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Steroid hormone analysis of adolescents and young women with polycystic ovarian syndrome and adrenocortical dysfunction using UPC² -MS/MS

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Abstract

Background: We recently identified 35 women with PCOS that exhibited features of micronodular adrenocortical hyperplasia. Steroid hormone analysis can be more accurate using state-of-the-art ultra-performance-convergence-chromatography-tandem-mass-spectrometry (UPC^2-MS/MS). We hypothesized that UPC^2-MS/MS may be used to better define hormonally this distinct subgroup of patients with PCOS.

Methods: Plasma from PCOS patients (n=35) and healthy volunteers (HV, n=19) who all received dexamethasone testing was analyzed. Samples were grouped per dexamethasone responses and UPC^2-MS/MS analysis followed. When insufficient, samples were pooled from patients with similar responses to allow quantification over the low end of the assay.

Results: The C11-oxy C_{19} (11β-hydroxyandrostenedione, 11keto-androstenedione, 11β-hydroxytestosterone, 11keto-testosterone): C_{19} (androstenedione, testosterone) steroid ratio was decreased by 1.75-fold in PCOS compared to HV. Downstream steroid metabolites 11β-hydroxyandrosterone and 11keto-androsterone metabolites were also measurable. The C11-oxy

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Patient consent: Informed consent was obtained from all healthy volunteers and adult patients; the patients' parents for all pediatric patients and older children signed an assent.

C_{21} steroids, 11-hydroxyprogesterone and 11keto-dihydroprogesterone levels were 1.2- and 1.7-fold higher in PCOS compared to HV, respectively.

Conclusions: We hypothesized that UPC^2-MS/MS may accurately quantify steroids, in vivo, and identify novel metabolites in a subgroup of patients with PCOS with adrenal abnormalities. Indeed, it appears that adrenal C11-oxy steroids have the potential of being used diagnostically to identify younger women and adolescents with PCOS who also have some evidence of micronodular adrenocortical hyperplasia.

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is a complex and multifactorial disorder in which identification of subpopulations of patients with distinct phenotypes may lead to elucidation of its many contributing pathophysiological mechanisms (1). We recently identified a pattern of glucocorticoid secretion in a subset of young women with PCOS which mimicked that of patients with micronodular adrenocortical hyperplasia (MAH). These patients had smaller adrenal volumes and higher steroid hormone secretion after dexamethasone (DEX) compared to the group of PCOS with appropriate responses to DEX (2). These patients were selected for the investigation due to their higher circulating dehydroepiandrosterone sulfate (DHEAS) and other adrenal steroids (2,3).

The role of adrenal steroid excess in PCOS has been debated for a number of years. There appears to be no significant difference in basal hormones or in DHEAS levels in response to adrenocorticotropic hormone (ACTH) stimulation between patients responding or not responding to DEX (4), as well as in DHEA, DHEAS, T and A4 levels in adrenal vein samples after ACTH stimulation (5). However, ACTH stimulation in women with PCOS has also been shown to result in increased levels of 17α-hydroxyprogesterone (17OHPROG), deoxycortisol and urinary free cortisol (UFC) (6,7). In our previous study (2), UFC was significantly higher in PCOS patients who were selected based on high adrenal androgen levels. Although urinary 17-hydroxycorticosteroids (17OHCS) and 17-ketocorticosteroids (17KS) overall were not significantly different from control subjects, within the PCOS group, UFC and/or 17OHCS were detected at or above the 75th percentile in the subset of women which indicated inadequate DEX suppression. We subsequently termed these patients high responders (HR) based on their adrenal steroid response to DEX.

The aforementioned studies clearly underlie the complexity of steroidogenesis in PCOS and suggest that the C_{19} steroids may not be the only hormones contributing to the PCOS phenotype. Given that ACTH stimulation increased deoxycortisol and UFC levels together with high adrenal androgen levels, it is evident that the adrenal's contribution to the steroid pool is more than only the C_{19} steroids generally considered (8). The adrenal C11-oxy androgens, which include 11β-hydroxyandrostenedione (11OHA4) and 11-ketotestosterone (11KT), were recently reported in patients with PCOS (9,10). These C11-oxy C_{19} steroids have also been detected in normal female control subjects shown both in adrenal vein samples and in the peripheral circulation (5,11), while we have reported their presence in prostate cancer (PCa) tissue and in circulation, both in normal and PCa patients (12,13). Furthermore, the C11-oxy C_{19} steroids are detected at higher levels than the C_{19} steroids,

androstenedione (A4) and testosterone (T), only when not taking dehydroepiandrosterone (DHEA) levels into account, in both the normal state and various diseases (5,9,13,14).

In the present study, we hypothesized that analyzing a comprehensive steroid hormone profile of the HR group against the remaining patients with PCOS, who we termed normal responders (NR), and comparing them both to healthy volunteers (HV), would lead to new diagnostic hormonal profile applicable to this subset of patients with PCOS. In addition, the information would potentially shed new light into the steroidogenic abnormalities associated with pathogenesis of PCOS in this specific group of patients with PCOS, a highly heterogeneous disorder.

SUBJECTS & METHODS

Subjects

The patients in this study have been previously described in detail (2). In brief, women 16–29 years old with PCOS defined as biochemical hyperandrogenism with associated findings of either menstrual irregularity and/or polycystic ovaries on ultrasound were eligible for participation. Hyperandrogenism was diagnosed if any of the following androgens were above the normal reference range: T, A4, DHEA, and DHEAS. A polycystic ovary on ultrasound was defined as having either 12 follicles measuring 2–9 mm in diameter, or an increased ovarian volume of 10 cc or greater. Amenorrhea was defined as absence of bleeding for at least three months; oligomenorrhea was defined as bleeding that occurred at an interval greater than 35 days. In order to participate in the study, patients were required to have had oligomenorrhea for at least six months prior to initial admission. Patients had to be off oral contraceptives or any other medications that alter steroidogenesis for at least one month prior to participating in the study. We also documented history of oligomenorrhea prior to initiation of oral contraceptives. We excluded patients with late onset CAH, untreated thyroid dysfunction, hyperprolactinemia, primary ovarian failure and menarche within two years prior to participation in the study.

