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Clinical Research Article

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Clinical and Hormonal Profiles Correlate With Molecular Characteristics in Patients With 11β-Hydroxylase Deficiency

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Abstract

Background: Given the rarity of 11β-hydroxylase deficiency (11βOHD), there is a paucity of data about the differences in clinical and biochemical characteristics of classic (C-11βOHD) and nonclassic 11βOHD (NC-11βOHD).

Objective: To characterize a multicenter pediatric cohort with 11βOHD.

Method: The clinical and biochemical characteristics were retrospectively retrieved. *CYP11B1* gene sequencing was performed. Seventeen plasma steroids were quantified by liquid chromatography-mass spectrometry and compared to that of controls.

Results: 102 patients (C-11βOHD, n = 92; NC-11βOHD, n = 10) from 76 families (46,XX; n = 53) had biallelic *CYP11B1* mutations (novel 9 out of 30). Five 46,XX patients (10%) were raised as males. Nineteen patients (19%) had initially been misdiagnosed with 21-hydroxylase deficiency. Female adult height was 152 cm [–1.85 SD score (SDS)] and male 160.4 cm (–2.56 SDS).None of the NC-11βOHD girls had ambiguous genitalia (C-11βOHD 100%), and none of the NC-11βOHD patients were hypertensive (C-11βOHD 50%). Compared to NC-11βOHD, C-11βOHD patients were diagnosed earlier (1.33 *vs* 6.9 years; $P < 0.0001$), had higher bone age-to-chronological age ($P = 0.04$) and lower adult height (–2.46 *vs* –1.32 SDS; $P = 0.05$). The concentrations of 11-oxygenated androgens and 21-deoxycortisol were low in all patients. The baseline ACTH and stimulated cortisol were normal in NC-11βOHD. Baseline cortisol; cortisone; 11-deoxycortisol; 11-deoxycorticosterone and corticosterone concentrations; and 11-deoxycortisol/cortisol, 11-deoxycorticosterone/cortisol, and androstenedione/cortisol ratios were higher in C-11βOHD than NC-11βOHD patients ($P < 0.05$). The 11-deoxycortisol/cortisol ratio >2.2, <1.5, and <0.1 had 100% specificity to segregate C-11βOHD, NC-11βOHD, and control groups.

Conclusion: NC-11βOHD can escape from clinical attention due to relatively mild clinical presentation. However, steroid profiles enable the diagnosis, differential diagnosis, and subtyping of 11βOHD.

Key Words: *CYP11B1*, congenital adrenal hyperplasia, steroid profiling, 11-oxygenated androgens, adrenal insufficiency, androgen excess, children

Steroid 11β-hydroxylase deficiency (11βOHD) (OMIM#202010) caused by biallelic mutations of *CYP11B1* is the second most common type of congenial adrenal hyperplasia (CAH) and accounts for 0.2% to 8% of all CAH cases (1-3). 11βOHD occurs in ~1:100 000 to 1:200 000 live births in general nonconsanguineous populations (3,4). However, we recently reported a higher incidence of 1:60 000 in the Turkish population (5).

The metabolic signature of 11βOHD includes raised concentrations of adrenocorticotropic hormone (ACTH), 11-deoxycortisol, 11-deoxycorticosterone (DOC), and adrenal androgen precursors together with low cortisol, corticosterone, and aldosterone. The clinical manifestations of cortisol deficiency are not apparent due to the glucocorticoid effect of high DOC concentrations, which also activates the mineralocorticoid receptor, leading to hypertension (2,3). In addition, an increased steroidogenic flux toward adrenal androgen precursors results in hyperandrogenemia. 11βOHD can present as classic (C-11βOHD) or nonclassic (NC-11βOHD) phenotypes depending on the degree of clinical severity and percentage loss of CYP11B1 activity (3). The clinical features of C-11βOHD include hyporeninemic hypokalemic hypertension, virilization of female external genitalia (46,XX difference/disorder of sex development), precocious pseudopuberty, accelerated skeletal maturation and short adult height. The frequency of NC-11βOHD is even rarer than the C-11βOHD, and only few genetically confirmed NC-11βOHD cases have been reported (6-13). NC-11βOHD is characterized by normal external genitalia at birth and can manifest later in life with milder virilization, precocious pseudopuberty, hirsutism, or menstrual irregularities, while arterial hypertension is less frequently observed (3).

Given the rarity of 11βOHD, there is a paucity of data about the differences in clinical and biochemical characteristics of C- and NC-11βOHD. In this study, we report a national cohort of 102 children with 11βOHD with extensive phenotyping, steroid profiling by mass spectrometry,

molecular genetic analysis of the *CYP11B1* gene and clinical data to study the differences between the patients with C- and NC-11βOHD. The aim was to expand our knowledge on the clinical, biochemical and molecular spectrum of 11βOHD.

Methods

Study Population

We performed a cross-sectional study recruiting children with clinical and biochemical features suggestive of 11βOHD from 16 Departments of Pediatric Endocrinology across Turkey. The medical records of the patients were reviewed for detailed clinical and biochemical information, in addition to other relevant medical and family history data.

Excess plasma samples from children examined for other conditions, such as well-controlled type 1 diabetes, euthyroid hypothyroidism or hyperthyroidism on treatment or simple growth retardation but without adrenal enzyme deficiency, polycystic ovary syndrome, or puberty disorder, were used as controls for adrenal steroid measurements. The control group included 210 children [119 girls and 91 boys, median age 11.4 (0.8-19.5) years]. Age and gender of control subjects were not different from the 11βOHD patients ($P = 0.98$ and $P = 0.78$, respectively).

