

The relationship between anti-Müllerian hormone, androgen and insulin resistance on the number of antral follicles in women with polycystic ovary syndrome

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BACKGROUND: Anti-Müllerian hormone (AMH) is a biomarker that predicts the number of antral follicles and is involved in follicle arrest for women with polycystic ovary syndrome (PCOS). We investigated the association between the characteristic hyperandrogenemia, insulin resistance (IR), AMH, and the morphology and size of ovaries for women with PCOS. **METHODS:** A total of 99 Taiwanese women with PCOS who were willing to undergo vaginal ultrasonography were enrolled in this cross-sectional study. **RESULTS:** The number of antral follicles and the ovarian volume showed a significant correlation with AMH, total testosterone and the free androgen index, but not with age, body mass index (BMI) or the homeostasis model assessment of insulin resistance (HOMA-IR). AMH had a significant negative association with both BMI and HOMA-IR. Multiple stepwise regression analysis demonstrated that AMH, BMI and total testosterone were independently related to the number of antral follicles. AMH and total testosterone were the main determinants for ovarian volume in a stepwise regression model. **CONCLUSIONS:** Our results suggest that not only the AMH level, but also obesity, IR and elevated androgen levels may relate to the development of the large size of antral follicle pool and ovarian volume in women with PCOS. Obesity and IR may enhance the follicular excess through the dysregulation of AMH or through the pathway of hyperandrogenemia. These findings might partly explain why adequate body weight management and improvement in IR can improve the ovulatory function for women with PCOS.

Keywords: polycystic ovary syndrome; anti-Müllerian hormone; obesity; insulin resistance; antral follicle count

Introduction

Since the Rotterdam consensus meeting in 2003 (The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004), the ultrasound criteria of increased ovarian volume (>10 ml) and/or the presence of 12 or more follicles, 2–9 mm in size, in at least a single ovary (Balen *et al.*, 2003) has been included in the triad definitive diagnostic criteria of the polycystic ovary syndrome (PCOS). However, this criterion for the definition of PCOS is still controversial since the relevance of polycystic ovary morphology to the characteristic endocrine syndrome in women with PCOS remains elusive.

PCOS is considered to be a syndrome of preserved (Poretsky, 2006), if not increased (Baillargeon and Nestler, 2006), ovarian sensitivity to insulin with systemic resistance to insulin action. Moreover, ovarian antral follicle counts and ovarian volume correlate positively with endogenous and

exogenous hyperinsulinemia, as reported in women with PCOS (Pache *et al.*, 1993; Carmina *et al.*, 2005) and with type 1 diabetes mellitus (Codner *et al.*, 2006). Biologically, hyperinsulinemia may stimulate the development of antral follicles, increase the sensitivity of granulosa cells to FSH, and thus increase the number of follicles and ovarian volume (Fulghesu *et al.*, 1997). Other studies, however, have not found such an association between polycystic ovarian morphology and insulin sensitivity (Loucks *et al.*, 2000; Legro *et al.*, 2005). Thus, there is still a debate on the association of obesity and insulin resistance (IR) with polycystic ovarian morphology.

Anti-Müllerian hormone (AMH), a member of the transforming growth factor- β superfamily, is derived specifically from the granulosa cells of early developing pre-antral and antral follicles (Weenen *et al.*, 2004), the number of which is

greater in polycystic ovaries as compared with normal ovaries (Jonard *et al.*, 2003; Pigny *et al.*, 2006). Serum AMH levels have an excellent correlation with the number of antral follicles as determined by vaginal ultrasound (Pigny *et al.*, 2003; Visser and Themmen, 2005; Visser *et al.*, 2006). The AMH assay is considered to have high reproducibility in repeated measurements (Fanchin *et al.*, 2005) and AMH concentration, unlike other ovarian hormones, has been reported to be constant throughout the menstrual cycle (Hehenkamp *et al.*, 2006). Because of this consistency and reliability, AMH has therefore been suggested to be a good surrogate marker for predicting the antral follicle count in women with and without PCOS (Feyereisen *et al.*, 2006; Pigny *et al.*, 2006). Further, AMH seems to inhibit the initiation of human primordial follicle growth (Carlsson *et al.*, 2006) and to act as one of the gatekeepers of the follicle cohort preventing multiple selection of a dominant follicle (Jonard and Dewailly, 2004). Therefore, dysregulation of AMH function may be involved in the failure to trigger ovulation.

