>
<td>94 ± 8.4</td>
<td>P &lt; 0.00001</td>
</tr>
</tbody>
</table>

All gene expression data have been multiplied by 1000. Spearman correlation coefficient test was used and a P < 0.05 was accepted as significant; NS, not significant. Spearman correlation coefficient test was used on the whole data set, but presented as quintiles.

*The measurements on progesterone were conducted on 71 follicles due to insufficient levels of progesterone in the remaining 16 follicles.

Table III In vivo modelling of circulating AMH concentration in relation to follicle distribution in the ovary.

<table>
<thead>
<tr>
<th>ID</th>
<th>Follicle size range</th>
<th>No of follicles</th>
<th>GLR model coefficient</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFC2</td>
<td>1.1–2 mm</td>
<td>128</td>
<td>0.021</td>
<td>0.666</td>
</tr>
<tr>
<td>AFC3</td>
<td>2.1–3 mm</td>
<td>228</td>
<td>0.005</td>
<td>0.859</td>
</tr>
<tr>
<td>AFC4</td>
<td>3.1–4 mm</td>
<td>225</td>
<td>0.053</td>
<td>0.180</td>
</tr>
<tr>
<td>AFC5</td>
<td>4.1–5 mm</td>
<td>247</td>
<td>0.018</td>
<td>0.654</td>
</tr>
<tr>
<td>AFC6</td>
<td>5.1–6 mm</td>
<td>226</td>
<td>0.133</td>
<td>0.003</td>
</tr>
<tr>
<td>AFC7</td>
<td>6.1–7 mm</td>
<td>180</td>
<td>0.067</td>
<td>0.126</td>
</tr>
<tr>
<td>AFC8</td>
<td>7.1–8 mm</td>
<td>115</td>
<td>0.115</td>
<td>0.086</td>
</tr>
<tr>
<td>AFC9</td>
<td>8.1–9 mm</td>
<td>68</td>
<td>-0.101</td>
<td>0.219</td>
</tr>
<tr>
<td>AFC10</td>
<td>9.1–10 mm</td>
<td>46</td>
<td>-0.039</td>
<td>0.689</td>
</tr>
<tr>
<td>ID</td>
<td>Follicle size range</td>
<td>No of follicles</td>
<td>GLR model coefficient</td>
<td>P-value</td>
</tr>
<tr>
<td>Small</td>
<td>1.1–5 mm</td>
<td>828</td>
<td>0.025</td>
<td>0.056</td>
</tr>
<tr>
<td>Medium</td>
<td>5.1–8 mm</td>
<td>521</td>
<td>0.103</td>
<td>0.00005</td>
</tr>
<tr>
<td>Large</td>
<td>8.1–10 mm</td>
<td>114</td>
<td>-0.063</td>
<td>0.305</td>
</tr>
</tbody>
</table>

1463 follicles were detected and in the top panel divided into follicle size with 1 mm interval. Top panel: initial analysis by Generalised Linear Regression model. In the lower panel the 1463 follicles were divided into three classes. Lower panel: grouped size ranges analysis by Generalised Linear Regression model.

*Denotes a significance level of less than 0.01.

illustrating the now well-established relationship to the concentration of AMH as measured in circulation. Despite this variation in the number of small antral follicles with age, the ovaries are capable of producing one pre-ovulatory follicle each menstrual cycle with high precision throughout most of the reproductive years. There exists a fine-tuned and delicate balance between the estradiol output by the pre-ovulatory follicle and gonadotrophin secretion by the pituitary to ensure that ovulation takes place at the right time (Baird, 1983). The variable number of small antral follicles does not appear to influence this delicate balance, mainly because they only secrete estradiol in small amounts. Based on the present and previous studies, it is suggested that one physiological function of AMH is to ensure that each small antral follicle produces little estradiol prior to selection (i.e. leading up to a follicular diameter of 8 mm) and therefore does not interfere with the ovarian/pituitary dialogue regulating the development of the selected follicle that will undergo ovulation.

This study also showed that the gene expression of AMH in GC is highly significantly associated with the expression of FSHR, AR and AMHR2, and inversely with the concentrations of estradiol, progesterone and testosterone in the corresponding follicular fluid. The highly significant association between AMH and AMHR2 expression in GC suggests that ligand and receptor are involved in autocrine mechanisms that may affect follicle development. This is further supported by the observation that AMHR2 and FSHR are significantly associated (Nielsen et al., 2010).