The study was performed with the approval of the Ethics Committee of Marmara University Faculty of Medicine, Istanbul, Turkey (09.2017.476). Patients and/or parents provided written informed consent.

Hormonal Assays

Blood samples were collected between 8:00 and 9:00 AM after 48 h off-treatment state in a hospital setting with continuous observation for general well-being, blood pressure, and the other vital signs. These procedures were performed after obtaining an informed consent from the patient and/or the parents. We did not observe any adverse events in the patients during the off-treatment period. Biochemical assessment included adrenal steroid profile measured by liquid chromatography-mass spectrometry (LC-MS/MS) as previously described (14). ACTH and other biochemical parameters were measured using commercial immunoassays.

Molecular Genetic Analysis of the *CYP11B1* Gene

Genomic DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN, Germantown, MD, USA) from peripheral blood lymphocytes according to standard protocols. The coding exons and exon-intron boundaries of the *CYP11B1* gene (NM_000497) were amplified as previously described (15), sequenced via Sanger sequencing using an ABI 3130XL DNA Sequencer (Thermo Fisher Scientific, Waltham, MA, USA), and analyzed by Seqscape sequencing analysis software, version 2.7 (Applied Biosystems, Foster City, CA, USA). Sequence variants were designated according to Human Genome Variation Society recommendations (www.hgvs.org/rec.html) using the reference sequences GenBank NC_000008 (g.DNA), NM_000497 (c.DNA), and NP_000488.3 (protein).

Patients were classified as C- or NC-11βOHD depending on the degree of clinical severity and percentage loss of CYP11B1 activity as reported previously (6-13). The differences between C- and NC-11βOHD were reviewed, focusing specifically on the clinical parameters and adrenal steroid profiles.

Statistical analysis

Statistical analysis was performed using GraphPad Prism® V5.0 software (GraphPad Software Inc, San Diego, California, USA). Data were tested for normality distribution using the Shapiro-Wilk test. Results are reported as frequencies and percentages, median with minimum-maximum, interquartile ranges (IQR) or 95% confidence intervals (95%CI) as appropriate. A nonparametric *t* test was used for comparison of numeric and χ^2 test for categorical variables. Statistical significance was set at $P < 0.05$.

Results

Clinical Characteristics of the Patients

We identified 102 patients (53 with 46,XX and 49 with 46,XY karyotypes) from 76 families (45 familial cases from 19 families and 57 singletons) diagnosed with 11βOHD and genetically confirmed using *CYP11B1* gene mutation analysis. The patients had been assessed from the neonatal period until 20.8 years of age (median: 9.9 years). The age at diagnosis and initiation of hydrocortisone treatment was 1.8 years (range: 0.01 to 15.8). Eighty patients (78%) were diagnosed before 4 years of age, 35 of 53 girls (66%) were presented before 1 year of age because of ambiguous genitalia, while 43 of 49 boys (87%) presented after 1 year of age, mainly due to precocious pseudopuberty and advanced growth (Fig. 1A). Thus, the girls were diagnosed earlier than the boys [0.04 (range: 0.01 to 4.5) *vs* 2 (range: 0.04 to 12) years, $P < 0.0001$]. Twenty-nine patients (28%) had hyperpigmentation, and 10 patients (10%) had mild hyponatremia at the presentation (sodium levels 131-134 mEq/L). Six of them erroneously used fludrocortisone. Five 46,XX

patients (10%) were raised as males. Nineteen patients (19%) had been initially misdiagnosed as 21-hydroxylase deficiency (21OHD). Premature adrenarche was present in 51 (50%) patients, and 46 patients (46%) required treatment with at least 1 antihypertensive drug for hypertension (1 [range: 1 to 3)] antihypertensive drugs). The age of the onset of antihypertensive therapy was 7.7 (range:1.5 to 16) years. The patients had markedly advanced bone age in both sexes [bone age/chronological age; females: 2.1 (range:0.80 to 15.00), n = 15 *vs* males: 2.4 (range:1.0 to 8.2), n = 37; $P = 0.67$] (Fig. 1B). Nineteen patients used gonadotropin-releasing hormone analogue in addition to hydrocortisone treatment to improve adult height. Adult height, adult height SD score (SDS), and corrected height SDS (adult height SDS – midparental SDS) were 152 (range: 136 to 162.5) cm, –1.85 (range: –4.62 to –0.10), and –1.07 (range: –3.45 to 0.79), respectively, for females (n = 18) and 160.4 (range: 134.2 to 175.3) cm, –2.56 (range: –6.81 to –0.15), and –1.85 (range: –4.87 to 1.64), respectively, for males (n = 24; $P = 0.22$ and $P = 0.11$ for adult height SDS and corrected height SDS, respectively). Fifteen of 49 boys (30%) had bilateral adrenal rest tumors [age at first detection 10 years (1.5 to 16)]. Detailed clinical features of the patients are provided in Supplemental Table 1 in (16).

