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Association between BMI and gene expression of anti-Müllerian hormone and androgen receptor in human granulosa cells in women with and without polycystic ovary syndrome

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Running Title*:* BMI and anti-Müllerian hormone

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Summary

Background: Anti-Müllerian hormone (AMH) is one of the most reliable markers of ovarian reserve. There is evidence which suggests that BMI may be associated with gene expression of AMH, AMH type II receptor (AMHR-II) and androgen receptor (AR) in human granulosa cells (GC) in women with and without polycystic ovary syndrome (PCOS).

Objective: To investigate the association between BMI and gene expression of AMH, AMHR-II and AR in human GC in women with and without PCOS.

Design, Patients and Measurements: In a cross-sectional study, hormonal profiles were measured among 38 patients with PCOS and 38 subjects without PCOS aged 18-40 years old.

AMH, AMHR-II and AR mRNA levels were quantified in cumulus GC. Pearson correlation and multiple linear regressions were used to assess the relationships.

Results: Quantitative RT-PCR demonstrated that AMH and AMHR-II expression were negatively correlated with BMI (r=-0.39, $P<0.001$ for AMH and r=-0.49, $P<0.001$ for AMHR-II), whereas AR expression was positively correlated with BMI (r=0.46, $P<0.001$).

Conclusions: There is a negative association between AMH, AMHR-II expression and BMI, and a positive association between AR expression and BMI in the GC of PCOS and non-PCOS women.

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders and affects 7-10% of women in reproductive age [1]. Recent studies have shown that up to 60% of women with PCOS have central obesity or abdominal adiposity [2]. Obesity is associated with a higher *in vitro* fertilization (IVF) cycle cancellation rate, a lower mature oocyte yield, lower number of cryopreservation cycles [3] as well as ovarian intra-follicular alterations at multiple cellular levels including steroidogenic, metabolic and inflammatory pathways [4]. Moreover, obese women have impaired response to ovarian stimulation and significantly lower live birth rate after IVF [5].

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor beta superfamily that includes the granulosa cell (GC) and theca cell-derived inhibins and activins as well as the oocyte-derived growth differentiation factor 9 [6]. The effects of AMH on ovarian function are not fully understood. It is also unclear if AMH is a marker of primordial follicle or later stage follicle development, or both [7]. Nevertheless, serum concentrations are a useful marker of the ovarian follicle pool [6]. Previous studies have reported that AMH may play an important pathogenic role in the follicular status of subjects with PCOS [6, 8]. Some

studies have demonstrated that obese women have a lower ovarian reserve than normal weight women; this is reflected by their lower serum AMH levels (up to 77% lower), suggesting that overweight/obesity may impair GC hormone production [9-10]. In addition, obese women have elevated levels of serum and follicular fluid leptin, and decreased levels of serum and follicular fluid adiponectin [11], which in turn may affect AMH levels, AMH gene expression and that of its receptor AMHR-II. Additionally, whilst some studies have examined the association between AMH and BMI, the results are contradictory. A few studies have reported a significant inverse correlation between AMH levels and BMI [10, 12], whereas others found no relationship between the two [13-14].

In the process of decision making, clinicians need to know whether obesity affects AMH levels, gene expression of AMH, its receptor AMHR-II, and the androgen receptor (AR). To date, there is a lack of studies which have assessed the relationships between BMI and these parameters in women with and without PCOS. Therefore, the aim of the current study was to evaluate the association between BMI and gene expression of AMH, its receptor AMHR-II, and AR in GC in women with and without PCOS.

Subjects and Methods

Participants

This cross-sectional prospective study was conducted from October 2013 to October 2014 among women aged 18-40 years old with PCOS (*N*=38) referred to the Alzara Clinic, Tabriz University of Medical Sciences (TUOMS), Tabriz, Iran, and women without PCOS (control group; *N*=38). The diagnosis of PCOS was made according to the Rotterdam criteria [15]: those with two of the following criteria were considered as having PCOS: oligo- and/or anovulation (defined as delayed menses >35 days or <8 spontaneous menstrual cycles/year), clinical (hirsutism defined by a modified Ferriman-Gallwey score of≥8) and/or biochemical signs of

