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Research paper

Elevated serum androstenedione is associated with a more severe phenotype in women with polycystic ovary syndrome (PCOS)

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ABSTRACT

OBJECTIVE: To evaluate the impact of elevated serum Δ_4A levels on the hormonal and metabolic features of the different phenotypes of PCOS. DESIGN: 1276 women with PCOS according to the Rotterdam criteria were included, in whom serum hormonal levels were determined. RESULTS: In PCOS women as a whole, as well as in patients presenting clinical and/or biochemical hyperandrogenemia (phenotypes I and II), Δ_4A levels >3.8 ng/ml were positively related to LH, LH/FSH ratio, T, DHEAS, 17 OH progesterone and FAI and negatively related to T/Δ_4A ratio. In the milder phenotype III, a positive correlation between Δ_4A levels >3.8 ng/ml and T, DHEAS, 17 OH progesterone and FAI and a negative one between increased Δ_4A and T/Δ_4A ratio were reported. In the whole PCOS group with androstenedione >3.8 ng/ml, an increased ovarian volume was observed, while a greater mean follicular number was found only in phenotypes I and II. CONCLUSIONS: Increased serum Δ_4A levels, which are associated with more severe PCOS phenotypes, possibly contribute to the worsening of PCOS features and therefore could be a valuable marker of biochemical hyperandrogenemia.

Key words: Adrenals, Androstenedione, Androstenedione/Testosterone ratio, Insulin resistance, PCOS

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy of reproductive-aged women.[1] According to the Rotterdam Conference sponsored by ESHRE/ASRM in 2003, the diagnosis of PCOS presupposes the presence of at least two out of the three following features: anovulation (ANOV), clinical and/or biochemical hyperandrogenism (HA) and polycystic ovaries on ultrasonography (PCO),[2] with other androgen excess disorders excluded. Subsequently, additional milder phenotypes of the syndrome

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have been included in PCOS;[3,4] still, phenotypes with biochemical hyperandrogenemia and chronic anovulation appear to be the most severe in terms of androgen levels, insulin resistance and obesity.[4] Biochemical hyperandrogenemia is based on the increase of serum testosterone (T) levels and/or serum free androgen index at levels higher than two standard deviations above the mean levels of a normal control population.[5] Δ_4Androstenedione (Δ_4A), a weaker androgen than T, is of both ovarian and adrenal origin. Although elevated Δ_4A levels are a frequent finding among women with PCOS, they are not included in the mainstream diagnostic criteria of biochemical hyperandrogenemia.

Heterogeneity in the responsiveness to weight loss among women with PCOS underlines the possible role of Δ_4Androstenedione as a clinical marker for the severity of the syndrome, based on the observation that PCOS women with higher baseline Δ_4Androstenedione levels tended to sustain PCOS phenotype after weight loss compared to those with lower Δ_4Androstenedione levels, who have been shown to recover.[6] An old theory suggests that the precursor of estrone (E1), that sensitizes GnRH (leading to exaggerated luteinizing hormone (LH) levels), is Δ_4Androstenedione,[7] this implying its non-negligible role in the pathogenesis of PCOS.

The aim of the present study was to evaluate the impact of elevated serum Δ_4A levels on the hormonal and metabolic features of the different phenotypes of PCOS.

MATERIALS AND METHODS

Materials

The study included 1276 Caucasian women with PCOS. The diagnosis of PCOS was based on the Rotterdam criteria.[2] Biochemical hyperandrogenemia was defined as serum androgens levels higher than two standard deviations above the mean levels of a normal control population (T levels >60 ng/ml and/or serum free androgen index >5 and/or Δ_4A levels >3.8 ng/ml). Clinical hyperandrogenism was defined as the presence of hirsutism and/or acne and/or androgenic alopecia. Hirsutism was assessed by the Ferriman-Gallwey scale[8] (patients with scores above or equal to 8 were considered as hirsute). Chronic anovulation was defined as a menstrual cycle of less than 21 or more than 35 days, with progesterone levels <3 ng/ml on days 18-21 of the cycle. Ovulation was defined as a menstrual cycle of 28±2 days and/or blood progesterone levels >8 ng/mL in two consecutive cycles.

