

Are There Any Sensitive and Specific Sex Steroid Markers for Polycystic Ovary Syndrome?

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Context: Despite the high prevalence of hyperandrogenemia, the principal biochemical abnormality in women with polycystic ovary syndrome (PCOS), a definitive endocrine marker for PCOS has so far not been identified.

Objective: To identify a tentative diagnostic marker for PCOS, we compared serum levels of sex steroids, their precursors, and main metabolites in women with PCOS and controls.

Design and Methods: In this cross-sectional study of 74 women with PCOS and 31 controls, we used gas and liquid chromatography/mass spectrometry to analyze serum sex steroid precursors, estrogens, androgens, and glucuronidated androgen metabolites; performed immunoassays of SHBG, LH, and FSH; and calculated the LH/FSH ratio.

Results: Androgens and estrogens, sex steroid precursors, and glucuronidated androgen metabolites were higher in women with PCOS than in controls. In multivariate logistic regression analyses, estrone and free testosterone were independently associated with PCOS. The odds ratios per SD increase were 24.2 for estrone [95% confidence interval (CI), 4.0–144.7] and 12.8 for free testosterone (95% CI, 3.1–53.4). In receiver operating characteristic analyses, the area under curve was 0.93 for estrone (95% CI, 0.88–0.98) and 0.91 for free testosterone (95% CI, 0.86–0.97), indicating high sensitivity and specificity.

Conclusion: Women with PCOS have elevated levels of sex steroid precursors, estrogens, androgens, and glucuronidated androgen metabolites as measured with a specific and sensitive mass spectrometry-based technique. The combination of elevated estrone (>50 pg/ml) and free testosterone (>3.3 pg/ml) appeared to discriminate with high sensitivity and specificity between women with and without PCOS. (*J Clin Endocrinol Metab* 95: 810–819, 2010)

Polycystic ovary syndrome (PCOS), the most common endocrinopathy in women of reproductive age, is characterized by polycystic ovaries, oligoovulation or anovulation, and hyperandrogenism (1–3). The most con-

stant and prominent of these characteristics is hyperandrogenism, manifested by hirsutism, persistent acne, alopecia, and biochemical abnormalities (2), including increased testosterone (T) secretion by ovarian theca cells

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Abbreviations: ADT-G, Androstane glucuronide; ANCOVA, analysis of covariance; AUC^{ROC}, area under the curve for ROC analyses; BMI, body mass index; CI, confidence interval; CMIA, chemiluminescence microparticle immunoassay; DHEA, dehydroepiandrosterone; DHEAS, DHEA sulfate; DHT, 5 α -dihydrotestosterone; 4-DIONE, androstenedione; 5-DIOL, 5-androstene-3 β , 17 β -diol E1, estrone; E1-S, E1 sulfate; E2, estradiol; FT, free T; 3G, androstane-3 α , 17 β -diol-3glucuronide; 17G, androstane-3 α , 17 β -diol-17glucuronide; GC-MS, gas chromatographic/mass spectrometry; LC-MS/MS, liquid chromatographic/tandem mass spectrometry; OR, odds ratio; PCOS, polycystic ovary syndrome; ROC, receiver operating characteristic; T, testosterone.

(4, 5) and possibly enhanced ovarian activity of 5α -reductase, which converts T to 5α -dihydrotestosterone (DHT) (5–7). Despite the prevalence of hyperandrogenism in women with the PCOS, a definitive diagnostic endocrine marker for the syndrome has not been identified.

Hyperandrogenemia in PCOS can be assessed in various ways. Commonly, this is done by measuring total T and/or free T (FT) and SHBG and calculating the bioavailable fraction by the free androgen index or mass equation (8, 9). Androstenedione (4-DIONE), which is synthesized in the adrenal cortex, liver, and ovarian theca cells, may be used to diagnose hyperandrogenemia and PCOS, but few prospective studies are available (3). Adrenal-derived dehydroepiandrosterone (DHEA) has limited diagnostic value because it varies diurnally and requires accurate and sensitive assays to measure (10). However, DHEA sulfate (DHEAS) has been used as a marker for adrenal androgen excess because it is almost exclusively ($\sim 98\%$) derived from the adrenal cortex, is stable throughout the day and the menstrual cycle (11), and is easy to measure.

Peripheral androgen synthesis may also contribute to hyperandrogenism in PCOS. Exogenous administration of DHEA in women with PCOS enhances serum DHT, which is generated from T by 5α -reductase, and its major metabolite androsterone glucuronide (ADT-G) (6). Thus, serum glucuronidated androgen metabolites measured by RIA might reflect peripheral androgen action in women with PCOS (12, 13).