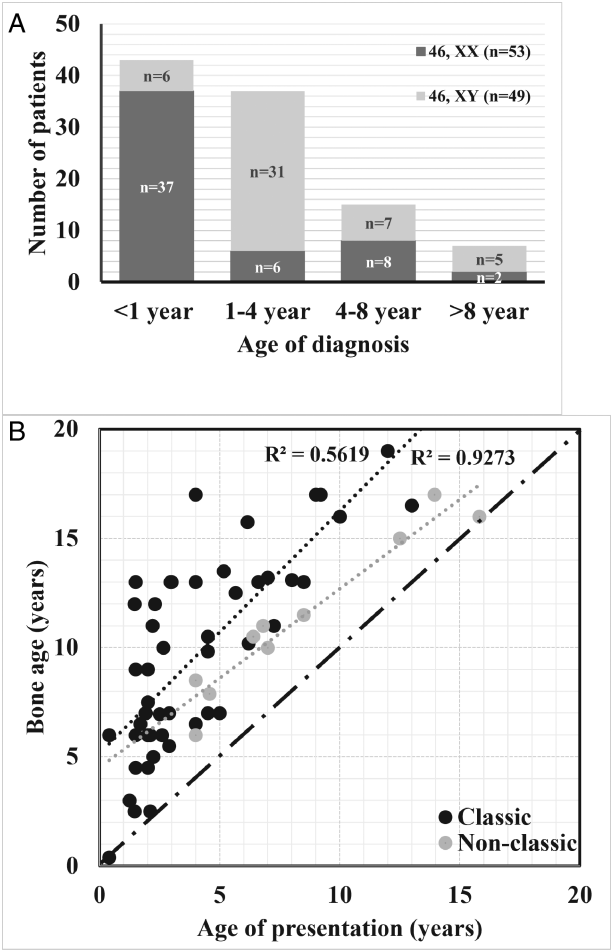

Figure 1. Clinical profile of 102 pediatric patients with 11β-hydroxylase deficiency. (A) The age at diagnosis in female and male patients with 11β-hydroxylase deficiency. Eighty patients (78%) were diagnosed before 4 years of age, 37 of 53 girls (70%) presented before 1 year of age because of ambiguous genitalia, while 43 of 49 boys (87%) presented after 1 year, mainly due to precocious pseudopuberty and advanced growth. (B) Bone age *vs* chronological age in classic (black) and nonclassic (grey) 11β-hydroxylase deficiency patients at the presentation. The black dotted line represents a slope of 1 (bone age = chronological age). Almost all patients showed advanced bone age, being more pronounced in patients with classic 11β-hydroxylase deficiency.

Molecular Analysis of *CYP11B1* Gene

Thirty sequence variations were identified including 15 missense (novel n = 5: p.Glu310Gln, p.His69Pro, p.Gly446Asp, p.Phe487Cys, p.Arg412Leu), 3 nonsense, 7 splice-site (novel n = 2:c.1122-1G>C, c.955-3C>A) changes; 1 intronic change (novel n = 1: c.239 + 150C>A); 3 small deletions (novel n = 1:p.Glu198del); and 1 small insertion and 1 small duplication (Table 1). Ninety-four patients were homozygous and 8 were compound heterozygous for *CYP11B1* mutations. The parents carried the mutant alleles in the heterozygous state.

All of the novel single nucleotide variants were predicted to be "damaging," "pathogenic," or "disease-causing" using multiple in silico analysis tools including Polyphen-2, SIFT, Mutationtaster and PROVEAN (29-32). None of the variants were found in the population databases GnomAD (Genome Aggregation Database), ExAC, 6500ESP, or 1000 Genomes (33).

The c.1179_1180dup (p.Asn394Argfs*37), c.896T>C (p.Leu299Pro), c.954G>A (p.Thr318=) and c.421C>T (p.Arg141*) were the most common mutations accounting for two thirds of the alleles with allele frequencies of 24.7%, 16%, 10%, and 7.2%, respectively. Ten patients were subsequently classified as NC-11βOHD based on the previous reports of mutations associated with NC-11βOHD in the literature (6-13).

Comparisons Between C-11βOHD and NC-11βOHD

The median ages of the 92 C-11βOHD (46,XX n = 45, 46,XY n = 47) and 10 NC-11βOHD (46,XX n = 8, 46,XY n = 2) patients at the time of assessment were 9.8 (range: 0.07 to 20.8) and 10.4 (range: 4.66 to 18.8) years, respectively ($P = 0.93$). None of the NC-11βOHD girls had ambiguous genitalia (C-11βOHD 100%, $P < 0.0001$), and none of the NC-11βOHD patients were hypertensive (C-11βOHD 50%, $P = 0.0019$). The C-11βOHD patients

Table 1. Characteristics, distribution, and the effect of CYP11B1 mutations in 102 patients with 11β-hydroxylase deficiency