hyperandrogenism (total testosterone>88 ng/dL) and polycystic ovaries (12 or more follicles in each ovary measuring 2-9 mm in diameter, and/or increased ovarian volume>10 ml^3). The PCOS group included 38 patients (18 patients of normal weight and 20 patients who were overweight) who were referred for IVF. The control group included 38 patients, without PCOS (20 of normal weight and 18 who were overweight), who were referred for IVF due to tubal and/or male infertility, or volunteers who were ovulatory with normal ovaries. Exclusion criteria were as follows: Cushing's syndrome, thyroid dysfunction, androgen-secreting tumour, decreased ovarian reserve, amenorrhoea and type 1 or type 2 diabetes mellitus. The study was performed after obtaining informed consent from each participant. The study protocol was approved by the Ethics Committee of TUOMS. Normal weight was defined as a BMI between 18.5 and 24.9 kg/m^2, and overweight between 25.0 and 29.9 kg/m^2.

Ovarian stimulation

In the current study, we used a standard long protocol with a gonadotrophin-releasing hormone (GnRH) analogue for ovarian stimulation. On cycle day 21, Buserelin acetate (Suprefact, Aventis, Germany) was commenced as a 0.5 mg daily subcutaneous injection until menstruation began and adequate suppression was attained [serum oestradiol (E2) levels <50 pg/mL and no ovarian cystic structures on ultrasound examination] [16]. At day 3 of the next menstrual cycle, the dose of Buserelin acetate was reduced to 0.2 mg, and recombinant follicle stimulating hormone (rFSH) (Gonal F, Serono, Switzerland) was commenced [16]. The starting dose for the first 5 days varied between 150-225 IU daily by subcutaneous injection depending on the age (< or >35 years) and history of the patient [16]. Thereafter, transvaginal ultrasonography was performed every other day and the dose was adjusted on the basis of follicle graph using Gonal-F and HMG (Menopur, Ferring, USA) [16]. Ovulation was induced

with a 10000 IU intramuscular injection of human chorionic gonadotrophin (HCG) (Ovidrel, Serono, Switzerland) when at least 2 follicles 18-20 mm were seen and serum oestradiol levels were between 1000 and 3000 pg/mL [16]. Oocyte aspiration was conducted 35-38 h after HCG administration under vaginal ultrasonography.

Collection of GC

Follicle size was determined immediately at the time of retrieval under ultrasound, and samples of follicular fluid from large follicles [LFs (≥12 mm)] were collected in separate tubes. LFs were evaluated separately to pinpoint differences between immature and mature follicles, respectively. After removal of the oocyte, fluid from each LF was pooled from the same woman, and cells were concentrated by centrifugation at 300 g and 600 g for 5 min each. The superficial layer of the pellet was collected and layered on 40% percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) then centrifuged at 500 g for 15 min to remove red and white blood cells. GC were collected from the layer interface and subjected to 5 mg/mL hyaluronidase for 2 min at room temperature to disperse cells. Cells were then washed with phosphate-buffered saline (PBS). After identification of the cumulus-oocyte complex in the aspirate, cumulus GC were mechanically collected by cutting the cumulus cell layer from each oocyte and washed with PBS. For each participant, mural and cumulus GC from LFs were pooled in order to extract sufficient RNA for quantification and RT-PCR analyses.

RNA extraction and cDNA synthesis

For each participant and for LFs, the RNA was isolated from the collected GC using Trizol reagent (RNX-plus, Tehran, Iran). The cells were then mixed with chloroform, followed by centrifugation at +4°C and 12000 *g* for 15 min. The aqueous phase containing RNA was separated. Total RNA was precipitated with isopropanol by centrifugation at +4°C and 12000

g for 15 min, washed with 75% ethanol followed by centrifugation at +4°C and 7500 *g* for 5 min, air-dried and reconstituted in diethylpyrocarbonate (DEPC)-treated water. The RNA was cleaned using the RNeasy mini kit (Qiagen, Valencia, CA, USA), and reverse transcription was conducted using Superscript III reverse transcriptase (Bioneer, Daejeon, Korea). RNA quality analysis was carried out using the Nanodrop Spectrophotometer. The cDNA was then stored at -80°C until further analysis. Gene-specific primers used for PCR were chosen according to published sequences of human AMH (Genbank accession no. NM_000479), AMHR-II (Genbank accession no. NM_001164690) and AR (Genbank accession no. NM_000044). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as a control (forward: 5′-AAGCTCATTTCCTGGTATGACAACG-3′; reverse: 5′-TCTTCCTCTTGTGCTCTTGCTGG-3′). The primer sequences of AMH, AMHR-II, AR and GAPDH are provided in Table 1.