Disassociations between serum levels of LH and FSH in PCOS have been attributed to chronic acyclic estrogen production characterized by a predominance of circulating estrone (E1) (14). Serum concentrations of E1 and estradiol (E2) are higher in women with PCOS than controls, but the ranges overlap widely (15, 16). It has also been suggested that impaired FSH release by chronic acyclic estrogen production derived from nonglandular aromatization of circulating androgens could be responsible for anovulation in PCOS (14). In addition, endometrial hyperplasia, an estrogen-specific response, is a common feature of PCOS, and higher bioavailability of estrogens in the endometrium has been reported in women with PCOS (17, 18). These women have high circulating levels of androgens and LH, and mild hyperandrogenemia might lead to hypersecretion of LH (19, 20). It is not known whether LH and the LH/FSH ratio might serve as diagnostic markers for PCOS (21–23).

The predictive power of endocrine variables in the diagnosis of PCOS has been assessed with receiver operating characteristic (ROC) analyses (21–25). Despite inclusion of similar serum endocrine variables, this approach has yielded different diagnostic discriminators (21–23, 25). This discrepancy may reflect the high variability of direct

assays of androgens (3, 26). Most assays for T are immunobased and were not designed or validated for the relatively low levels normally present in women (9), raising concerns about their diagnostic value in PCOS (27). Moreover, the range considered healthy for women is broad and includes many hyperandrogenic women, even those with severe hirsutism (27). Serum analysis fails to identify biochemical hyperandrogenism in 20–40% of patients with PCOS (28).

Measurement of glucuronidated metabolites of androgens is the best way to accurately estimate the total androgen pool in women (29, 30). Three such metabolites— androstane- 3α , 17β -diol-3glucuronide (3G), androstane- 3α , 17β -diol-17glucuronide (17G), and ADT-G— can be analyzed separately by liquid chromatographic/tandem mass spectrometry (LC-MS/MS) (31), raising the possibility of tissue-specific analysis of androgen glucuronidation (32–34). LC-MS/MS and gas chromatographic/mass spectrometry (GC-MS) are highly accurate and have been suggested for clinical research (26, 35).

Mass spectrometry has been used to measure serum T, DHT, and urinary metabolites in women with PCOS and controls (36–38), but this technique has not, to our knowledge, been used to obtain more comprehensive profiles of serum sex steroid hormones (Fig. 1). We hypothesized that serum levels of estrogens, androgen precursors, bioactive androgens, and glucuronidated androgen metabolites, measured by sensitive, specific, and validated

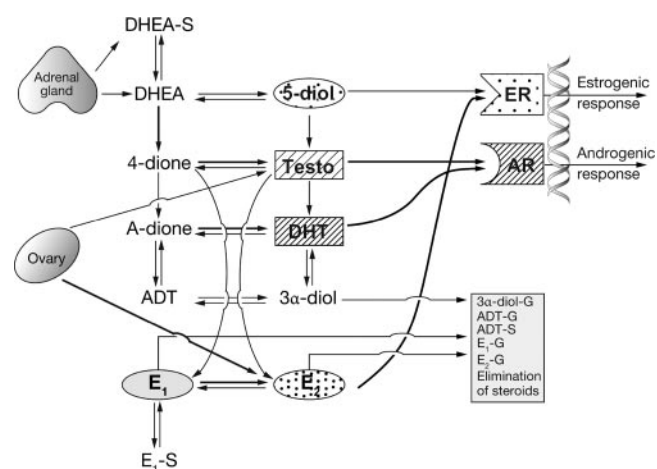


FIG. 1. Human steroidogenic and steroid-inactivating enzymes in peripheral intracrine tissues. The adrenal glands produce DHEA and DHEAS, which are transported in the general circulation to peripheral target tissues, where they are converted into estrogens, androgens, or both, depending upon the enzymatic machinery in each tissue. The active hormones made locally are the estrogens E2 and 5-DIOL and the androgens T (Testo) and DHT. To exert their specific effects, estrogens act through the estrogen receptor (ER) and androgens through the androgen receptor (AR). The pathways leading to hormone inactivation are also shown. A-DIONE, Androstenedione; ADT, androsterone; ADT-G, ADT glucuronide; ADT-S, ADT sulfate; E₁-G, estrone glucuronide; E₁-S, estrone sulfate; E₂-G, estradiol glucuronide; E₂-S, estradiol sulfate.

mass spectrometry techniques, would be higher in women with PCOS than in controls and might discriminate between women with and without PCOS. To test this hypothesis, we conducted a cross-sectional study to assess the utility of these variables in the diagnosis of PCOS.

Subjects and Methods

Study population

Women with PCOS ($n = 74$) and controls ($n = 31$) were recruited from advertisements in two local newspapers. The criteria for PCOS were polycystic ovary (PCO) morphology (12 or more 2- to 9-mm ovarian follicles or ovarian volume > 10 ml in one or two ovaries by two-dimensional vaginal ultrasound), and/or clinical signs of hyperandrogenism (HA) and/or oligo/amenorrhea (Oligo). PCO+HA+Oligo was considered classic PCOS; PCO+HA or PCO+Oligo was considered nonclassic PCOS. PCOS women with congenital adrenal hyperplasia, Cushing's syndrome, or androgen-secreting tumors were excluded (1, 39). Controls were excluded if they had PCO morphology or menstrual irregularities (cycles < 28 or > 35 d), hirsutism with a Ferriman-Gallwey score greater than zero (40) and answered "yes" to the question "Do you have acne?" All subjects who had physical or psychiatric disease or reported pharmacological treatment within 12 wk or breast feeding within 24 wk before enrollment were excluded.

