human reproduction

ORIGINAL ARTICLE *Reproductive endocrinology*

The relationships between AMH, androgens, insulin resistance and basal ovarian follicular status in non-obese subfertile women with and without polycystic ovary syndrome

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BACKGROUND: Hyperandrogenaemia and insulin resistance are prominent features of polycystic ovary syndrome (PCOS) and influence the process of folliculogenesis in women with the endocrinopathy. Anti-Müllerian hormone (AMH) levels are elevated in women with PCOS and studies including IVF subjects have shown that this is a reliable marker of ovarian performance. The aims of this prospective study were to assess the relationship between insulin resistance, androgens and AMH, and whether AMH contributes to altered folliculogenesis in non-obese women with PCOS.

METHODS: A total of 232 IVF candidates, 49 of whom had PCOS according to the Rotterdam 2003 consensus criteria, were recruited. AMH levels and ovarian morphology were assessed. The relationships between AMH and insulin resistance and androgenaemia in patients with and without PCOS were studied.

RESULTS: PCOS patients were slightly older than controls (median ages 34 and 30 years, respectively). AMH generally increased with antral follicle count (AFC), insulin, homeostatic model assessment of tissue insulin sensitivity (HOMA-IR), testosterone, free androgen index and luteinising hormone, and decreased with chronological age, homeostatic model assessment of steady state beta cell function (HOMA-B) and serum sex hormone binding globulin (SHBG). For these relationships there were no significant differences in the slopes between PCOS and non-PCOS patients. The ratio of AMH per antral follicle (AMH/AF) was higher in PCOS patients. Both PCOS and non-PCOS groups showed a very similar increase in AMH with increases in AFC, but the PCOS patients had consistently higher AMH across all AFC levels.

CONCLUSIONS: These observations indicate that AMH is similarly related to insulin resistance and androgens in women with and without PCOS. This effect appears to be independent of age although an indirect causal effect due to ageing or some other mechanism cannot be ruled out. Excessive granulosa cell activity may be implicated in the abnormal follicular dynamic of the syndrome.

Key words: polycystic ovary syndrome / anti-Müllerian hormone / insulin resistance / antral follicle count / androgens

Introduction

Increased insulin resistance is a prominent feature of polycystic ovary syndrome (PCOS) (Robinson *et al.*, 1993; Chang *et al.*, 2005). Though insulin resistance is amplified by increasing obesity, women with PCOS are more insulin resistant than can be accounted for by their obesity alone (Dunaif *et al.*, 1989, 1992). Insulin has been shown to promote *in vitro* secretion of androgens by ovarian theca and stromal tissue (Barbieri *et al.*, 1984), and in PCOS subjects it stimulates testosterone biosynthesis by binding to its own receptor in theca cells (Nestler *et al.*, 1998). Ovarian sensitivity to insulin may be enhanced in PCOS (Baillargeon and Nestler, 2006) and measures that reduce insulin metabolism ameliorate androgen secretion in women with the syndrome (Taylor, 2000).

Intra-ovarian androgen receptors are widely distributed in granulosa cells of small pre-antral and antral follicles, as well as theca and stromal cells (Hillier *et al*., 1997) and decrease with further follicular development (Rice *et al*., 2007). Quantitative ultrasound evaluation of follicle distribution in polycystic ovaries suggests a possible role for insulin in the dysregulation of folliculogenesis (Jonard *et al*., 2003). Fleming and collaborators (Fleming *et al*., 2006) have shown that follicular development in response to the induction of ovulation with gonadotrophins is qualitatively different in insulin-resistant PCOS patients. Treatment with the insulin sensitizer metformin reduces the number of antral follicles, ovarian volume and circulating anti-Müllerian hormone (AMH) levels (Piltonen *et al*., 2005).

Androgens have been shown to stimulate early stages of follicle growth and to increase the number of antral follicles in rat ovary (Tetsuka *et al*., 1995). There appears to be a degree of reciprocal control between androgens and follicle stimulating hormone (FSH) in follicle development. FSH-receptor expression is up-regulated by androgens, and FSH increases androgen receptor expression in primary follicles (Weil *et al*., 1999). To further demonstrate what drives folliculogenesis in PCOS, some researchers have recently found a relationship between insulin resistance and AMH (La Marca *et al*., 2004), and more recently between antral follicles and free androgen index (FAI) (Chen *et al*., 2008). However, a significant relationship between AMH and androgens could not be found. Of note, the available evidence highlights a direct involvement of androgens in follicular development and in the dysregulated folliculogenesis of PCOS (Jonard and Dewailly, 2004).

