11-oxygenated C19 steroids as circulating androgens in women with polycystic ovary syndrome

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Abstract. 11-oxygenated C19 steroids (11oxC19s) are newly specified human androgens. Although median serum levels of 11oxC19 were reported to be higher in patients with polycystic ovary syndrome (PCOS) than in unaffected women, inter-individual variations in androgen levels among PCOS patients have poorly been investigated. Here, we quantified four 11oxC19s, i.e., 11-ketotestosterone (11KT), 11β-hydroxytestosterone (11OHT), 11β-hydroxyandrostenedione (11OH Δ4A), and 11-ketoandrostenedione (11K Δ4A), in blood samples of 28 PCOS patients and 31 eumenorrheic women using liquid chromatography-tandem mass spectrometry. We referred to our previous data of classic androgens in these individuals. We found that 11OHT levels were higher in the PCOS group than in the eumenorrheic group. Moreover, although the median values of 11KT, 11KΔ4A, and 11OHΔ4A were comparable between the two groups, these steroids were markedly increased in some patients. Of the 28 patients, 8 had high levels of both 11oxC19s and classic androgens, whereas 4 had an increase only in 11oxC19 levels, and 12 had an increase only in classic androgen levels. Intragroup variations in androgen levels were relatively large in the PCOS group. Levels of 11OHT and 11KT were significantly higher in overweight/obese patients than in normal weight patients and correlated with body mass indexes. These results highlight the clinical significance of 11oxC19s as circulating androgens in PCOS patients and indicate that the accumulation of 11oxC19s and/or classic androgens is an essential feature of PCOS. The profiles of circulating androgens appear to vary among patients. In particular, overweight/obesity likely enhances the 11oxC19s accumulation in PCOS, although this notion awaits further validation.

Key words: Liquid chromatography-tandem mass spectrometry, Non-classic androgen, Polycystic ovary syndrome, 11β-hydroxytestosterone, 11-ketotestosterone

II-OXYGENATED C19 STEROIDS (11oxC19s) represent non-classic androgens that have recently been specified in humans [1, 2]. 11oxC19s are predicted to be produced predominantly in the adrenal gland from andro-
and 11KΔ4A [3-5]. In vitro reporter assays revealed that androgenic activity of 11KDH and 11KT is almost comparable to that of DHT and T respectively, while the activity of 11OHΔ4A and 11KΔ4A is ~50% of that of DHT and T respectively [3]. 11OHΔ4A and 11KΔ4A have minimal androgenic activity [3]. Since 11oxC19s are nonaromatizable androgens and serum levels of 11KT in females are as high as those of T [2], these steroids likely play a significant role in women’s health.

Polycystic ovary syndrome (PCOS) is a relatively common endocrinopathy affecting 6–20% of women at reproductive ages [6-8]. Patients with PCOS typically present with hyperandrogenic symptoms and obesity, in addition to ovarian dysfunction [6]. We have previously shown that multiple steroidogenic pathways are involved in the overproduction of Δ4A and T in PCOS patients [9]. In our previous study, however, we also identified several PCOS patients who retained normal serum levels of Δ4A, T, and DHT, despite having hyperandrogenic symptoms. Similarly, other studies have reported that only ~68% of PCOS patients have increased serum levels of Δ4A or T [10, 11]. These results indicate that Δ4A, T, and DHT cannot account for the entire etiology of hyperandrogenism in PCOS.

In 2017, O’Reilly et al. quantified serum levels of classic and non-classic androgens and urinary levels of androgen metabolites in 114 PCOS patients and 49 unaffected women [12]. The authors found that the levels of 11KT, 11OHT, 11OHΔ4A and 11KΔ4A were significantly higher in the PCOS group than in the control group. This study provided the first evidence that accumulation of 11oxC19s contributes to the phenotype in PCOS. However, O’Reilly et al. focused primarily on the median steroid values in the patient and control groups, and did not describe inter-individual variations in the PCOS group. Thus, the frequency of biochemical hyperandrogenemia among PCOS patients and modifiers of androgen accumulation in PCOS remain to be clarified. To address these unsolved issues, we measured androgen levels in 28 PCOS patients and 31 eumenorrheic women.

