Differences of adrenal-derived androgens in 5α-reductase deficiency versus androgen insensitivity syndrome

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Abstract
Steroid 5α-reductase type 2 deficiency (5α-RD2) and androgen insensitivity syndrome (AIS) are difficult to distinguish clinically and biochemically, and adrenal-derived androgens have not been investigated in these conditions using modern methods. The objective of the study was to compare Chinese patients with 5α-RD2, AIS, and healthy men. Sixteen patients with 5α-RD2, 10 patients with AIS, and 39 healthy men were included. Serum androgen profiles were compared in these subjects using liquid chromatography/tandem mass spectrometry (LC-MS/MS). Based on clinical features and laboratory tests, 5α-RD2 and AIS were diagnosed and confirmed by genotyping. Dihydrotestosterone (DHT) and testosterone (T) were both significantly lower in patients with 5α-RD2 than AIS (p < 0.0001). The T/DHT ratio was higher in 5α-RD2 (4.5–88.6) than AIS (13.4–26.7) or healthy men (7.6–40.5). Using LC-MS/MS, a cutoff T/DHT value of 27.3 correctly diagnosed 5α-RD2 versus AIS with sensitivity 93.8% and specificity 100%. Among the adrenal-derived 11-oxygenated androgens, 11β-hydroxyandrostenedione (11OHA4) and 11-ketoandrostenedione (11KA4) were also lower in patients with 5α-RD2 than those of patients with AIS. In contrast, 11β-hydroxytestosterone (11OHT) was higher in 5α-RD2 than AIS. Furthermore, a 11OHT/11OHA4 cutoff value of 0.048 could also distinguish 5α-RD2 from AIS. Thus, both elevated T/DHT values above 27.3 and the unexpected 11-oxygenated androgen profile, with a 11OHT/11OHA4 ratio greater than 0.048, distinguished 5α-RD2 from AIS. These data suggest that the metabolism of both gonadal and adrenal-derived androgens is altered in 5α-RD2.

Study Highlights
WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
Steroid 5α-reductase type 2 deficiency (5α-RD2) and androgen insensitivity syndrome (AIS) are difficult to distinguish.

Bing Han and Hui Zhu contributed equally to this work.

These data were presented previously in abstract form (J Endocr Soc. April 15, 2019; 3(Suppl 1): SUN-362).

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INTRODUCTION

The 5α-reductase type 2 deficiency (5α-RD2; OMIM #264600) is an autosomal recessive disorder caused by impairment of steroid 5α-reductase type 2, which converts testosterone (T) to dihydrotestosterone (DHT) in target tissues, such as genital skin and the prostate.1 Undervirilization of the male external genitalia at birth in 5α-RD2 results from low DHT synthesis during fetal development. The actions of T and DHT are mediated through the androgen receptor (AR), a member of the nuclear hormone receptor superfamily, and its co-activator and co-repressor proteins. Androgen insensitivity syndrome (AIS; OMIM #300068) is most often caused by loss-of-function mutations in the AR gene, which encodes the AR protein. To date, over 500 different AR mutations have been reported in patients with AIS.2 The spectrum of phenotypes manifest in patients with AIS ranges from completely female external genitalia and absent body hair (complete AIS [CAIS]), to some degree of undervirilization (partial AIS [PAIS]), to male infertility with normal virilization (mild AIS [MAIS]), depending on the degrees of AR functional impairment. In up to 90% of patients with CAIS, mutations in the AR gene can be identified, whereas PAIS is often a diagnosis of exclusion after alternative conditions are reasonably excluded, and AR mutations are identified in less than half of patients with PAIS.3 Among patients with 46,XY disorders of sexual development (46,XY DSD), 5α-RD2 and AIS are the most common etiologies; however, these two conditions can be difficult to distinguish clinically and biochemically, particularly before puberty. Even with modern approaches, a specific molecular diagnosis is only achieved in up to 50% of patients with 46,XY DSD.4