AMH is secreted by the granulosa cells of small antral and pre-antral follicles in the ovary. AMH diminishes aromatase induction by FSH in antral follicles and inhibits recruitment of primordial follicles (Durlinger *et al*., 2002; Weenen *et al*., 2004; Carlsson *et al*., 2006). Previous studies have shown that circulating AMH levels are significantly higher in subjects with PCOS and correlate with circulating androgens (Cook *et al*., 2002; Pigny *et al*., 2003) but not with fasting insulin (Pigny *et al*., 2003). In keeping with this observation, it has been demonstrated that granulosa cells from PCOS patients secrete more AMH *in vitro* (Pellatt *et al*., 2007). Furthermore, the increase in circulating AMH is accompanied by, and is related to, an increase in the antral follicle count (AFC) (Fanchin *et al*., 2003). The concentration of AMH is higher in the follicular fluid from unstimulated ovaries of women with PCOS (Das *et al*., 2008). The production of AMH by granulosa cells of stimulated follicles of women with PCOS is increased, and the genes for the AMH, FSH and androgen receptors are all overexpressed (Catteau-Jonard *et al*., 2008).

This prospective study was designed to explore further the relationship between insulin resistance, androgens and AMH in women with and without PCOS, and to gain understanding of the mechanisms implicated in the dysregulated folliculogenesis in a distinct group of PCOS subjects in whom obesity is not an overriding influence.

Materials and Methods

A total of 232 patients aged 22–41 years undergoing infertility work-up prior to their first IVF treatment were recruited. The inclusion criteria were: (a) presence of both ovaries, (b) no use of hormone therapy in the 6 months preceding the study, (c) no history of premature ovarian failure, (d) no previous ovarian surgery, (e) no exposure to cytotoxic drugs or pelvic radiation therapy and (f) body mass index (BMI) >19 <30 kg/m^2 (to satisfy eligibility criteria for government-funded IVF treatment in Greater Manchester, UK). PCOS was diagnosed according to the criteria from The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group (2004). The project was approved by the Local Research Ethics Committee (UK-NHS 05/QI409/50) and all subjects gave written informed consent prior to entering the study.

On day 2–3 of a spontaneous menstrual cycle or after a withdrawal bleed, blood samples for measurement of AMH concentrations were obtained by venepuncture at ∼08:00 h. On the same morning, a transvaginal ultrasound scan of the ovaries was performed using a 6 MHz transducer (PVM-651VT, Nemio 20, Toshiba, Japan) in order to determine the total number of early antral follicles measuring 2–5 mm in diameter (AFC). The intra-analysis coefficient of variation for follicular diameter measurements was <5% and the lower limit of detection was 0.5 mm.

Biochemical analyses

Serum testosterone was measured on unextracted serum using an Abbott Architect automated immunoassay analyzer. Samples giving initial results >2.5 nmol/l were re-assayed following solvent extraction with diethyl-ether and the extracted results recorded. Serum sex hormone binding globulin (SHBG) was measured on a Perkin-Elmer Auto-DELFIA immunoassay analyzer. FAI was calculated using the standard formula testosterone/SHBG × 100. Serum estradiol (E_2), luteinising hormone (LH) and FSH were measured on a Roche E-170 automated immunoassay analyzer. Between-batch coefficients of variation for these assays were <10%.

All blood samples for measurements of AMH levels were collected in a lithium-heparin tube. Plasma was separated within 2 h of venepuncture, frozen in aliquots at −70°C until thawed and assayed in batches. Measurements of AMH were determined in duplicate using the ultra sensitive enzyme-linked immunosorbent assay (ELISA), as previously reported (Nardo *et al*., 2008). The functional sensitivity of the assay was 0.05 ng/ml. The intra- and inter-assays coefficient of variation were <10 and <15%, respectively. Conversion factor to ng/ml = pmol/l ÷ 7.143.