PCOS women were further divided into four groups, corresponding to four different phenotypes. Phenotype I (n=645) included PCOS women with biochemical hyperandrogenemia and/or clinical hyperandrogenism, chronic anovulation and polycystic ovaries on PCO. Phenotype II (n=401) included PCOS women with biochemical hyperandrogenemia and/or clinical hyperandrogenemia and chronic anovulation. Phenotype III (n=130) included PCOS women with biochemical hyperandrogenemia and/or clinical hyperandrogenism and polycystic ovaries on PCO. Phenotype IV (n=100) included PCOS women with chronic anovulation and polycystic ovaries on ultrasound.

PCOS women as a whole and each one of the four different phenotypes separately were divided into those with serum Δ_4A levels >3.8 ng/ml and those with serum Δ_4A levels <3.8 ng/ml. Phenotype IV is not presented since there were no women in this subgroup with Δ_4A levels >3.8 ng/ml.

Exclusion criteria were congenital adrenal hyperplasia, androgen secreting tumors and Cushing's syndrome. All subjects had normal thyroid, kidney and liver function, no excessive alcohol intake and no medication that could interfere with normal function of the hypothalamic-pituitary-gonadal axis during the last semester. All subjects gave written informed consent and the study was performed according to the guidelines of the Institutional Review Board; the study met the requirements of the 1975 Helsinki guidelines.

Methods

Weight, height as well as waist and hip circumferences were measured in all women. Body weight was measured using analogue scales and in light clothing; height was measured barefoot using a stadiometre. Body mass index (BMI, kg/m^2) was calculated by dividing weight by height squared (kg per square

metre) to assess obesity. Waist circumference (W) was obtained as the smallest circumference at the level of the umbilicus and waist to hip ratio (WHR) was calculated dividing weight by hip values.

Baseline blood samples were collected between days 3 and 7 of the menstrual cycle in the control group and after a spontaneous bleeding episode in the PCOS group, following an overnight fast. On the same day, transvaginal ultrasound examination was performed.

Circulating levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), Δ_4-Androstenedione (Δ_4A), dehydroepiandrosterone-sulfate (DHEA-S), 17-OH progesterone (17-OH-P), prolactin (PRL), sex-hormone-binding globulin (SHBG), glucose (Glu) and insulin (Ins) were measured. Free Androgen Index (FAI) was calculated as T (nmol/L)X100/SHBG (nmol/L).[9] The QUICKI index was calculated as the product of the equation 1 / log (fasting insulin) + log (glucose),[10] while the HOMA2IR index calculator was downloaded from www.OCDEM.ox.ac.uk.

All assays of hormonal levels and plasma glucose determination were carried out at the Department of Biochemistry of the Aristotle University of Thessaloniki School of Medicine.

Plasma glucose concentrations were measured using a glucose oxidase technique with an autoanalyser (Roche/Hitachi 902; Roche Diagnostics GmbH, Mannheim, Germany). LH and FSH levels were measured with an enzyme-linked immunoassay (EIA) using commercial kits (Nichols Institute Diagnostics, CA, USA). Testosterone was measured with a Direct RIA kit (Sorin, Biomedica); Δ4-Androstenedione with a Gamma Coat [125I] RIA kit (Incstar Corp.); DHEA-S with direct RIA solid-phase coated tubes (Zer Science Based Industries Ltd); 17-OH progesterone with an ImmuChem Double Antibody [125I] RIA kit (ICN Pharmaceuticals, Inc.); insulin with a Coat-A-count Insulin kit (Diagnostic Products Corp.); and SHBG with an immunoradiometric assay (IRMA) kit (SHBG: [125I] IRMA Kit, Orion Diagnostica). The intra-assay coefficients of variation (CV) were 1.5% for FSH, 0.7% for LH, 2.7% for prolactin, 3.8% for insulin, 4.1% for 17-OH progesterone, 1.3% for testosterone, 5.9% for androstenedione, 9.4% for DHEA-S and 5.8% for SHBG. The average inter-assay CV were 3.2% for FSH, 1.7% for LH, 4.4% for insulin, 6.3% for 17-OH progesterone, 2.2% for testosterone, 9.2% for Δ_4A, 12.1% for DHEA-S, and 7.8% for SHBG.

