Anti-Mullerian hormone is increased in follicular fluid from unstimulated ovaries in women with polycystic ovary syndrome

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BACKGROUND: Anti-Mullerian hormone (AMH) may have a role in disordered folliculogenesis in polycystic ovary syndrome (PCOS). Though there have been several investigations into circulating AMH levels in patients with PCOS, no previous studies have compared AMH concentrations in the follicular fluid of unstimulated ovaries in women with PCOS with that of normally ovulating women. METHODS: Follicular fluid was aspirated from 4–8-mm follicles of unstimulated ovaries during routine laparoscopy or laparotomy from women with anovulatory PCOS (n = 11) and those with regular ovulatory cycles (n = 8). Follicular AMH was compared in the two groups. Serum samples were analysed for AMH and endocrine profile. RESULTS: Follicular fluid AMH levels were significantly higher (P < 0.0001) in women with anovulatory PCOS (median: 466.2 ng/ml) compared with normal-ovulatory controls (median: 78.0 ng/ml). Mean follicular fluid AMH levels in PCOS patients were 60 times higher than in the serum. Moreover, there was a significant correlation between the follicular fluid and serum concentrations of AMH in the PCOS group (r = 0.86; P = 0.007) but not in controls. CONCLUSIONS: Highly elevated AMH in follicular fluid from PCOS patients in contrast to age-matched normal controls suggests that increased circulating concentrations of AMH are partly due to the increased production of AMH by individual follicles and not simply attributable to the increased number of small antral follicles. This suggests an intrinsic abnormality in the ovarian follicles themselves in PCOS, which could contribute to disordered folliculogenesis.

Keywords: polycystic ovary syndrome; anti-Mullerian hormone; follicular fluid; granulosa cell.

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine abnormality affecting 6.6–8% of women of reproductive age (Azziz et al., 2004) and is the main cause of anovulatory infertility (Franks, 1995). The pathogenesis of PCOS remains largely unknown though recent studies have suggested that anti-Mullerian hormone (AMH) may have a role to play in the disordered folliculogenesis in PCOS (Pigny et al., 2003; Laven et al., 2004).

AMH, also known as Mullerian-inhibiting substance, is a member of the transforming growth factor beta super family (Cate et al., 1986) and is expressed only post-natally in the ovary and in granulosa cells of growing follicles (Hirobe et al., 1992; Durlinger et al., 2002a). Recent studies in mice lacking the AMH gene have suggested that AMH is important in regulating initiation and progression of follicular growth (Durlinger et al., 1999). AMH expression is highest in the large pre-antral and small antral follicular stages, whereas its expression is not observed at the primordial stage or in atretic follicles (Weenen et al., 2004). Moreover, FSH-induced follicle growth was reported to be increased in the absence of AMH (Durlinger et al., 2001). It has been proposed that AMH participates in two critical selection points of follicle development; it inhibits the recruitment of primordial follicles into the pool of growing follicles and also decreases the responsiveness of growing follicles to FSH (Durlinger et al., 2002b). Circulating concentrations of AMH are significantly higher in women with PCOS than in age-matched controls (Cook et al., 2002; Pigny et al., 2003; Laven et al., 2004; LaMarca et al., 2004a). It has been suggested that serum AMH concentrations correlate with the number of small pre-antral and early antral follicles (Pigny et al., 2003; Laven et al., 2004). Serum AMH levels were also reported to correlate with elevated testosterone or LH levels in PCOS and an increased follicle number and ovarian volume on ultrasound examination (Laven et al., 2004).

Though there have been several investigations into circulating AMH levels in patients with PCOS, no previous studies...
comparing AMH concentrations in the follicular fluid of unstimulated ovaries in women with PCOS with that of normally ovulating women have been reported. The level of AMH in the follicular fluid and serum of patients with PCOS undergoing IVF was significantly higher than in patients with endometriosis or pelvic adhesions (Fallat et al., 1997). Experiments utilizing in vitro culture of granulosa cells have shown that the mean level of AMH is much higher in conditioned media from anovulatory PCOS-derived granulosa cell culture than from normal ovaries. In addition, exogenous FSH results in decreased in vitro production of AMH by granulosa cells obtained from PCOS ovaries, whereas the addition of LH increased AMH production. This effect was not seen in granulosa cells obtained from normal ovaries (Pellatt et al., 2007).

