Adrenal-derived 11-oxygenated 19-carbon steroids are the dominant androgens in classic 21-hydroxylase deficiency

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Abstract

Objective: To comprehensively characterize androgens and androgen precursors in classic 21-hydroxylase deficiency (21OHD) and to gain insights into the mechanisms of their formation.

Design: Serum samples were obtained from 38 patients (19 men) with classic 21OHD, aged 3–59, and 38 sex- and age-matched controls; 3 patients with 11β-hydroxylase deficiency; 4 patients with adrenal insufficiency; and 16 patients (8 men) undergoing adrenal vein sampling. Paraffin-embedded normal (n = 5) and 21OHD adrenal tissues (n = 3) were used for immunohistochemical studies.

Methods: We measured 11 steroids in all sera by liquid chromatography-tandem mass spectrometry. Immunofluorescence localized 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) and cytochrome b5 (CYB5A) within the normal and 21OHD adrenals.

Results: Four 11-oxygenated 19-carbon (11oxC19) steroids were significantly higher in male and female 21OHD patients than in controls: 11β-hydroxyandrostenedione, 11-ketoandrostenedione 11β-hydroxytestosterone, and 11-ketotestosterone (3–4-fold, P < 0.0001). For 21OHD patients, testosterone and 11-ketotestosterone were positively correlated in females, but inversely correlated in males. All 11oxC19 steroids were higher in the adrenal vein than in the inferior vena cava samples from men and women and rose with cosyntropin stimulation. Only trace amounts of 11oxC19 steroids were found in the sera of patients with 11β-hydroxylase deficiency and adrenal insufficiency, confirming their adrenal origin. HSD3B2 and CYB5A immunoreactivities were sharply segregated in the normal adrenal glands, whereas areas of overlapping expression were identified in the 21OHD adrenals.

Conclusions: All four 11oxC19 steroids are elevated in both men and women with classic 21OHD. Our data suggest that 11oxC19 steroids are specific biomarkers of adrenal-derived androgen excess.

Introduction

Steroid 21-hydroxylase deficiency (21OHD) accounts for the majority of congenital adrenal hyperplasia cases and is one of the most common autosomal recessive diseases (1). As a consequence of the steroid 21-hydroxylase (P450c21, CYP21A2) dysfunction, upstream steroids are diverted toward androgenic pathways. Severe or classic 21OHD leads to in utero virilization and ambiguous genitalia of affected girls (1). Females with mild or nonclassic 21OHD may present with hirsutism, acne, and irregular menses (2). The excessive adrenal androgen production...
can lead to premature pubarche, rapid somatic growth, advanced bone age, and subfertility in both males and females (3, 4, 5, 6).

Normalization of adrenal androgen synthesis is difficult to achieve (7) without supraphysiological doses of glucocorticoids. Furthermore, reliable biomarkers that accurately distinguish adrenal from gonadal androgen synthesis are lacking, and as a consequence, biochemical targets of disease control are not well defined, especially after the onset of puberty (8, 9). Dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS), the most abundant 19-carbon (C19) steroids produced by the adrenal glands (10), are disproportionally suppressed by glucocorticoid treatment and are not good indicators of hyperandrogenism in classic 21OHD (11, 12). Similarly, there is no good correlation between the routinely measured androgens, androstenedione (AD) and, in women, testosterone (T), and clinical evidence of androgen excess in 21OHD patients (13, 14), suggesting that other unrecognized androgens might be produced by the adrenal gland.

We previously found that 11β-hydroxyandrostenedione (11OHD) is the most abundant unconjugated C19 steroid in the human adrenal vein blood samples and that its synthesis is adrenocorticotropin (ACTH)-dependent (10). In teleost fishes, 11-ketotestosterone (11KT) is the major androgen, and its synthesis involves the 11β-hydroxylation of AD to 11OHD with subsequent oxidation and reduction (15, 16). Furthermore, 11KT is a potent agonist of the human androgen receptor (NR3C4), with an affinity comparable to testosterone (10). Given the profound accumulation of testosterone precursors in the adrenal glands of patients with 21OHD, we reasoned that 11-oxygenated C19-steroids (11oxC19) might be abundant adrenal products and a major source of active androgens. The goals of the current study were to provide a detailed characterization of the androgens and androgen precursors in classic 21OHD and to gain insights into the mechanisms and pathways of their formation (Fig. 1A).