#	Nucleotide change (genome assembly GRCh37.p13, *CYP11B1* (NM_000497)	Protein	Alleles (n)	Homozygous patients (n)	Heterozygous patients (n)	Localization	Activity (%)	Clinical phenotype	Reference	MAF in GnomAD (%)	RefSNPs (rs)
1	c.1179_1180dup	p.Asn394Argfs*37	51	24	3	e7/K-L loop	ND	C	17	0.0008	rs758714890
2	c.896T>C	p.Leu299Pro	33	15	3	e5/H-I loop	1	C	18	0.0004	rs387907573
3	c.954G>A	p.Thr318=	21	10	1	e5/I helix	ND	C	19	0.0012	rs753774484
4	c.421C>T	p.Arg141*	15	7	1	e3/C helix	ND	C	20	0.0008	rs775479837
5	**c.928G>C**	**p.Glu310Gln**	**14**	**7**	**0**	**e5/I helix**	**ND**	**C**	**This study**	**NA**	
6	c.372delG	p.His125Thrfs*8	12	6	0	e2/B-C loop	ND	C	21	NA	rs1554653520
7	c.1342C>T	p.Arg448Cys	4	2	0	e8/Cys pocket	0	C	22	0.0004	rs1221010438
8	**c.239 + 150C>A**	**p.?**	**4**	**2**	**0**	**i1**	**ND**	**C**	**This study**	**NA**	
9	c.1012C>T	p.Gln338*	4	2	0	e6/J helix	ND	C	23	0.0008	rs1214983921
10	**c.206A>C**	**p.His69Pro**	**4**	**2**	**0**	**e1/A helix**	**ND**	**C**	**This study**	**NA**	
11	c.563_566insTCCA	p.Gln189Hisfs*71	4	2	0	e3/E helix	ND	C	24	NA	
12	c.595 + 12G>A	p.?	3	1	1	i3	ND	C	25	0.0088	
13	**c.1337G>A**	**p.Gly446Asp**	**2**	**1**	**0**	**e8/Cys pocket**	**ND**	**C**	**This study**	**NA**	
14	c.1449_1451delGGT	p.Met483Ile	2	1	0	e9/C-term	ND	C	26	NA	
15	c. 1398 + 2T>C	p.?	2	1	0	i8	ND	C	27	0.0008	rs577022490
16	**c.593_595delAAG**	**p.Glu198del**	**2**	**1**	**0**	**e3/E helix**	**ND**	**C**	**This study**	**NA**	
17	c.348G>C	p.Trp116Cys	2	1	0	e2/B-C loop	3	C	18	0.0007	rs772003869
18	c.1201-9C>A	p.?	2	1	0	i7	ND	C	28	NA	
19	**c.1122-1G>C**	**p.?**	**2**	**1**	**0**	**i6**	**ND**	**C**	**This study**	**NA**	
20	**c.1460T>G**	**p.Phe487Cys**	**1**	**0**	**1**	**e9/C-term**	**ND**	**C**	**This study**	**NA**	
21	c.1398 + 5G>C	p.?	1	0	1	i8	ND	C	24	NA	rs1563867837
22	c.946G>A	p.Val316Met	6	3	0	e5/I helix	ND	NC	13	0.0032	rs375833424
23	c.1466T>C	p.Leu489Ser	4	2	0	e9/C-term	ND	NC	8	0.00 039	rs750428278
24	c.427C>T	p.Arg143Trp	4	2	0	e3/C helix	10	NC	10	0.016	rs140336749
25	c.281C>T	p. Pro94Leu	2	1	0	e2/B helix	0-2	NC	7	0.0004	rs104894070
26	c.890C>T	p.Ala297Val	1	0	1	e5/H-I loop	27	NC	11	0.0016	rs375892072
27	c. 1066C>T[a]	p.Gln356*	1	0	1	e6/J-K loop	ND	NC[a]	23	0.00 005	rs146124466
28	**c.955-3C>A[a]**	**p.?**	**1**	**0**	**1**	**i5**	**ND**	**NC[a]**	**This study**	**NA**	
29	**c.1235G>T[b]**	**p.Arg412Leu**	**1**	**0**	**1**	**e8/K-L loop**	**ND**	**NC[b]**	**This study**	**NA**	
30	c.799 + 2T>C[b]	p.?	1	0	1	i4	ND	NC[b]	NA	NA	rs193922541

Novel mutations are in bold.

Abbreviations: MAF in GnomAD, minor allele frequency in Genome Aggregation Database; NA, variant does not have a gnomAD genomes entry; ND, Not done.

[a]In patient #76 (16), clinical and biochemical phenotype was NC-11βOHD with compound heterozygosity for [c.1066C>T];[c.955-3C>A] mutations in *CYP11B1*. c.1066 C>T was reported to cause a truncated, nonfunctional protein. The enzymatic activity of c.955-3 C>A variant has not been tested in vitro. Nonetheless, this variant was predicted to cause a milder gene defect and NC-11βOHD phenotype was presumably related to the residual activity of this milder allele.

[b]In patient #72 (16), clinical and biochemical phenotype was NC-11βOHD with compound heterozygosity for [c.1235G>T];[c.799 + 2T] mutations in *CYP11B1*. The enzymatic activities of c.1235G>T and c.799 + 2T>C variants have not been tested in vitro. Nonetheless, at least 1 of these variants was predicted to cause a milder gene defect and NC-11βOHD phenotype was presumably related to the residual activity of this milder allele.

were diagnosed earlier than NC-11βOHD patients (median, 95%CI, and range: 1.33, 1.19 to 2.19, and 0.01 to 13 years *vs* 6.90, 5.3 to 11.2, 4.0 to15.8 years, respectively; $P < 0.0001$). The bone age was more advanced in C-11βOHD patients (bone age/chronological age ratio median, 95%CI, and range: 2.33, 2.40 to 3.74, and 0.80 to 15.00 for C-11βOHD and 1.50, 1.27 to 1.78, and 1.01 to 2.18 years, respectively, for NC-11βOHD; $P = 0.04$) (Fig. 1B). All of the C-11βOHD and NC-11βOHD patients in our study were on hydrocortisone replacement therapy. Hydrocortisone was initiated in NC-11βOHD patients to control androgen excess. The median age of starting hydrocortisone treatment was 1.3 (range: 0.01 to 13) and 6.9 (range: 4.0 to 15.8) years in C- and NC-11βOHD patients, respectively ($P < 0.0001$). Hydrocortisone treatment doses were not statistically different in C- and NC-11βOHD patients (16.8 ± 5 and 14.4 ± 2.8 mg/m^2/d in C- and NC-11βOHD patients, respectively ($P = 0.14$)). Adult height SDS and corrected height SDS values were lower in C-11βOHD (n = 37) than in NC-11βOHD (n = 5) patients [–2.46 (range: – 6.81 to –0.1) *vs* –1.32 (range –1.55 to –0.19), $P = 0.05$ and –1.5 (range: –4.87 to 1.64) *vs* –1.14 (range: –1.40 to –0.57), $P = 0.39$; respectively].