The HV control group, 18–29 years old, were also recruited; all healthy controls had a medical history and a physical exam by a physician and were found to be otherwise healthy besides their obesity. A diagnosis of PCOS was excluded in all HV by clinical and biochemical evaluation.

Clinical protocol

All patients were evaluated under the clinical protocol 11-CH-0119 (Clinical Trials Number: NCT01313455) that was approved by the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Institutional Review Board (IRB). This was a prospective case-control study. Informed consent was obtained from all HV and adult patients; the patients' parents for all pediatric patients and older children signed an assent.

Participants were admitted as inpatients at the Clinical Research Center (CRC) at NIH for a total of 8 full days. Each subject provided 24-hour urine collections for a total of 6 days. This study was done in the inpatient setting, as previously described (2): day 1 was the baseline; during days 2 and 3 subjects received low dose DEX (0.5 mg) *per os* every 6 hours,

(LDDST) and during days 4 and 5 they received 2 mg *per os* every 6 hours (HDDST). Final blood and urine collection were done on day 6. In the present study, we report plasma steroid analyses only since UFCs and urine 17OHCS and 17KS levels were previously reported (2).

Steroid analyses

Plasma samples were thawed on ice and vortexed after which 0.5 mL was aliquoted. Internal standards testosterone-D2 (D2T; 1.5 ng), cortisol-D4 (D4CSOL; 15 ng), progesterone-D9 (D9PROG; 15 ng), 11β-hydroxyandrostenedione-D7 (D711OHA4; 15 ng) and androstenedione-D7 (D7A4; 15 ng) were prepared in deionized water and added to samples (total volume 100 μL). The samples were vortexed in 5 mL methyl tert-butyl ether (MTBE) for 26 min, and subsequently frozen at −80°C. Thereafter the organic phase was removed, dried under nitrogen, and the dried residues dissolved in 150 μL 50% methanol in water (15). Subsequent to UPC^2-MS/MS analysis, samples were combined within the three groups according to the Liddle's test (PCOS HR, PCOS NR and HV) to allow analyses of steroid metabolites present at concentrations below the limit of quantification (LOQ). Four samples were combined, evaporated to dryness under a stream of nitrogen and the residue resuspended in 100 μL 50% methanol in water for analyses. In each of the three groups, 3 sets of samples (n=3; representing 12 serum samples) were prepared.

UPC^2–MS/MS quantification of steroid metabolites

Steroids (A4, T, 5α-androstanedione (5αDIONE), dihydrotestosterone (DHT), androsterone (AN), 5α-androstan-3α,17β-diol (3αDIOL), dihydroprogesterone (DHPROG), DHEA, 17α-hydroxyprogesterone (17OHPROG), cortisone, cortisol and corticosterone (CORT)) and MTBE were purchased from Sigma-Aldrich (St. Louis, MO, USA). 11OHA4, 11β-hydroxytestosterone (11OHT), 11keto-androstenedione (11KA4), 11KT, 11keto-dihydrotestosterone (11KDHT), 11β-hydroxyandrosterone (11OHAN), 11keto-androsterone (11KAN), 11keto-dihydroprogesterone (11KDHPROG), 5α-pregnan-17α-ol-3,20-dione (pdione), 5β-androstan-3β-ol-17-one (epietiocholanolone), 11keto-progesterone (11KPROG), 5β-androstan-3α-ol-11,17-dione (11K-etiocholanolone), 11β-hydroxyprogesterone (11βOHPROG), 5α-pregnan-3α,17α-diol-20-one (pdiol), 5β-androstan-3β,11β-diol-17-one (11OH-epietiocholanolone), 11α-hydroxyprogesterone (11αOHPROG), 21-deoxycortisol (21dF) and 5β-pregnan-3α,17α,20α-triol (pregnanetriol) were purchased from Steraloids Inc. (Newport, RI, USA). PROG was purchased from BDH Chemicals Ltd (Poole, England) and 16α-hydroxyprogesterone (16OHPROG) was purchased from TRC (Toronto Research Chemicals, Toronto, Canada). Fetal bovine serum (FBS) superior was obtained from Biochrom GmbH (Berlin, Germany). All deuterated steroids were purchased from either Cambridge Isotope Laboratories Inc. (Andover, MA, USA) or CDN Isotopes Inc. (Quebec, Canada). See supplemental Fig. 1 for a steroid metabolic pathway (classical, alternative and backdoor) with the steroids included in this work.

Stock solutions of all steroids were dissolved in absolute ethanol (2 mg/mL) and stored at −20°C. Standards ranging from 0.01 to 1000 ng/mL were extracted from FBS (500 μL) using the protocol described above. Linearity of the standard curves was assessed using the

Runs test in GraphPad Prism and determined acceptable at r^2-values (>0.9947) for all steroids.