A large proportion of women with PCOS are obese and insulin resistant. It has been demonstrated that weight loss can improve the recovery of spontaneous ovulation in obese women with PCOS (Norman *et al.*, 2004). Whether or not obesity or IR interferes with the effects of AMH on polycystic ovary morphology has not been reported. This study was designed to investigate the roles of serum AMH, obesity and IR in the regulation of the antral follicle count of women with PCOS.

Materials and Methods

Subjects

A total of 99 women with PCOS who had a chief complaint of irregular menstrual cycles and/or clinical hyperandrogenism, and who were willing and able to undergo vaginal ultrasound, were recruited from our reproductive endocrinology clinic. The study was approved by the Institutional Review Board of the National Taiwan University Hospital. Informed consent was obtained from all of the subjects and/or their parents. The inclusion and exclusion criteria for the enrollment of the subjects have been described in detail previously (Chen *et al.*, 2006, 2007). Briefly, the diagnosis of PCOS was based on the Rotterdam criteria, in which at least two of the following three criteria were met: Oligomenorrhea (<8 spontaneous menstrual cycles per year for at least 3 years before enrollment) or amenorrhea, biochemical hyperandrogenemia (serum total testosterone level >0.8 ng/ml), and polycystic ovaries (>12 follicles in the 2–9 mm range and/or an ovarian volume >10 ml per ovary by vaginal ultrasound). The diagnosis of PCOS was retained after excluding hyperprolactinemia, thyroid dysfunction, Cushing's syndrome, congenital adrenal hyperplasia, an adrenal tumor, an ovarian tumor, current or previous pregnancy within 1 year of enrollment, autoimmune disease, malignancy, central nervous system disease, current or previous use of oral contraceptives within 6 months of enrollment, or the use of medications known to affect the hypothalamic–pituitary–ovarian axis, such as anti-androgens, ovulation induction agents, anti-diabetic medications, anti-obesity medications or glucocorticoids.

Serum sampling

Overnight fasting blood samples were collected randomly from PCOS subjects with amenorrhea exceeding 3 months without

hormone-induced withdrawal bleeding and/or between Days 3 and 7 of the menstrual cycle for those women who ovulated spontaneously. Blood samples were collected before anthropometric measurements and after transvaginal ultrasound. If a dominant follicle (>10 mm) was present by vaginal ultrasound, the subject's blood sample was not collected; if the serum estradiol level was >150 pg/ml or the serum progesterone was >2 ng/ml, the collected blood samples were discarded and the subject's blood sample was collected following menstruation after spontaneous ovulation according to the subject's recorded basal body temperature. Blood was processed within 30 min of collection. Blood glucose and insulin samples were stored at 4°C and analysed the day of sampling. Serum and plasma were aliquoted and frozen at –70°C until assayed. The body mass index (BMI) was calculated as the weight in kilograms divided by the height in meters squared.

Assay methods

The concentration of plasma glucose, the serum levels of insulin and the hormone profiles (i.e. FSH, LH, estradiol, progesterone and sex hormone-binding globulin [SHBG]) were measured as described previously (Chen *et al.*, 2006). Serum total testosterone and dehydroepiandrosterone sulfate were measured by radioimmunoassay (Diagnostic Systems Laboratories). The homeostasis model assessment (HOMA) and free androgen index (FAI) were applied to estimate the degree of IR and bioavailable testosterone, respectively. HOMA-IR and FAI were calculated as described previously (Chen *et al.*, 2007). Serum AMH levels were assessed using a second generation enzyme immunoassay (AMH-EIA kit; reference number: A16 507), according to the supplier's instructions (Immunotech A Beckman Coulter Company, Marseilles, France). The intra-assay and inter-assay coefficients of variation of the AMH-EIA were <10%.