Statistics

The data were presented homogeneously as mean ±S.D, regardless of their distribution (normal or non-normal).

All variables (anthropometric, clinical and hormonal) were tested for normal distribution using the Kolmogorov-Smirnov test. For normally distributed variables the independent t-test was used to compare means, while for non-normally distributed variables the non-parametric Mann-Whitney test was used to compare the medians. Comparisons of hormonal levels in the total PCOS population and phenotype I women were adjusted to the effect of anthropometric characteristics (age, BMI and WHR and BMI, respectively), using the multivariate general linear model. The values of variables that were non-normally distributed were naturally log-transformed in order to perform the univariate general linear model. Bonferroni correction for multiple comparisons was applied.

The Spearman's moment correlation coefficient was used to assess all studied correlations. Stepwise linear regression analysis was used to ascertain the independent predictive value and impact of each parameter proved to significantly correlate with Δ_4A according to Spearman's moment correlation coefficients.

Values were considered to be statistically significant at $p < 0.05$. Statistical analysis was done using PASW 19 for windows (IBM SPSS Statistics, IBM software).

RESULTS

In the total group of 1276 PCOS women defined according to the previously described criteria, phenotype I accounted for 645 (50.54%) of the total PCOS women, phenotype II for 401 (31.42%), phenotype III for 130 (10.18%) and phenotype IV for 100 (7.83%). In the total group of PCOS women, 1073 (84%) had Δ_4A levels <3.8 ng/ml and 203 (16%) had Δ_4A levels >3.8 ng/ml. Specifically, among PCOS women of

phenotype I, 493 (76%) had Δ_4A levels <3.8 ng/ml and 152 (24%) had Δ_4A levels >3.8 ng/ml. Among 401 PCOS women of phenotype II, 366 (91%) had Δ_4A levels <3.8 ng/ml and 35 (9%) had Δ_4A levels >3.8 ng/ml. Among PCOS women of phenotype III, 114 (86%) had Δ_4A levels <3.8 ng/ml and 16 (14%) had A levels >3.8 ng/ml. Androstenodione levels >3.8 ng/ml as the sole criterion of biochemical hyperandrogenemia were found at non-negligible rates: in 12 women of the total PCOS group (0.9%), in 6 women of phenotype I (0.9%), in 3 of phenotype II (0.7%) and in 3 of phenotype III (2%).

The anthropometric and hormonal features of all PCOS women are summarized in Table 1. The anthropometric and hormonal features of phenotypes I, II and III are presented in Tables 2-4. Tables 1-4 summarize the results of the comparison between women with Δ_4A levels <3.8 ng/ml and Δ_4A levels >3.8 ng/ml. Phenotype IV is not presented since there were no women in this subgroup with Δ4A levels >3.8 ng/ml.

In the whole PCOS population the comparisons have been adjusted to the effect of age, BMI and WHR, while in phenotype I women the comparisons have been adjusted to the effect of BMI. On the other hand, the comparisons regarding phenotypes II and III involve the groups not differing in age, BMI or WHR.

Hormonal features

In the total PCOS group, women with Δ_4A levels >3.8 ng/ml had higher levels of LH, LH/FSH ratio, T, DHEAS, 17-OH progesterone, FAI and prolactin and lower levels of SHBG and T/Δ_4A ratio compared to PCOS women with Δ_4A levels <3.8 ng/ml. On the other hand, no differences were observed regarding FSH levels (Table 1).

In phenotype I, women with Δ_4A levels >3.8 ng/ml had higher levels of LH, LH/FSH ratio, T, DHEAS, 17-OH progesterone and FAI and lower values of T/Δ_4A ratio compared to PCOS women with Δ_4A levels <3.8 ng/ml. Conversely, no differences were noted regarding FSH, prolactin and SHBG levels (Table 2).