Subjects and methods

Human serum samples

We enrolled 38 patients with classic 21OHD (19 women), ages 3–59 (Supplemental data file and Supplementary Table 1, see section on supplementary data given at the end of this article). In 34 of these patients, peripheral serum was obtained during routine clinical visits, while on their usual glucocorticoid replacement (Supplemental data file and Supplementary Table 1). In addition, four samples...
were obtained at 8 AM, before the first morning dose of hydrocortisone, from women with a serum AD greater than 345 ng/dL (>12 nmol/L), who had participated in another study (17). Patients who were potentially overtreated with glucocorticoids, as evidenced by a 17OHP <20 ng/dL, were excluded. We also enrolled 38 age- and sex-matched controls who were not receiving glucocorticoids, hormonal contraceptives, or chemotherapy. In addition, we obtained peripheral serum from three patients with 11β-hydroxylase deficiency (11OHD), and four patients with adrenal insufficiency (two with classic 21OHD who underwent bilateral adrenalectomy and two with Addison’s disease).

Adrenal vein (AV) samples were obtained as part of standard of care from patients undergoing evaluation for primary aldosteronism. Leftover serum from 16 patients (8 men) with aldosterone-producing adenomas, ages 32–75, was used for these studies. Only the inferior vena cava (IVC) and AV samples contralateral to the aldosterone-producing adenoma were used, in order to minimize the influence of dysregulated tumor steroidogenesis on these profiles. The samples were obtained from the IVC and AV before and 20 min after administration of 0.25 mg bolus cosyntropin. Successful catheterization was confirmed by a minimum AV/IVC cortisol gradient of two at baseline and five after cosyntropin stimulation. All samples were collected under Institutional Review Board (IRB) approved protocols. Written informed consent was granted by all participants who underwent AV sampling, those with 11OHD and adrenal insufficiency, and 17 patients with 21OHD. A waiver of consent was granted by the IRB for using any leftover serum collected as part of standard clinical care for the control group and 21 of the 21OHD patients.

**Steroid quantitation by LC-MS/MS**

Unlabeled and deuterium-labeled steroid standards were obtained from Sigma-Aldrich, Steraloids, Cerilliant, C/D/N Isotopes, and Cambridge Isotope Laboratories or synthesized (Supplementary data file, Steroid synthesis and Supplementary Table 2). A 10–100 µL aliquot of serum was deproteinated with 225 µL acetonitrile containing 100–200 µL internal standard deuterated steroids at known concentrations, followed by 150 µL methanol. The suspension was mixed and centrifuged for 5 min at 15 000 rpm. For measurement of 3-keto-Δ4-5 (Δ4, such as AD) steroids, the supernatant was mixed with 300 µL water and 1 mL of methyl-t-butyl ether (MTBE) for 4 min. After 10 min, the organic phase was separated and concentrated under nitrogen. For measurement of 3β-hydroxy-Δ5-6 (Δ5, such as DHEA) steroids, a separate aliquot was first extracted with MTBE, dried, resuspended in 50 µL 1 M ammonium hydroxide and 100 µL 1 M hydroxylamine hydrochloride, incubated at 90 °C for 30 min, and subsequently re-extracted with MTBE and dried as described above. Steroid sulfates were extracted with 1 mL of 1:1 chloroform:2-butanol from a serum aliquot after mixing with 200 µL 1 M ammonium sulfate. The dried extracts were reconstituted with 100–200 µL of methanol/deionized water (1:1) and transferred to a 0.25 mL vial insert. Steroids quantitation was performed as previously described (18); Supplementary Table 2 gives the retention times and precursor/product ion pairs for the targeted steroids. The lower limit of detection for each steroid, defined as the minimum concentration achieving an extrapolated signal-to-noise ratio of 3, ranged from 0.8 to 27 ng/dL (Supplementary Table 2). Intra-assay coefficients of variability (CV) ranged from 2 to 4% for steroid concentrations >100 ng/dL and from 2–11% for steroid concentrations <100 ng/dL. Inter-assay CV ranged from 2 to 8%. The linearity of response was assessed by measuring four separate dilutions per sample (n=3 samples), which rendered r² values consistently >0.95.