The higher serum AMH seen in PCOS may be a consequence of the increased number of follicles, each follicle from a polycystic ovary secreting similar amounts of AMH as those from normal ovaries (Pigny et al., 2003). In contrast, it has been proposed that AMH production in anovulatory PCOS is significantly higher per granulosa cell, and that there is an exponential fall in AMH as the follicle size increases, particularly apparent once the follicle reaches 10 mm in diameter, which is the size at which follicle selection normally occurs. This decrease in AMH may be important for the dominant follicle to be selected and AMH may inhibit follicle growth (Pellatt et al., 2007). However, the increased granulosa cell production of AMH from PCOS ovaries has been demonstrated in vitro, in the presence of various other ill-defined mitogenic factors. It is still uncertain whether the increased levels of AMH in PCOS are a result of the increased number of small antral follicles or whether individual follicles produce a higher amount of AMH.

We have therefore compared AMH levels in the follicular fluid from 4–8-mm follicles from unstimulated ovaries of age-matched patients with anovulatory PCOS and normo-ovulatory women. We have also examined the correlation of follicular fluid AMH in both groups to the serum LH and free testosterone levels.

Patients and methods

Patients

Ethical approval was obtained from the local research ethics committee and all women gave their written informed consent before participation. Patients from Newham University Hospital, London, UK, were recruited between September 2003 and March 2005. Women with PCOS who were undergoing laparoscopic investigation for infertility or ovarian drilling for ovulation induction were recruited (n = 11). The diagnosis of PCOS was established according to the revised Rotterdam ESHRE/ASRM criteria (2004). All women had oligomenorrhea or amenorrhea (eight or fewer menstrual cycles in a year), had clinical (hirsutism) and/or biochemical (raised free androgen index) evidence of hyperandrogenism and polycystic ovaries on ultrasound scan. Patients with congenital adrenal hyperplasia, Cushing’s syndrome, androgen-secreting tumours and thyroid disease were excluded and all patients had normal prolactin levels. The control group consisted of normally menstruating women, who were undergoing laparoscopic sterilization, hysterectomy for benign conditions and diagnostic laparoscopy for pelvic pain (n = 8). All controls had a normal pelvis apart from uterine fibroids. None of the patients in either group had taken any fertility drugs or hormonal medication for 3 months prior to sample collection. Endocrine parameters in control subjects were all within the normal range. Transvaginal ultrasonography was performed before the procedure in all patients to assess the size and number of ovarian follicles. Any potential size bias during the later laparoscopic sampling would be randomly distributed between the two groups, so that the size matching as seen by ultrasound would be reflected in the follicles aspirated. All aspirated follicles were between 4 and 8 mm. Ultrasound scans were performed by an independent clinician who was not involved in the rest of the project.

Hormone samples

Blood samples for subsequent biochemical analyses were obtained in the morning just prior to the operation. Samples were analysed for FSH, LH, progesterone, testosterone, sex hormone-binding globulin (SHBG), androstenedione, prolactin, thyroxine, thyroid-stimulating hormone, 17-hydroxyprogesterone and dehydroepiandrosterone sulphate.

Free testosterone was calculated using total testosterone and SHBG according to the formula of Vermeulen et al. (1999).

Follicular fluid collection

Follicular fluid was collected from individual follicles at the time of laparoscopy, using a single lumen 17-gauge needle (Casmed, UK). Each sample was collected into a sterile tube without culture medium. No flushing medium was used. Follicular fluid was selected from follicles 4–8 mm in diameter, free from blood contamination. From the women with regular cycles, only size-matched follicles were taken to avoid bias due to the increased number of small follicles in PCOS ovaries. The follicular fluid from all aspirated follicles (4–8 mm size) from each patient was pooled and was immediately centrifuged at 700g for 10 min. Supernatants were removed and stored at −20°C prior to analysis. Three to four follicles were aspirated from each patient in both groups.