In phenotype II, women with Δ_4A levels >3.8 ng/ml had higher levels of LH, LH/FSH ratio, T, DHEAS, 17-OH progesterone and FAI and lower levels of T/Δ_4A ratio compared to PCOS women with Δ_4A levels

Table 1. Anthropometric and hormonal features of TOTAL PCOS women regarding the levels of Androstenedione. The PCOS subpopulations significantly differed in age, BMI and WHR, thus the comparison was performed via the multivariate general linear model, adjusting for the effect of these factors

	PCOS (n=1276)	**PCOS Δ4>3.8 (n=203)**	**PCOS Δ4≤3.8 (n=1073)**	**p value**
Age	24.25±5.79	23.28±4.45	24.43±5.99	0.002
BMI (Kgr/m^2)	26.80±7.03	25.47±5.83	27.05±7.21	0.001
WHR	0.79±0.26	0.81±0.22	0.77±0.23	0.000
FSH (mIU/ml)	5.89±1.80	5.88±1.68	5.87±1.82	0.942
LH (mIU/ml)	7.78±5.53	10.88±6.48	7.20±5.13	0.000
R	1.40±1.23	1.95±1.25	1.23±1.20	0.000
PRL (ng/ml)	14.14±7.28	15.93±8.53	13.81±6.97	0.004
Testo (ng/dl)	74.77±30.81	98.08±33.98	70.36±28.09	0.000
Δ4 Andro (ng/ml)	2.80±1.12	4.77±0.92	2.43±0.68	0.000
Testo/Δ4	28.46±11.69	20.94±7.46	29.88±11.81	0.000
DHEAS (μg/dl)	2938±1218	3645±1348	2804±1220	0.000
17OH (ng/ml)	1.13±0.55	1.59±0.63	1.05±0.49	0.000
SHBG (nmol/l))	41.78±25.51	37.16±21.24	42.66±26.16	0.008
FAI	8.70±7.15	12.38±9.34	7.99±6.44	0.000
Insulin (μIU/ml)	12.78±12.04	12.31±8.96	12.86±12.54	0.498
Glucose (mg/dl)	97.08±14.13	96.34±12.14	97.22±14.47	0.827
HOMA2-IR	1.58±1.29	1.60±1.13	1.58±1.32	0.380
Quicki	0.34±0.03	0.34±0.03	0.34±0.03	0.086
Glucose/ Insulin	11.58±7.76	10.99±6.09	11.68±8.03	0.072
Mean Follicular Number	11.06±4.83	12.68±5.15	10.76±4.71	0.012
Mean Ovarian Volume (cm^3)	7.96±3.58	8.62±3.49	7.83±3.58	0.298

Table 2. Anthropometric and hormonal features of PCOS women of Phenotype I regarding the levels of Androstenedione. The PCOS sub-groups significantly differed in BMI, thus the comparison was performed via the multivariate general linear model, adjusting for the effect of BMI