Assessment of anthropometric measures

Body weight was measured in a fasting state by a trained midwife in the gynaecology clinic, using a digital balance (Seca, Hamburg, Germany) and after bladder emptying. Height was quantified with the use of a wall-mounted stadiometer (Seca, Hamburg, Germany). BMI was determined as weight in kg divided by height in metres squared.

Biochemical assessment

Fasting blood samples (10 mL) were collected at Tabriz reference laboratory in the early morning after an overnight fast. Each participant was examined within 3 and 5 days of her menstrual period. To separate serum, blood samples were immediately centrifuged (Hettich D-78532, Tuttlingen, Germany) at 3500 rpm for 10 min. The samples were then stored at -80$^{\circ C}$ before analysis at the TUOMS reference laboratory. ELISA kits (Siemens, Erlangen,

Germany) were used to quantify serum follicle-stimulating hormone (FSH) and luteinising hormone (LH), oestradiol and progesterone. Serum AMH levels were also assessed using ELISA (Beckman Coulter Immunotech, Villepinte, France).

Statistical analysis

To ensure the normal distribution of variables, we applied the Kolmogorov-Smirnov test. All variables had a normal distribution. To detect differences in serum levels of hormones between the two groups of PCOS (normal weight and obese) or non-PCOS (normal weight and obese) patients, we used the independent sample Student's t test. We performed Pearson correlations and multivariate linear regression analysis to assess the relationship between variables. The linear regression analyses were conducted in crude and adjusted models which were controlled for mutual effects of other biochemical variables as covariates. *P* values <0.05 were considered statistically significant. SPSS version 17 (SPSS Inc., Chicago, Illinois, USA) was used for data analysis.

Results

Quantitative RT-PCR demonstrated that AMH and AMHR-II expression were negatively correlated with BMI (r=-0.39, *P*<0.001 for AMH and r=-0.49, *P*<0.001 for AMHR-II), and AR expression was positively correlated with BMI (r=0.46, *P*<0.001) (Table 2). Comparison of serum LH, LH/FSH ratio, oestradiol and AMH levels showed a significant difference between normal weight and overweight women with PCOS (*P*<0.001 for LH, LH/FSH ratio, oestradiol and *P*=0.003 for AMH) (Table 3). In addition, a significant difference in serum AMH levels between normal weight and overweight controls was observed (*P*<0.001).

Our study indicated that FSH (r=-0.48, *P*=0.003), LH (r=-0.81, *P*<0.001), LH/FSH ratio (r=-0.53, *P*=0.001) and oestradiol (r=0.68, *P*<0.001) were significantly associated with BMI among PCOS women (Table 4). Furthermore, we found that FSH (r=-0.36, *P*=0.02) and oestradiol (r=0.36, *P*=0.02) were significantly associated with BMI in controls.

Simple linear regression analysis demonstrated that among the biochemical measures in PCOS women, serum FSH, LH and FSH/LH ratio were negatively correlated with BMI (β=-1.50, *P*=0.002 for FSH, β=-1.29, *P*<0.001 for LH and β=-6.03, *P*=0.001 for LH/FSH ratio) and serum oestradiol levels were positively correlated with BMI (β=0.23, *P*<0.001) (Table 5). These relationships remained significant even after controlling for other biochemical indicators (β=-1.72, *P*=0.01 for LH and β=0.14, *P*<0.001 for oestradiol) but the association of FSH and LH/FSH ratio disappeared. Multiple linear regression analysis revealed that there was no association between progesterone with BMI among women with PCOS. In controls, serum FSH levels were negatively correlated with BMI (β=-0.94, *P*=0.02) whereas serum oestradiol levels were positively correlated with BMI (β=0.14, *P*=0.02). These relationships remained significant even after controlling for other biochemical measures (β=-8.11, *P*=0.004 for FSH and β=0.14, *P*=0.01 for oestradiol). Multiple linear regression analysis revealed that there was no association between LH/FSH ratio and progesterone with BMI among controls.

Discussion

The results of this cross-sectional study demonstrated that AMH and AMHR-II expression in both PCOS and non-PCOS women was negatively correlated with BMI, and AR expression was positively correlated with BMI. It must be kept in mind that in the current study, we used a standard long protocol with GnRH analogue for ovarian stimulation. Although we recognise that the agonist protocol is accompanied by a series of complications including

hypo-oestrogenaemia, requirement for a prolonged period of down-regulation and an increase in FSH and LH in primary administration compared with natural cycle follicles, this method is well accepted in clinical practice [17].