Plasma glucose was measured on fluoride oxalate samples by a glucose oxidase method on a Roche Modular clinical chemistry analyzer with a between batch coefficient of variation of <2.5%. Serum insulin was measured by radioimmunoassay with charcoal separation, as previously described (Yates and Laing, 2002). The lower limit of detection was calculated to be <0.4 mU/l. The intra- and inter-assay coefficients of variation were 6.3 and 6.1%, respectively, at an insulin concentration of 3.8 mU/l. Cross reactivity of pro-insulin in this assay was 38%. The Homeostatic Model Assessment (HOMA 2) algorithm was used to give estimates of steady state beta cell function (HOMA-B) and tissue insulin sensitivity (HOMA-IR), calculated from the fasting glucose and insulin pairs obtained for each patient (Levy *et al*., 1998). Using this algorithm a fit, healthy, young individual would have a HOMA-B of approximately 100% and a HOMA-IR of approximately 1.0%.

Statistical analysis

The software package R version 2.7 (R Development Core Team, 2008) was used for statistical analysis. Continuous variables are summarized by their medians and interquartile ranges. Differences between groups were assessed using the Mann–Whitney *U*-test and analysis of covariance adjusting for age. The relationship between the biochemical markers was assessed using an analysis of covariance (ANCOVA) stratifying by PCOS status. Models were fitted with and without an interaction term allowing for different slopes between groups. Additional models included age as a linear covariate.

Where appropriate, regression slopes are reported along with a significance level for slope (without interaction). Additionally, a test for differing

slopes between the two groups based on the interaction term in the model is shown. Highly skewed variables were log-transformed and the slopes presented per doubling of the independent variable. A 5% significance level was used throughout.

Results

Of the 232 subjects who participated in this study, 49 (21.1%) had PCOS and the remaining 183 subjects were referred to IVF because of male factor, tubal factor or more ≥2 years unexplained subfertility. The data for both patient groups are shown in Table I. AMH levels were measurable in all patients with values ranging from 0.1 to 12.5 ng/ml. Of note, there was a 3-fold increase in circulating AMH levels in women with PCOS compared with those without the syndrome. The AFC was also significantly higher in the PCOS group. Fasting insulin was marginally higher in PCOS patients, as was the derived index of insulin resistance. These effects just failed to reach statistical significance if we adjusted for the age difference between the groups. The derived index of beta cell function was not different. Testosterone was higher and SHBG lower in the PCOS group, and differences in androgenaemia were magnified when FAI was compared between the groups. The SHBG decreased with increasing BMI ($P < 0.001$), HOMA-IR ($P < 0.001$) and insulin ($P < 0.001$), but there was no evidence that this decline differed between the two groups (ANCOVA interaction test).

The relationships between AMH and other demographics and biochemical markers are summarized in Table II. AMH increased with AFC, LH, insulin, HOMA-IR, testosterone and FAI, and decreased with chronological age, E_2, HOMA-B and SHBG. No significant relationships were found between circulating AMH levels and BMI or glucose. For all of the above variables, there was no significant difference between the slopes for PCOS and non-PCOS patients.

However, AMH showed a different relationship with FSH in the two groups (interaction test $P = 0.021$), with a negative association in the non-PCOS group and a positive, albeit non-significant, association in the PCOS patients.

If we additionally adjusted for age the relationships between AMH and AFC, LH, testosterone, E_2, SHBG and FAI remain unchanged, but the relationships between AMH and insulin, HOMA-B and HOMA-IR are slightly weakened and statistical significance is lost.

The relationship between AMH and AFC is illustrated in Fig. 1. Both PCOS and non-PCOS groups show a very similar increase in AMH as the AFC increases, but the PCOS patients have consistently higher AMH levels (difference 2.05 ng/ml, SD ± 0.26; $P < 0.001$). Although the non-PCOS patients show a line that is close to passing through the origin (intercept 0.42, SD ± 0.20; $P = 0.04$), the PCOS patients extrapolate back to a level of intercept 2.47 (SD ± 0.35; $P < 0.001$) at zero AFC. Additionally, adjusting for age does not affect these results (difference in AMH levels = 2.00 ± 0.26), and Fig. 1 shows no evidence of differential effects between younger and older patients.