All participants gave informed consent. The study was conducted at the Sahlgrenska Academy in accordance with the Declaration of Helsinki and was approved by the University of Gothenburg Ethics Committee.

Study procedure

The initial evaluation, including case history and two-dimensional vaginal ultrasound (HDI 5000; ATL Ultrasound Inc., Bothell, WA), was performed by gynecologists. Blood samples for endocrine measurements were obtained between 0730 and 0830 h and stored at -80 C; in controls, blood samples were obtained during the follicular phase of the menstrual cycle (d 1–7) to match the hormonal milieu of PCOS subjects and to avoid the preovulatory estrogen rise. Blood samples were obtained independently of cycle day in women with PCOS because 57 of the 74 women with PCOS had oligoanovulation. Age, height, body weight (light clothing, no shoes), body mass index (BMI), excess facial and body hair, acne, and menstrual cycle frequency were also assessed. BMI (kilograms per square meter) was calculated as weight divided by height squared. Amenorrhea was defined as absent menstrual bleeding in the past 90 d. Oligomenorrhea was defined as > 35 d between cycles with fewer than eight menstrual periods in the past year. Hirsutism was defined as a Ferriman-Gallwey score above 8 (40, 41).

Biochemical analyses

GC-MS

DHEA, 5-androstene- 3β , 17 β -diol (5-DIOL), 4-DIONE, T, DHT, E1, and E2 were measured with a validated GC-MS system, which uses a 50% phenyl-methyl polysiloxane (DB-17HT) capillary column (30 m \times 0.25 mm internal diameter; 0.15- μ m film thickness) and helium as carrier gas. The analytes and in-

ternal standard are detected with an HP5973 quadrupole mass spectrometer equipped with chemical ionization source. The limits of detection were 0.10 ng/ml for DHEA, 30 pg/ml for 5-DIOL, 0.05 ng/ml for 4-DIONE, 0.02 ng/ml for T, 5.00 pg/ml for DHT, 5.00 pg/ml for E1, and 1.00 pg/ml for E2.

LC-MS/MS

A validated LC-MS/MS system with a TurboIonSpray source was used to analyze DHEAS (limit of detection, 0.075 μ g/ml), E1 sulfate (E1-S) (0.075 ng/ml), ADT-G (2.00 ng/ml), 3G (0.50 ng/ml), and 17G (0.50 ng/ml).

Immunoassay

SHBG, LH, and FSH were analyzed by chemiluminescence microparticle immunoassay (CMIA) at an accredited laboratory at the Department of Clinical Chemistry, Sahlgrenska University Hospital, using Architect reagent kits from Biokit (Barcelona, Spain) for SHBG (limit of detection, 0.1 nmol/liter) and from Abbott Laboratories (Chicago, IL) for LH (0.07 IU/liter) and FSH (0.05 IU/liter). Serum FT was measured by RIA using Coat-A-Count FT (limit of detection, 2.0 pmol/liter) from Diagnostic Products Corporation (Los Angeles, CA). Total serum T was measured by competitive immunochemistry with chemiluminescence technology using ADVIA Centaur TSTO Ready Pack primary reagents (limit of detection, 0.35 nmol/liter) from Bayer Health Care LLC (Tarrytown, NY).

Calculation of FT

FT was calculated as described (9, 42), using the total T concentration determined by GC-MS and SHBG determined by CMIA and assuming a fixed albumin concentration of 43 g/liter.

Statistics

Differences between women with PCOS and controls were analyzed with *t* tests and by analysis of covariance (ANCOVA) considering age and BMI. Crude and age- and BMI-adjusted differences between women with classic and nonclassic PCOS and controls were analyzed by ANOVA and ANCOVA followed by Bonferroni *post hoc* tests.

Univariate associations among sex steroids were assessed by Pearson correlation. The relative independent effects of adrenal-derived DHEAS and mainly ovarian-derived T on serum levels of glucuronidated androgen metabolites were determined by linear regression analyses, with T and DHEAS as well as age and BMI as covariates. The predictive values of sex steroids for PCOS were determined by logistic binary regression with adjustment for age and BMI. For ROC analysis, women with PCOS were considered affected, and controls as nonaffected. ROC curves were constructed by plotting the sensitivity (true-positive) on the ordinate as a function of the complement of specificity (false-positive) for all possible cutoff values of the diagnostic test (26). Greater deviation toward the left upper corner of the curve indicates better detection of disease (43).