**Immunofluorescence analysis**

Paraffin-embedded adrenal glands from patients with 21OHD (n=3) and deceased renal transplant donors without any adrenal pathology (n=5) were obtained under IRB approval. Immunostaining studies were performed using antibodies for human cytochrome b₅ (CYB5A, mouse monoclonal, Acris) and anti-human 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2, also recognizes type 1 isoenzyme) (rabbit polyclonal, kindly provided by CR Parker, University of Alabama at Birmingham) antibodies. For immunofluorescence double-staining, the tissues were incubated with the primary antibody solutions overnight (1:3000 dilution for the CYB5A and 1:1000 dilution for the HSD3B2 antibodies), washed with phosphate-buffered saline, and subsequently incubated with species-specific secondary fluorescent antibodies for 1 h (Alexa Fluor 488-conjugated anti-mouse and Alexa 594 anti-rabbit dilution 1:100). Immunofluorescence was viewed under an Olympus FV 500 Confocal microscope.

**Statistical analysis**

Nonparametric Mann–Whitney U test was applied to compare the 21OHD patients and controls, using GraphPad
Prism6. Correlation between pairs of steroids was assessed using the nonparametric Spearman’s correlation test. A P<0.05 was considered to be statistically significant.

Results

Androgens and androgen precursors in sera of 21OHD patients

Using LC-MS/MS, we performed a targeted analysis of 11 steroids in sera of both 21OHD patients and controls, including seven unconjugated C19-steroids and four steroid sulfates. The four 11oxC19 steroids: 11OHAD, DHEAS 18 744 (7847–64 308) 139 784 (58 409–186 697) 0.1 17OHPregS 416 (290–1174) 481 (370–683) 0.9 0.6 PregS 10 600 (3400–25 305) 3738 (2853–7769) 2.8 0.001 DHEA 29 (16–85) 175 (118–318) 0.2 11KAD 96 (58–143) 31 (20–42) 3.1 0.0271 for DHEAS.

11KT 171 (105–366) 50 (29–78) 3.4 0.0001 11OHAD 351 (188–792) 118 (70–154) 3.0 0.0001

α-hydroxypregnenolone sulfate (17OHPregS); 0.027 for androst-5-ene-3β,17β-diol sulfate correlated tightly with DHEAS in both 21OHD patients (r=0.96, P<0.0001) and controls (r=0.94, P<0.0001). Pregnenolone sulfate also correlated with DHEAS (r=0.44, P=0.0065 in 21OHD patients; r=0.79, P<0.0001 in controls). These data demonstrate that the precursor steroids upstream of DHEA and DHEAS are diverted to 11oxC19 and to pregnenolone sulfate in patients with 21OHD.

Adrenal gland production of 11oxC19 steroids

To study the origins of 11oxC19 steroids, we measured these steroids in paired IVC and AV samples from AVS studies, before and after cosyntropin stimulation. We used only AV samples contralateral to an aldosterone-producing adenoma to minimize deviations from normal adrenal steroid production. Compared with the IVC, the AV concentrations at baseline were 33-fold higher for 11OHAD, 3.3-fold higher for 11OHT, 2.5-fold higher for 11KAD, and 1.8-fold higher for 11KT (Table 2). Cosyntropin stimulation further increased the AV/IVC gradient to 196-fold for 11OHAD, 17-fold for 11OHT,

<table>
<thead>
<tr>
<th>Steroid</th>
<th>21OHD (n = 38)</th>
<th>Controls (n = 38)</th>
<th>Fold</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td>155 (72–390)</td>
<td>42 (22–63)</td>
<td>3.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Testosterone</td>
<td>80 (38–162)</td>
<td>26 (12–309)</td>
<td>3.0</td>
<td>0.09</td>
</tr>
<tr>
<td>11OHD</td>
<td>351 (188–792)</td>
<td>118 (70–154)</td>
<td>3.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>11KAD</td>
<td>96 (58–143)</td>
<td>31 (20–42)</td>
<td>3.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>11OHT</td>
<td>59 (21–104)</td>
<td>15 (9–21)</td>
<td>4.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>11KT</td>
<td>171 (105–366)</td>
<td>50 (29–78)</td>
<td>3.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DHEA</td>
<td>29 (16–85)</td>
<td>175 (118–318)</td>
<td>0.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PregS</td>
<td>10 600 (3400–25 305)</td>
<td>3738 (2853–7769)</td>
<td>2.8</td>
<td>0.001</td>
</tr>
<tr>
<td>17OHPPregS</td>
<td>416 (290–1174)</td>
<td>481 (370–683)</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>DHEAS</td>
<td>18 744 (7847–64 308)</td>
<td>139 784 (58 409–186 697)</td>
<td>0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AdiolS</td>
<td>2711 (1228–9723)</td>
<td>25 576 (12 095–35 882)</td>
<td>0.1</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