Overweight is associated with several symptoms of PCOS including hirsutism, a clinical marker of elevated androgens [18], higher rates of oligo- and amenorrhoea, infertility and miscarriage compared with lean women [19]. We found that AMH and AMHR-II expression both PCOS and non-PCOS women was negatively correlated with BMI. The observed inverse association between AMH, AMHR-II expression and BMI suggests that obesity-related hormonal dysfunction of adipose tissue is a factor leading to the reduction in ovarian reserve in both obese PCOS and non-PCOS women. Obesity is associated with poor outcomes in IVF cycles [20]. The mechanisms by which obesity may influence ovarian function are not clear. Furthermore, obesity may decrease AMH levels via increased catabolism of AMH, reduced ovarian potential, and ovarian dysfunction [21]. It is well known that obese women of late reproductive age have more anovulatory and longer cycles than non-obese women [22]. One of the potential mechanisms is through the interaction of adiponectin with granulosa cells [9]. Adiponectin is secreted from white adipose tissue and its serum concentrations are decreased in obese women [23]. It is present in the follicular fluid of porcine ovaries and its receptors are present on granulosa cells. In addition, it induces vascular endothelial growth factor synthesis and modifies steroid synthetic enzyme pathways by increasing steroidogenic acute regulatory protein mRNA and decreasing aromatase expression [24]. Lower LH levels in obese women with PCOS are a result of increased aromatisation of androgens to oestrogens in peripheral fat tissue, which in turn may result in LH suppression [25], and decreased AMH and AMHR-II expression. In a study by Panidis et al. [26] AMH and LH levels were positively correlated. Elevated leptin levels in obese PCOS

women may also influence AMH and AMHR-II expression. Merhi et al.[27] demonstrated that leptin suppressed AMH mRNA expression via the JAK2/STAT3 pathway, as treatment with a JAK2/STAT3 inhibitor prevented leptin-induced changes in AMH mRNA in all compartments but not AMHR-II mRNA expression in cumulus GC. Therefore, weight loss, and hence reduced levels of leptin, may improve AMH production.

We demonstrated that AR expression in both PCOS and non-PCOS women was positively correlated with BMI. In agreement with our study, Zhang et al.[28] demonstrated that higher BMI was associated with an increased risk of AR positive breast tumours in postmenopausal women. In addition, a few studies have indicated that the free androgen index in women with PCOS is positively correlated with body weight and BMI [29-30]. Androgens, via the AR, play a direct role in the normal growth of ovarian follicles, follicle health, development and ovulation [31]. High serum androgen concentrations are associated with hirsutism, inhibition of follicular development, anovulation and irregular menstruation [32]. However, there are some PCOS patients whose serum testosterone concentration is normal, suggesting possible differences in AR gene activities. Because of these inter-relationships, it has been suggested that the AR might be one of the genetic predictors of an individual's susceptibility to PCOS [33].

In conclusion, our study demonstrated that there was a negative association between AMH, AMHR-II expression and BMI, and a positive association between AR expression and BMI in the GC of PCOS and non-PCOS women.

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Table 1: Specific primers used for real-time quantitative PCR

Gene	Primer	Product size (bp)	Annealing temperature (°C)
GAPDH	F: AAGCTCATTTCCTGGTATGACAACG R: TCTTCCTCTTGTGCTCTTGCTGG	126	61.3
AMH	F: TTCCGAGAAGACTTGGACTGG	87	54

	R: GCTGCTGCCATTGCTGTC		
AMHR-II	F: TGTGTTTCTCCCAGGTAATCCG	164	62.1
	R: AATGTGGTCGTGCTGTAGGC		
AR	F: CAACTCCTTCAGCAACAG	158	61
	R: GACACTGCCTTACACAAC		

AMH, anti-Müllerian hormone; AMHR-II, AMH type II receptor; AR, androgen receptor; GAPDH, glyceraldehyde-3-Phosphate dehydrogenase.