	Phenotype I			
	PCOS (n=645)	**Δ4>3.8 (n=152)**	**Δ4≤3.8 (n=493)**	**p value**
Age	23.48±5.27	23.04±4.19	23.62±5.56	0.234
BMI (Kgr/m^2)	26.93±7.13	25.47±5.94	27.40±7.40	0.001
Waist to Hip ratio	0.79±0.26	0.80±0.22	0.79±0.23	0.203
FSH (mIU/ml)	5.70±1.70	5.74±1.71	5.69±1.69	1.000
LH (mIU/ml)	8.801±6.02	11.36±6.59	7.98±5.55	0.000
R	1.65±1.53	2.06±1.23	1.52±1.58	0.002
PRL (ng/ml)	14.28±7.45	15.79±8.57	13.82±7.03	0.070
Testo (ng/dl)	81.13±30.83	100.24±33.95	75.22±27.30	0.000
Δ4 Andro (ng/ml)	3.07±1.18	4.68±0.81	2.56±0.66	0.000
Testo/Δ4	28.37±11.38	21.769±7.66	30.42±11.55	0.000
DHEAS (μg/dl)	3027±1267	3573±1348	2861±1194	0.000
17OH (ng/ml)	1.20±0.57	1.60±0.66	1.08±0.0.47	0.000
SHBG (nmol/l))	38.48±23.17	36.80±21.06	28.98±23.80	0.104
FAI	9.94±7.72	12.93±9.85	9.02±6.70	0.000
Insulin (μIU/ml)	13.84±14.05	12.61±9.38	14.22±15.20	0.896
Glucose (mg/dl)	97.51±16.21	96.70±12.19	97.74±17.27	0.782
HOMA2-IR	1.76±1.46	1.64±1.18	1.80±1.54	0.877
Quicki	0.34±0.03	0.34±0.03	0.33±0.04	0.227
Glucose/ Insulin	11.04±7.39	11.02±6.29	11.05±7.71	0.190
Mean Follicular Number	13.29±4.66	14.06±4.95	13.04±4.53	0.096
Mean Ovarian Volume (cm^3)	9.13±3.59	9.31±3.15	9.07±3.71	0.626

Table 3. Anthropometric and hormonal features of PCOS women of Phenotype II regarding the levels of Androstenedione. Mann-Whitney test was used to assess the differences in the hormonal and metabolic profile of PCOS sub-groups

	Phenotype II			
	PCOS (n=401)	**Δ4>3.8 (n=35)**	**Δ4≤3.8 (n=366)**	**p value**
Age	25.55±6.19	23.10±4.55	24.68±6.31	0.177
BMI (Kgr/m^2)	27.18±7.39	25.61±5.54	27.33±7.53	0.332
Waist to Hip ratio	0.78±0.07	0.78±0.06	0.78±0.07	0.944
FSH (mIU/ml)	6.06±1.89	5.95±1.43	6.08±1.93	0.711†
LH (mIU/ml)	6.98±5.09	8.95±5.09	6.79±5.05	0.004
R	1.20±0.82	1.69±1.40	1.15±0.73	0.012
PRL (ng/ml)	13.76±7.04	15.58±8.70	13.59±6.85	0.298
Testo (ng/dl)	74.06±28.57	89.49±31.57	72.58±27.57	0.000†
Δ4 Andro (ng/ml)	2.59±0.98	4.79±0.83	2.38±0.70	0.000
Testo/Δ4	30.58±12.22	18.75±6.15	31.71±12.06	0.000
DHEAS (μg/dl)	2995±1265	3663±1146	2931±1258	0.004†
17OH (ng/ml)	1.06±0.52	1.49±0.51	1.02±0.50	0.000
SHBG (nmol/l))	41.05±25.90	36.19±18.00	41.52±26.51	0.602
FAI	8.66±6.93	11.11±8.37	8.42±6.74	0.028
Insulin (μIU/ml)	12.45±10.26	11.89±7.15	12.51±10.52	1.000
Glucose (mg/dl)	96.06±11.86	94.54±11.23	96.20±11.93	0.310†
HOMA2-IR	1.61±1.23	1.54±0.93	1.61±1.26	0.800
Quicki	0.34±0.03	0.34±0.03	0.34±0.03	0.764†
Glucose/ Insulin	11.25±6.77	9.99±4.34	11.37±6.95	0.522
Mean Follicular Number	6.96±2.06	7.06±1.73	6.95±2.10	0.699†
Mean Ovarian Volume (cm^3)	5.55±1.91	5.32±1.46	5.58±1.95	0.488

† The independent t-test was used.

<3.8 ng/ml. No differences in FSH, prolactin and SHBG levels were observed (Table 3).

In phenotype III, women with Δ_4A levels >3.8 ng/ml had higher levels of T, DHEAS, 17-OH progesterone and FAI and lower T/Δ_4A ratio compared to PCOS women with Δ_4A levels <3.8 ng/ml. LH, SHBG, PRL levels and LH/FSH ratio did not differ between the subgroups (Table 4).