To convert ng/dL to nmol/L, multiply by 0.0347 for testosterone; 0.0349 for androstenedione; 0.0328 for 11β-hydroxytestosterone (11OHT); 0.0331 for 11-ketotestosterone (11KT); and 11β-hydroxyandrostenedione (11OHD); 0.0333 for 11-ketoandrostenedione (11KAD); 0.0347 for DHEA; 0.0252 for Pregnenolone sulfate (PregS); 0.0242 for 17α-hydroxypregnenolone sulfate (17OHPPregS); 0.027 for androst-5-ene-3β,17β-diol sulfate (AdiolS); and 0.0271 for DHEAS.
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6-fold for 11KAD, and 3.3-fold for 11KT. Following cosyntrin stimulation, the AV concentrations of 11OHAD were augmented 12-fold, those of 11OHT 4.3-fold, while those of 11KAD and 11KT approximately 2-fold each. The IVC concentrations for all 11oxC19 steroids were similar between men and women both at baseline, as well as after cosyntrin stimulation. These data indicate that 11OHAD is a major, ACTH-stimulated product of the adrenal gland in men and women, and suggest that 11OHT is also a minor adrenal product, whereas 11KAD and 11KT are primarily peripheral metabolites from their 11β-hydroxylated precursors. To confirm that adrenal 11β-hydroxylase enzymes are responsible for their synthesis, we measured 11oxC19 steroids in three patients with 11OHAD and in four patients with adrenal insufficiency. Only trace amounts of 11OHD, 11OHT, 11KAD, and 11KT were found in sera of all seven patients (0–22 ng/dL).

Immunostaining of key enzymes in androgen synthesis in 21OHD

The robust synthesis of 11β-hydroxylated, 19-carbon, Δ4-steroids in 21OHD constitutes a paradox, because their synthesis requires enzymes and cofactor proteins segregated to the zona fasciculata (HSD3B2) and zona reticularis (CYB5A) in the normal adrenal. To explain this conundrum, we performed immunohistochemistry for these two key proteins in adrenal glands from patients with 21OHD (n = 3) and from deceased renal transplant donors (n = 5) with normal adrenal function. Representative images of HSD3B2 and CYB5A immunofluorescence in 21OHD and normal adrenal glands are shown in Fig. 3. In normal adrenal glands, HSD3B2 and CYB5A immunoreactivities are precisely segregated between zona fasciculata and zona reticularis, respectively (Fig. 3A). In contrast, the 21OHD adrenals exhibited areas containing a mixture of HSD3B2 and CYB5A immunoreactivities (Fig. 3B).