All variables except T, DHEAS, age, and height were skewed and underwent logarithmic transformation before statistical analysis. Values are presented as mean \pm SD or median and interquartile range if skewed. $P < 0.05$ was considered significant. All statistical analyses were conducted with Prediction Application Software (version 17.0 for Windows; SPSS, Chicago, IL).

Results

Characteristics of women with PCOS

Anthropometric characteristic and sex steroid profiles

Anthropometric characteristics and sex steroid levels measured with mass spectrometry are summarized in Tables 1 and 2. Among all women with PCOS (Table 1), age and BMI were higher than in controls. Therefore, age and BMI were included as covariates in regression analyses. Levels of all measured sex steroids and glucuronidated androgen metabolites were significantly higher in women with PCOS before and after adjustment for age and BMI (Table 1). LH and the LH/FSH ratio were higher, and FSH and SHBG were lower, in women with PCOS than in controls (Table 1).

In women with classic PCOS, the concentrations of all sex steroid variables except DHEA and 5-DIOL were higher than in controls, as were LH and the LH/FSH ratio, and SHBG was significantly lower (Supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>). Women with nonclassic PCOS had significantly higher levels of E1, E1-S, E2, 4-DIONE, T, FT, DHT,

ADT-G, 3G, and 17G, a higher LH/FSH ratio, and lower FSH than controls (Supplemental Table 1). Women with classic PCOS displayed higher concentrations of 4-DIONE, T, FT, DHT, 3G, and LH, a higher LH/FSH ratio, and lower E2 concentrations than women with nonclassic PCOS. E2 was higher in women with nonclassic PCOS, but E1 did not differ between the classic and nonclassic groups. Seventeen women with nonclassic PCOS had a regular bleeding pattern. To exclude the possibility that the high E2 concentrations in the nonclassic PCOS group reflected the timing of blood sampling, PCOS women with irregular cycles ($n = 57$) were compared with controls to exclude the influence of cycle day. The results were the same as in women with classic PCOS (data not shown).

Correlation analyses of sex steroid precursors, androgens, estrogens, and glucuronidated androgen metabolites

In PCOS women (Supplemental Table 2) and controls (Supplemental Table 3), glucuronidated androgen metabolites (ADT-G, 3G, and 17G) were strongly and positively associated with each other ($r = 0.5–0.83$; $P < 0.001$) and moderately associated with adrenal-derived DHEAS ($r =$

TABLE 1. Anthropometry and sex steroids measured with mass spectrometry in PCOS women and controls

Variable	PCOS women vs. controls				
	PCOS (n = 74)	Controls (n = 31)	Difference from control (%)	Crude P value ^a	Adjusted P value
Anthropometry					
Age (yr)	30.0 ± 4.4	27.8 ± 3.5	8	0.018	
Weight (kg)	70.9 (62.7–93.6)	67.5 (58.8–78.2)	5	0.059	0.471
BMI (kg/m ²)	25.3 (22.1–33.3)	23.6 (21.0–27.2)	7	0.020	
Height (m)	1.67 ± 0.07	1.69 ± 0.06	–1	0.283	0.486
Mass spectrometry					
E1 (pg/ml) (GC)	71.8 (54.4–92.8)	34.8 (29.2–43.9)	106	<0.001	<0.001
E1-S (ng/ml) (LC)	1.20 (0.80–1.77)	0.36 (0.27–0.67)	200	<0.001	<0.001
E2 (pg/ml) (GC)	63.7 (44.4–103.2)	35.7 (26.0–54.0)	78	<0.001	<0.001
DHEA (ng/ml) (GC)	6.48 (5.08–8.34)	4.84 (3.82–6.28)	35	0.003	0.001
DHEAS (μg/ml) (LC)	1.80 ± 0.81	1.25 ± 0.48	44	0.001	<0.001
4-DIONE (ng/ml) (GC)	1.58 (1.34–2.40)	1.02 (0.82–1.22)	60	<0.001	<0.001
5-DIOL (pg/ml) (GC)	652 (484–852)	530 (394–670)	23	0.010	0.015
T (ng/ml) (GC)	0.44 ± 0.18	0.21 ± 0.08	110	<0.001	<0.001
FT (pg/ml) ^a	7.50 (4.39–10.4)	2.15 (1.72–2.82)	241	<0.001	<0.001
DHT (pg/ml) (GC)	117 (84–152)	77.8 (63.4–86.0)	50	<0.001	<0.001
ADT-G (ng/ml) (LC)	52.0 (33.8–73.4)	23.0 (18.1–32.9)	126	<0.001	<0.001
3G (ng/ml) (LC)	2.01 (1.34–2.90)	0.97 (0.60–1.40)	100	<0.001	<0.001
17G (ng/ml) (LC)	1.81 (1.12–2.68)	1.04 (0.59–1.46)	80	<0.001	<0.001
Immunoassay					
SHBG (nmol/liter) (CMIA)	38.0 (26.0–53.2)	75.0 (42.0–90.0)	–49	<0.001	<0.001
LH (IU/liter) (CMIA)	6.85 (3.85–9.45)	4.10 (2.70–5.20)	66	<0.001	<0.001
FSH (IU/liter) (CMIA)	4.15 (3.00–5.00)	4.80 (4.20–6.00)	–12	0.004	0.005
LH/FSH ratio	1.64 (1.14–2.62)	0.80 (0.60–1.02)	100	<0.001	<0.001