All patients underwent the procedure in the early follicular phase of the menstrual cycle as assessed by the last day of the menstrual cycle, by their endocrine profile and by transvaginal ultrasound scan. In the PCOS group, the day of serum collection was either after a spontaneous period or randomly chosen because they were amenorrhoeic. All patients underwent a transvaginal ultrasound scan prior to the procedure in addition to an endocrine profile to confirm that they were in the follicular phase, as well as to confirm the absence of a dominant follicle.

AMH enzyme-linked immunosorbent assay

Follicular fluid and serum were assayed for AMH using an ultrasensitive enzyme-linked immunosorbent assay (Diagnostic Systems Laboratories, Oxon, UK) according to the manufacturer’s instructions. The sensitivity of the assay was 0.1 ng/ml; intra- and inter-assay coefficients of variation were <5 and 8%, respectively. Samples were assayed in triplicate.

Statistical analysis

As the data were not normally distributed, the non-parametric Mann–Whitney U-test was used to analyse data using the Statistical Package for the Social Sciences software version 14 for Windows. Correlations were expressed as Spearman’s correlation coefficients. A value of P < 0.05 was considered statistically significant.
Results

Clinical and endocrine parameters
Clinical and endocrine parameters in normo-ovulatory controls and in patients with anovulatory PCOS are summarized in Table I. There was no significant difference in mean ages between patients in the anovulatory PCOS group and controls, nor did BMI differ between the two groups. There were no significant differences in follicle size between the two groups (4.8 ± 0.5 mm in PCOS and 5.3 ± 0.5 mm for controls, \( P = 0.19 \)). The free testosterone level was significantly higher in the PCOS group (\( P < 0.001 \)) as were circulating levels of LH (\( P < 0.001 \)). However, plasma concentrations of FSH did not differ between the two groups (\( P = 0.97 \)).

Table I. Clinical and endocrine parameters in normo-ovulatory controls and in patients with anovulatory PCOS.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PCOS (n = 11)</th>
<th>Control (n = 8)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>28 (25–33)</td>
<td>32 (30–34)</td>
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<tr>
<td>BMI (kg/m(^2))</td>
<td>27 (26–28)</td>
<td>26 (26–28)</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>5.1 (3.8–5.7)</td>
<td>4.8 (4–6)</td>
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<tr>
<td>LH (IU/l)</td>
<td>14.9 (9.3–20.8)</td>
<td>5.5 (4.1–6.5)</td>
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<tr>
<td>Testosterone (nmol/l)</td>
<td>2.8 (2.4–3.2)</td>
<td>1.9 (1.5–2.1)</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>31.5 (25.1–34.4)</td>
<td>46.6 (40.4–51.0)</td>
</tr>
<tr>
<td>Free testosterone (pmol/l)</td>
<td>53.5 (50.6–57.1)</td>
<td>28.9 (21.9–30.7)</td>
</tr>
<tr>
<td>DHEAS (nmol/l)</td>
<td>5.5 (3.6–6.2)</td>
<td>4.5 (2.9–6.2)</td>
</tr>
<tr>
<td>Androstenedione (nmol/l)</td>
<td>11.5 (9.1–18.5)</td>
<td>6.9 (5.4–11.3)</td>
</tr>
<tr>
<td>Progesterone (nmol/l)</td>
<td>2.1 (1.6–2.2)</td>
<td>1.7 (1.3–2.3)</td>
</tr>
<tr>
<td>AMH in follicular fluid (ng/ml)</td>
<td>466.2 (301–707.8)</td>
<td>78.0 (26.3–119.9)</td>
</tr>
<tr>
<td>AMH in plasma (ng/ml)</td>
<td>8.5 (4.7–11.6)</td>
<td>1.13 (0.63–3.4)</td>
</tr>
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</table>

The values given are median and inter-quartile range.
DHEAS, dehydroepiandrosterone sulphate.
\(^a\)\( P < 0.001 \); \(^b\)\( P < 0.01 \).

Follicular fluid AMH in anovulatory PCOS and normal controls
Follicular fluid AMH levels were significantly higher in women with anovulatory PCOS compared with normal-ovulatory controls (\( P < 0.0001 \); Fig. 1). Levels of AMH from anovulatory PCOS ovaries ranged from 159.4 to 923.7 ng/ml, with a mean of 504.3 ng/ml (median 466.2 ng/ml). In contrast, the follicular fluid AMH levels in normo-ovulatory controls ranged from 7.4 to 386.5 ng/ml, with a mean of 107.0 ng/ml (median 78.0 ng/ml).