Discussion

Adrenal androgen excess is a hallmark of 21OHD, but the traditional serum steroid biomarkers, including AD, testosterone, DHEA, and DHEAS, do not serve as consistent, linear indicators of disease severity or treatment response in all patients (11, 13, 14). Furthermore, DHEAS and AD are not bioactive androgens themselves, but constitute a pool of precursors for potent androgens, such as testosterone and DHT. Previous studies found elevated 11OHAD concentrations in women with nonclassic 21OHD (19, 20, 21). In this study, we have shown that four 11oxC19 steroids, 11OHAD, 11KAD, 11OHT, and 11KT, are significantly higher in both male and female patients with classic 21OHD than in age-matched controls. Using in vitro cell-based luciferase reporter systems, we have previously shown that both 11OHT and 11KT activate the human androgen receptor similar to testosterone (10, 22, 23). Conversely, AD and 11KAD led to only modest activation of the androgen receptor, and 11OHAD demonstrated no androgen receptor activation at concentrations up to 1000 nmol/L (10, 22). These data indicate that 11KT is an important androgen in patients with 21OHD.
Unlike AD and testosterone, which also derive from the gonads, 11oxC19 steroids derive primarily from the adrenals and thus strongly reflect the adrenal contribution to the circulating androgens. The synthesis of 11OHT occurs predominately in the adrenal gland from AD (Fig. 1), through the action of steroid 11β-hydroxylase (CYP11B1), and small amounts might be produced from cortisol (24). Consistent with previous reports that measured only 11OHT (25, 26), we found negligible amounts of all 11oxC19 steroids in the sera of patients with 11OHD, which confirms that their synthesis relies on CYP11B1. In vitro studies with radiolabeled substrates showed that the ovarian granulosa cells cannot synthesize 11OHT from AD (27). We have previously shown that 11OHT is the most abundant unconjugated C19 steroid produced by the adrenal glands in women, and that the adrenal was also a source of 11KAD, 11OHT, and 11KT (10). In this study, we extend these findings to show that the adrenal contribution to the circulating 11OHT and 11KT pool is similar between men and women, supporting the fact that gonadal testosterone is not an important precursor, if at all, for 11OHT and 11KT. Furthermore, in our 21OHD males, 11KT correlated directly and tightly with 11OHT but tended to correlate inversely with testosterone, as 11KT will suppress gonadotropins and testosterone production from the testes in men with poorly controlled 21OHD. This latter result suggests that the testosterone/11KT ratio might be an ideal parameter for titrating therapy in men with 21OHD. These findings further suggest that adrenal-derived 11OHT, rather than gonadal-derived testosterone, is the precursor of 11KT. In addition, we found only trace amounts of 11oxC19 steroids in two 21OHD patients who had undergone adrenalectomy and in two patients with Addison’s disease, further supporting the central role of the adrenal in their synthesis. Based on its high AV/IVC gradients and cosyntropin stimulation, our data suggest that 11OHT is the major direct 11oxC19 product of the adrenal along with some 11KAD and 11KT are primarily formed in peripheral tissues.

DHEAS, the dominant C19 steroid product of the adrenal, is often paradoxically low or low-normal in 21OHD patients even without treatment, thus limiting its clinical utility in these patients (11). Even though we excluded patients with a suppressed 17OHP, we found that DHEA and DHEAS were 6- to 7-fold higher in controls than in 21OHD patients. The mechanisms underlying this

| Table 2 | Steroid concentrations and ratios in adrenal vein and inferior vena cava serum samples (ng/dL). Concentrations of testosterone, androstenedione (AD), and their 11-oxygenated derivatives in adrenal veins (AV) and inferior vena cava (IVC) of 16 sample sets (8 male), AV/IVC ratios at baseline and following cosyntropin (post-ACTH) stimulation, and fold stimulation. |
|---------|----------------------------------|-----------------|-----------|-----------|-----------------|-----------|
|         | 11OHT  | 11KT  | 11OHAD  | 11KAD  | Testosterone  | AD        |
| AV baseline |        |       |         |        |                |           |
| 72       | 47     | 3119   | 37      | 55      | 375            |
| AV post-ACTH | 357     | 112     | 27 731  | 83      | 128            | 16 194    |
| IVC baseline | 15      | 31     | 100     | 15      | 40             | 29        |
| IVC post-ACTH | 17      | 31     | 146     | 17      | 32             | 35        |
| AV/IVC baseline | 3.3      | 1.8    | 33      | 2.5     | 1.0            | 15        |
| AV/IVC post-ACTH | 17      | 3      | 196     | 6       | 5              | 451       |
| Fold stimulation post-ACTH AV | 4        | 2.0    | 12      | 2.4     | 3.4            | 48        |
| Fold stimulation post-ACTH IVC | 1.0      | 0.9    | 1.3     | 1.0     | 1.0            | 1.2       |
| P values, women vs men | | | | | | |
| AV/IVC baseline | 0.3      | 0.6    | 0.7     | 0.2     | 0.0002         | 0.6        |
| AV/IVC post-ACTH | 0.2      | 0.6    | 0.6     | 0.2     | <0.0001        | 0.6        |
| Fold stimulation post-ACTH AV | 0.8      | 0.8    | 1.0     | 0.2     | 0.1            | 0.5        |
| Fold stimulation post-ACTH IVC | 0.4      | 0.7    | 0.3     | 0.8     | 0.8            | 0.1        |