The C_{19} and C_{21} steroids analyzed by ACQUITY UPC ®–MS/MS (Waters Corporation, Milford, USA; also termed ultra-high performance supercritical fluid chromatography tandem mass spectrometry) were separated using a Waters ACQUITY UPC^2® BEH column (3 × 100 mm, 1.7 μm particle size), with a 4.9 min linear elution gradient from 2% to 25% methanol used to separate the steroids (supplemental table 1). The mobile phase consisted of CO_2 modified with methanol (+1% formic acid) in which steroids, in an injection volume of 2 μL, were eluted at a flow rate of 2 mL/min in a total run time of 6 min per sample. Mass transitions, limit of detection (LOD) and LOQ of all steroids are shown in supplemental Table 1, indicating LODs and LOQs ranging from 0.01 to 10 ng/mL and from 0.01 to 20 ng/mL, respectively, the elution profile of the steroids are shown in supplemental Fig. 2, and other MS related settings have been previously published (12,13,16). Method validation has been published, including recovery, matrix effect (including the quantification of endogenous steroids in FBS), process efficiency, together with accuracy and precision (15), with the exception of CORT, which was quantified using the mass transition of 21dF in the present study. The method shows the recovery, matrix effect and process efficiency of steroids isolated from a serum matrix to be within acceptable limits. Good accuracy, repeatability and reproducibility were also shown, with acceptable %RSDs (< 20%) obtained for all the steroids for both the accuracy (ranging from 1.5% to 20.0%) and precision (1.6% to 19.9% tested over a three-day period) parameters. Recovery ranged from 45.9% to 116.3%, matrix effect from −50.7% to 28.1% and process efficiency from 36% to 118.7% (15). Data were collected and analyzed with the MassLynx 4.1 (Waters Corporation) software program.

Statistical analyses

Statistics were performed using an unpaired t-test using GraphPad Prism (version 6) software (GraphPad Software, San Diego, California). One-way ANOVA and Two-way ANOVA were used to test for possible differences between the three groups. Differences were considered statistically significant at $P<0.05$. Principal Component Analysis (PCA) biplots were used to visually depict correlations between variables, as well as differences between the groups, and Spearman's nonparametric tests were used to assess correlations between pairs of steroids. Furthermore, normality assumptions were reviewed by inspecting normal probability plots and were judged to be acceptable.

RESULTS

Patients

The demographic and clinical characteristics of women with PCOS and HVs have been previously reported (2). In brief, the average age of PCOS patients was 20.4±3.9 while HVs were 23.6±2.6 years old. They had similar homeostasis model assessment of insulin resistance (HOMA-IR; 3.5±3.6 vs 2.3±1.5, PCOS vs. HV, respectively). Thirteen of the women with PCOS (40%) had UFC and/or 17OHCS values at or above the 75th percentile (HR group). There were no statistically significant differences between this PCOS subsets vs

the remaining PCOS patients that we termed NR (n = 22) in terms of their metabolic parameters, body mass index, and baseline values of T, DHEA, DHEAS, A4, and 17OH-pregnenolone (2). The group was distinguished, in addition, by the percentage change between baseline and day 5 for 17OHCS (HR–29.5% ± 25.7% vs NR–55.4% ± 17.5%, P = 0.001), suggesting that the HRs not only had higher glucocorticoids but also suppressed less in response to DEX. Their adrenal volumes were also smaller, pointing to their overall similarity to the phenotype of patients with MAH (2).

Analyses of the C_{19} steroids: classic androgens and C11-oxy androgens

Considering PCOS as a single group (Figure 1), analysis of unconjugated C_{19} steroid levels showed that circulating DHEA, A4 and T were all significantly higher in PCOS patients (P<0.001) than in the HV and while 11KA4 levels were higher in the HV (P=0.078), 11KT was the only C11-oxy androgen significantly higher in the PCOS group (P<0.05). Data also shows that C_{19} steroids, including DHEA, were present at higher levels than the C11-oxy C_{19} steroids, with T present at similar levels compared to 11OHT and 11KT combined.

DHEA, A4 and T levels in the PCOS women as HR and NR (Figure 2a, c, d and Table 1) indicated no difference between these two groups regarding these C19 steroids with comparable A4:T ratios of 5.02 and 5.16, respectively, 1.2-fold greater than HV controls (4.42) (Figure 2b). While 11OHA4, 11KA4 and 11OHT levels were also similar within the HR and NR groups, 11KT levels were higher in the NR group than in the HR group and significantly higher (P=0.01607) than in the HV (Figure 2e and Table 1). The levels of the downstream metabolite, 5αDIONE, the precursor to DHT in the alternative biosynthesis pathway, were similar in the HR and NR groups and comparable to the HV (Table 1). Of interest is 3αDIOL (5α-androstane-3α, 17β-diol), the inactive metabolite of DHT, which was marginally lower in the HR group compared to the HV group, while in the NR group, 3αDIOL levels were lower, approaching significance (P=0.06) (Figure 2f).

Analyses of the C11-oxy C_{21} steroids

Comparative analyses of the combined PCOS group and the HV group showed that only cortisol (P<0.05) and cortisone (P<0.001) were significantly higher and different from the controls (HV) (Table 1). However, when analyzing the PCOS group as HR and NR groups, it was only in the HR group that cortisol levels were elevated (P<0.05) when compared to HVs (Figure 3a), while cortisone levels of both HR and NR groups were significantly increased (Figure 3c) with cortisol:cortisone ratios nevertheless comparable at 2.79 (NR), 3.64 (HR) and 3.46 (HV) (Table 1). CORT levels were, however, higher in the HR group (P<0.05) compared to the NR group and to the HV (P<0.01) (Figure 3e). Analyses of PROG and its 16- and 17-hydroxylated metabolites (Figure 3b, -d, -f) showed that these three steroids were lower in the NR group compared to the HR group (Table 1).