Transvaginal ultrasound

Ultrasound examination was performed with a 7 MHz transvaginal probe. The ultrasound measurements were obtained in real-time by a single physician according to a standardized protocol and before blood sampling. The ovaries were examined under the highest possible magnification. After the longest medial axis of the ovary had been determined, the second dimension was measured, and then the vaginal probe was rotated 90 degrees to obtain the third dimension. Ovarian volume was calculated according to a simplified formula for an ellipsoid ($0.5 \times \text{length} \times \text{width} \times \text{thickness}$; Balen *et al.*, 2003). Ovarian volume per ovary (OVPO) was defined as the average of the ovarian volume obtained from both ovaries. Each ovary was scanned in both longitudinal and transverse cross-section from the inner to the outer margins to enumerate the total number of follicles. All follicles between 2 and 9 mm in diameter were counted. If there was a dominant follicle (>10 mm), we repeated the transvaginal scan and blood sampling during the next cycle. The follicle number per ovary (FNPO) was defined as the average for the total number of follicles counted from both ovaries.

Statistical analysis

The data are presented as the median with 5–95th percentiles, unless indicated otherwise. The Shapiro–Wilk W test was used to identify whether all the variables were normally distributed. Log transformation was performed on variables with significant deviation from a normal distribution before further analysis. Pearson correlation coefficients were calculated to determine the correlations between the variables. Forward stepwise multiple linear regression analysis was performed using FNPO or OVPO as the dependent variable, and age, BMI, HOMA-IR, total testosterone, FAI and AMH as the

independent variables, as indicated. The probability of *F* for entering the model was chosen as 0.05 and for removal from the model was chosen as 0.10. A *P*-value <0.05 was considered statistically significant. All statistical analyses were performed using the PC version of the Statistical Analysis System (SAS, version 9.1, SAS Institute, Inc., Cary, NC, USA).

Results

The clinical and endocrine characteristics of the subjects are depicted in Table I. Among the subjects in this study, 39 subjects (39.4%) were overweight or obese (BMI ≥25 kg/m²), and 89 subjects (90%) were nulliparous. The prevalence of oligomenorrhea, biochemical hyperandrogenism and polycystic ovarian morphology in this study were 86, 68 and 97%, respectively. FNPO and OVPO showed a significant correlation with each other and with AMH, total testosterone and FAI, but not with age, BMI or HOMA-IR (Table II). AMH had a significant negative association with both BMI (γ = -0.213; *P* = 0.035) and HOMA-IR (γ = -0.220; *P* = 0.030).

Age-adjusted multivariable linear regression analysis was used to determine the significant independent association between FNPO and BMI (β = 0.498; *P* = 0.0005), HOMA-IR (β = 0.117; *P* = 0.0007), testosterone (β = 0.192; *P* = 0.01) or FAI (β = 0.166; *P* < 0.0001) after adjusting for the confounding effect of AMH. The interaction term between variables was not significant in the linear regression model. Using stepwise multiple linear regression analysis, only AMH, BMI and total testosterone were significantly related to FNPO in the final model after the stepwise introduction of age, AMH, BMI, HOMA-IR and total testosterone as independent variables (Table III). However, when adding FAI as one of the independent variables to the stepwise regression model, only AMH and FAI maintained a significant relationship to FNPO (Table IV).

The HOMA-IR (β = 0.071; *P* = 0.024), testosterone (β = 0.187; *P* = 0.0046) and FAI (β = 0.106; *P* = 0.0009) were

Table I. Clinical and endocrine characteristics of the PCOS (*n* = 99) subjects.