Insulin resistance

In the total PCOS group and in each phenotype separately, no differences were documented regarding insulin resistance indices (fasting glucose, fasting insulin, glucose/insulin ratio, HOMA-IR and QUICKI) between women with Δ_4A levels >3.8 ng/ml and PCOS women with Δ_4A levels <3.8 ng/ml (Tables 1-4).

Ultrasonography of the ovary

In the total PCOS group and in each phenotype separately, no differences were documented regarding mean ovarian volume between women with Δ_4A levels >3.8 ng/ml and PCOS women with Δ_4A levels <3.8 ng/ml (Tables 1-4).

However, in the total PCOS group and in phenotypes I and II, women with Δ_4A levels >3.8 ng/ml had greater mean follicular number compared to PCOS women with Δ_4A levels <3.8 ng/ml. No such differences were documented in phenotype III (Tables 1-4).

Correlations and multiple regression analysis

The results of Spearman's moment correlation coefficients between Δ_4A and anthropometric, hormonal and ultrasonographic features are the following: age, BMI and SHBG levels were significantly inversely correlated with Δ_4A levels (Age: ϱ=-0.117, p<0.001. BMI: ϱ=-0.107, p=0.01. SHBG: ϱ=-0.141, p<0.001). On the other hand, T, DHEAS and 17-OH progesterone levels were strongly positively correlated, while LH, PRL, mean follicular number and mean ovarian volume exerted weaker but significant positive correlation with Δ_4A levels. (T: ϱ=0.549, p<0.001. DHEAS: ϱ=0.403, p<0.001. 17-OH progesterone: ϱ=0.507, p<0.001. LH: ϱ=0.342, p<0.001. PRL: ϱ=0.142, p<0.001. Mean follicular number: ϱ=0.187, p<0.001. Mean ovarian volume: ϱ=0.128, p=0.001).

Table 4. Anthropometric and hormonal features of PCOS women of Phenotype III regarding the levels of Androstenedione. Mann-Whitney test was used to assess the differences in the hormonal and metabolic profile of PCOS sub-groups

	Phenotype III			
	PCOS (n=130)	Δ4>3.8 (n=16)	Δ4≤3.8 (n=114)	p value
Age	25.82±5.72	26.31±5.63	25.75±5.75	0.714†
BMI (Kgr/m^2)	26.65±5.62	25.52±5.73	26.81±5.62	0.395†
Waist to Hip ratio	0.77±0.06	0.78±0.06	0.77±0.07	0.571†
FSH (mIU/ml)	6.26±1.72	7.08±1.44	6.14±1.73	0.040†
LH (mIU/ml)	6.08±3.90	9.51±6.42	5.60±3.16	0.000
R	0.98±0.51	1.31±0.68	0.93±0.46	0.010
PRL (ng/ml)	14.73±6.92	17.93±8.32	14.28±6.62	0.112
Testo (ng/dl)	73.63±27.32	95.85±39.11	70.51±23.86	0.020
Δ4 Andro (ng/ml)	2.80±1.06	5.08±0.78	2.48±0.61	0.000
Testo/Δ4	28.29±11.47	18.66±6.74	29.64±11.37	0.000†
DHEAS (μg/dl)	3147±1319	4334±1656	2980±1180	0.000†
17OH (ng/ml)	1.14±0.57	1.70±0.52	1.06±0.83	0.000
SHBG (nmol/l))	42.85±23.64	41.98±29.52	42.97±22.86	0.862
FAI	7.51±4.38	10.32±5.87	7.11±4.01	0.120
Insulin (μIU/ml)	10.42±6.38	10.46±8.94	10.41±5.99	0.684
Glucose (mg/dl)	96.46±11.59	96.38±14.11	96.47±11.26	0.275†
HOMA2-IR	1.36±0.82	1.37±1.12	1.36±0.78	1.000
Quicki	0.34±0.03	0.35±0.03	0.34±0.03	0.676†
Glucose/ Insulin	12.60±7.68	13.19±7.44	12.53±7.74	1.000
Mean Follicular Number	11.87±3.93	11.43±2.83	11.92±4.05	1.000†
Mean Ovarian Volume (cm^3)	8.15±3.35	8.92±5.40	8.04±2.96	0.680†

† The independent t-test was used.