To the best of our knowledge, this is the first study where a highly significant positive correlations between the gene expression of AMH and the expression of FSHR, AR and AMHR2 have been shown in GC from human small antral follicles. A positive correlation between AR expression and mitosis in GC from antral primate follicles and an inverse correlation between AR gene expression and GC apoptosis have been demonstrated (Weil et al., 1998, 1999). Healthy follicles thus have a high AR expression, consistent with in vivo data that androgen treatment promotes early follicle growth in primates (Vendola et al., 1998). Considering these findings, the highly positive association
between AMH and AR in the present study may indicate that AMH expression is linked to follicular health and GC proliferation, which provides further evidence supporting this suggestion (Nielsen et al., 2011). Further, Weenen et al. (2004) showed that AMH is expressed predominantly in GC of healthy human small antral follicles and that atretic follicles lacked AMH protein expression (Weenen et al., 2004). Low or absent follicular expression of AMH has been linked to atresia in mouse follicles (Durlinger et al., 2001, 2002). In this study we were not able to distinguish whether the correlations between AMH gene expression and FSHR, AR and AMH-R2 gene expression are an effect of follicular health and/or follicular size. In mice, AMH appears to inhibit progression of pre-antral follicles to the early antral follicle stage by reducing FSH sensitivity of the follicles (Durlinger et al., 2001, 2002) and recent data suggest that AMH also inhibits ovine, porcine and human GC responses to FSH (di Clemente et al., 1994; Pellatt et al., 2011; Campbell et al., 2012). The present study was unable to address the regulation of early recruitment of follicles, but at the antral stage of human follicular development, there is a strong positive association between gene expression of AMH and FSHR, confirming the results of earlier studies (Jeppesen et al., 2012a, b). The mechanism involved in this interrelationship is not revealed by the present study and could potentially in principle be unrelated, but potential mechanisms include inhibition of AMH by FSH or that AMH indirectly results in an increase in the expression of FSHR through a rise in androgen levels (Weil et al., 1998, 1999), because of a decrease in CYP19a1 expression. The rise in AMH and FSHR expression observed here and in earlier studies until follicles selection (8 mm in diameter) (Jeppesen et al., 2012a, b) seems to be a reflection of a delicate balance between the rising of FSHR expression until selection and the decrease in expression of FSHR in follicles larger than 8 mm in diameter.

The negative association between the concentration of AMH (FF) and the expression of CYP19a1 in the corresponding GC found in this study confirms and expands previous results (Andersen and Byskov, 2006; Grossman et al., 2008; Yding Andersen et al., 2008; Pellatt et al., 2011) and supports the suggestion that a possible major role of AMH within small follicles is to prevent premature estrogen production. Results from cultures of human GC from pre-ovulatory follicles also showed that AMH caused a down-regulation of CYP19a1 at both gene and protein levels (Grossman et al., 2008; Pellatt et al., 2011). In the present study we were unable to demonstrate a significant association between AMH expression and CYP19a1 expression, but there was a strong negative relationship between follicular fluid estradiol and AMH expression, i.e. indicating that once follicles have become estrogenic, AMH production is suppressed, perhaps directly by estrogen.

The in vivo modelling data show striking concordance with the direct measurements obtained in vitro and support the concept that AMH is involved in a switch between the small antral, preselection stages of folliculogenesis and follicles selected for further growth. The close correlation between serum AMH and AFC is well recognized (La Marca et al., 2010; Anderson et al., 2012), and both give good prediction of the ovarian reserve whether measured functionally as the follicular response to ovolation induction (Seifer et al., 2002; Andersen and Byskov, 2006) or histologically as primordial follicle population (Hansen et al., 2011). There is, however, no consensus on whether the total AFC or subsets within the 2–10 mm range should be assessed. Few studies have tried to assess with greater precision which follicles are the main contributors to AMH concentrations, although that might help the precision of prediction of the ovarian response in IVF. In bovine it has recently been shown that the small antral follicles (3–7 mm in diameter) contribute the most to the circulating AMH concentration (Rico et al., 2009, 2011; Ireland et al., 2011). In a previous analysis (Jayaprakasan et al., 2010) we demonstrated that there were significant correlations between the number of follicles in each of the single-mm increment groups (2.1–8 mm) with serum AMH. A more significant correlation between 2.1–5 mm to serum AMH was observed. In that study the number of follicles within each group was analysed independently of the rest of the follicles present. In the present analysis we further take the woman’s whole follicle cohort into account when correlating with serum AMH, which is the key novelty of the present modelling. We here demonstrate that circulating AMH was strongly associated with the number of 5.1–8 mm follicles, with a weaker association with the number of follicles smaller than that, and no association with the number of larger (>8 mm) diameter follicles. This analysis cannot assess the possible contribution of pre-antral follicles but the much weaker apparent contribution of small than medium-sized follicles and the much smaller number of granulosa cells in pre-antral follicles (McNatty et al., 1979; Gougeon, 1996) suggests that this is likely to be of little importance. We calculate that 60% of measured AMH is produced by 5.1–8 mm follicles, 20–25% by 2.1–5 mm follicles and 15–20% by >8 mm follicles.