The phenotype of patients with 5α-RD2 varies widely from complete female external genitalia to nearly complete male phenotype with mild evidence of undermasculinization (hypospadias, micropenis, and/or cryptorchidism),5 which could be easily confused with PAIS.6 Assessment of the baseline and hCG-stimulated serum T/DHT ratio has been widely used as a diagnostic strategy7–9 in undervirilization. The previous suggested cutoff value of T/DHT for 5α-RD2 diagnosis ranges from 8.5 to 30.10,11 In a recent study, Abacı et al.12 found that cutoff values yielding the best sensitivity for stimulated T/DHT ratio were greater than or equal to 8.5 for mini-pubertal, greater than or equal to 10 for prepubertal, and greater than or equal to 17 for pubertal patients. These data, however, are based on immunoassay values, and cross-reactivity of the antibodies used might impact the accuracy of the measurements used for narrowing the differential diagnosis. Liquid chromatography/tandem mass spectrometry (LC-MS/MS) has been used for steroid assays with obvious advantages, including improved specificity, simultaneous measurement of several analytes, and often better sensitivity than immunoassays.

Substantial evidence suggests that 11-oxygenated C19 adrenal-derived androgens, 11β-hydroxyandrostenedione (11OHA4), 11-ketoandrostenedione (11KA4), 11β-hydroxytestosterone (11OHT), and 11-ketotestosterone (11KT), are clinically important androgens. In treated patients with classic 21OHD, all 11-oxygenated C19 androgens are three-fold to four-fold higher than in age-paired and sex-paired controls.13 The levels of 11-oxygenated C19 androgens also reflect the status of long-term disease control.14 Moreover, in 114 women with polycystic ovary syndrome (PCOS), all four 11-oxygenated C19 androgens were significantly higher than those of controls. In addition, 11OHA4 and 11KA4 correlated with insulin resistance,15 and increased expression of aldo-keto reductase 1C3 (AKR1C3 or 17β-hydroxysteroid dehydrogenase type 5) in adipocytes of women with PCOS increases the synthesis of active androgens from adrenal-derived precursors.16 Furthermore, hyperandrogenemia is a characteristic of both premenarchal daughters of affected women with PCOS and obese girls; however, 11-oxygenated C19 steroid profiles cannot be used to differentiate these groups.17 In

WHAT QUESTION DID THIS STUDY ADDRESS?

This study investigated adrenal-derived androgens in 5α-RD2, AIS, and healthy men by liquid chromatography/tandem mass spectrometry.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

The 11β-hydroxyandrostenedione (11OHA4) and 11-ketoandrostenedione (11KA4) were lower in patients with 5α-RD2, whereas 11β-hydroxytestosterone (11OHT) was higher in patients with 5α-RD2.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

The cutoff value of 11OHT/11OHA4 could be used to distinguish 5α-RD2 from AIS.
prepubertal children, androgens derive from the adrenals during adrenarche, which manifests as the appearance of axillary and pubic hair. In girls, premature adrenarche is a risk factor for developing PCOS and ovarian hyperandrogenemia after puberty. Rege et al. found that 11-oxygenated C19 androgens, including 11OHA4 and 11KT, were significantly higher in children with premature adrenarche than in controls. Consequently, primary disorders of gonadal androgen production often influence adrenal-derived androgen synthesis and vice-versa via multiple mechanisms. Testicular androgens are elevated at puberty in patients with 5α-RD2 and patients with AIS, but profiles of 11-oxygenated C19 androgens have not been studied in 5α-RD2 and AIS. In the current study, we compared gonadal and adrenal-derived androgens in patients with 5α-RD2 and patients with AIS, as well as in healthy male subjects, using LC-MS/MS.

**MATERIALS AND METHODS**

**Patients**

Sixty-five postpubertal subjects were enrolled, including 26 patients with 46,XY DSD, aged from 11 to 34 years at first presentation to our hospital, and 39 healthy male subjects. The phenotypes of the 16 patients with 5α-RD2 and 10 patients with AIS (2 CAIS and 8 PAIS) ranged from nearly female external genitalia to hypospadias and 10 patients with AIS (2 CAIS and 8 PAIS) ranged from nearly female external genitalia to hypospadias and 10 patients with AIS (2 CAIS and 8 PAIS) ranged from nearly female external genitalia to hypospadias and 10 patients with AIS (2 CAIS and 8 PAIS). Genomic DNA of the patients was extracted from peripheral blood leukocytes (TIANGEN Biotech, Beijing, China). All exons of SRD5A2 and AR genes were amplified by polymerase chain reaction (PCR), and the products were purified and sequenced, as previously described. When a novel mutation was found, PCR fragments, amplified from the genomic DNA of 100 healthy subjects, were also analyzed to exclude polymorphisms.