	Median	Range (5–95%)
Age (years)	26	21–35
FNPO	24.5	12–47.5
OVPO	9.29	5.72–17.23
FSH (mIU/ml)	5.64	3.76–8.49
LH (mIU/ml)	11.05	3.12–24.4
Estradiol (pg/ml)	39.60	18.11–89.60
Total testosterone (ng/ml)	1.05	0.43–1.91
SHBG (nmol/l)	31.3	11.30–84.90
FAI (%)	10.79	2.10–41.98
BMI (kg/m ²)	23.05	17.61–37.11
Fasting insulin (μU/ml)	9.10	2.2–44.0
Fasting glucose (mg/dl)	82.0	70–122
HOMA-IR	1.87	0.44–11.72
AMH (pM)	94.67	34.54–237.58

AMH, anti-Müllerian hormone; BMI, body mass index; FAI, free androgen index; FNPO, follicle number per ovary; HOMA-IR, homeostasis model assessment of insulin resistance; OVPO, ovarian volume per ovary; SHBG, sex hormone-binding globulin.

Table II. Correlations among variables in women with PCOS.

	ln(FNPO)	ln(OVPO)	ln(BMI)	ln(HOMA-IR)	Ln(testosterone)	ln(FAI)	ln(AMH)
Ln(age)							
ln(FNPO)	0.02652 (NS)	0.01891 (NS)	0.09848 (NS)	0.01726 (NS)	-0.07894 (NS)	-0.12993 (NS)	0.10734 (NS)
ln(OVPO)	-	0.69025 (<i>P</i> < 0.0001)	0.12338 (NS)	0.11683 (NS)	0.29562 (<i>P</i> = 0.003)	0.32852 (<i>P</i> = 0.0009)	0.62739 (<i>P</i> < 0.0001)
ln(BMI)	-	-	-0.01982 (NS)	0.05211 (NS)	0.32802 (<i>P</i> = 0.0014)	0.26928 (<i>P</i> = 0.0094)	0.55726 (<i>P</i> < 0.0001)
ln(HOMA-IR)	-	-	-	0.72670 (<i>P</i> < 0.0001)	0.18219 (<i>P</i> = 0.071)	0.53962 (<i>P</i> < 0.0001)	-0.21253 (<i>P</i> = 0.035)
ln(testosterone)	-	-	-	-	0.20966 (<i>P</i> = 0.038)	0.55693 (<i>P</i> < 0.0001)	-0.21971 (<i>P</i> = 0.030)
ln(FAI)	-	-	-	-	-	0.75785 (<i>P</i> < 0.0001)	0.14742 (NS)
							-0.04572 (NS)

Values are expressed as the correlation coefficient (γ); *P*-values are indicated in parentheses; NS indicates non-significance at *P* ≥ 0.1.

Table III. Forward stepwise multiple linear regression models of dependent variable FNPO and OVPO for women with PCOS.

ln(FNPO)				ln(OVPO)			
Variable	Estimate	F	P-value	Variable	Estimate	F	P-value
ln(AMH)	0.48962	72.16	<0.0001	ln(AMH)	0.33311	37.47	<0.0001
ln(BMI)	0.41539	8.96	0.0035	ln(testosterone)	0.19176	8.91	0.0037
ln(testosterone)	0.14953	4.31	0.0406				
Model R ² = 48.65%				Model R ² = 37.42%			

Covariates considered for selection in each model: age, AMH, BMI, HOMA-IR and testosterone.

significantly related to the OVPO after adjustment for age and AMH. Using stepwise multiple linear regression analysis, only AMH and total testosterone were significantly related to OVPO in the final model after the stepwise introduction of age, AMH, BMI, HOMA-IR and total testosterone as independent variables (Table III). When adding FAI as one of the independent variable to the stepwise regression model, only AMH and FAI maintained a significant relationship to OVPO (Table IV). It is noteworthy that the BMI and/or HOMA-IR had a positive association with FNPO and/or OVPO after adjustment for the confounding effect of AMH. However, the associations between BMI/HOMA-IR and FNPO/OVPO were weakened after adding the FAI variable in the models. It might be that FAI integrates both androgenic and insulinic effects, the latter through diminution of SHBG. Therefore, FAI masks other relationships when introduced in the model.