The mean AMH concentration in the follicular fluid in PCOS patients was 60 times higher than in the serum. Moreover, there was a significant correlation between the follicular fluid and serum concentrations of AMH in the PCOS group (\( r = 0.86; P = 0.007 \)). There was no significant relationship between serum and follicular fluid concentrations of AMH in controls.

There was no correlation between age and follicular fluid AMH levels in either group. Similarly, no correlation between BMI and AMH levels in either the PCOS or the normal control group was observed.

A positive and significant relationship between serum AMH and the free testosterone levels (\( r = 0.92; P = 0.001 \)) was seen in anovulatory PCOS patients. There was no statistically significant relationship between follicular fluid AMH and the free testosterone levels in either the PCOS (\( r = 0.301; P = 0.19 \)) or the control (\( r = 0.095; P = 0.82 \)) groups. No significant relationship between follicular fluid AMH levels and circulating LH in either the PCOS group or the normo-ovulatory controls was observed; similarly no relationship between AMH and circulating FSH or estradiol (E\(_2\)) levels was apparent. No significant relationship between serum AMH and circulating LH, FSH or estradiol levels was observed in either the PCOS or the control groups.

Discussion
The finding of increased AMH levels in the follicular fluid of unstimulated anovulatory PCOS patients compared with age-matched normal-ovulatory controls is consistent with recent observations that levels of AMH in media conditioned in vitro by granulosa cells obtained from anovulatory PCOS are 75 times higher than in media conditioned by granulosa cells from normal ovaries (Pellatt et al., 2007). This suggests that actual AMH production by granulosa cells in anovulatory PCOS is significantly higher.

Our findings are also in agreement with those of Fallat et al. (1997), who reported that the level of AMH in the follicular fluid and serum of patients with PCOS undergoing IVF was significantly higher than that in patients with endometriosis or pelvic adhesions.

We found no correlation between age and follicular fluid AMH in either the anovulatory PCOS or the normal control group. Similarly, there was no significant correlation between AMH and BMI in either study group. These findings are broadly in accordance with previous reports (Cook et al., 2002; Pigny et al., 2003; Chu et al., 2005).

Figure 1: Box and whisker plots depicting the follicular fluid AMH levels in anovulatory women with PCOS and norm-o-ovulatory controls. Solid lines inside boxes depict the median value, whereas the upper and lower limits of the boxes and whiskers indicate the 75th, 25th, 95th and 5th percentiles, respectively (PCOS: \( n = 11 \); controls: \( n = 8 \)).
Follicular fluid AMH concentrations in anovulatory PCOS patients in our study were typically over 60 times higher than in serum, implicating the follicle as the major site of synthesis, further supported by the observed correlation between the follicular fluid and serum concentrations of AMH in the PCOS group ($r = 0.86$).

Several investigations have suggested that the increased number of follicles in PCOS are the source of elevated serum AMH levels due primarily to the increased number of small antral follicles, assuming that each follicle produces a normal amount of AMH (Cook et al., 2002; Pigny et al., 2003). However, these studies were confined to circulating AMH levels of PCOS patients and did not include follicular fluid determinations. It is not possible to ascertain from these studies whether the increase in AMH concentrations was simply due to the higher number of follicles or whether it was because individual follicle production was increased.

Given that follicular fluid from size-matched follicles was pooled from each patient, it is not possible to determine whether the elevated follicular fluid AMH concentrations in anovulatory PCOS that we observed were due to much greater production of AMH by each individual follicle or whether only a few follicles in PCOS ovaries produce even greater quantities of AMH when compared with the rest. Future studies that would involve analysing follicular fluid from individual follicles may help to resolve this question. Either way, these data indicate that the elevated serum concentrations of AMH in PCOS patients are not simply due to an increase in the number of small antral follicles, but also due to the fact that either each individual follicle or a few follicles produce greatly increased amounts of AMH. We suggest that the follicles themselves in PCOS are intrinsically different from those of women with regular cycles in terms of their AMH production. It may be that high, localized concentrations of AMH could then act on the surrounding growing follicles to decrease their responsiveness to FSH, thereby contributing to follicular arrest. Indeed, it has been hypothesized that the excess of AMH at the level of selectable follicles could contribute to the follicular arrest of PCOS, mainly by an inhibitory action on FSH-induced aromatase expression (Pigny et al., 2003; Jonard and Dewailly, 2004). Further studies are required to settle these issues.