Values are expressed as mean ± SD or median (25th–75th interquartile ranges). Crude P values were determined by ANOVA. P values adjusted for age and BMI were determined by ANCOVA followed by Bonferroni *post hoc* tests. $P < 0.05$ is considered statistically significant and is shown in bold. GC, Gas chromatography-mass spectrometry; LC, liquid chromatography-tandem mass spectrometry.

^a FT was calculated by taking total T and SHBG into account and assuming a fixed albumin concentration of 43 g/liter (9, 42).

TABLE 2. Associations between PCOS and estrogens, androgen precursors, bioactive androgens, and glucuronidated androgens determined by logistic binary regression analyses adjusted for age and BMI

Regression models and covariates	PCOS vs. controls		Classic PCOS vs. controls	
	OR ^a (95% CI)	P value ^b	OR ^a (95% CI)	P value ^b
Model 1 (estrogens)				
E1	11.33 (2.34–54.89)	0.003	15.34 (2.56–92.13)	0.003
E1-S	1.05 (0.37–2.93)	0.930	1.35 (0.41–4.44)	0.625
E2	2.21 (0.73–6.67)	0.159	1.80 (0.50–6.41)	0.367
Model 2 (sex steroid precursors)				
DHEA	1.01 (0.44–2.31)	0.981	0.71 (0.24–2.04)	0.520
DHEAS	2.57 (0.94–7.01)	0.065	2.97 (0.83–0.57)	0.094
4-DIONE	8.04 (2.90–22.65)	<0.001	13.38 (3.32–53.89)	<0.001
5-DIOL	0.77 (0.31–1.91)	0.574	1.01 (0.30–3.39)	0.984
Model 3 (androgens)				
T	1.28 (0.16–9.97)	0.811	4.91 (0.35–69.68)	0.240
FT	11.0 (1.96–62.28)	0.007	8.73 (1.29–59.20)	0.027
DHT	1.77 (0.78–4.05)	0.173	2.59 (0.83–8.09)	0.101
Model 4 (glucuronidated androgen metabolites)				
ADT-G	1.52 (0.53–4.42)	0.439	2.08 (0.56–7.66)	0.275
3G	3.83 (1.15–12.75)	0.029	3.53 (0.85–14.62)	0.082
17G	1.07 (0.44–2.63)	0.878	0.024 (0.46–3.36)	0.112

^a Per sd increase.

^b Significant associations are shown in *bold*.

0.38–0.58; $P < 0.05$ –0.001). These metabolites were also associated with FT in PCOS women and controls ($r = 0.42$ –0.54; $P < 0.01$ –0.001); their association with T was less pronounced, with a significant association in PCOS ($r = 0.29$ –0.36; $P < 0.05$ –0.001) but not in controls ($r = 0.26$ –0.34), except for 3G ($r = 0.38$; $P < 0.05$).

E2 was significantly and negatively associated with T and FT in PCOS women but not in controls (T, PCOS $r = -0.25$, $P < 0.05$; and controls $r = 0.20$; FT, PCOS $r = -0.40$, $P < 0.001$; and controls $r = 0.01$; Supplemental Tables 3 and 4). Plotting of E2 vs. T revealed two PCOS populations, one with high aromatase activity (high E2 and normal T) and one with low aromatase activity (high T and normal E2) (Fig. 2A). Plotting of DHT vs. T showed

no major difference between PCOS women and controls, indicating normal 5- α reductase activity (Fig. 2B) in PCOS women, consistent with the lack of difference in the DHT/T ratio between the groups (data not shown).

Independent effects of adrenal-derived sex steroid precursors and ovarian-derived T on serum concentrations of glucuronidated androgen metabolites

Glucuronidated androgen metabolites have been proposed as indicators of the total androgen pool in women (12, 13). In multiple linear regression analyses of T and DHEAS, with age and BMI as covariates, DHEAS was a stronger independent predictor of glucuronidated androgen metabolites than T (Supplemental Table 4). Thus, an-