**Materials and Methods**

**Subjects**

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and performed after obtaining written informed consent from all participants. The study group included 28 Japanese patients with PCOS and 31 Japanese women with regular menstrual cycles. These participants were recruited in our previous study [9]. The PCOS patients were diagnosed based on the Rotterdam criteria. The median age and body mass index (BMI) were similar between the PCOS and eumenorrheic groups (29.5 years vs. 24.0 years, p = 0.114, and 23.0 kg/m² vs. 21.0 kg/m², p = 0.39, respectively) [9].

The PCOS and eumenorrheic groups were further divided into overweight or obese and normal weight subgroups. Individuals with BMI of 25.0–29.9 kg/m² and those of greater than 30.0 kg/m² were regarded overweight and obese, respectively [13]. Our participants comprised 5 obese, 6 overweight, and 17 normal weight PCOS patients, and 8 overweight and 23 normal weight eumenorrheic women. Furthermore, we also subdivided...
the PCOS group according to the presence or absence of clinical symptoms of hyperandrogenism. Patients were assessed for hirsutism, acne, and male-type alopecia; 18 women were found to have one or more of these symptoms.

Blood samples
Blood samples were collected in our previous study [9]. Samples of the eumenorrheic women were collected during the mid-follicular phase and those of PCOS patients were obtained during the course of diagnostic evaluations when the patients had no follicles with a diameter of >9 mm. In that study, we measured serum levels of classical androgens using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and dehydroepiandrosterone-sulfate (DHEA-S) using chemiluminescent enzyme immunoassay.

Steroid quantification
In the present study, we quantified levels of 11OHΔ4A, 11KΔ4A, 11OHT, and 11KT using LC-MS/MS (ASKA Pharmaceutical Medical Corporation, Kanagawa, Japan). Approximately 20 μL of each sample after extraction and derivatization was analyzed using an API-5000 triple stage quadrupole mass spectrometer equipped with a positive electrospray ionization (ESI) source (AB SCIEX, Foster City, CA, USA) and an Agilent 1290 Infinity II LC system (Agilent Technologies, Santa Clara, CA, USA). The temperature of the Capcellcore C18 column, Capcellcore ADME (Osaka SODA company, Osaka, Japan) or the Xselect HSS C18 SB column (Milford, MA, USA) was maintained at 50°C. N₂ was used as the collision gas. The mobile phase consisting of 0.1% formic acid (solvent A) and methanol (solvent B) was used for gradient elution. Solvents A/B were used from 45:55 to 0:100 between 0 and 14.0 minutes. The following ESI conditions were used: ion spray voltage, 5,500 eV; collision gas, 11 psi; curtain gas, 22 psi; ion source temperature, 500°C; ion source gas 1, 70 psi; ion source gas 2, 80 psi.

The inter-assay coefficients of variabilities calculated from the data of five replicates of three pools ranged from 5.7% to 14.8%. The intra-assay coefficients of variabilities calculated from five replicates of three samples ranged from 1.7% to 13.4%. Analytical recovery ranges were between 82.2% and 110.0%. The lower detection limits were 50 pg/mL for 11OHA4A and 11KΔ4A, 10 pg/mL for 11OHT, and 20 pg/mL for 11KT.

Data analyses
We examined the differences in serum androgen levels between the PCOS and eumenorrheic groups. We also investigated a possible correlation between serum levels of 11oxC19s and DHEA-S, a marker for adrenal androgen production [14]. In this context, we referred to our previous data regarding DHEA-S and classic androgens of the participants [9].

Next, we analyzed the frequency of hyperandrogenemia among PCOS patients. Hyperandrogenemia was defined as serum values of 11oxC19s or classic androgens that were above the 95th percentile of the eumenorrheic group.

Then, we examined differences in serum levels of 11oxC19s and classic androgens between overweight/obese and normal weight individuals. The possible correlation between BMI and androgen values was also investigated. Furthermore, we analyzed the differences in androgen levels between patients with and without clinical symptoms of hyperandrogenism.