Plasma ACTH concentrations were higher in C-11βOHD than in NC-11βOHD patients [median (IQR): 142 (47 to 213) *vs* 10 (6 to 22) pmol/L, $P = 0.02$; normal range: 2.2 to 13.2 pmol/L). Cortisol response to standard intravenous cosyntropin test (250 µg) was 533 nmol/L (range: 450 to 687) in NC-11βOHD patients (n = 4). Table 2 shows the comparison of adrenocortical steroid hormone measurements by LC-MS/MS between C- and NC-11βOHD patients compared to the control group. The most significant elevations were in 11-deoxycortisol, DOC, 17OH-pregnenolone, and the adrenal androgen precursor (androstenedione) in decreasing order in 11βOHD patients compared to controls. The most notable decrease was in cortisol, cortisone, and corticosterone concentrations in patients compared to controls. The 11-oxygenated androgens were also significantly lower in patients compared to controls. Cortisol, cortisone, 11-deoxycortisol, DOC, corticosterone, and DHEA concentrations were the main adrenal steroids, which differed between C- and NC-11βOHD patients (Table 2). Among the steroid concentrations adjusted for cortisol, 11-deoxycortisol/cortisol, DOC/cortisol, and androstenedione/cortisol ratios were specific to discriminate C- and NC-11βOHD patients, in decreasing order (Table 2, Fig. 2A, 2B, and 2F). The 17OH-pregnenolone/cortisol, 17OH-progesterone/cortisol, and DHEA/cortisol ratios were similar between C- and NC-11βOHD patients (Table 2, Fig. 2C, 2D, and 2E). The ratio of 11-deoxycortisol/cortisol was significantly different between C-11βOHD patients with or without hypertension and NC-11βOHD patients; [median (IQR): 42.3 (22.7-69.3) for C-11βOHD with hypertension, 18.4 (8.15-37.7) for C-11βOHD without hypertension and 0.35 (0.24-0.91) for NC-11βOHD patients; $P < 0.0001$].

Discussion

This study describes the largest pediatric group, as well as the largest national cohort, of patients with C- and NC-11βOHD and outlines the spectrum of this rare disease. Our findings demonstrate a concordance of clinical and metabolic profile of 11βOHD patients with their molecular characteristics. Our study shows, in particular, the utility of the multisteroid profiling data that may predict the 11βOHD phenotype (classic *vs* nonclassic).

Ambiguous genitalia was the leading sign of C-11βOHD girls in our study, which is similar to 21OHD (34). On the other hand, we noticed that lack of salt-wasting delayed the time of diagnosis in C-11βOHD boys further than boys with classic 21OHD (34) unless severe hypertension comes to clinical attention. This also applied to 10% of the 46,XX patients in our cohort with severe genital virilization (Prader 5) who had delayed diagnosis of C-11βOHD and raised as males. Nevertheless, we found that C-11βOHD patients were diagnosed before an age of 2.5 years, while the clinical presentation of a NC-11βOHD before 5 years of age is unlikely. The absence of ambiguous genitalia in girls and the normal blood pressure were the main clinical differences between C- and NC-11βOHD, explaining the delayed diagnosis in those with NC-11βOHD. Instead, those patients were initially followed for other diagnoses such as premature pubarche, hirsutism, polycystic ovary syndrome, or NC-21OHD, similar to previous reports (6-11). Indeed, NC-11βOHD should be an important diagnosis of exclusion in the clinical and biochemical work-up of disorders of androgen excess (3).

Short adult height is a feature of 11βOHD patients (35). Our results showed that the adult height was approximately 16 cm (2.7 SD) shorter in C-11βOHD and 6 cm (1.0 SD) shorter in NC-11βOHD patients compared to healthy Turkish population (36,37). Impaired adult height maybe attributed to a delay in diagnosis and possible overreplacement with glucocorticoids. However, our patients with NC-11βOHD were diagnosed significantly later than the ones with C-11βOHD, and both groups were treated with the similar doses of hydrocortisone. We have also observed that the adult height is more compromised in 11βOHD patients compared to 21OHD. The adult height of our patients with 11βOHD were shorter than Turkish classic and NC-21OHD patients by

approximately 1.1 and 1.5 SD (6.6-9.0 cm), respectively (36,38,39). In line with this, bone age to chronological age ratio was >2 in almost all C-11βOHD patients, and it was below 1.8 in almost all NC-11βOHD patients. This may suggest that not only delayed diagnosis, but severity of the disease may contribute to impaired adult height in 11βOHD. There were significantly increased 17OH-pregnenolone and androstenedione concentrations in both C- and NC-11βOHD patients. The extent of 17OH-pregnenolone elevation was even more prominent than that of 17OH-progesterone. As 17OH-pregnenolone is the preferred substrate for the17,20-lyase activity of CYP17A1, this allows for an increase in the biosynthesis of adrenal androgen precursors via the delta 5 pathway. Furthermore, the loss of CYP11B1 activity prevents the 11β-hydroxylation of androstenedione thereby reducing the production of 11-oxygenated androgens and potentially leading to more androstenedione being available for the peripheral aromatization to estrogens. This may contribute to accelerated bone age, premature epiphyseal closure, and short adult height in these patients. This is the first study to confirm low 11-oxygenated androgens in this disorder due to loss of CYP11B1 activity and therefore the inability to convert A4 into 11OHA4.