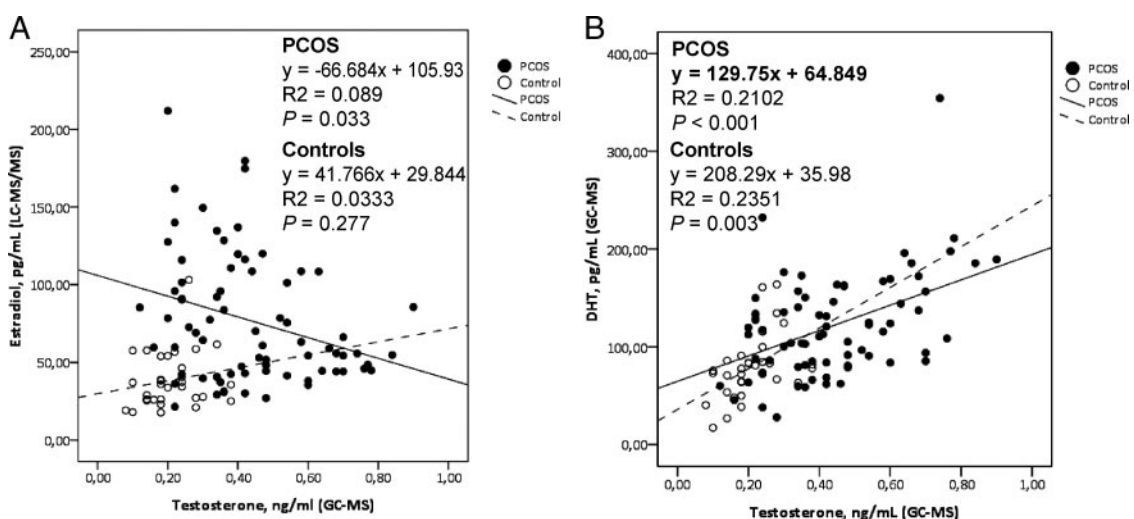


FIG. 2. Scatterplots of E2 vs. (A) and DHT vs. T (B) in PCOS women and controls.

drogens were largely derived from the adrenal gland in both women with PCOS and controls.

Associations between PCOS and serum sex steroids

To evaluate independent associations between PCOS and sex steroid precursors, androgens, estrogens, and glucuronidated androgen metabolites, we performed logistic binary regression analyses (enter) with adjustments for age and BMI. We first evaluated these parameters in separate models (Table 2). In model 1 (estrogens), PCOS was associated with E1 [odds ratio (OR) per SD increase, 11.3; 95% confidence interval (CI), 2.3–54.9], but not E1-S or E2. In model 2 (androgen precursors), PCOS was associated with 4-DIONE (OR per SD increase, 8.0; 95% CI, 2.9–22.6) but not DHEA, DHEAS, or 5-DIOL. In model 3 (bioactive androgens), PCOS was strongly associated with FT (OR per SD increase, 35.5; 95% CI, 4.3–295.7) but not T or DHT. In model 4 (glucuronidated androgen metabolites), PCOS was moderately associated with 3G (OR per SD increase, 3.8; 95% CI, 1.2–12.8) but not ADT-G or 17G. In general, similar results were obtained in women with classic PCOS (Table 2, *right column*), although 3G was not significantly associated with classic PCOS in this subgroup (Table 2).

To determine whether E1, 4-DIONE, FT, and 3G are independently associated with PCOS, we included these parameters in logistic binary regression analyses (forward conditional) with adjustment for age and BMI. PCOS was independently associated with E1 (OR per SD increase, 23.8; 95% CI, 4.1–139.7) and FT (OR per SD increase, 12.0; 95% CI, 3.0–49.0) but not 4-DIONE or 3G. Inclusion of SHBG and the LH/FSH ratio, both proposed to discriminate for PCOS (21, 23), in the model did not affect the significant associations for E1 and FT or reveal significant independent associations with PCOS (data not shown).

In ROC analyses of the ability of E1 and FT to detect PCOS (Fig. 3), the area under the curve (AUC^{ROC}) was 0.93 (95% CI, 0.88–0.98) for E1 and 0.91 (95% CI, 0.86–0.97) for FT. The combination of E1 and FT resulted in a higher AUC^{ROC} of 0.94 (95% CI, 0.90–0.98). A decision threshold of E1 greater than 50.0 pg/ml resulted in a sensitivity of 84% and a specificity of 94% in diagnosing PCOS. A threshold of FT greater than 3.3 pg/ml resulted in a sensitivity of 84% and a specificity of 87% (Figs. 3 and 4).