Lastly, we calculated ratios between two androgens in the PCOS and eumenorrheic groups. Calculations of steroid metabolite ratios are often employed to estimate the activity of enzymes involved in the steroid biosynthesis [15]. In the present study, we analyzed 11OHA4A/Δ4A and 11OHT/T as markers for CYP11B1 activity, T/Δ4A and 11KT/11KΔ4A as markers for AKR1C3 activity, and 11KΔ4A/11OHA4A and 11KT/11OHT as markers for HSD11B2 activity (Fig. 1).

Statistical analyses
Statistical analyses were performed using the EZR software (version 1.36; Saitama Medical Center, Jichi Medical University, Saitama, Japan). Normality was assessed via a visual check on histograms and by the Schapiro-Wilk test. The Mann-Whitney U test was used to analyze non-normally distributed data. The quantitative correlation among steroids was examined by the Spearman’s correlation test. The Fisher’s exact test was used to assess the prevalence of rare (expected frequency of less than 5) events.

Results
Serum levels of 11oxC19s in the PCOS and eumenorrheic groups
All samples analyzed in this study yielded 11oxC19 values above the lower detection limits. As shown in Fig. 2, levels of 11OHT were significantly higher in the
PCOS group than in the eumenorrheic group. Furthermore, the median values of 11KΔ4A and 11KT were higher in the PCOS group than in the eumenorrheic group, although the intergroup differences were not significant. Levels of 11OHΔ4A, which was the most abundant 11oxC19s examined in this study, were comparable between the two groups. The intragroup variations in the levels of the four 11oxC19s were larger in the PCOS group than in the eumenorrheic group (Supplementary Table 1). In both groups, 11KT levels were significantly correlated with DHEA-S levels (r = 0.457, p = 0.0146 in the PCOS group and r = 0.589, p = 0.000485 in the eumenorrheic group).

**The frequency of hyperandrogenemia in PCOS patients**

Of the 28 PCOS patients, 24 showed increased serum levels of 11oxC19s and/or classic androgens (Table 1, Supplementary Table 2). T and Δ4A were both increased in more than 50% of the patients, while the levels of each of the remaining androgens were increased in 4–8 patients. Consequently, 8 patients had high levels of both 11oxC19s and classic androgens, whereas 4 patients showed an increase only in 11oxC19 levels, and 12 patients showed an increase only in classic androgen levels (Supplementary Table 2).

**Serum levels of androgens in overweight/obese and normal weight individuals**

Levels of 11OHT and 11KT were significantly higher in overweight/obese PCOS patients than in normal weight patients, whereas such difference was not apparent in eumenorrheic women (Fig. 3). In the PCOS group but not in the eumenorrheic group, levels of 11OHT and 11KT were correlated with BMI (Fig. 4). In both PCOS and eumenorrheic groups, obesity had no effect on levels of 11OHΔ4A or 11KΔ4A (Fig. 3).

**Serum levels of androgens in PCOS patients with and without clinical symptoms of hyperandrogenism**

Levels of 11oxC19s and/or classic androgens were increased in 16 of the 18 patients with clinical symptoms of hyperandrogenism, and also in 8 of 10 patients without such symptoms (Table 1). No significant differences were observed in 11oxC19 levels between the two subgroups, although the median values of 11OHA4A and 11KA4A were slightly higher in PCOS patients with symptoms of hyperandrogenism than in those without symptoms (Supplementary Fig. 1). Similarly, the levels of classic androgens measured in our previous study were comparable between PCOS patients with and without hyperandrogenic symptoms.

**Estimated activities of enzymes involved in the 11oxC19s production**

As shown in Fig. 5, the enzymatic activity of AKR1C3 estimated from T/Δ4A and 11KT/11KΔ4A and HSD11B2 activity estimated from 11KA4A/11OHA4A and 11KT/11OHT were comparable between the PCOS
and eumenorrheic groups. 11OHA4A/Δ4A was lower in the PCOS group than in the eumenorrheic group, suggesting slightly decreased CYP11B1 activity in PCOS. Mildly decreased 11OHT/T in the PCOS group supports this notion, although the intergroup difference was not statistically significant.

11KΔ4A/11OHΔ4A was lower in overweight/obese PCOS patients than that in normal weight patients, suggesting a relatively low HSD11B2 activity in overweight/obese patients. 11KT/11OHT, the other marker for HSD11B2 activity, was also relatively low in overweight/obese patients. Increased 11KT/11Δ4A in overweight/obese PCOS patients was suggestive for increased enzymatic activity of AKR1C3; however, T/Δ4A ratio, the other marker for AKR1C3 activity, was relatively low in overweight/obese PCOS patients. There were no differences in the estimated CYP11B1 activity between overweight/obese and normal weight patients.