Similar to some previous reports, one fifth of our patients were initially misdiagnosed and treated as 21OHD, the main differential diagnosis of 11βOHD due to its clinical similarities and high 17OH-progesterone (40). The 11β-hydroxylated products of 17OH-progesterone (21-deoxycortisol), androstenedione (11β-hydroxyandrostenedione), and testosterone (11β-hydroxytestosterone) have recently been shown to be increased in 21OHD patients (41,42). In contrast, our findings clearly show that low 21-deoxycortisol, 11β-hydroxyandrostenedione, and 11β-hydroxytestosterone can be regarded as a signature steroid profile of 11βOHD during the differential diagnosis of 21OHD. High concentrations of 11-deoxycortisol, DOC, androstenedione, and low concentrations of cortisol, cortisone, and corticosterone should also be considered as a typical steroid fingerprint in 11βOHD. Furthermore, 11-deoxycortisol, DOC, 17OH-pregnenolone, and androstenedione were the most markedly raised hormones in 11βOHD patients compared to controls by 300, 150, 90, and 15 times, respectively. These results altogether suggest that simultaneous measurement of these steroids by LC-MS/MS are the most convenient method for diagnosis of 11βOHD providing high accuracy and reducing the need for ACTH stimulation test. Similarly, diagnostic ratios calculated using gas chromatography mass spectrometry, which measures quantitative urinary steroid hormone profiles can readily establish the diagnosis of 11βOHD. Indeed, a single spot urine measuring 100*tetrahydrodeoxycortisol/(tetrahydrocortisol + tetrahydrocortisone + 5α-tetrahydrocortisol) measured in a single spot urine establishes the diagnosis of 11βOHD with a high confidence in any age (43).

Unlike C-11βOHD, baseline ACTH and stimulated cortisol levels were normal or near-normal in our patients with NC-11βOHD. Nevertheless, there was a significant difference between basal measurements of 11-deoxycortisol, cortisol, DOC, corticosterone, cortisone, and DHEA concentrations, in descending order of significance, between C- and NC-11βOHD patients. This suggests that steroidogenesis is less severely impaired in the glucocorticoid and mineralocorticoid pathways than in the adrenal sex steroid biosynthesis in NC-11βOHD compared to C-11βOHD. However, the high DOC, and adrenal androgens in NC-11βOHD compared to controls indicate that these patients should have appropriate treatment and require surveillance to avoid long-term consequences of hyperandrogenism and potential hypertension.

Diagnostic specificity for the subgroups of 11βOHD was increased using steroid concentrations in proportion to cortisol. Especially 11-deoxycortisol/cortisol ratio emerged as the best biochemical marker to differentiate C- and NC-11βOHD patients. This ratio was around 75 times higher in C-11βOHD than in NC-11βOHD, compared to 5- to 6-fold difference in 11-deoxycortisol concentrations alone. No overlap was found in the 11-deoxycortisol/cortisol ratio, and the lower and upper 95%CIs were 27 to 40, 0.18 to 1.00, and 0.002 to 0.003 in C-11βOHD and NC-11βOHD patients and control groups, respectively. Furthermore, 11-deoxycortisol/cortisol ratio was significantly higher in the subset of C-11βOHD patients with hypertension compared to those without. Our results support the concordance between genotype, clinical phenotype, and steroid metabolism in 11βOHD patients.

We identified 9 novel and 21 previously reported mutations in our 102 children with 11βOHD. There was an enrichment of four rare pathogenic alleles in *CYP11B1* (p.Asn394Argfs*37, p.Leu299Pro, p.Thr318 = and p.Arg141*) in 58% of 11βOHD patients, which represents a significant mutational load in the Turkish population. These 4 variants were the most common mutations previously described in Turkish patients with 11βOHD (23,25). This may allow a more focused clinical genetic screening program for 11βOHD to be established in Turkey.

There is no distinct demarcation between C- and NC-11βOHD, and this is often a spectrum due to relative loss of CYP11B1 function as in the case of 21OHD CAH. The categorization of C- and NC-11βOHD should be based on clinical, biochemical, and molecular grounds. There are less than 20 mutations associated

Table 2. Comparison of plasma adrenocortical steroid hormone measurements by LC-MS/MS between patients with classic and nonclassic 11β-hydroxylase deficiency compared to the controls