Discussion

In the present study, we observed a significant positive association between the antral follicle count and BMI, HOMA-IR, serum testosterone levels and FAI levels after adjustment for age and AMH levels in women with PCOS. HOMA-IR, serum total testosterone and FAI levels also presented as major determinants for ovarian volume after adjustment for age and AMH levels. The findings of the present study suggest that not only the AMH level, but also obesity, IR and elevated total or bioavailable testosterone levels are related to the number of antral follicles and ovarian size in women with PCOS.

The biologic mechanisms whereby BMI exerts its effect on modulating the association between AMH and the antral follicle count are still under investigation. It is reasonable to speculate that IR may play a pivotal role. AMH is produced exclusively in the gonads from the granulosa cells and is involved in the regulation of follicular growth and

development (La Marca and Volpe, 2006). AMH is undetectable in women who have undergone bilateral oophorectomy or in those with menopause (La Marca *et al.*, 2005). AMH is expressed predominantly in the small antral follicles and is not expressed in atretic follicles or theca cells (Rey *et al.*, 2000; La Marca and Volpe, 2006). Therefore, AMH measurements may represent both a quantitative and qualitative marker of granulosa cell activity and the ovarian follicle pool (Feyereisen *et al.*, 2006; La Marca and Volpe, 2006). For the above reason, factors that may disturb granulosa cell function may also affect the production of AMH. Prospective studies have demonstrated that although antral follicle count does not change over time (4–7.3 years), AMH levels decline significantly (de Vet *et al.*, 2002; van Rooij *et al.*, 2005). That finding may be attributed to the aging of the granulosa cell. Not only age-related variables, but also obesity and IR, have been reported to have a negative effect on ovarian granulosa cell function (Franks *et al.*, 1996; Cortet-Rudelli *et al.*, 2002; Gracia *et al.*, 2005; Freeman *et al.*, 2007). The negative effect of obesity and IR on ovarian granulosa cell function has not only been reported in women during the menopausal transition or late reproductive stage (Gracia *et al.*, 2005; Freeman *et al.*, 2007), but has also been reported specifically for women with (Pigny *et al.*, 2000) and without PCOS (Cortet-Rudelli *et al.*, 2002), possibly explaining the negative association between BMI/HOMA-IR and AMH demonstrated in this study.

The inverse association between obesity and AMH levels has been reported in healthy women without PCOS (Pigny *et al.*, 2003) and in women of advanced reproductive age (Freeman *et al.*, 2007). However, such a relationship has not been reported for women with PCOS. Recently, AMH was reported to be one of the local inhibitors of FSH action (Josso *et al.*, 2001) in inhibiting granulosa cell proliferation, and is involved in the inhibition of the selection process of the dominant follicle (Durlinger *et al.*, 2002; Jonard and

Table IV. Forward stepwise multiple linear regression models of dependent variable FNPO and OVPO for women with PCOS.

ln(FNPO)				ln(OVPO)			
Variable	Estimate	F	P-value	Variable	Estimate	F	P-value
ln(AMH)	0.48188	82.17	<0.0001	ln(AMH)	0.36230	46.67	<0.0001
ln(FAI)	0.16578	25.34	<0.0001	ln(FAI)	0.10736	12.08	0.0008
Model R ² = 52.18%				Model R ² = 39.41%			

Covariates considered for selection in each model: age, AMH, BMI, HOMA-IR, testosterone and FAI.

Dewailly, 2004). Since the antral follicles from AMH knockout mice are more sensitive to FSH than those from the wild type, AMH may also exert its effect by decreasing granulosa cell sensitivity to FSH (Durlinger *et al.*, 2001). Due to the negative association between AMH and IR/BMI shown in this study, we speculate that IR might exert its negative effect on AMH directly or indirectly to down-regulate the inhibitory action of AMH on follicular development and therefore, to increase the sensitivity of granulosa cells to FSH. However, this hypothesis requires further biological investigation.