### Discussion

In this study, we found that the PCOS group had higher serum levels of 11OHT than the eumenorrheic group. Moreover, although there were no significant intergroup differences in the levels of 11KT, 11KΔ4A, or 11OHΔ4A, these steroids were markedly increased in some of the PCOS patients. Consequently, 12 of the 28 PCOS patients showed increased levels of at least one of the four 11oxC19s analyzed. In particular, 8 patients had increased levels of 11KT, a steroid whose in vitro binding activity to the androgen receptor is comparable to that of T [3-5]. The results of this study highlight the clinical importance of 11oxC19s as circulating androgens in PCOS. Notably, serum levels of 11KT in the PCOS and eumenorrheic groups were closely correlated with those of DHEA-S, indicating that both basal and excessive 11oxC19s are produced primarily in the adrenal gland. The functional significance of the adrenal gland as the source of 11oxC19s is supported by recent findings that 11oxC19s are overproduced in the adrenal gland.
gland of patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency [16]; median serum values of 11oxC19s of these patients were much higher than those of our cases.

Of the 28 PCOS patients, 24 showed increased serum levels of 11oxC19s and/or classic androgens. Importantly, 4 patients had increased levels of 11oxC19s but retained normal levels of classic androgens. These findings would explain why several PCOS patients have been found to develop clinical symptoms of hyperandrogenism in the absence of accumulation of classic androgens [11, 17, 18]. On the other hand, 20 of our PCOS patients showed increased serum levels of classic androgens. Thus, both 11oxC19s and classic androgens seem to play significant roles in the phenotype of PCOS.

Our data suggest that the patterns of androgen excess are variable among PCOS patients. Intragroup variations in serum androgen levels were larger in the PCOS group than in the eumenorrheic group. These results likely reflect heterogeneous enzymatic defects in PCOS patients. Indeed, PCOS is known as an etiologically heterogeneous condition caused by several genetic and environmental factors [19-21]. Therefore, measurement of circulating 11oxC19s and classic androgens may support the diag-

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Fig. 3 Serum levels of androgens

Steroid values in overweight/obese (O, shown in red) and normal weight (N, shown in blue) polycystic ovary syndrome (PCOS) patients and eumenorrheic women (Eumenorrheic) are shown. The median values of each steroid are indicated by horizontal lines and the 95th percentile of the eumenorrheic group is depicted by broken lines. P-values of statistically significant differences are shown. Δ4A, androstenedione; T, testosterone; DHEA-S, dehydroepiandrosterone-sulfate; 11OΔ4A, 11β-hydroxyandrostenedione; 11OHT, 11β-hydroxytestosterone; DHT, dihydrotestosterone; 11KΔ4A, 11-ketoandrostenedione; 11KT, 11-ketotestosterone. The conversion factors to the SI unit: Δ4A, 0.00347 (nmol/L); T, 0.00347 (nmol/L); DHEA-S, 2.720 (nmol/L); and DHT, 0.00344 (nmol/L).
nostic classification of PCOS patients according to their etiologies.

Serum levels of 11OHT and 11KT were much higher in overweight/obese PCOS patients than in normal weight patients, while such difference was absent in eumenorrheic women. Furthermore, serum levels of these 11oxC19s in the patients were significantly correlated with BMI. These findings imply that overweight/obesity enhances enzymatic abnormalities of PCOS patients, resulting in greater accumulation of 11OHT and 11KT. Since no differences were observed in serum levels of classic androgens between overweight/obese and normal weight patients, increased BMI may exert more significant effects on the production of 11oxC19s than that of classic androgens. In this regard, it is known that weight loss in PCOS patients frequently ameliorates clinical features [22]. Such beneficial effects of weight loss may be, at least in part, ascribed to the suppression of 11oxC19 accumulation.