In conclusion the present study showed a significant positive correlation between the expression of AMH in GC and AMH levels in the corresponding follicular fluid, suggesting that local AMH concentrations reflect contemporaneous production. Further, the circulating concentrations of AMH reflect the actual constitution of follicles in the ovaries, predominantly those 5–8 mm in diameter. We propose that high AMH production within a follicle is likely to be part of the mechanism suppressing estrogen production by that follicle and that the abrupt decline of AMH production at diameters above 8 mm is functionally linked with increased estrogen production and selection of the pre-ovulatory follicle.

Acknowledgements

The excellent technical help from Tiny Roed is greatly appreciated.

Authors’ roles

J.V.J., C.Y.A. and R.A.A. were involved in conception and design of the study, data acquisition and analysis and interpretation, drafting of the manuscript and revision of the article. T.W.K., S.L.C. and S.G.K. were involved in data acquisition and analysis and interpretation of the data and revision of the article. K.J. and NR-F contributed with conception and design and manuscript drafting. All the authors were involved in the final approval of the version to be published.

Funding

This work was supported by the Danish Cancer Society (DP05112/R2-A41-09-S2); The Danish Medical Research Council (271-07-0452;
09-072265); the UK Medical Research Council (G1 100357 to RAA); the
Novo Nordic Foundation; Sophus Carl Emil Friis and wife Olga Doris
Friis’ foundation; The Lundbeck Foundation and the University Hospital
of Copenhagen is also gratefully acknowledged.

Conflict of interest

None declared.