Table 2: Pearson correlation coefficients between BMI and gene expression of anti-Müllerian hormone, AMHR-II and the androgen receptor in PCOS and non-PCOS subjects

Variables	All (*N*=76)		PCOS (*N*=38)		Non-PCOS (*N*=38)	
	BMI	$P^{\dagger}$	BMI	$P^{\dagger}$	BMI	$P^{\dagger}$
AMH	-0.39	<0.001	-0.50	0.001	-0.59	<0.001
AMHR-II	-0.49	<0.001	-0.54	<0.001	-0.60	<0.001
AR	0.46	<0.001	0.50	0.001	0.72	<0.001

AMH, anti-Müllerian hormone; AMHR-II, AMH type II receptor; AR, androgen receptor.

† Obtained from Pearson's correlation analysis.

Table 3: Serum hormone concentrations in PCOS and non-PCOS subjects

	PCOS				Non-PCOS			
	All (*N*=38)	Normal weight (*N*=18)	Overweight (*N*=20)	$P^{\dagger}$	All (*N*=38)	Normal weight (*N*=20)	Overweight (*N*=18)	$P^{\dagger}$
FSH (IU/L)	6.0±1.0	6.2±0.9	5.8±1.1	0.29	6.6±1.2	6.5±1.3	6.6±1.0	0.77
LH (IU/L)	8.7±2.0	10.2±1.4	7.3±1.4	<0.001	6.7±1.2	6.5±1.2	6.8±1.1	0.52
LH/FSH ratio	1.5±0.3	1.7±0.2	1.3±0.2	<0.001	1.0±0.1	1.0±0.1	1.0±0.1	0.96
Oestradiol (pg/mL)	59.3±8.7	53.6±8.3	64.5±5.2	<0.001	89.9±7.7	81.6±7.9	84.3±7.4	0.28
Progesterone (ng/mL)	1.2±0.2	1.2±0.2	1.1± 0.2	0.39	0.9±0.2	0.9±0.1	0.9±0.2	0.50
AMH (ng/mL)	4.3±2.3	5.5±2.5	3.3±1.5	0.003	2.1±1.3	2.7±1.3	1.3±1.0	<0.001

All values are means± SDs.

† P values derived from the independent t test.

AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; LH, luteinising hormone; PCOS, polycystic ovary syndrome.

Table 4: Pearson correlation coefficients between hormonal measures and BMI in PCOS and non-PCOS subjects.

Variables	PCOS		Non-PCOS	
	BMI	$P^{\dagger}$	BMI	$P^{\dagger}$
FSH (IU/L)	-0.48	0.003	-0.36	0.02
LH (IU/L)	-0.81	<0.001	-0.28	0.08
LH/FSH ratio	-0.53	0.001	0.11	0.48
Oestradiol (pg/mL)	0.68	<0.001	0.36	0.02
Progesterone (ng/mL)	-0.16	0.32	0.01	0.91

† Obtained from Pearson's correlation analysis.

FSH, follicle-stimulating hormone; LH, luteinising hormone; PCOS, polycystic ovary syndrome.

Table 5: Multiple regression analysis between BMI and hormonal measures in PCOS and non-PCOS subjects

Variables	PCOS			Non-PCOS		
	β	95% CI	$P^{\dagger}$	β	95% CI	$P^{\dagger}$
FSH (IU/L)						
Crude	-1.50	-2.40,-0.59	0.002	-0.94	-1.76, -0.12	0.02
Adjusted††	0.83	-1.13, 2.81	0.39	-8.11	-13.48, -2.74	0.004
LH (IU/L)						
Crude	-1.29	-1.60, -0.97	<0.001	-0.74	-1.61, 0.11	0.08
Adjusted	-1.72	-3.14, -0.30	0.01	7.61	2.08, 13.13	0.008

LH/FSH						
Crude	-6.03	-9.27, -2.78	0.001	4.95	-9.28, 19.19	0.48
Adjusted	4.55	-4.19,13.30	0.29	-22.33	-45.41, 0.73	0.05
Oestradiol (pg/mL)						
Crude	0.23	0.14, 0.33	<0.001	0.14	0.01, 0.27	0.02
Adjusted	0.14	0.08, 0.20	<0.001	0.14	0.02, 0.26	0.01
Progesterone (ng/mL)						
Crude	-2.50	-7.26, 2.26	0.29	0.28	-5.15, 5.72	0.91
Adjusted	-1.50	-3.82, 0.80	0.19	-0.28	-4.98, 4.41	0.90

† Obtained from multiple regression analysis.

BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinising hormone; PCOS, polycystic ovary syndrome.

†† After controlled mutual effect of other biochemical indicators as covariates.