**Genetic diagnosis and genotyping**

Genomic DNA of the patients was extracted from peripheral blood leukocytes (TIANGEN Biotech, Beijing, China). All exons of SRD5A2 and AR genes were amplified by polymerase chain reaction (PCR), and the products were purified and sequenced, as previously described. When a novel mutation was found, PCR fragments, amplified from the genomic DNA of 100 healthy subjects, were also analyzed to exclude polymorphisms.

**Steroids tested 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. Serum (100 µl) was mixed with 200 µl deionized water and 100 µl internal standard mix containing 50–1000 pg each steroid, proportionate to their typical concentrations in 40% aqueous methanol (Table S1). At known concentrations in 40% aqueous methanol. The mixtures were loaded onto ISOLUTE SLE columns (Biotage, Charlotte, NC) using nitrogen gas at 3 psi pressure applied for 5 s in a Biotage PRESSURE+48 (Biotage, Uppsala, Sweden). After equilibrating for 5 min, steroids were eluted from the columns with two rinses of 700 µl methyl-tert-butyl ether (MTBE) for 5 min each under gravity, followed by application of nitrogen gas at 10 psi pressure for 30 s to complete elution. The solvent was evaporated under nitrogen, and the dried extracts were reconstituted with 100 µl of 40% aqueous methanol and transferred to a 250 µl vial insert. Samples (10 µl) were injected and resolved by two-dimensional chromatography with a C4 10 × 2.1-mm column (Thermo Fisher Scientific, Waltham, MA) on an Agilent 1260 binary pump, and Kinetex 50 × 2.1-mm, 2.6-mm particle-size biphenyl column (Phenomenex, Torrance, CA) on an Agilent 1290 binary pump, respectively, using gradient elution with 0.2 mmol/L ammonium fluoride and methanol. Steroid quantitation was performed with an Agilent 6495 triple quadrupole tandem mass spectrometer (Agilent Technology, Santa Clara, CA) using mass ratio monitoring in positive ion mode. Based on the previous method, we modified the parameters to shorten the first dimension from 3.6 to 3.0 min and added DHT at 8.2 min in the second dimension (m/z for precursor/product ions, DHT = 291.2/255.2 and 105.0 qualifier; DHT-d3 = 294.2/258.2 and 105.1 qualifier).

**Statistical analysis**

ANOVA with post hoc test (LSD) was used to compare differences among SRD5A2, AIS and HEALTHY male subjects using SPSS version 21 software (IBM Inc., Chicago, IL, USA). Receiver operating characteristic (ROC) analysis was performed to evaluate the diagnostic performance. The optimal cutoff values of each predictor were set at the closest point to the upper left corner of the ROC curve plot. We also performed power analysis. According to mean value of three groups, the power to distinguish them was 0.8470 when each group had nine samples (PASS 15.0, one-way analysis of variance f-tests). However, according to mean value of two groups (SRD5A2 and AIS), the power to distinguish them was 0.9267 when each group had nine samples (PASS 15.0, two sample t-test). A p < 0.05 was considered to be statistically significant.
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RESULTS

Clinical characteristics of participants

The ages of the patients with 5α-RD2, AIS, and healthy men were 20.6 ± 5.5, 21.4 ± 4.0, and 25.3 ± 1.7 years. The clinical manifestation of 5α-RD2 were variable. Nine of the patients were born with female external genitalia or micropenis with hypospadias, underwent virilization after puberty, and were diagnosed as 5α-RD2 clinically. The diagnosis of CAIS was relatively easy, whereas patients with PAIS had similar clinical manifestations as patients with 5α-RD2, including micropenis, hypospadias, cryptorchidism, as well as different degrees of breast development. Patient 1 of the 5α-RD2 group was being treated with DHT gel.

