ORIGINAL ARTICLE

Serum Cortisol and Cortisone as Potential Biomarkers of Partial 11β-Hydroxysteroid Dehydrogenase Type 2 Deficiency

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BACKGROUND
Pathogenic variations in HSD11B2 gene triggers the apparent mineralocorticoid excess syndrome (AME). There is scarce information regarding the phenotypes of subjects carrying heterozygous pathogenic variants in HSD11B2 gene. We investigated if serum cortisol/cortisone (F/E) ratio and cortisone are useful for identifying partial 11βHSD2 deficiency in those heterozygous subjects.

METHODS
We studied two patients diagnosed with AME and their families carrying either D223N or R213C mutation. We also evaluated 32 healthy control subjects (13 children and 19 adults) to obtain normal references ranges for all measured variables. Case 1: A boy carrying D223N mutation in HSD11B2 gene and Case 2: A girl carrying R213C mutation. We assessed serum F/E ratio and cortisone by HPLC-MS/MS, aldosterone, plasma-renin-activity(PRA), electrolytes, and HSD11B2 genetic analyses.

RESULTS
The normal values (median [interquartile range]) in children for serum F/E and cortisone (μg/dl) were 2.56 [2.21–3.69] and 2.54 [2.35–2.88], and in adults were 4.42 [3.70–4.90] and 2.23 [1.92–2.57], respectively. Case 1 showed a very high serum F/E 28.8 and low cortisone 0.46 μg/dl. His mother and sister were normotensives and heterozygous for D223N mutation with high F/E (13.2 and 6.0, respectively) and low cortisone (2.0 and 2.2, respectively). Case 2 showed a very high serum F/E 175 and suppressed cortisone 0.11 μg/dl. Her parents and sister were heterozygous for the R213C mutation with normal phenotype, but high F/E and low cortisone. Heterozygous subjects showed normal aldosterone, PRA, but lower fractional excretion of sodium and urinary Na/K ratio than controls.

CONCLUSION
Serum F/E ratio and cortisone allow to identify partial 11βHSD2 deficiencies, as occurs in heterozygous subjects, who would be susceptible to develop arterial hypertension.

Keywords: 11βHSD2 deficiency; AME syndrome; blood pressure; cortisol; cortisone; HSD11B2 mutation; hypertension.

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Arterial hypertension (AHT), especially associated with monogenic alterations, has been linked to pathological conditions affecting the synthesis, metabolism, or action of endocrine hormones, such as peptides, mineralocorticoids, and glucocorticoids.[1–8] Impairment of endocrine metabolic pathways associated with blood pressure regulation, as occurs due to deficiency of 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) enzyme, is being studied as a pathognomonic example of this type of alteration. 11βHSD2 is associated with the pre-receptor metabolism of the mineralocorticoid receptor (MR) and management of sodium/water equilibrium in renal epithelia and is therefore involved in the control of blood pressure. 11βHSD2 is expressed mainly in specific epithelial tissues where mineralocorticoid action is exerted, such as kidney (i.e., distal convoluted tubule (DCT)), colon, and placenta, and it is also expressed in nonepithelial tissues, such as vascular endothelium. 11βHSD2 catalyzes the conversion of cortisol (F) to cortisone (E) in the presence of cofactor NAD+, thereby avoiding the agonist action of cortisol over the MR and the

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potential activation of MR-dependent genes downstream, which can ultimately induce changes in blood pressure.[9–13]

Studies have shown that a complete deficit of the 11βHSD2 enzyme secondary to mutations in coding regions of HSD11B2 triggers severe hypertension in association with the early suppression of plasmatic renin levels and aldosterone.[2,13–15] This severe congenital form, described more than 40 years ago, it is known as "apparent mineralocorticoid excess" (AME).[16,17] AME is caused by a homozygous gene mutation in the HSD11B2 gene and is classically characterized by low-renin AHT, low aldosterone, and hypokalemia.[18] AME reports describe approximately 35 point mutations in intronic and exonic regions of the HSD11B2 gene in 66 families around the world, most of them partially or totally affecting the activity of the 11βHSD2 enzyme.[18] Furthermore, the presence of polymorphisms, variations in microsatellite regions[19,20] and epigenetic modifications in the HSD11B2 gene can affect its expression.[21,22] In 2003, we published a case of a Chilean pediatric patient with AME syndrome and AHT (OMIM 614232.0009, HSD11B2, ASP223ASN).[2] In this patient, we detected two homozygous mutations in the HSD11B2 gene: G to A transition in exon 4, resulting in the change Asp223Asn (D223N), and a C to T substitution in intron 3 (7279C-T; OMIM 614232.0001).