The calculation of AMH production per antral follicle (AMH/AF) showed that there was a significant difference in median AMH/AF between PCOS and non-PCOS (0.327 and 0.20, $P < 0.0001$). The ratio appeared to decline with age in the non-PCOS group but remained steady with age in the PCOS group. When correlating AMH/AF with other parameters, the ratio increased with testosterone, SHBG and FAI, and decreased with FSH. There was no effect of BMI, insulin, HOMA-IR or HOMA-B.

Discussion

In the current study the PCOS patients did not have higher BMI than those without the syndrome and demonstrated only a minor degree of

Table I Biochemical and demographic data for women with and without PCOS

Marker	Non-PCOS (n = 183)		PCOS (n = 49)		Unadjusted P-value*	Age-adjusted P-value**
	median	[IQ range]	median	[IQ range]		
Age (years)	33.7	[30.6−35.9]	29.9	[27.3−33.4]	<0.001	−
BMI (kg/m^2)	23.5	[21−27]	25	[22−28]	0.092	0.094
Antral follicles	10	[7−14]	17	[14−22]	<0.001	<0.001
FSH (IU/l)	7.6	[6.5−9.2]	6.0	[5.4−7.1]	<0.001	0.002
E_2 (pmol/l)	149	[121−182]	143	[121−176]	0.95	0.97
LH (IU/l)	6.1	[4.8−7.4]	8.0	[5.9−9.5]	<0.001	<0.001
Glucose (mmol/l)	5.0	[4.7−5.2]	5.0	[4.7−5.3]	0.25	0.52
Insulin (mU/l)	7.2	[5.7−9.9]	8.4	[6.6−11.3]	0.037	0.059
HOMA-B	94.2	[77.3−117.6]	94.6	[82.2−124]	0.16	0.17
HOMA-IR	0.93	[0.75−1.26]	1.08	[0.86−1.43]	0.033	0.077
Testosterone (nmol/l)	1.6	[1.2−2.1]	2.2	[1.7−2.7]	<0.001	<0.001
SHBG (nmol/l)	67	[51−87]	39	[27.5−50.5]	<0.001	<0.001
FAI	2.3	[1.6−3.3]	6.4	[3.1−9.1]	<0.001	<0.001
AMH (ng/ml)	1.98	[1.03−3.54]	6.10	[4.25−6.78]	<0.001	<0.001

AMH: anti-Mullerian hormone; E_2: estradiol; FAI: free androgen index; HOMA-B: homeostatic model assessment of steady state beta cell function; HOMA-IR: homeostatic model assessment of tissue insulin sensitivity; PCOS: polcystic ovary syndrome; SHBG: serum sex hormone binding globulin.
*Comparing groups using a Mann−Whitney U-test.
**Comparing groups using analysis of covariance adjusting for age.

Table II Relationship between AMH and the biochemical and demographic markers

Marker	Slope expressed as	Slope of AMH versus Marker (SE)				Significance	
		Non-PCOS		PCOS		Overall slope	Difference in slopes
Age (years)	per year	−0.14	(0.03)	−0.11	(0.06)	<0.001	0.70
BMI (kg/m^2)	per kg/m^2	−0.04	(0.04)	−0.02	(0.08)	0.82	0.78
Antral follicles	per follicle	0.19	(0.02)	0.19	(0.03)	<0.001	0.99
FSH (IU/l)	per IU/l	−0.24	(0.05)	0.11	(0.14)	<0.001	0.021
E$_2$ (pmol/l)	per doubling	−0.69	(0.26)	−0.22	(0.47)	0.012	0.38
LH (IU/l)	per doubling	0.22	(0.26)	0.78	(0.39)	<0.001	0.23
Glucose (mmol/l)	per mmol/l	−0.04	(0.27)	0.72	(0.52)	0.22	0.19
Insulin (mU/l)	per doubling	0.28	(0.21)	−0.03	(0.40)	0.004	0.49
HOMA-B	per doubling	0.38	(0.30)	−0.60	(0.58)	0.046	0.14
HOMA-IR	per doubling	0.27	(0.21)	0.00	(0.40)	0.004	0.55
Testosterone (nmol/l)	per doubling	0.32	(0.21)	1.14	(0.45)	<0.001	0.10
SHBG (nmol/l)	per doubling	0.12	(0.22)	−0.34	(0.33)	<0.001	0.26
FAI	per doubling	0.13	(0.17)	0.52	(0.25)	<0.001	0.19