Genetic diagnosis

Fifteen different mutations were identified in 16 patients with 5α-RD2. Compound heterozygous mutations were found in 12 patients and homozygous mutations in four patients. The most frequent mutations were p.G203S, p.R227Q, and p.Q6X, found on one allele of four patients, respectively. Eight mutations were detected in patients with AIS, and p.R841C was the most frequent mutation found in three patients (Table 1).

Steroid profiles of SRD5A2, AIS, and healthy men

In patients with 5α-RD2, 18-hydroxycortisol (12.9 ± 4.8 vs. 17.9 ± 7.4 nmol/L), 17-hydroxyprogesterone (3.35 ± 1.83 vs. 6.68 ± 3.61 nmol/L), 11-deoxycorticosterone (0.11 ± 0.08 vs. 0.24 ± 0.12 nmol/L), progesterone (0.20 ± 0.10 vs. 0.32 ± 0.17 nmol/L), 16α-hydroxyprogesterone (0.94 ± 0.75 vs. 1.78 ± 1.07 nmol/L), and androstenedione (2.89 ± 1.20 vs. 4.72 ± 2.04 nmol/L) were significantly lower than in patients with AIS. However, there were no significant differences in cortisol, cortisone, 11-deoxycorticisol, corticosterone, and estrone between patients with 5α-RD2 and patients with AIS (Table 2).

The 11-oxygenated androgens comparison in three groups

The 11OHA4 (6.39 ± 2.15 vs. 9.63 ± 3.98 nmol/L) and 11KA4 (0.68 ± 0.21 vs 1.69 ± 0.68 nmol/L) were lower in patients with 5α-RD2 than patients with AIS (Figure 1a,b). In contrast, 11OHT was found to be higher in patients with 5α-RD2 compared with patients with AIS (0.53 ± 0.26 vs. 0.29 ± 0.09 nmol/L; Figure 1c). There was no significant difference in 11KT (1.21 ± 0.51 vs. 1.07 ± 0.75 nmol/L) between patients with 5α-RD2 and patients with AIS (Figure 1d). In addition, compared with healthy men, 11OHA4 and 11KA4 were significantly decreased in patients with 5α-RD2 (Figure 1a,b), whereas 11OHT and 11KT were elevated (Figure 1c,d). In addition, 11OHT/11OHA4 ratio in patients with 5α-RD2 was higher in patients with AIS and healthy men (Figure 1e).

Androgens comparison of three groups

Both testosterone (41.74 ± 23.70 vs. 15.64 ± 4.47 nmol/L) and DHT (1.92 ± 0.95 vs. 0.39 ± 0.17 nmol/L) were increased in the patients with AIS, compared with those of patients with 5α-RD2 (Figure 2a,b). However, patients with 5α-RD2 have similar testosterone levels (15.64 ± 4.47 vs. 15.98 ± 5.09 nmol/L) but significantly decreased DHT level (0.39 ± 0.17 vs. 1.02 ± 0.47 nmol/L) when compared with healthy subjects, indicating the impairment of the conversion from testosterone to DHT (Figure 2a,b). Similarly, the ratio of testosterone/DHT (T/DHT) in patients with 5α-RD2 was higher than that of patients with AIS or healthy men (46.50 ± 19.49 vs. 21.64 ± 4.68 or 17.72 ± 6.75, respectively; Figure 2c).

TABLE 1 Gene mutations of patients with 5α-RD2 and patients with AIS

| 5α-RD2   | SRD5A2 mutation | AIS  | AR mutation |
|----------|-----------------|------|-------------|
| P1       | p.Q6X/p.R227Q   | P1   | p.L907V     |
| P2       | p.G203S/p.G203S | P2   | p.L907V     |
| P3       | p.G203S/p.R227Q/p.G34R | P3   | p.V904M     |
| P4       | p.L20/p.R246Q   | P4   | p.C620R     |
| P5       | p.Q6X/p.H162P   | P5   | p.G590R/p.R841C |
| P6       | p.A228V/       | P6   | p.R841C     |
| P7       | p.Q6X/p.H162P   | P7   | p.L295P     |
| P8       | p.Q6X/p.N193S   | P8   | p.R841C     |
| P9       | p.R171S/G196V   | P9   | p.L813F     |
| P10      | IVS4+2T>C/IVS4+2T>C | P10  | p.V686A     |
| P11      | p.L20/p.R227X   |      |             |
| P12      | p.Y136X/p.Y136X |      |             |
| P13      | p.G203S/p.S234C |      |             |
| P14      | p.R227Q/p.R227Q |      |             |
| P15      | p.G203S/p.G246R |      |             |
| P16      | p.R227Q/p.S234C |      |             |