Serum levels of classic androgens and/or 11oxC19s were increased in 24 patients, including 8 patients who had no clinical symptoms of hyperandrogenism. While four of our 28 PCOS patients showed normal serum values of all tested androgens, these patients may have elevated levels of 11OHDHT or 11KDHT, two potent androgens whose levels were not measured in this study. The results of this study challenge the current understanding that substantial percentages of PCOS patients retain normal androgen levels [10, 11]. It appears that hyperandrogenemia is an essential feature of PCOS. However, since there were no significant differences in the serum levels of 11oxC19s and classic androgens between PCOS patients with and without clinical symptoms of hyperandrogenism, the phenotypes of the patients could not be simply determined by the circulating androgen levels.

Relatively low 11OHΔ4A/Δ4A and 11OHT/T in the PCOS group suggest that the activity of CYP11B1, an enzyme that catalyzes the conversion of classic androgens into 11oxC19s (Fig. 1), is not enhanced in most PCOS patients. Moreover, estimated activities of AKR1C3 and HSD11B2 were not particularly high in the
patient group. Thus, other mechanisms, such as overproduction of Δ4A and T in the adrenal gland or decreased metabolism of 11oxC19s in peripheral tissues, may largely contribute to the accumulation of 11oxC19s in PCOS. However, considering the heterogeneity of enzymatic defects in PCOS (mentioned above), it is possible that CYP11B1, AKR1C3, or HSD11B2 activity is increased in a small fraction of PCOS patients.

Relatively high 11KT/11KΔ4A in the overweight/obese patients implies that obesity can enhance AKR1C3 activity in PCOS. In addition, there was a correlation between 11KT levels and BMI only in the PCOS group. These results may reflect an excessive conversion of 11KΔ4A to 11KT in adipose tissues of the PCOS patients, because Wang et al. have detected a high AKR1C3 activity in subcutaneous fat of PCOS patients [23]. Furthermore, since AKR1C3 expression in adipose tissues of PCOS patients is known to be induced by insulin [24], high 11KT/11KΔ4A in our overweight/obese patients may be associated with insulin resistance. However, this notion needs to be confirmed in future studies by quantification of blood insulin levels in the patients. In this regard, while T/Δ4A, the other marker for AKR1C3 activity, was relatively low in our overweight/obese patients, this may be consistent with the prior findings that 11KΔ4A is a much better substrate for AKR1C3 than Δ4A [3]. Alternatively, high 11KT/11KΔ4A in the overweight/obese patients may reflect a reduction in the activity of 17β-hydroxysteroid dehydrogenase type 2 (HSD17B2) which catalyzes reverse reactions against AKR1C3 (Fig. 1), because HSD17B2 was shown to be downregulated in the liver of obese individuals [25]. Similarly, while relatively low 11KΔ4A/11OHΔ4A in our overweight/obese patients was indicative of a reduction of HSD11B2 activity, these results can also be ascribed to an increase in the activity of 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) which catalyzes reverse reactions against HSD11B2. Indeed, HSD11B1 is known to be upregulated in the adipose tissue of obese individuals [26].

The results of this study provided both similarities and differences with those of the previous study by O’Reilly...
First, both studies documented an accumulation of 11oxC19s and classic androgens in PCOS patients; however, there were differences in the median values of each androgen. In the European patients, serum values of Δ4A and its 11ox-metabolites were markedly higher than those in our cases, while the levels of T and its 11ox-metabolites were comparable to or slightly lower. Such a discrepancy may be due to ethnic differences. Indeed, it is known that serum levels of Δ4A tend to be higher in East Asian PCOS patients than in Caucasian patients [10], although the mechanism underlying such difference remains to be clarified. Since there was significant difference in serum Δ4A levels of the control groups between the previous and present studies, Caucasian women may have higher basal Δ4A production than Japanese women. Second, unlike the results of the present study, O`Reilly et al. found no correlation between BMI and serum levels of 11OHT or 11KT. This may be attributable to the relatively high BMI in non-obese patients of the previous study compared to our cases (26.0 kg/m² vs. 20.6 kg/m²). Actually, non-obese patients reported by O`Reilly et al. exhibited a more significant increase in 11oxC19 levels than in our normal weight patients. Alternatively, the discrepancy may have resulted from the ethnic difference in enzymatic defects of PCOS.