The AUC^{ROC} for FT measured with RIA was 0.84 (95% CI, 0.76–0.92) compared with 0.91 (95% CI, 0.86–0.97) for FT measured with GC-MS, and the AUC^{ROC} for T measured with RIA was 0.85 (95% CI, 0.77–0.93) compared with 0.89 (95% CI, 0.82–0.95) for T measured with

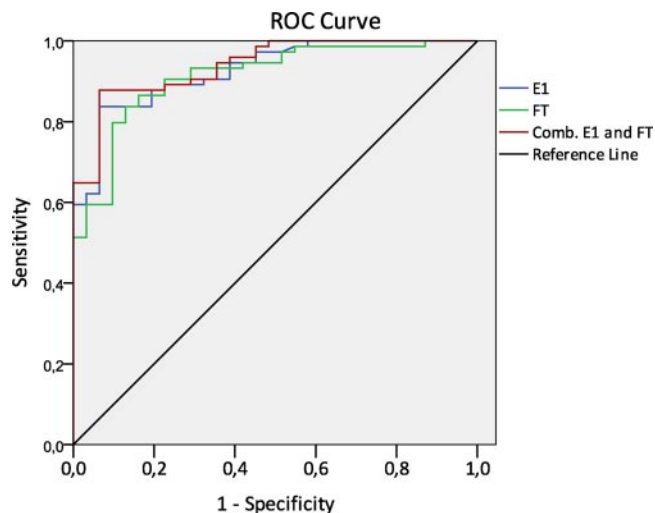


FIG. 3. ROC curves for the detection of PCOS using E1 and FT. AUC^{ROC} was 0.93 (95% CI, 0.88–0.98) for E1, 0.91 (95% CI, 0.86–0.97) for FT, and 0.94 (95% CI, 0.90–0.98) for the combination of E1 and FT.

GC-MS. These data demonstrate that the ability to detect PCOS is higher for sex steroids measured with mass spectrometry compared with RIA.

Discussion

This comprehensive analysis of serum sex steroid levels determined by specific and validated mass spectrometry techniques shows that sex steroid precursors, androgens, and glucuronidated androgen metabolites, and also estrogens, can distinguish women with PCOS from controls. E1 and FT were independently associated with PCOS; E1 levels greater than 50 pg/ml and FT levels above 3.3 pg/ml discriminated between women with and without PCOS with high sensitivity and specificity.

The ovaries and adrenal glands are both thought to contribute to hyperandrogenemia in PCOS. Ovarian hyperandrogenemia is likely to be especially important, as demonstrated by the intrinsic abnormality of thecal cell steroidogenesis in polycystic ovaries (4, 5, 44). Although the ovary is the principal source of androgen

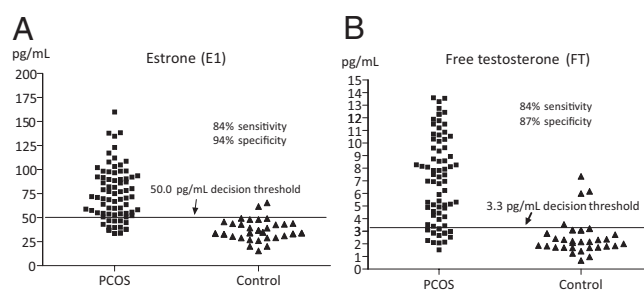


FIG. 4. Levels of E1 (A) and FT (B) in women with PCOS and controls. Proposed decision thresholds and corresponding sensitivities and specificities for the diagnosis of PCOS are shown.

excess, 40–70% of women have high levels of adrenal-derived androgen precursors, particularly DHEAS, suggesting a potential role of the adrenals (45).

Sex steroid precursors and bioactive androgens were all higher in women with PCOS than controls, ranging from a 44% increase for DHEAS to a 202% increase for FT. The differences were similar in women with classic PCOS but less pronounced in those with nonclassic PCOS, indicating the heterogeneity in the syndrome. FT, the bioactive androgen variable that differed most in PCOS women and controls, was independently associated with PCOS. Although some studies show increased 5 α -reductase activity in PCOS (6, 7, 37, 46), we found no difference in DHT/total T ratios in PCOS women and controls, confirming reports of normal 5 α -reductase activity in PCOS (38, 47). Of the adrenal-derived androgen precursors, 4-DIONE was the strongest predictor of PCOS, but multiple logistic regression analyses showed no independent association.

In men and women, androgens are eliminated by inactivation into androsterone and 3 α -diol and their subsequent glucuronidation into ADT-G, 3G, and 17G. Thus, assessment of glucuronidated androgen metabolites has been suggested as the best means of evaluating total androgenic activity (29–31). We used LC-MS/MS to separately analyze ADT-G, 3G, and 17G (Fig. 1), raising the possibility of evaluating tissue-specific glucuronidation of androgen metabolites (32–34). Despite the increased role of the ovary in PCOS women, a substantial part of the total androgenic pool in both PCOS women and controls is likely to be derived from the adrenal gland, consistent with our previous finding in elderly men (33, 48). Indeed, both in women with PCOS and in controls, DHEAS was a much stronger independent predictor of glucuronidated androgen metabolites than T. However, we cannot exclude that FT drives a significant part of the total androgenic pool in absolute terms because it may be speculated that DHEAS is more directly metabolized to glucuronidated androgen metabolites when compared with FT.