Abbreviations: 5α-RD2, 5α-reductase type 2 deficiency; AIS, androgen insensitivity syndrome; AR, androgen receptor.
When compared with healthy subjects, the cutoff T/DHT value for diagnosing 5α-reductase deficiency of 93.8% and specificity of 100%, respectively (Figure 3a,b). Moreover, a 11OHT/11OHA4 cutoff value of 0.048 correctly diagnosed 5α-reductase type 2 deficiency, corresponding to a sensitivity of 87.5% and specificities of 100% (Figure 4a,b).

**DISCUSSION**

To our knowledge, this is the first report to measure T, DHT, and 11-oxygenated C19 androgens by LC-MS/MS in patients with 5α-RD2 and patients with AIS. Using a modification of our prior method, we compared the steroid profiles of patients with 5α-RD2, AIS, and healthy men. The 11OHA4 and 11KA4 were decreased but 11OHT was increased in patients with 5α-RD2. Then, we established T/DHT and 11OHT/11OHA4 ratios for differential diagnosis of patients with 5α-RD2 and AIS.

The differentiation of male external genitalia requires adequate levels of T and intracellular conversion to DHT during male fetal development. Both androgens exert their effects by binding to AR, with different consequences in specific target tissues, such as the genital skin and prostate. Androgen biosynthesis and receptor defects are main causes of 46,XY DSD. It is well known that 5α-RD2 is caused by biallelic loss-of-function mutations of the SRD5A2 gene, which is located in 2p23.1 and encodes a 254 amino acid protein. Previously, 5α-RD2 was considered a rare etiology for 46,XY DSD; however, with expanded genotyping and lowered screening of the T/DHT ratio to 8.5, more than 120 different pathogenic variants have been reported, including mutations found in patients with 46,XY DSD without a biochemical diagnosis. AIS, inherited as an X-linked recessive disorder, most often results from mutations in the AR gene located on chromosome Xq11.2–q12 and containing eight exons. According to the degree of undermasculinization, AIS can be divided into CAIS, PAIS, and MAIS, and AR mutations are found in almost all patients with CAIS.

Some of the patients with PAIS showed an undervirilization phenotype similar to 5α-RD2. Therefore, the diagnosis of AIS should exclude 46,XY DSD caused by other etiologies, which could be the result from defects in gonadal development or androgen biosynthesis (17-hydroxylase deficiency, 17β-hydroxysteroid dehydrogenase type 3 deficiency, and 5α-RD2). Moreover, variable external genitalia virilization occurs in both patients with PAIS and patients with 5α-RD2, which is rare in patients with CAIS. Previous studies have shown that the T/DHT ratio in serum was not always sufficiently sensitive in the differential diagnosis of PAIS and 5α-RD2, particularly in prepubertal children. Therefore, molecular diagnosis based on Sanger or next-generation sequencing is often necessary to establish the diagnosis of patients with 46,XY DSD.

The previous diagnostic criteria first defined the cutoff T/DHT ratio as 30:1 and then 10:1, whereas a recent study reported that a cutoff of 10:1 in newborns and young infants