We also investigated further downstream products of the C11-oxy C_{19} steroids, PROG, as well as the C11-oxy C_{21} steroids. Since these steroids were present below the LOQ, samples with similar steroid metabolite profiles were combined, therefore enabling our analysis of estimated levels. However, certain steroids remained too low to quantify and only those shown in Table 2 could be quantitated accurately. In addition, 11OH-epietiocholanolone and

11OHAN as well as 11K-etiocholanolone and 11KAN could not be resolved chromatographically and were therefore quantified collectively as indicated in Table 2. These downstream metabolites were not statistically different when comparing the PCOS group to the HV group, however, 11OHAN and 11OH-epietiocholanolone, DHPROG and 11KDHPROG were ~2-fold higher in the PCOS group (Table 2).

Steroids below the LOQ in the combined samples, detected in the three groups, were: DHT, 11KDHT, AN, epietiocholanone, 11KPROG, 11αOHPROG, pdione, pdiol and pregnanetriol.

Relationships between steroids

We carried out a principal component analysis (PCA) to identify all relevant steroids other than those generally analyzed in the diagnosis of PCOS patients. Depicted in the PCA biplot is an overview of the statistical analyses of the HV and the two PCOS groups–a deviation from the cohort of control samples (Figure 4, black circle) is indicative of a relationship between steroids correlated with the HR (grey circle) and NR (dashed circle) groups of PCOS patients. Both HR and NR groups lie askance from the HV group–indicating a shift in the relationship between certain steroids. As expected, increased A4 and DHEA steroid levels are positively correlated (supplemental Fig. 3 and 4), reflecting the increased androgen production in hyperandrogenism characteristic of these PCOS patients. In addition, A4 and cortisone are visually correlated, as is 11OHA4 and cortisol (supplemental Fig. 3 and 4). Cortisone, cortisol, CORT and 16OHPROG are also positively correlated with each other in our PCOS group (supplemental Fig. 3 and 4), suggesting elevated levels of one would be associated with increased levels of the other. 11KT was also identified as a relevant steroid in PCOS and may be of importance when compared to the HV group, but more so in the NR group than the HR group, reflected in the higher 11KT levels detected in our NR group (Figure 2e). Interestingly, while PROG and 17OHPROG were strongly correlated (supplemental Fig. 3 and 4), 11KT was negatively correlated with these C_{21} steroids, (Figure 4) reflecting the differences observed between the C_{21} and C_{19} steroid levels in the HR and NR groups within the PCOS group.

DISCUSSION

The significance of adrenal androgen biosynthesis in the pathogenesis of PCOS has been the subject of intense investigation over the last half century. In this work our results, by the use of a novel high-throughput UPC^2-MS/MS method, point out that C11-oxy steroids have the potential of being used diagnostically to identify younger women and adolescents with PCOS who also have some evidence of micronodular adrenocortical hyperplasia.

Our data show that circulating C_{19} steroid levels are significantly increased in the PCOS group with DHEA (±2.5-fold) and A4 (±2.1-fold) higher than in our healthy controls. While the ovary would also contribute to these steroids in circulation, the origin of the C11-oxy C_{19} steroids is attributed to the adrenals with the liver also potentially contributing towards 11OHT levels (17). 11KT was significantly higher (±1.4-fold) in the PCOS group and considering the C11-oxy C_{19} steroids collectively, these steroids were more abundant than A4 and T, when not taking DHEA into account. It should be noted that adrenal DHEA is

also peripherally converted to A4 and T. In our investigation, we included the analyses of the downstream inactivated metabolites of DHT (3αDIOL and AN) and of 11OHA4 and 11KA4 (11KAN, 11OHAN, 11keto-etiocholanolone and 11OH-epietiocholanolone). DHT was detected below the LOQ in both groups despite the fact that A4 and T levels were higher in PCOS patients. AN was detected below the LOQ, while 3αDIOL was ±1.5-fold lower in PCOS compared to HVs. It may be that these metabolites together with T and DHT, are conjugated and excreted efficiently in the PCOS group or that the inactivation by 3α-hydroxysteroid dehydrogenases (3αHSDs), such as AKR1C2 (3αHSD type 3) is less efficient in these patients. In addition, 5αDIONE levels were similar in the PCOS group compared to the control group suggesting the alternative pathway in the production of DHT may be comparable in both groups. Similar findings regarding the C11-oxy C_{19} steroids, in which 11KDHT was also detected below the LOQ, support the downstream metabolism of 11OHA4 and 11KA4 to their AN and etiocholanolone derivatives, via 5α- and 5β-reductases (SRD5A) and AKR1C2.

The data show that the C11-hydroxy downstream metabolites were detected at higher levels than the C11-keto derivatives, in line with the higher 11OHA4 and lower 11KA4 precursor levels. Interestingly, even though 11OHA4 levels were not statistically different in PCOS patients, our PCA biplot indicated that the steroid was prominent in the HR group as is reflected in the shift towards elevated 11OHA4 levels in this group in comparison to the HV group, suggesting a potential role for this steroid in PCOS.

Further analysis of adrenocortical steroidogenesis between the two PCOS subgroups that we identified showed that while DHEA, T and A4 levels were similar in the HR and NR groups, differences were apparent with 11KT levels being higher in the NR group, together with lower 3αDIOL, 11OHAN and 11OH-epietiocholanolone levels (1.5-fold), while 11KAN and 11keto-etiocholanolone were 4.4-fold higher. It would seem that the shunt towards androgen production and, in particular, adrenal androgen production as indicated by the C11-keto metabolites, is greater in the NR group in comparison to the HR group. Furthermore, our PCA biplot clearly showed that 11KT is negatively correlated with PROG and 17OHPROG, suggesting an increase in 11KT may lead to a decrease in PROG and 17OHPROG, as is reflected in their levels (Table 1). This is expected, as a shift in the production of androgens will decrease precursor steroids in the production of progesterone metabolites. Interestingly, 16OHPROG is positively correlated with the PCOS group (specifically the HR group), suggesting increases in this steroid could be a biomarker that may be studied in these patients in the future.