On the contrary, women with PCOS have been reported to have a positive correlation between AMH and the 2-h insulin level (Crisosto *et al.*, 2007). A plausible explanation may be that in our population, women with a higher BMI and HOMA-IR receive more influence from obesity itself than from the negative effect of AMH, and those women with a lower BMI and HOMA-IR experience more influence from the negative effect of AMH to suppress ovulation. Therefore, obese PCOS women with lower baseline AMH levels have been reported to have a better response, with respect to a restoration of normal menstrual status, after weight loss compared with BMI- and HOMA-IR-matched women with PCOS who have a higher AMH level (Moran *et al.*, 2007). Short-term metformin treatment was reported to result in improvements in hyperandrogenism, menstrual cyclicity, reduction in the number of antral follicles and IR (Bayrak *et al.*, 2007), while long-term metformin administration is required for a reduction in the AMH level (Fleming *et al.*, 2005; Piltonen *et al.*, 2005). Not only the negative effect of AMH, but also insulin and androgen may lead to follicle excess. Therefore, the reduction in the number of antral follicles associated with metformin treatment may outweigh the negative effect of insulin on the granulosa cell production of AMH, finally leading to a decrease in the AMH level. However, such a putative mechanism requires further investigation and validation.

Testosterone was found to promote follicular growth by augmenting the actions of FSH (Weil *et al.*, 1999) and insulin-like growth factor-1 (Vendola *et al.*, 1999). Furthermore, not only in an animal model (Vendola *et al.*, 1998), but also in humans (Pache and Fauser, 1993), exogenous androgen treatment has been reported to increase the number of non-ovulatory antral follicles similar to the situation observed in women with PCOS. In this study, the serum total testosterone and FAI levels were positively correlated with the FNPO and OVPO. This positive correlation still existed, even after adjustment for AMH and BMI/HOMA-IR. Compared with the number of antral follicles, ovarian volume seems to be under greater influence by hyperandrogenemia. Hyperinsulinemia or obesity might share the common pathway of hyperandrogenemia to affect the size of the antral follicle pool and ovarian volume (Jonard and Dewailly, 2004).

There are still several flaws in this study. Jonard *et al.* (2003) reported that the FNPO within the 6–9 mm range was significantly and negatively related to the BMI and the fasting insulin level. In this study, we did not differentiate between the 2–5 and 6–9 mm follicles, therefore, we can not ascertain if the association was related to different follicle size or not. It was also difficult for us to collect an adequate, non-biased

representative age-matched control group in order to show if the effect of BMI on AMH was specific for women with PCOS. However, previous studies have demonstrated this inverse relationship between BMI and AMH in women without PCOS (Freeman *et al.*, 2007; Pigny *et al.*, 2003). Finally, because the phenotype of clinical hyperandrogenism is not distinct in the Asian population (Carmina *et al.*, 1992) and the diagnosis is very subjective, we included only biochemical hyperandrogenemia in the diagnostic criteria of PCOS in our study; this could preclude the extrapolation of our findings to the entire PCOS spectrum and amplify the effect of hyperandrogenemia on FNPO and OVPO in this study.

In conclusion, our results are consistent with the hypothesis that not only elevated AMH, but also obesity, IR and elevated androgen levels may contribute to the large size of the antral follicle pool and to increase ovarian volume in women with PCOS. These findings suggest that weight loss, amelioration of IR and resolution of hyperandrogenemia may attenuate polycystic ovary morphology and modulate the role of AMH, contributing to improved ovulatory function in women with PCOS. However, further studies are still necessary to elucidate further aspects of the roles of IR and hyperandrogenemia in the production or activity of AMH and in the regulation of ovulatory function in PCOS.

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