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**TABLE 2** Steroid profile of patients with SRD5A2, AIS, and HEALTHY men

| Steroids                  | SRD5A2 (n = 16) | AIS (n = 10) | HEALTHY (n = 39) | SRD5A2 vs. AIS | SRD5A2 vs. HEALTHY | AIS vs. HEALTHY |
|---------------------------|----------------|-------------|-----------------|---------------|-------------------|-----------------|
| 18-Hydroxycortisol        | 12.9 ± 4.8     | 17.9 ± 7.4  | 19.24 ± 6.62    | 0.058         | 0.001             | 0.549           |
| Cortisol                  | 290.28 ± 95.38 | 370.18 ± 114.66 | 350.10 ± 86.17  | 0.037         | 0.034             | 0.545           |
| Cortisone                 | 39.58 ± 10.67  | 45.41 ± 14.05 | 39.44 ± 6.76    | 0.120         | 0.960             | 0.071           |
| 11-Deoxycorticosterone    | 0.11 ± 0.08    | 0.24 ± 0.12 | 0.92 ± 0.50     | 0.078         | 0.965             | 0.042           |
| 16α-Hydroxyprogesterone   | 0.94 ± 0.75    | 1.78 ± 1.07 | 1.07 ± 0.68     | 0.009         | 0.561             | 0.012           |
| Corticosterone            | 13.01 ± 9.24   | 15.48 ± 11.00 | 11.37 ± 7.55    | 0.477         | 0.522             | 0.181           |
| Estrone                   | 0.10 ± 0.04    | 0.10 ± 0.04 | 0.11 ± 0.04     | 0.859         | 0.683             | 0.889           |
| 17-Hydroxyprogesterone    | 3.35 ± 1.83    | 6.68 ± 3.61 | 4.10 ± 1.74     | 0.000         | 0.241             | 0.000           |
| 11-Deoxycorticosterone    | 0.11 ± 0.08    | 0.24 ± 0.12 | 0.12 ± 0.06     | 0.000         | 0.385             | 0.000           |
| Progesterone              | 0.20 ± 0.10    | 0.32 ± 0.17 | 0.23 ± 0.08     | 0.005         | 0.307             | 0.016           |
| Androstenedione           | 2.89 ± 1.20    | 4.72 ± 2.04 | 2.75 ± 0.75     | 0.000         | 0.695             | 0.000           |

**Note:** Values are represented in mean ± SD. ANOVA with post hoc test (LSD) was used to compare differences among three groups. Abbreviations: 5α-RD2, 5α-reductase type 2 deficiency; AIS, androgen insensitivity syndrome.
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It was reported that 16 of 18 (88.9%) cases had an hCG-stimulated T/DHT ratio above 10.7 In our previous study, however, we found the cutoff value of 10 could not diagnose all genotype-proven patients, and we found overlap between patients with PAIS and patients with 5α-RD2. In a Turkish study, the cutoff values of T/DHT have a sensitivity of 72.7%.10

**FIGURE 1** Comparison of 11-oxygenated androgens in three groups. (a, b) The 11OHA4 and 11KA4 in the SRD5A2 group was significantly lower than the AIS or HEALTHY groups. (c) The 11OHT in the SRD5A2 group was significantly higher than the AIS or HEALTHY groups. (d) The 11KT in the HEALTHY group was significantly lower than the SRD5A2 or AIS groups. (e) The 11OHT/11OHA4 ratio in SRD5A2 group was significantly higher than the AIS or HEALTHY groups. ANOVA with post hoc test (LSD) was used to compare differences among three groups. Conversion factors for ng/dL: multiply by 30.24 for 11OHA4 and 11KT, 30.04 for 11KA4, and 30.44 for 11OHT. *p < 0.05, **p < 0.001. Values are represented in mean ± SD. AIS, androgen insensitivity syndrome

**FIGURE 2** Androgen comparison of three groups. (a) T in the AIS group was higher than the SRD5A2 group. Whereas, the SRD5A2 group has similar T levels compared to the HEALTHY group. (b) DHT in the SRD5A2 group was lower than AIS or HEALTHY groups. (C) T/DHT ratio was higher in the SRD5A2 group than in the AIS or HEALTHY groups. Conversion factors for ng/dl: multiply by 28.84 for T and 29.04 for DHT. ANOVA with post hoc test (LSD) was used to compare differences among three groups. *p < 0.05, **p < 0.001. Values are represented in mean ± SD. AIS, androgen insensitivity syndrome; DHT, dihydrotestosterone; T, testosterone
ratio were different in minipubertal, prepubertal, and pubertal groups. Previous reports have suggested that the DHT levels can reach into the normal range after puberty owing to the activities of the peripheral type 1 isoform. In our study, we only investigated postpubertal patients with SRD5A2 mutations. Because DHT was traditionally measured by radioimmunoassay, cross-reaction of the antibody with testosterone might confound DHT measurements. In steroid measurements, LC-MS/MS is more specific and often more sensitive than immunoassays and has been successfully used in the diagnosis of congenital adrenal hyperplasia (CAH). Thus, we implemented LC-MS/MS to analyze the steroid profiles in patients with 5α-RD2 and patients with AIS.