Stepwise multiple regression analysis showed that T alone could predict 25.7% of Δ_4A variation ($p<0.001$). Testosterone together with 17-OH progesterone could predict 38.5% of Δ_4A variation ($p<0.001$). The addition of LH increased the predicted value to 40.9% ($p<0.001$) and the addition of DHEAS increased the predicted value to 42.6% ($p<0.001$). Further addition of correlated variables (mean follicular number, BMI, SHBG and age) minimally affected the predicted value of Δ_4A levels reaching 43.6% ($p<0.001$), while PRL levels and mean ovarian volume were excluded from the predictive model, indicating a non-significant effect on Δ_4A variance.

DISCUSSION

In the present study, we investigated the specific impact of elevated serum Δ_4A levels on the hormonal and metabolic features of PCOS.

Although Δ_4A is a weaker androgen than testosterone, its net androgenic potency is rather considerable, given that its serum levels are 10-fold higher than those of testosterone.

However, high serum Δ_4A levels are not included in the mainstream diagnostic criteria of biochemical hyperandrogenemia of PCOS, while the effect of increased Δ_4A levels on the hormonal profile of the different PCOS phenotypes has not been thoroughly studied so far. In fact, Δ_4A overproduction from theca cells of PCO ovaries along with higher Δ_4A concentration in the follicular fluid of PCOS patients have been confirmed by previous studies.[11] Additionally, in the study of Knochenhauer et al,[12] increased serum Δ_4A levels were detected in 18% of PCOS women, diagnosed according to the National Institutes of Health (NIH) 1990 criteria (hyperandrogenism and/or hyperandrogenemia and oligo/anovulation). It is noteworthy that increased Δ_4A levels were the sole abnormal biochemical finding in 9% of the total PCOS women enrolled in this study.[12] Therefore, it seems that there is a proportion of PCOS cases which could be underdiagnosed when using the NIH diagnostic criteria, unless Δ_4A levels are taken into account. In the present study, Δ_4A levels >3.8 ng/ml as the sole criterion of biochemical hyperandrogenemia were found at non-negligible rates.

In the present study, we took into account the recent new criteria of the Androgen Excess-PCOS Society report on PCOS phenotype (2009).[13] Subsequently, we expanded the definition of biochemical hyperandrogenemia by including increased serum Δ_4A. Consequently, inclusion of increased Δ_4A levels in the criteria of biochemical hyperandrogenemia could account for the aforementioned 9% of PCOS women that are underestimated by the NIH definition.[12] Additionally, it could lead to minimization of the prevalence of the overestimated phenotype IV according to the Rotterdam criteria. In fact, the rates of each one of the four PCOS phenotypes by the Rotterdam definition are generally configured as follows: 44.09% for phenotype I, 22.84% for phenotype II, 14.17% for phenotype III and 18.9% for phenotype IV.[14] In our study, only 7.83% of PCOS were categorized as phenotype IV.

The findings of the present study revealed a remarkable incidence of increased Δ_4A levels in women with PCOS. It is notable that in phenotype I, the most critical finding in terms of hormonal and metabolic features, the incidence of increased Δ_4A levels, was much higher compared to other phenotypes.

Furthermore, increased Δ_4A levels were associated with both hormonal and ovarian ultrasonographic features of PCOS. In the total PCOS group and phenotypes I, II and III, increased Δ_4A levels were positively correlated with testosterone, FAI, DHEAS and 17-OH progesterone levels. A strong relationship between Δ_4A and testosterone was demonstrated based on stepwise multiple regression analysis, while overall, testosterone, 17-OH progesterone, DHEAS and LH levels accounted for more than 50% of Δ_4A variance.