Based on the ANCOVA analysis, the table gives the regression slopes for AMH versus each parameter for the PCOS or non-PCOS groups along with a test for an overall association and for a difference in the relationship between the two groups. Skewed variables were log-transformed and the slopes expressed per doubling of marker level as indicated.
AMH: anti-Mullerian hormone; E$_2$: estradiol; FAI: free androgen index; HOMA-B: homeostatic model assessment of steady state beta cell function; HOMA-IR: homeostatic model assessment of tissue insulin sensitivity; PCOS: polycystic ovary syndrome; SHBG: serum sex hormone binding globulin.
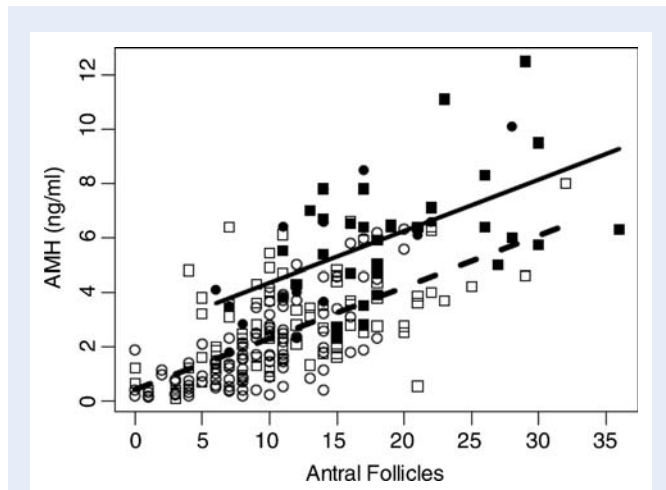
Figure 1 Relationship between AMH and AFC for PCOS subjects (solid symbols and line) and non-PCOS subjects (open symbols and broken line), and for younger (below median age of 33 years; squares) and older (above median age of 33 years; circles) subjects.Lines represent linear regression fits to each patient group. AFC: Antral follicle count; AMH: Anti-Müllerian hormone; PCOS: Polycystic ovary syndrome.

concentrations in women with PCOS. Differences in study populations, clinical setting and local guidelines for treatment have to be borne in mind to explain discrepancies between the studies. For instance, the pre-selection of patients based on BMI cut-offs, which was the case in our study owing to local eligibility criteria and national guidelines (NICE, 2004), may have jeopardized the analysis of the relationship between circulating AMH and BMI, thus not offering evidence of the correlation of obesity with reproductive hormone levels, which has been reported previously by other authors (Clark *et al.*, 1995). The same holds true for the lack of significant difference in the HOMA-B between subjects with and without PCOS. It is however, interesting to note that within the same BMI range and insulin metabolism status, PCOS women have enhanced granulosa cell activity compared with controls. Whether this effect would be any different if obese PCOS women were included could not be tested.

The patients exhibited appropriate biochemical characteristics of PCOS, with increased testosterone, FAI, lower SHBG and increased LH and AFC. Notwithstanding the exclusion of obese (BMI ≥ 30 kg/m^2) subjects from the study, we found a positive relationship of AMH with fasting insulin and the derived indices of beta cell function and insulin sensitivity. This relationship could not be demonstrated if we adjusted for age, a not surprising result given that both AMH and insulin sensitivity show a strong age-dependence. Larger datasets will be needed to determine the true causal relationships. This was evident in spite of the modest degree of insulin resistance in the patients and non-PCOS controls. There was also a positive relationship between AMH and LH. These observations suggest the possibility of a direct effect of both insulin and LH on AMH secretion or may reflect the fact that both insulin and LH contribute to ovarian androgen secretion (Nestler, 1993; Baillargeon and Nestler, 2006). Of all

insulin resistance, which is different from that of typical PCOS populations (Conway *et al.*, 1989; Jayagopal *et al.*, 2003; Chang *et al.*, 2005). We could find no relationship between AMH and BMI, confirming our earlier observations in a similar group of subfertile subjects (Nardo *et al.*, 2007) although this was in contrast to other studies (Freeman *et al.*, 2007; Chen *et al.*, 2008). Pigny and colleagues (2003) also found that BMI did not influence the circulating AMH

the markers studied only basal serum FSH appeared to be differently related to AMH in PCOS and non-PCOS patients. Whereas FSH and AMH were inversely correlated in non-PCOS women, the two biochemical indices had a positive correlation in the PCOS group. This observation leads to speculation on the role of AMH as direct modulator of the FSH sensitivity of individual follicles in PCOS, which in turn may also explain previous data on follicular recruitment and delayed ovarian ageing in women with the endocrinopathy (Mulders et al., 2004).