References

Andersen CY, Byskov AG. Oestradiol and regulation of anti-Mullerian
hormone, inhibin-A, and inhibin-B secretion: analysis of small antral
and preovulatory human follicles’ fluid. J Clin Endocrinol Metab 2006;
91:4064–4069.
Andersen CY, Schmidt KT, Kristensen SG, Rosendahl M, Byskov AG,
Ernst E. Concentrations of AMH and inhibin-B in relation to follicular
diameter in normal human small antral follicles. Hum Reprod 2010;
Anderson RA, Nelson SM, Wallace WH. Measuring anti-Mullerian
hormone for the assessment of ovarian reserve: When and for whom
is it indicated? Maternitas 2012;71:28–33.
Baird DT. Factors regulating the growth of the preovulatory follicle in the
Broer SL, Mol BW, Døllemann M, Fauser BC, Broekmans JMF. The role of
anti-Mullerian hormone assessment in assisted reproduction technology
Campbell BK, Clinton M, Webb R. The role of anti-mullerian hormone (AMH)
during follicle development in a monovulatory species (sheep). Endocrinol
di Clemente N, Goxe B, Remy Jj, Cate RL, Josso N, Vigier B, Sallese R.
Inhibitory effect of AMH upon the expression of aromatase and LH
receptors by cultured granulosa cells of rat and porcine immature
Donahoe PK, Clarke T, Teixeira J, Maheswaran S, MacLaughlin DT.
Enhanced purification and production of Mullerian inhibiting substance
Durlinger AL, Crijns Jd M, Kramer P, Karels B, Kumar TR, Matzuk MM,
Rose UM, de Jong FH, Ultenbroek JT, Themmen AP et al. Anti-Mullerian hormone attenuates the effects of FSH on follicle
Durlinger AL, Visser JA, Themmen AP. Regulation of ovarian function as
Gougeon A. Regulation of ovarian follicular development in primates: facts
Grendahl ML, Nielsen ME, Dal Canto MB, Radin R, Rasmussen IA,
Westergaard LG, Kristensen SG, Yding Andersen C. Anti-Mullerian
hormone remains highly expressed in human cumulus cells during the final
Grossman MP, Nakajima ST, Fallat ME, Siow Y. Mullerian-inhibiting
substance inhibits cytochrome P450 aromatase activity in human
Hansen KR, Hodnett GM, Knowton N, Craig LB. Correlation of ovarian
reserve tests with histologically determined primordial follicle number.
Ireland JJ, Smith GW, Scheetz D, Jimenez-Krassel F, Folger JK, Ireland JL,
Mossa F, Lonergan P, Evans AC. Does size matter in females? An
overview of the impact of the high variation in the ovarian reserve on
ovarian function and fertility, utility of anti-Mullerian hormone as a
diagnostic marker for fertility and causes of variation in the ovarian
Jayaprakasan K, Deb S, Batcha M, Hopkisson J, Johnson I, Campbell B,
Raine-Fenning N. The cohort of antral follicles measuring 2–6 mm
reflects the quantitative status of ovarian reserve as assessed by
serum levels of anti-Mullerian hormone and response to controlled
Jeppesen JV, Nielsen ME, Kristensen SG, Yding Andersen C.
Concentration of activin A and follistatin in follicular fluid from human
antral follicles associated to gene expression of the corresponding
Jeppesen JV, Kristensen SG, Nielsen ME, Humaidan P, Canto MD, Fadini R,
Schmidt KT, Ernst E, Yding Andersen C. LH-receptor gene expression in
human granulosa and cumulus cells from antral and preovulatory
Knight PG, Grist C. TGF-β superfamilly members and ovarian follicle
La Marca A, Broekmans Fj, Volpe A, Fauser BC, Macklon SN.
Anti-Mullerian hormone (AMH): what do we still need to know? Hum
Reprod 2009;24:2264–2275.
La Marca A, Sighinolfi G, Radi D, Argento C, Baraldi E, Artesino AC,
Stabile G, Volpe A. Anti-Mullerian hormone (AMH) as a predictive
marker in assisted reproductive technology (ART). Hum Reprod
McNatty KP, Smith DM, Makris A, Osathanondh R, Ryan KJ. The
microenvironment of the human antral follicle: interrelationships
among the steroid levels in antral fluid, the population of granulosa
cells, and the status of the oocyte in vivo and in vitro. J Clin Endocrinol
Anti-Mullerian hormone as a predictive endocrine marker for embryo
Nelson SM, Anderson RA, Broekmans Fj, Raine-Fenning N, Fleming R, La
Marca A. Anti-Mullerian hormone: clairvoyance or crystal clear? Hum
Nielsen ME, Rasmussen IA, Fujioka M, Westergaard LG, Yding
Andersen C. Concentration of anti-Mullerian hormone in fluid from
small human antral follicles show a negative correlation with CYP19
mRNA expression in the corresponding granulosa cells. Mol Hum
Nielsen ME, Rasmussen IA, Kristensen SG, Christensen ST, Malsgård K,
Wreford Andersen E, Byskov AG, Yding Andersen C. In human
granulosa cells from small antral follicles, androgen receptor mRNA
and androgen level in follicular fluid correlate with FSH receptor
mRNA. Mol Hum Reprod 2011;17:63–70.
Pellatt L, Rice S, Dilaver N, Heshi A, Galea R, Brincat M, Brown K,
Simpson ER, Mason HD. Anti-Mullerian hormone reduces follicle
sensitivity to follicle-stimulating hormone in human granulosa cells.
Pepinsky RB, Sinclair LK, Pingchang CE, Mattaliano RJ, Mangonaro TF,
Donahoe RK, Cate RL. Proteolytic processing of Mullerian inhibiting
substrate produces a transforming growth factor-β like fragment. J Biol
Rico C, Fabre S, Médigue C, di Clemente N, Clément F, Bontoux M,
Touzé J-L, Dupont M, Briant E, Rémy B et al. Anti-Mullerian hormone
is an endocrine marker of ovarian gonadotropin responsive follicles
and can help to predict superovulatory responses in the cow. Biol
Rico C, Médigue C, Fabre S, Jarrier P, Bontoux M, Clément F,
Monniaux D. Regulation of anti-Mullerian hormone production in the
Which follicles make the most AMH?