It seems that increased Δ_4A levels are associated with a more severe phenotype of PCOS. Thus, a possible implication of Δ_4A in the pathogenesis of PCOS and its contribution to the severity of the syndrome could be further speculated.

The ovary and the adrenal gland equally contribute to serum Δ_4A levels. In fact, adrenal androgen excess (mainly increased Δ_4A and DHEAS levels) has been reported in 40-70% of PCOS patients.[15] Apart from the insulin-mediated over-activation exclusively of the ovarian CYP17 enzyme pathway,[16] CYP17 mutations and natural polymorphisms are a

frequent finding among women with PCOS.[17] Since P450c17 is expressed both in the adrenal gland and the ovary, in affected PCOS women genetic CYP17 derangements do not result only in adrenal but also in ovarian androgen overproduction.

The results of the present study demonstrated that in total PCOS women as well as in phenotypes I and II, increased serum Δ_4A levels were positively correlated with LH levels and LH/FSH ratio. Altered hypothalamic GnRH input along with increased pituitary sensitivity to GnRH is responsible for the abnormal gonadotropin levels, which is an extremely common finding reported in PCOS,[18] leading to increased LH/FSH ratio.[19] Increased Δ_4A levels in the context of high-normal estradiol levels have been proposed as contributing to the neuroendrocrine dysfunction characterizing PCOS.[7,20] Androstenedione physiologically modulates the hypothalamic GnRH pulse generator, keeping it uncoupled from the excessive inhibitory effect, exerted by estrogens, thus playing a central role in the normal desensitization process. Increased Δ_4A levels are therefore associated with accelerated activity of the pulse generator.[20]

In the present study, increased Δ_4A levels were associated with higher ovarian volume and mean follicular number in PCOS women. Increased serum Δ_4A levels have been shown to inhibit insulin and LH-induced estrogen and progesterone production from granulosa cells of PCO follicles, contributing to chronic anovulation.[21] Furthermore, a study in Δ_4A treated rat ovaries has *in vitro* confirmed premature luteinization of granulosa cells and follicular cyst development.[22] Moreover, increased serum Δ_4A levels further contribute to follicular arrest. Androstenedione is the substrate of 5-α-reductase, whose activity has been shown to be 4-fold higher in granulosa cells of PCO compared to controls.[23] The product of 5-α reduction, 5alpha-androstane-3,17-dione, has been demonstrated to inhibit aromatase activity in a competitive manner, interfering with the establishment of an estrogen microenvironment,[24] thus blocking the selection of the dominant follicle.

Given the conversion of androstenedione to testosterone by the enzyme 17beta HSD, it seems plausible that the effect of androstenedione could be attributed to its conversion to testosterone. Since PCOS has been considered as a form of functional ovarian hyperandrogenism, increased androgen production could be attributed to transcriptional dysregulation of the enzymes involved in steroidogenesis.[25] For instance, dysregulation of 17βHSD could lead to increased testosterone levels. It seems that the androstenedione/testosterone ratio is differentially modulated, given the identification of polymorphisms of the 17βHSD gene which lead to increased transcriptional activity that strongly favors androstenedione conversion to testosterone, whereas different polymorphisms have been reported to favor milder conversion. For example, the -71G HSD17B5 variant has been shown to be positively associated with increased testosterone levels in women with PCOS and biochemical hyperandrogenism.[26] Consequently, there is a substantial proportion of androstenedione levels whose hormonal effect is mediated by androstenedione *per se* and not by conversion to testosterone.

Finally, serum Δ_4A levels were inversely correlated with BMI in the present study, a finding which is in consistency with the results of previous studies.[27] The inverse relationship between androstenedione and obesity could be attributed to elevated free IGF-I levels that have been reported in non-obese PCOS women compared to obese PCOS and are responsible for adrenocortical stimulation leading to increased adrenal hyperandrogenism.

In conclusion, increased serum Δ_4A levels are associated with more severe PCOS phenotypes, possibly contributing to the deterioration of PCOS features, and therefore could be a valuable marker of biochemical hyperandrogenemia.

DISCLOSURE

The authors have no conflict of interest to declare.

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