Figure 3

Double immunofluorescence of 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) (red) and cytochrome b₅ (CYB5A) (green). Nuclei are counterstained in blue. In the normal adrenal gland (A), HSD3B2 and CYB5A are sharply segregated to the zona fasciculata and zona reticularis, respectively, while in the 21OHD adrenal (B), areas with intermingled expression of both HSD3B2 and CYB5A were identified.
phenomenon are poorly understood. Androst-5-ene-3β,17β-diol sulfate and DHEAS concentrations varied in parallel, in both 21OHD patients and controls. In contrast, 21OHD patients produced significantly higher amounts of pregnenolone sulfate as compared with controls (Fig. 1B). Although not as robustly, pregnenolone sulfate correlated directly, rather than inversely, with DHEAS. Combined with the data for 11oxC19 steroids, these results suggest that 21-carbon steroids are diverted along several ordinarily minor pathways in the 21OHD adrenal. Several enzymes, including HSD3B2, CYP17A1, CYP11B1, and SULT2A1, compete for these accumulating common substrates. The kinetic interplay between these multiple reactions is difficult to predict and requires further study.

Another intriguing aspect of adrenal steroid biosynthesis is the mechanism by which the production of active androgens becomes sufficient to cause severe virilization in females with 21OHD. For the synthesis of AD and downstream androgens, both HSD3B2 and CYB5A are required. These two key factors in androgens synthesis are co-expressed in the testicular Leydig and ovarian theca cells (28, 29). In the normal adrenal gland, HSD3B2 and CYB5A are segregated to the zona glomerulosa and fasciculata or the zona reticularis, respectively, such that the major adrenal C19 steroids are DHEA and DHEAS. Double-immunohistochemical analysis of HSD3B2 and CYB5A in the normal adrenal glands identified a small number of cells where these two proteins overlap at the interface of the zonae fasciculata and reticularis, which might be responsible for the adrenal AD and testosterone syntheses (30). Comparison between age groups in this study showed that the co-localization of HSD3B2 and CYB5A is most prominent in 13–20-year-old group, following adrenarche. We hypothesized that the adrenal glands of patients with 21OHD exhibit larger areas of overlapping HSD3B2 and CYB5A expression, which would confer to these cells greater androgenic production efficiency, normally present only in the gonads. Indeed, we found islands of cells with overlapping expression of HSD3B2 and CYB5A in the adrenal glands from patients with classic 21OHD, but not in normal adrenals (Fig. 3).

Despite a large number of both males and females with matched controls, this initial study of androgens in classic 21OHD has several limitations. Most of our serum samples were obtained randomly, from patients on various glucocorticoid replacement regimens, and accurate clinical assessment of disease control was not possible in many participants. Prospective studies will be needed to assess the correlation of adrenal androgens in 21OHD with the clinical phenotype and response to treatment across the life span. Nevertheless, our data suggest that these 11oxC19 steroids are promising biomarkers of adrenal androgen excess in 21OHD and might be superior to AD and testosterone. Because AD and testosterone also derive from the gonads, these traditional biomarker steroids are problematic not only in men but also in women with 21OHD, who often secondarily develop polycystic ovarian syndrome (31). An important strength of our study is the inclusion of both males and females with classic 21OHD. Although clinical stigmata of adrenal androgen excess can be subtle in males, they suffer from sexual precocity (12, 32, 33, 34) and infertility (4, 5, 6) similar to females. The inclusion of males in both our comparison of 21OHD with unaffected controls, as well as in our AV analysis, allowed us to conclude that the major source of 11oxC19 steroids is the adrenal gland.

In summary, we have shown that four 11oxC19 steroids are similarly elevated in patients with classic 21OHD of both sexes. As 11KT is a potent androgen, it might be the most clinically relevant adrenal-derived androgen in 21OHD patients. In addition, our findings suggest that pregnenolone sulfate might serve as an additional biomarker for disease control in patients with 21OHD. With the expanded use of LC-MS/MS, future prospective studies will allow the characterization of steroid biomarkers that accurately reflect disease control and facilitate treatment monitoring.
serum samples from study 21208HPL1002 under written informed consent for research purposes.

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