Considering the C_{21} steroids, cortisol and cortisone were significantly increased in the PCOS group, CORT levels were significantly higher in the HR group (2.24-fold) compared to the NR group. These data suggest that there is a greater steroid flux in the glucocorticoid and possibly the mineralocorticoid pathways of the HR group in comparison to the NR group. This was further supported by PROG levels being higher in the HR group (5.75-fold) compared to the NRs, as well as the levels of 17OHPROG (1.38-fold) and 16OHPROG (2-fold). We previously reported that recombinant cytochrome P450 17α-hydroxylase (CYP17A1) catalyzes 17OHPROG and 16OHPROG production at 4:1 with a 3.25:1 ratio shown in adult testicular microsomal preparations, and deviations from this ratio is

associated with clinical conditions characteristic of adrenal disorders such as Cushing syndrome, congenital adrenal hyperplasia (CAH), and in 21-hydroxylase deficient (21OHD) patients(18–20). The increased 16OHPROG and 17OHPROG levels in our patients with PCOS clearly skewed the normal ratio which is markedly smaller in the HR group (2.98) compared to the NR group (3.94). Furthermore, our *in vitro* studies recently identified downstream metabolites of 16OHPROG following the backdoor pathway, comparable to 17OHPROG (20,21), suggesting these metabolites may have a role to play in PCOS. 17OHPROG and 21dF levels (median), although elevated in PCOS, lie within the normal range for these steroids in all of the groups (22,23) ruling out other adrenal disorders such as CAH. 17OHPROG and 21dF are generally considered as markers in CAH characterized by 21OHD. Both 21dF and 11OHPROG were detected at higher levels, 2- and 1.3-fold, respectively in the HR group, levels nevertheless were not within abnormal CAH ranges.

Contributing to circulating cortisol levels is the activation of cortisone; dysregulation of 11βHSD1 in PCOS has been implicated in the increased production of cortisol (24). Although our data show similar cortisone levels in both the HR and NR groups, we detected higher cortisol levels in the HR group, however, ratios did not differ significantly. It has been suggested that increased cortisol in PCOS patients leads to an upregulation of adrenal steroidogenesis resulting in C_{21} steroid production at the expense of C_{19} steroids (7), as is the case in the HR group in terms of the aforementioned steroids. Of note is 11βOHPROG which was detected at higher levels in the HR group, with this steroid presenting as a potential substrate for peripheral 11βHSD2 and SRD5A enzymes yielding 11KPROG and subsequently 11KDHPROG, which we also detected to be present at higher levels (1.88-fold) in the HR group. In addition, the SRD5A reduced metabolite of PROG, DHPROG was also higher in the HR group (1.3-fold higher), although these levels cannot be attributed solely to the adrenal production of PROG. While it has been reported that increased UFC together with that of adrenal androgen excretion is associated with increased peripheral conversion by SRD5A, not associated with body mass and insulin, SRD5A has also been reported to be upregulated in PCOS (25).

The aforementioned C_{21} and C11-oxy C_{21} steroid metabolites, present at higher levels in the HR group, strongly suggest a greater steroid shunt towards the C_{21} steroids in this subset of PCOS women, also supported by data showing the peripheral conversion of PROG and 11OHPROG by SRD5A. We have shown that 21dF is converted to 11KDHT via 11KAN in the backdoor pathway (26) together with 11OHPROG and 11KPROG also yielding 11KDHT via the same metabolic route (27). It is therefore possible that the C11-oxy C_{21} steroids, 21dF and 11OHPROG, and more specifically 11KDHPROG may add to the androgen pool via the backdoor pathway thus contributing to androgen excess in PCOS, more so in the HR group than in the NR group. Our data therefore suggests that in addition to measuring A4, T and DHEA (and DHEAS) levels in PCOS patients, 11OHA4, 11KT and perhaps DHPROG and 11KDHPROG should be included as biomarkers to analyze adrenal androgen excess in these patients. Furthermore, as CORT, together with cortisol and cortisone, also show a strong correlation in our PCOS patients, the overproduction of mineralocorticoids and glucocorticoids, including 16OHPROG, could also be monitored in these patients.

A limitation of the current study is that most of the steroid metabolites, necessary to fully characterize steroid panels in PCOS patients, are commercially unavailable and therefore the analyses of the complete C_{21} and C_{19} steroids, together with the C11-oxy C_{21} and C11-oxy C_{19} cannot be fully assessed in the metabolic pathways of patients. However, as this technology becomes cheaper and more available, studies like this can serve as guides for novel disease markers in PCOS and other disorders of aberrant steroidogenesis.

In conclusion, there appears to be indeed a subgroup of women with PCOS and abnormal adrenocortical steroidogenesis that may be detected by the measurement of specific novel biomarkers such as the C11-oxy C_{19} and C_{21} steroids. This study represents a small step towards the characterization of a distinct subgroup of women with PCOS who may in fact be suffering from an adrenocortical disorder. Whether this is also accompanied by a primary defect in ovarian steroidogenesis or their ovarian dysfunction is secondary to the abnormal adrenal steroids remains unknown. In any case, treatment of these women with PCOS may be fundamentally different in the future, as these patients could benefit from pharmacologic manipulation of their adrenocortical function to mitigate the signs and symptoms of PCOS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Article impact points

- Adrenal C11-oxy steroids may be clinically important in identifying young patients with PCOS and adrenal abnormalities.
- The steroids presented in our manuscript have not yet been considered in the clinical setting so far, and we believe that this study could represent a first focused step towards the characterization of a distinct subgroup of women with PCOS who may in fact be treated differently than the average patient with PCOS.
- This paper can change the understanding of PCOS as one disorder: it is in fact a heterogeneous condition. In addition, for the subgroup of PCOS associated with adrenocortical dysfunction, our paper provides novel hormonal markers that can be used diagnostically. Finally, the paper also adds to the basic pathophysiological understanding of adrenocortical-ovarian interactions in steroidogenesis of young women and adolescent girls with PCOS.