It has been suggested that the increased production of AMH may be due to the elevated concentrations of androgens that are characteristically found in patients with PCOS. Indeed, previous studies have indicated a relationship between AMH and circulating testosterone and androstenedione levels in PCOS patients (Fallat et al., 1997; Eldar-Geva et al., 2005; Pigny et al., 2006). We observed a positive and significant relationship between serum AMH, free testosterone levels and androstenedione levels in anovulatory PCOS patients. However, we did not observe a statistically significant relationship between follicular fluid AMH and the free testosterone levels. Given that androgen treatment of adult rhesus monkeys was shown to stimulate growth and proliferation of small antral follicles (Vendola et al., 1998), it may be argued that this accounts for the increased circulating levels of AMH. However, our observation that the concentration of AMH is greatly increased in the follicular fluid suggests that the androgen-induced excess in the number of follicles is not the sole reason for the high circulating AMH levels in PCOS. It is possible that androgens may stimulate individual follicles to produce more AMH. Future studies focusing on the association between follicular fluid AMH and androgens may help to clarify this matter further.

Though it is still debatable whether there is an association between AMH levels and hyperinsulinaemia (La Marca et al., 2004a; La Marca et al., 2004b; Fleming et al., 2005; Bayrak et al., 2007), elevated levels of LH and/or amplification of LH action on granulosa cells of the developing follicle by hyperinsulinaemia could explain the arrest of follicle growth (Franks et al., 1996; Willis et al., 1998). Laven et al. (2004) reported a positive correlation between serum AMH and LH levels in normogonadotrophic anovulatory women. The appearance of LH receptors on follicles of a smaller size in polycystic when compared with normal ovaries could be an important factor in the arrest of their future development (Jakimiuk et al., 2001). This issue has not been definitively settled, however, as some studies report no clear relationship between AMH and LH levels (Pigny et al., 2003). More recent studies by the same group demonstrated that serum LH levels were significantly related to the decrease in serum AMH levels, independently from FSH and E2 variations (Catteau-Jonard et al., 2007). The high circulating levels of LH in our PCOS patients could have contributed to the increased levels of AMH we report. However, we did not observe a significant correlation between follicular fluid AMH and serum LH levels in PCOS patients. Similarly, no significant relationship between follicular fluid AMH levels and circulating LH in normal-ovulatory controls was observed. Though no significant relationship between follicular AMH and these circulating hormones was found, it remains possible that one would emerge in a larger study. However, we report significantly increased AMH production in the PCOS follicles themselves, suggesting that the previously reported elevations in circulating levels of AMH are not solely due to increased numbers of AMH-producing follicles. Irrespective of the relationship between AMH and other circulating hormones, the observation that the PCOS follicles themselves are behaving in a fundamentally abnormal way regarding AMH production is, we believe, physiologically relevant.

In PCOS, there is a defect in the selection of a dominant preovulatory follicle, resulting in the accumulation of multiple small antral follicles (Fallat et al., 1997). As an exponential fall in AMH was observed as the size of the follicle increased, especially above 10 mm in diameter, which is the size at which follicle selection would normally occur (Pellatt et al., 2007), declining AMH levels could therefore have an important role to play in the selection of the dominant follicle.

To summarize, we present data showing for the first time that the concentrations of AMH in unstimulated size-matched ovarian follicles from anovulatory PCOS patients are significantly higher than in age-matched subjects with regular ovariatory cycles. This suggests that the increased concentrations of AMH in PCOS are partly due to the increased production of AMH by individual follicles and not just due to the increased
number of small antral follicles characteristically seen in PCOS. This indicates that there is an intrinsic abnormality in the ovarian follicles themselves in PCOS, which cause them to produce more AMH thereby contributing to the follicular arrest of PCOS.

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