We were able to demonstrate a direct positive relationship between AMH and AMH/AF with circulating levels of testosterone and FAI, but could not detect any significant difference in the associations between PCOS and non-PCOS patients. It is plausible to speculate that the intra-ovarian hormonal milieu is not dependent exclusively on the PCOS status. Although some studies (Piltonen et al., 2005) have demonstrated that serum testosterone and androstenedione correlated with AMH concentrations in both women with PCOS and controls, others (Pigny et al., 2003; Eldar-Geva et al., 2005) have found this relationship to hold only in the PCOS groups. Our findings concur with those of Piltonen et al. (2005) in that a relationship of AMH to circulating androgens is demonstrated by lack of difference in the slopes of the relationship between PCOS and non-PCOS patients.

The relative contribution of the different follicles sizes to the circulating levels of AMH is still a matter of much debate. The association between early antral follicles, granulosa cell function and follicular development arrest has been reported (Pigny et al., 2003; Stubbs et al., 2005; Dewailly et al., 2007). It is plausible to speculate that follicles entering the cyclic recruitment from secondary follicles may produce more AMH as a reflection of granulosa cell numbers and as they are less involved in the FSH-dominance process responsible for cyclic selection. Based on the available data, we only considered early antral follicles measuring 2–5 mm in diameter. A recent study (Weenen et al., 2004) demonstrated that AMH expression is highest in small antral follicles and gradually disappeared in granulosa cells of large-sized FSH-dependent antral follicles. Both in PCOS women and in controls the 2–5 mm follicles, but not the 6–9 mm follicles, correlated to AMH concentrations (Pigny et al., 2003) and androgen serum levels (Jonard et al., 2003), thus showing the effect of intra-ovarian hormones on follicle dynamics. In agreement with other studies (van Rooij et al., 2002; Pigny et al., 2003; Laven et al., 2004; Piltonen et al., 2005), AMH and AFC were higher in PCOS and positively correlated with each other. This confirms that the large number of small follicles generally associated with polycystic appearance of the ovary is the source of increased AMH production. We found that AMH increases with AFC at a consistent rate of 0.2 ng/ml per follicle and that there is an increased level of AMH per follicle in PCOS, thus shedding light on the difference in the ratio of AMH to AFC between the two groups. Both AFC and AMH decline with age, but this relationship is unchanged if we adjust for age, suggesting that this is not a simple consequence of reproductive ageing. However, it is still plausible that age, or some other confounder may affect these relationships and that the effects seen are a consequence of some unmeasured or incompletely allowed for confounders. These findings are relevant in the context of recent work showing that granulosa cells of patients with PCOS secrete more AMH in vitro (Pellatt et al., 2007) and that AMH

concentrations are higher in unstimulated ovaries of women with PCOS (Das et al., 2008). The understanding that smaller follicles, which might not always be detected on ultrasound scan, do contribute to the levels of AMH could explain in part our observations.

In conclusion, AMH is similarly related to insulin resistance and circulating androgens both in PCOS and non-PCOS subjects as shown by the lack of difference in the slopes. AMH and the ratio AMH/AF are significantly higher in non-obese women with PCOS compared with those without PCOS, suggesting an increased granulosa cell activity in vivo, which may not be bodyweight-related. Furthermore, AMH, like insulin resistance and androgens, seems to be implicated in the abnormal follicular development of the syndrome. Although many believe that the ovary is central to the complexity of PCOS, whether dysregulation of AMH, insulin or androgen action is the primary event in the abnormal folliculogenesis remains a matter of much debate. Although the relationship between circulating AMH and androgens was demonstrated in this study, we were unable to clarify whether the ovarian or adrenal contribution to the hyperandrogenaemia is the more important. Without doubt, available studies, including this one, are limited as peripheral hormone concentrations may not necessarily be a reflection of their action within the ovary. Further studies in ovarian tissue are needed to better define the roles of both androgens and AMH in normal folliculogenesis and in the abnormal follicular dynamic in women with PCOS.

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