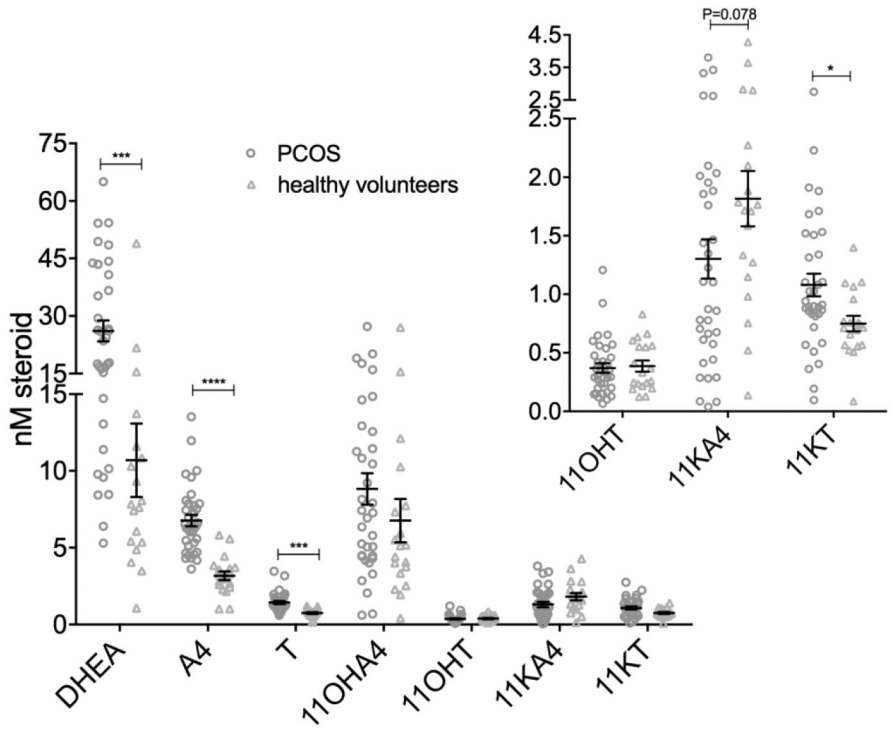

Figure 1:

Analysis of circulating classic C_{19} and C11-oxy C_{19} steroids in PCOS women and healthy controls. DHEA, dehydroepiandrosterone A4, androstenedione; T, testosterone; 11OHA4, 11 β-hydroxyandrostenedione; 11OHT, 11 β-hydroxytestosterone; 11KA4, 11-ketoandrostenedione; 11KT, 11-ketotestosterone. Statistical significance was determined by multiple t tests of two-way ANOVA (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

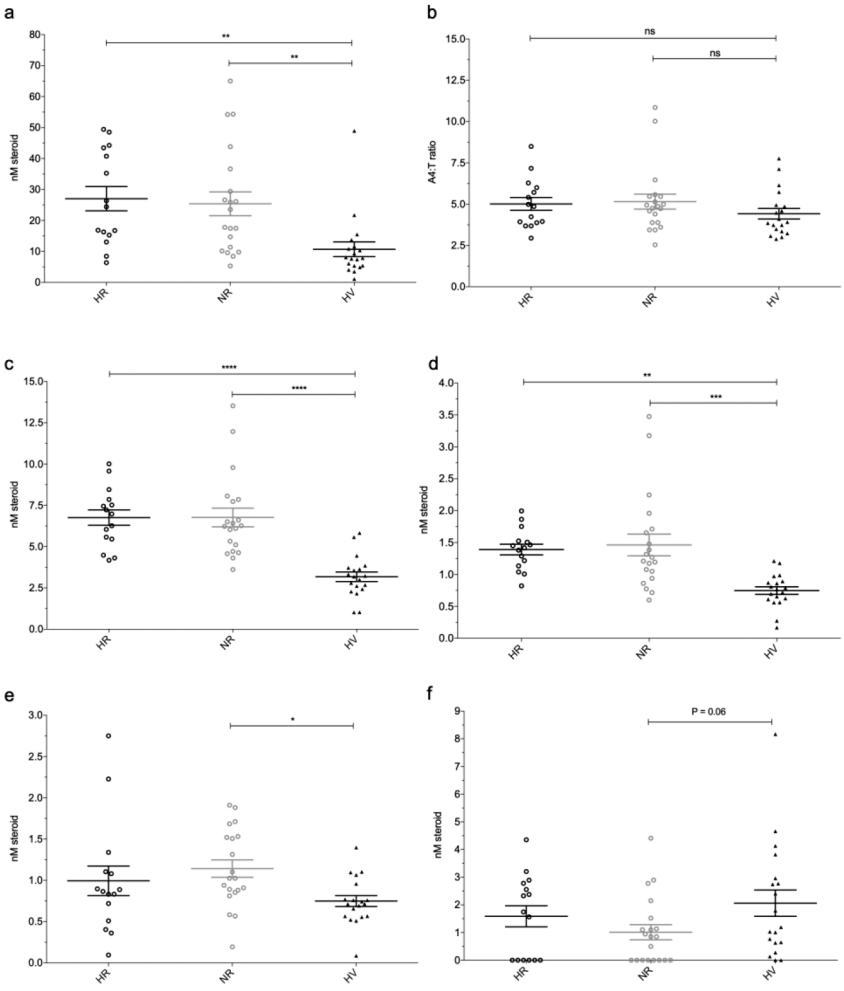

Figure 2:

Comparison of significantly different circulating C19 steroids (nM) in PCOS women and healthy controls. PCOS women: HR, high responders, n=15; NR, normal responders, n=20; and HV, healthy sex- and age-matched volunteers, n=20. (a) dehydroepiandrosterone (DHEA); (b) A4(androstenedione):T(testosterone) ratio; (c) A4; (d) T; (e) 11-ketotestosterone (11KT); (f) 5α-androstane-3α,17β-diol (3αDIOL). Statistical significance was determined by One-way ANOVA and unpaired t-tests. (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns= not significant).