Elevated levels of urinary glucuronidated androgen metabolites have been reported in women with PCOS (12, 13, 49). In the present study—the first to evaluate all three major glucuronidated androgen metabolites in relation to PCOS—the levels of these metabolites were higher (94–102%) in women with PCOS than in controls. Of these metabolites, 3G was the strongest predictor of PCOS. However, 3G was not associated with PCOS when included in the regression analyses together with E1, FT, 4-DIONE, SHBG, and the LH/FSH ratio. Evidently, levels of glucuronidated androgen metabolites do not further discriminate between women with and without PCOS.

Levels of all estrogens were higher in PCOS women than in controls, ranging from approximately 100% higher for E2 and E1 to 182% higher for E1-S. Women with nonclassic PCOS had significantly higher E2 concentrations than women with classic PCOS, but E1 and E1-S levels were similar in the two groups. Interestingly, two populations were identified, one with high E2 and low T, indicating high aromatase activity, and another with high T and low E2. An old theory suggests that 4-DIONE is aromatized peripherally to E1, which increases LH (50). High E1 is thought to sensitize GnRH to secrete excess LH. However, exogenously administered E1 does not increase basal GnRH-stimulated LH release (14), and peripheral aromatase inhibitors do not reduce LH pulse frequency (51). On the other hand, the observation that antiestrogens (*e.g.* clomiphene citrate) alter abnormal gonadotropin secretion in women with PCOS suggests that E1 excess contributes to the pathogenesis of PCOS (52). In our study, E1 was the estrogen that was clearly and independently associated with PCOS and added useful information to discriminate between women with and without PCOS. Further studies are required to determine the possible role of E1 in PCOS.

Use of ROC curves to investigate the predictive power of endocrine parameters in the diagnosis of PCOS (21–25) has identified various discriminators. Total T was proposed as the best discriminator between PCOS and mild Cushing's syndrome, with an AUC^{ROC} of 0.87 (25). SHBG, FT, the free androgen index, and DHEAS have been proposed as discriminators of PCOS, with AUC^{ROC} of 0.875 for SHBG, 0.867 for free androgen index, and 0.832 for DHEAS (21). Valid discriminators proposed in other studies include combinations of E2, SHBG, LH, FSH, and the LH/FSH ratio as well as insulin (22, 23, 53). Thus, there is a variation of previously suggested discriminators for the diagnosis of PCOS.

In our study, E1 and FT measured with mass spectrometry were independent predictors of PCOS, and the combination of E1 and FT was even stronger. A decision threshold of more than 50 pg/ml for E1 and more than 3.3 pg/ml for FT discriminated between women with and without PCOS with high sensitivity and specificity. Moreover, E1 and FT may detect PCOS independently of sampling conditions (*e.g.* menstrual cycle day), which is critical for gonadotropins and E2. The AUC^{ROC} values for E1 (0.93) and FT (0.91) were higher than previously reported (21, 23, 25). This discrepancy may reflect the high variability of direct immunoassays for androgens (3, 26) and the fact that commercial assays for total T are not designed to detect the low levels normally seen in women (9). The study by Pall *et al.* (25) is an exception because they used mass spectrometry to analyze total T with the hypothesis

that total T, bioavailable T, or the free androgen index would be lower in women with Cushing's syndrome than in women with PCOS. The AUC^{ROC} for detection of Cushing's syndrome with total T was 0.87, but would most likely be higher if PCOS were compared with controls. However, they did not calculate FT by the same method as used in the present study (9, 42).

Despite the considerable variation between hormone assays and the lack of accurate ranges for well-defined non-PCOS populations, circulating androgens are elevated in 60–80% of women with PCOS (54), and serum analysis fails to show biochemical hyperandrogenism in 20–40% of PCOS patients (28). Our findings suggest that the mass spectrometry might be useful for analyses of sex steroids in future studies.

Although sample size is a limitation of the present study, strengths include the community recruitment procedure, which allowed inclusion of PCOS women without previous health care contact, and rigorous inclusion/exclusion criteria. All potential participants underwent gynecological examination and vaginal ultrasound to confirm PCOS/control status. For the diagnostic criteria, we followed the Rotterdam consensus recommendation, except that all women should have polycystic ovaries. This means that there is no subgroup with oligo/anovulation + hyperandrogenism. Thus, it is possible that there is a selection bias compared with other studies. However, the nonclassic PCOS group differed significantly from controls in all estrogens, androgens, and glucuronidated androgen metabolites, indicating that although they did not have a Ferriman-Gallwey score of at least 8, they might still have hyperandrogenism.

In conclusion, women with PCOS have elevated levels of sex steroid precursors, estrogens, androgens, and glucuronidated androgen metabolites as measured with a specific and sensitive mass spectrometry technique. In combination, elevated E1 and FT, determined with this approach, can discriminate with high sensitivity and specificity between women with and without PCOS. The significance of this finding for routine clinical work remains to be shown, but it may serve as a valuable adjunct to the diagnostic criteria in scientific studies on this heterogeneous syndrome.

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