This study has three limitations. First, we were unable to measure 11OHDHT and 11KDHT levels, although these 11oxC19s are predicted to have strong androgenic activity [3, 5]. Since the activity of 5α-reductase, an enzyme that mediates the biosynthesis of 11OHDHT and 11KDHT, is known to be increased in PCOS [27], these 11oxC19s may be significantly accumulated in PCOS patients. Second, clinical information of our patients was limited. Thus, we could not examine the possible association between 11oxC19 levels and insulin resistance, which was suggested by O`Reilly et al. [12]. Indeed, relatively low 11KΔ4A/11OHΔ4A in our overweight/obese patients may be associated with increased activity of HSD11B1, which underlies insulin resistance by enhancing the conversion of cortisone to cortisol [28]. Third, the number of subjects was small. In the future studies, we need to study a large number of patients with variable degree of obesity.

In conclusion, the results of this study highlight the clinical significance of 11oxC19s as circulating androgens in PCOS and suggest that accumulation of 11oxC19s or classic androgens is an essential feature in PCOS. The profiles of circulating androgens appear to vary among patients, possibly reflecting heterogeneous enzymatic defects in PCOS. In particular, overweight/obesity likely enhances the accumulation of 11oxC19s, although this notion awaits further validation.

**Disclosure**

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<table>
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<tr>
<th>11oxC19s</th>
<th>F-test</th>
<th>p-value</th>
<th>Standard deviation (pg/mL)</th>
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<td>11OHΔ4A</td>
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<td>PCOS group (n = 28) 759.2</td>
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<td>11KΔ4A</td>
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<td>11OHT</td>
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<tr>
<td>11KT</td>
<td>0.000261</td>
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<td>440.8</td>
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11oxC19s, 11-oxygenated C19 steroids; PCOS, polycystic ovary syndrome; 11OHΔ4A, 11β-hydroxyandrostenedione; 11KΔ4A, 11-ketoandrostenedione; 11OHT, 11β-hydroxytestosterone; 11KT, 11-ketotestosterone.
**Supplementary Table 2** Serum levels of classic androgens and 11-oxygenated C19 steroids in the polycystic ovary syndrome group

<table>
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<tr>
<th>Patients</th>
<th>Clinical symptoms of hyperandrogenism&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Body mass index (kg/m²)</th>
<th>Classic androgens&lt;sup&gt;b&lt;/sup&gt;</th>
<th>11-oxygenated C19 steroids</th>
<th>11OHΔA4&lt;sup&gt;c&lt;/sup&gt;</th>
<th>11KΔA4A&lt;sup&gt;c&lt;/sup&gt;</th>
<th>11OHT&lt;sup&gt;c&lt;/sup&gt;</th>
<th>11KT&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>2.20&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>1.49&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>863.8</td>
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<td>1,421.2</td>
<td>332.9</td>
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Cut-off value<sup>c</sup>

Δ4A, androstenedione; T, testosterone; DHT, dihydrotestosterone; 11OHΔA4, 11β-hydroxyandrostenedione; 11KΔA4A, 11-ketoandrostenedione; 11OHT, 11β-hydroxytestosterone; 11KT, 11-ketotestosterone.

Androgen values above the cut-off values are boldfaced.

<sup>a</sup>Hirsutism, acne and/or male-type alopecia.

<sup>b</sup>Serum levels of classic androgens were measured in our previous study [9].

<sup>c</sup>The 95th percentile value of the eumenorrheic group.
Supplementary Fig. 1  Serum levels of steroids
Steroid values in polycystic ovary syndrome (PCOS) patients with clinical symptoms of hyperandrogenism (P/HA+) and without those symptoms (P/HA−) are shown in filled and open boxes, respectively. Steroid values in eumenorrheic women (E) are shown in open circles. The median values of each steroid are indicated by horizontal lines. The values of 95th percentile of the eumenorrheic group are depict by broken lines. Asterisks indicate significant differences (*: p-value < 0.05, **: p-values < 0.01). DHEA-S, dehydroepiandrosteronesulfate; 11OΔ4A, 11β-hydroxyandrostenedione; 11OHT, 11β-hydroxytestosterone; DHT, dihydrotestosterone; 11KΔ4A, 11-ketoandrostenedione; 11KT, 11-ketotestosterone.

References
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