	Classic 11βOHD (n = 78), median (IQR)	Nonclassic 11βOHD (n = 10), median (IQR)	Control (n = 210), median (IQR)	*P*-value			Fold change (X) (pt/control)
				C *vs* NC	NC *vs* control	Pt *vs* control	
Pathway/steroid hormone (nmol/L)[a]							
Minerolocorticoid							
Pregnenolone	2.54 (0.31-6.82)	4.99 (0.50-13.3)	0.15 (0.03-0.50)	0.06	<0.0001	<0.0001	5.26
Progesterone	1.71 (0.76-3.56)	0.41 (0.25-1.33)	0.19 (0.09-0.35)	0.21	0.75	0.00 006	3.42
11-deoxycorticosterone	25.7 (7.93-42.0)	5.87 (1.27-7.27)	0.12 (0.06-0.18)	0.007	<0.0001	<0.0001	149.28
Corticosterone	0.66 (0.26-1.87)	3.15 (0.98-14.7)	14.5 (9.30-21.2)	0.01	0.03	<0.0001	0.15
Aldosterone	0.22 (0.08-0.36)	0.13 (0.02-0.27)	0.05 (0.02-0.11)	0.45	0.48	0.0001	2.83
Glucocorticoid							
17OH-pregnenolone	9.93 (1.41-124.3)	231 (31.9-448)	0.66 (0.18-2.55)	0.73	<0.0001	<0.0001	94.16
17OH-progesterone	6.43 (2.83-12.4)	2.38 (2.11-3.74)	0.90 (0.42-1.41)	0.31	0.0009	<0.0001	10.07
11-deoxycortisol	348 (138.6-500)	68.2 (47.2-100)	0.72 (0.37-1.26)	0.001	<0.0001	<0.0001	314.44
Cortisol	8.91 (5.83-14.9)	190 (158-254)	285 (199-427)	<0.0001	0.06	<0.0001	0.12
Cortisone	0.60 (0.25-1.77)	61.7 (46.7-140.9)	67.1 (49.8-81.9)	<0.0001	0.003	<0.0001	0.36
21-Deoxycortisol	0.05 (0.02-0.20)	0.08 (0.02-0.46)	0.11 (0.05-0.28)	0.09	0.71	0.15	1.03
Androgen							
DHEA	4.61 (0.97-10.8)	37.1 (9.82-52.0)	3.64 (0.79-8.50)	<0.0001	<0.0001	0.0001	2.11
Androstenedione	19.8 (4.36-32.1)	8.96 (7.01-24.3)	0.87 (0.38-2.51)	0.22	<0.0001	<0.0001	13.24
Testosterone	2.35 (0.79-7.35)	1.38 (0.76-4.30)	0.31 (0.13-1.24)	0.41	0.94	0.002	1.98
Androsterone	4.46 (1.25-10.7)	5.61 (2.54-6.70)	0.41 (0.10-1.64)	0.31	0.02	<0.0001	5.45
11β-hydroxyandrostenedione	0.76 (0.39-1.85)	0.59 (0.39-1.25)	27.8 (17.0-42.3)	0.68	0.007	<0.0001	0.04
11β-hydroxytestosterone	0.06 (0.03-0.19)	0.06 (0.03-0.16)	0.16 (0.06-0.39)	0.73	0.31	0.0001	0.51
Steroid hormone ratios							
11-deoxycortisol/cortisol	27.75 (12.22-47.92)	0.35 (0.24-0.91)	0.002 (0.001-0.003)	0.0004	<0.0001	<0.0001	10 849
11-deoxycorticosterone/cortisol	2.43 (0.89-3.60)	0.02 (0.01-0.03)	0.0003 (0.0001-0.0005)	0.0016	<0.0001	<0.0001	5561
17OH-pregnenolone/cortisol	1.38 (0.18-18.18)	1.05 (0.29-2.66)	0.002 (0.0005-0.008)	0.17	<0.0001	<0.0001	3228
17OH-progesterone/cortisol	0.68 (0.26-1.35)	0.01 (0.009-0.04)	0.002 (0.001-0.004)	0.10	<0.0001	<0.0001	343
DHEA/cortisol	0.38 (0.11-1.18)	0.14 (0.08-0.16)	0.01 (0.002-0.020)	0.21	<0.0001	<0.0001	55
Androstenedione/cortisol	1.20 (0.41-2.97)	0.03 (0.02-0.11)	0.002 (0.001-0.006)	0.01	<0.0001	<0.0001	399

Abbreviations: C, classic; DHEA, dehydroepiandrosterone; IQR, interquartile range; NC, nonclassic; Pt, patient.

[a]To convert nmol/L to ng/mL, divide by 2.77 for aldosterone, 2.89 for corticosterone, 3.03 for 11-deoxycorticosterone, 3.10 for pregnenolone, 3.02 for 17α-hydroxyprogesterone, 3.18 for progesterone, 3.01 for 17α-hydroxypregnenolone, 2.88 for 21-deoxycortisol and 11-deoxycortisol, 2.75 for cortisol, 2.77 for cortisone, 3.47 for DHEA, 2.71 for DHEAS, 3.49 for androstenedione and androsterone, 3.47 for testosterone, 3.31 for 11β-hydroxyandrostenedione, and 3.28 for 11β- hydroxytestosterone.