Adrenal-derived androgen precursors include dehydroepiandrosterone, 5-androstenediol, and their respective sulfates. The adrenal is also the source of 11-oxygenated C19 steroids, which were formerly identified as major androgens in teleost fishes. Recently, their importance in human beings has become gradually appreciated. The 11OHA4 is the major direct 11-oxygenated C19 product of adrenal, whereas 11KA4 and 11KT are primarily formed in peripheral tissues. 11OHA4 and 11OHT can be oxidized to 11KA4 and 11KT by 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2). The 11KA4 is an excellent substrate for AKR1C3 and is efficiently reduced to 11KT. Both 11KT

![Figure 3](image3.png)

**Figure 3** Receiver operating characteristic (ROC) curves for analysis of the diagnostic value of T/DHT for differentiation of patients with 5α-RD2 from patients with AIS and healthy men. (a) SRD5A2 versus AIS; (b) SRD5A2 versus HEALTHY. 5α-RD2, 5α-reductase type 2 deficiency; DHT, dihydrotestosterone; T, testosterone

![Figure 4](image4.png)

**Figure 4** Receiver operating characteristic (ROC) curves for analysis of the diagnostic value of 11OHT/11OHA4 for differentiation of patients with 5α-RD2 from patients with AIS and healthy men. (a) SRD5A2 versus AIS; (b) SRD5A2 versus HEALTHY. 5α-RD2, 5α-reductase type 2 deficiency
and less so 11OHT activate the human androgen receptor, and 11KT is almost as potent as T. In patients with 21OHD, all four 11-oxygenated C19 steroids are significantly elevated versus controls, which reflects their adrenal origin stimulation by ACTH or cosyntri- to the loss of the type 2 isoenzyme might contribute to this finding (Figure 5). In addition, insulin induces expression of AKR1C3 in adipocytes, and this mechanism has been proposed as a reason for increased adipocyte T and DHT in women with PCOS, who also exhibit elevated 11-oxygenated C19 androgens. We did not study insulin sensitivity in our cohort, but increased AKR1C3 activity in patients with 5α-RD2 could also contribute to our findings.

The limitations to our study include the modest number of patients, particularly with PAIS, the single blood samples without dynamic testing, and the lack of 3β-hydroxy-D5-steroid measurements in our LC-MS/MS panel. The strengths of the study include the use of LC-MS/MS, the inclusion of 11-oxygenated androgens in our panel, and the restriction of our cohort to postpubertal subjects. In addition, all blood samples were obtained at 8 a.m., to account for diurnal variations, particularly for adrenal-derived steroids. Future studies should incorporate measures of 5α-reduced metabolites of 11-oxygenated C19 androgens and measures of insulin sensitivity.

In summary, we compared the steroid profiles, including 11-oxygenated C19 androgens, in patients with 5α-RD2, AIS, and healthy subjects using LC-MS/MS. A cutoff T/DHT value of 27.6 and 27.5 correctly diagnosed 5α-RD2 compared with AIS or healthy men. In addition, the 11OHT/11OHA4 ratio might be another sensitive biomarker for the diagnosis of 5α-RD2. The simultaneous measurement of traditional and 11-oxygenated androgens LC-MS/MS appears to be a sensitive method to differentiate patients with 5α-RD2 from patients with AIS using a single small blood sample.

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CONFLICT OF INTEREST
The authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS
B.H., R.J.A., and J.Q. wrote the manuscript. R.A. and J.Q. designed the research. B.H. and H.Z. performed the research. H.Y., H.W., W.Z., and T.C. analyzed the data. J.R. and P.O.D. contributed new reagents/analytical tools.

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