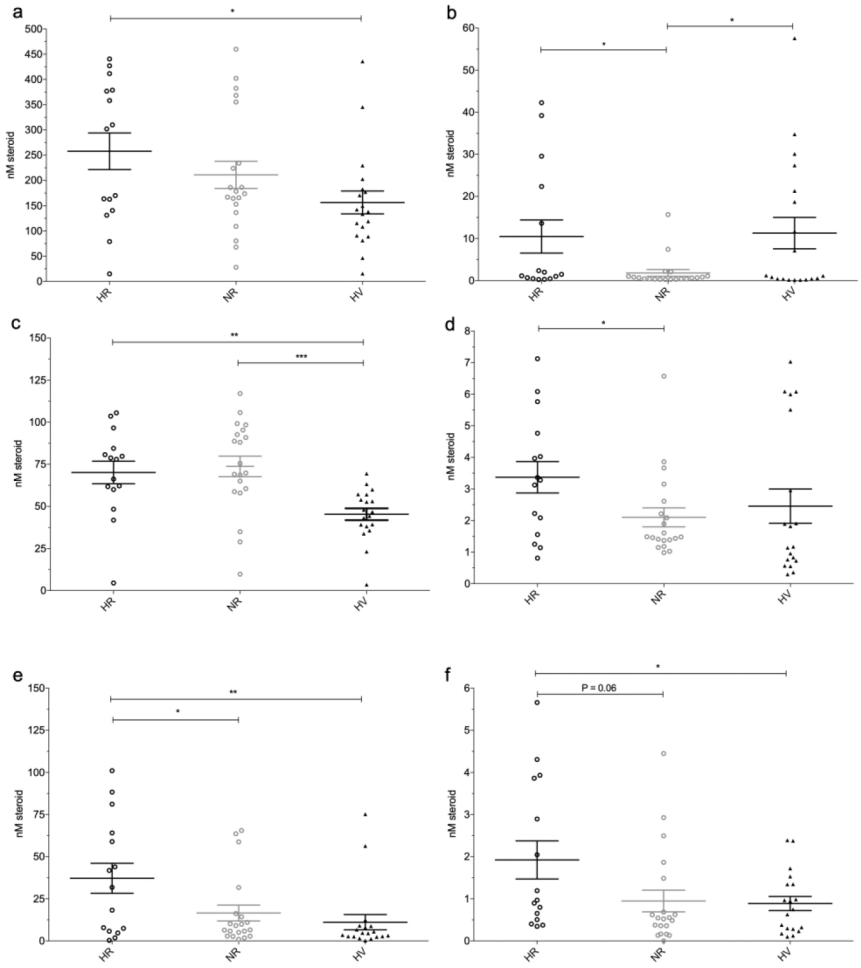

Figure 3:

Comparison of significantly different circulating C21 steroids (nM) in PCOS women and healthy controls. PCOS women: HR, high responders, n=15; NR, normal responders, n=20; and HV, healthy sex- and age-matched volunteers, n=20. (a) cortisol; (b) progesterone (PROG); (c) cortisone (d) 17α-hydroxyprogesterone (17OHPROG); (e) corticosterone (CORT); (f) 16α-hydroxyprogesterone (16OHPROG). Statistical significance was determined by One-way ANOVA and unpaired t-tests. (*P<0.05, **P<0.01, ***P<0.001)

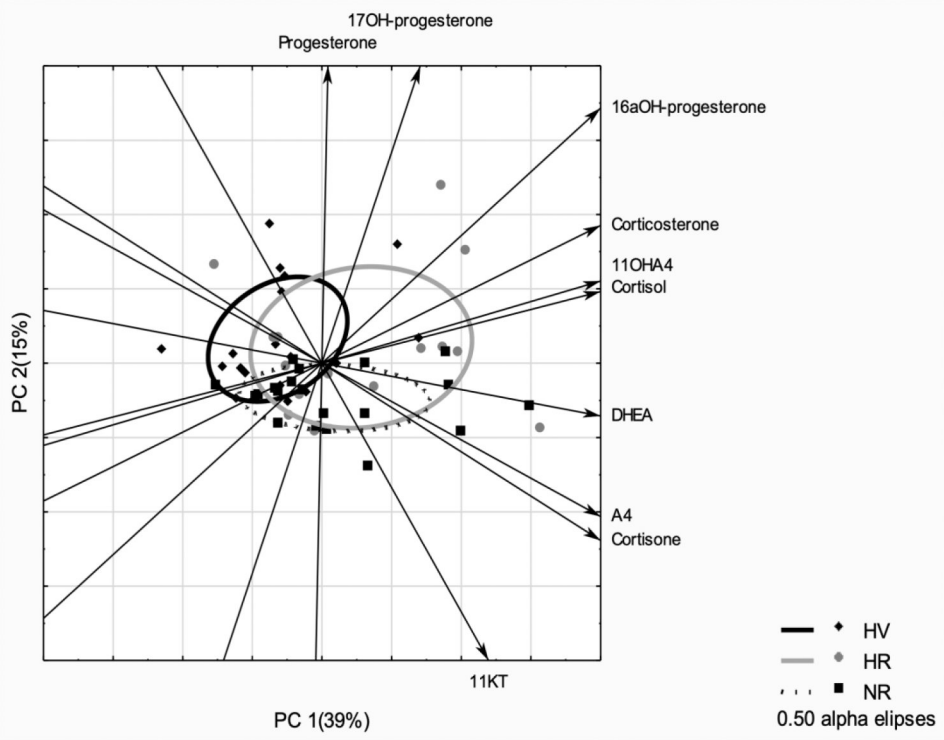

Figure 4:

PC2 plot versus PC1 plot, visually indicating relationships between steroids in the three groups. HV - healthy volunteers; NR - PCOS normal responders; HR, PCOS high responders.

Table 1.

Plasma steroid concentrations (nmol/L) and steroid ratios.

Steroid metabolite	PCOS *n*=35	PCOS HR *n*=15	PCOS NR *n*=20	HV *n*=19
DHEA	26.09 ± 15.93[a]	27.03 ± 14.66[b]	25.38 ± 16.79[b]	10.69 ± 10.14
A4	6.77 ± 2.19[a]	6.76 ± 1.74[a]	6.77 ± 2.47[a]	3.18 ± 1.21
T	1.43 ± 0.60[a]	1.39 ± 0.31[a]	1.46 ± 0.74[a]	0.75 ± 0.26
11OHA4	8.82 ± 5.99	10.27 ± 7.55	7.73 ± 4.15[a]	6.76 ± 5.98
11KA4	1.30 ± 0.98	1.31 ± 1.07	1.30 ± 0.91	1.82 ± 1.00
11OHT	0.37 ± 0.24	0.35 ± 0.27	0.39 ± 0.21	0.39 ± 0.20
11KT	1.08 ± 0.56[c]	0.99 ± 0.67	1.14 ± 0.46[c]	0.75 ± 0.28
5αDIONE	0.67 ± 0.44	0.66 ± 0.29	0.68 ± 0.53	0.73 ± 0.35
3αDIOL	1.26 ± 1.33	1.59 ± 1.43	1.01 ± 1.19	2.06 ± 2.02
PROG	5.53 ± 10.88	10.48 ± 14.72	1.82 ± 3.54	11.28 ± 15.82
17OHPROG	2.64 ± 1.70	3.37 ± 1.87	2.10 ± 1.31	2.45 ± 2.30
16OHPROG	1.37 ± 1.48	1.92 ± 1.69[cd]	0.95 ± 1.12	0.89 ± 0.71
CORT	25.39 ± 28.63	37.17 ± 33.33[cd]	16.56 ± 20.48	11.12 ± 19.24
Cortisone	72.21 ± 25.99[a]	70.15 ± 25.10[b]	73.75 ± 26.54[a]	45.35 ± 14.84
Cortisol	231.16 ± 127.44[c]	257.82 ± 135.51[c]	212.17 ± 117.13	156.44 ± 96.11
Steroid ratios				
A4: T	5.09 ± 1.77	5.02 ± 1.46	5.16 ± 1.97	4.42 ± 1.37
Cortisol: cortisone	3.15 ± 1.19	3.64 ± 1.37	2.79 ± 0.87	3.46 ± 1.85
17OHPROG: 16OHPROG	3.53 ± 3.12	2.98 ± 2.63	3.94 ± 3.38	2.81 ± 1.10

Data are expressed as median ± standard deviation. Statistical significance was determined by One-way ANOVA and unpaired t-tests.

[a] $P<0.001$,

[b] $P<0.01$,

[c] $P<0.05$ compared to healthy volunteers (HV) and

[d] $P<0.05$ comparison between high responders (HRs) and normal responders (NRs).

To convert nmol/L to ng/dL, multiply by 28.84 for dehydroepiandrosterone (DHEA); testosterone (T) and 5α-androstanedione (5αDIONE); 28.64 for androstenedione (A4); 30.24 for 11β-hydroxyandrostenedione (11OHA4) and 11keto-testosterone (11KT); 30.04 for 11keto-androstenedione (11KA4); 30.44 for 11β-hydroxytestosterone (11OHT); 29.24 for 3α-androstanediol (3αDIOL); 31.45 for progesterone (PROG); 33.05 for 17α-hydroxyprogesterone (17OHPROG); 16α-hydroxyprogesterone (16OHPROG); 34.65 for corticosterone (CORT); 36.04 for cortisone; and 36.25 for cortisol.

Table 2.

Circulating C_{19} and C_{21} steroids (nmol/L) in PCOS women and healthy controls below the low.

Steroid metabolite	PCOS		HV
	HR *n*=3	NR *n*=3	*n*=3
11OHAN and 11OH-epietiocholanolone	2.24 ± 1.27	1.49 ± 0.82	0.97 ± 0.30
11KAN and 11K-etiocholanolone	1.69 ± 1.46	7.38 ± 9.80	6.78 ± 4.63
DHPROG	7.82 ± 2.09	5.83 ± 4.23	3.95 ± 0.84
11KDHPROG	2.22 ± 1.28	1.18 ± 1.16	1.01 ± 0.40
21dF	0.70 ± 0.62	0.33 ± 0.20	0.14 ± 0.12
11βOHPROG	0.60 ± 0.46	0.45 ± 0.48	0.43 ± 0.44

Data are expressed as median (±SD). HR, high responders; NR, normal responders; and HV, healthy volunteers (n=1 represents 4 combined samples).