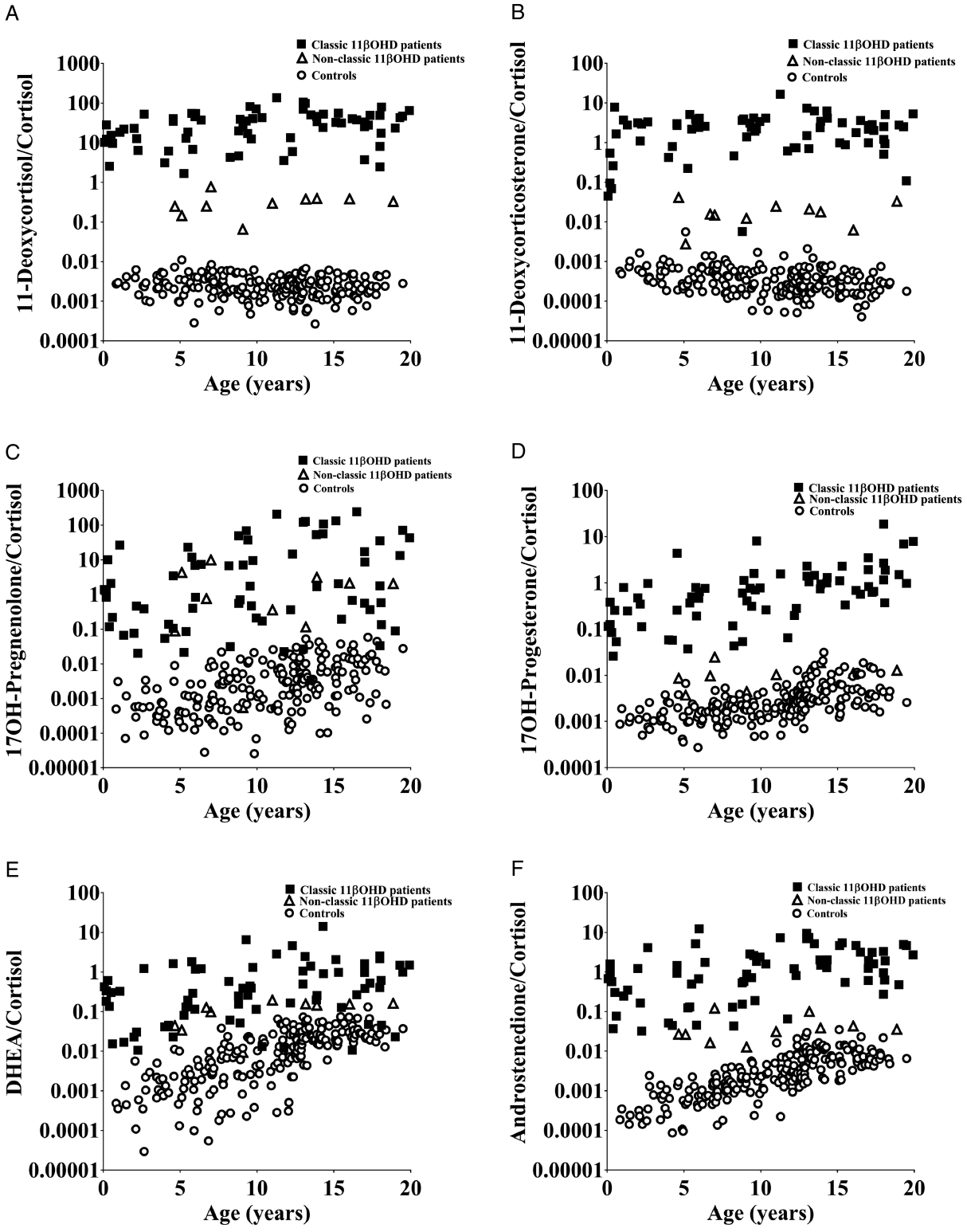

Figure 2. Steroid ratios across ages in patients with classic and nonclassic 11β-hydroxylase deficiency. Data are shown as linear regressions of steroids normalized to cortisol concentrations as a function of age (in years). All ratios showed a significant difference between patients and controls. 11-deoxycortisol/cortisol (A), 11-deoxycorticosterone/cortisol (B) and androstenedione/cortisol (F) ratios, but not 17OH-pregnenolone/cortisol (C), 17OH-progesterone/cortisol (D), and DHEA/cortisol (E) ratios could discriminate classic and nonclassic 11β-hydroxylase deficiency.

with NC-11βOHD in the literature (3,11). A residual CYP11B1 activity of above 10% to 25% is associated with a clinical phenotype of NC-11OHD similar to NC-21OHD or mild simple virilizing 21OHD (3,11). As in 21OHD patients (44), we observed in our patients with NC-11βOHD that a compound heterozygosity for

1 mild and 1 severe mutation in *CYP11B1* had a less severe clinical presentation than those patients with 2 severe mutations (ie, the phenotype was determined by the least severe mutation).

The main limitation of our study was the retrospective nature of the study. As we collected the clinical and biochemical characteristics retrospectively from medical files, some data regarding initial presentation were incomplete. Another limitation was the relatively small subgroup size of NC-11βOHD patients. Despite the large number of study individuals, comparisons of some characteristics between C- and NC-11βOHD patients should be interpreted with caution.

In conclusion, 11βOHD is an important diagnosis of exclusion in hypertension and androgen excess states in childhood. Nevertheless, it is not always straightforward to establish the diagnosis due to its broad clinical spectrum depending on the genotype. Especially NC-11βOHD can escape from clinical attention due to relatively mild clinical presentations and near-normal first-line adrenal function tests. However, the retained enzyme activity of CYP11B1 showed a good correlation with LC-MS/MS-based steroid profiles, which enable the diagnosis and subtyping of 11βOHD.

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Author contributions: TG, MY, and AB designed the study. TG, MY, EI, ZYA, MK, MNO, FB, AU, MB, CK, HO, FD, AC, EU, AA, HD, TC, FD, GC, ST, and AB recruited and clinically characterized the patients. TG, KHS, AY, and GH conducted and analyzed biochemical and LC-MS/MS measurements. TG, ZYA, and TB performed and analyzed the Sanger sequencing of *CYP11B1*. TG, MY, ZYA, HF, and AB prepared the draft manuscript. All authors contributed to the discussion of results and edited and approved the final manuscript.

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