Although the clinical and biochemical features of AME syndrome have been extensively reported, the phenotypical presentation of heterozygous subjects has received little attention. Animal studies have revealed the potential pathogenic behavior of heterozygous subjects in the presence of a second hit,[23] such as a high salt diet, leading to a hypertensive phenotype. The aim of the present study was to describe the clinical and biochemical characteristics of subjects carrying heterozygous pathogenic variants of either the R213C or D223N mutation in the HSD11B2 gene, and highlight the serum F/E ratio and cortisone as useful metrics for identifying 11βHSD2 partial activity.

SUBJECTS AND METHODS

Subjects

In this study, we evaluated two patients diagnosed with AME and their families as well 32 healthy control subjects (13 children and 19 adults) to obtain references ranges for all measured variables (Table 1). All of the subjects were receiving an *ad libitum* sodium diet and declared they had not ingested any herbal products (i.e., chewing tobacco, licorice, or grapefruit) or diet supplements (i.e., high potassium diet) during the month preceding the analysis. The blood pressure (BP) of children was classified according to the Seventh Report of Task Force.[24] BP index was calculated to compare BP in children of different genders, ages, and statures. The BP index was determined by using the observed BP/50th percentile BP for gender, age, and stature using the standard reported values. Normal values, reference ranges (RR), and percentiles for all biochemical variables including serum and urinary F, E, F/E ratio, and (THF+alloTHF)/THE ratio were determined from 32 control subjects. The study and protocol were explained to all of the participants, and written informed consent was obtained. The protocol followed in this study was in accordance with the guidelines of the Declaration of Helsinki and was approved by the Ethical Committee of the Faculty of Medicine, Pontificia Universidad Catolica de Chile (CEC-MEDUC#14–268).

We studied two patients diagnosed with AME and their families. Briefly, the Family 1 members consist of a 17-year-old male (Index case 1), his mother (33 years old) and his

Table 1. Clinical and biochemical characteristics of healthy pediatric and adult control subjects participating in the current study

	Children	Adults
N	13	19
Age (years)	12.50 [10.32–13.90]	40.15 [29.12–51.99]
BMI, kg/m^2	21.60 [19.80–27.20]	26.00 [24.53–27.48]
zBMI	1.14 [0.51–1.84]	
SBP, mm Hg	111.0 [105.2–115.0]	112.3 [108.7–116.0]
SBP index	1.0 [0.9–1.05]	
DBP, mm Hg	65.00 [61.00–68.30]	72.30 [69.00–75.30]
DBP index	1.0 [1.0–1.15]	
Aldosterone, ng/dl	7.60 [4.25–12.15]	8.60 [6.100–12.20]
Plasma renin activity, ng/ml × h	2.10 [1.35–3.34]	1.54 [1.10–2.35]
Serum Cortisol, µg/dl	7.01 [5.52–9.67]	9.80 [7.44–12.00]
Serum Cortisone, µg/dl	2.54 [2.35–2.88]	2.23 [1.92–2.57]
Serum F/E ratio	2.56 [2.21–3.69]	4.42 [3.70–4.90]
Urinary F/E ratio	0.33 [0.27–0.41]	0.35 [0.28–0.46]
(THF + alloTHF)/THE ratio	0.63 [0.45–0.81]	0.86 [0.64–1.07]

Values are shown as the median [interquartile range]. Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; DBPi, index DBP; E, cortisone; F, cortisol; PRA, plasma renin activity; SBP, systolic blood pressure; SBPi, index SBP; THE, tetrahydrocortisone; THF, tetrahydrocortisol.

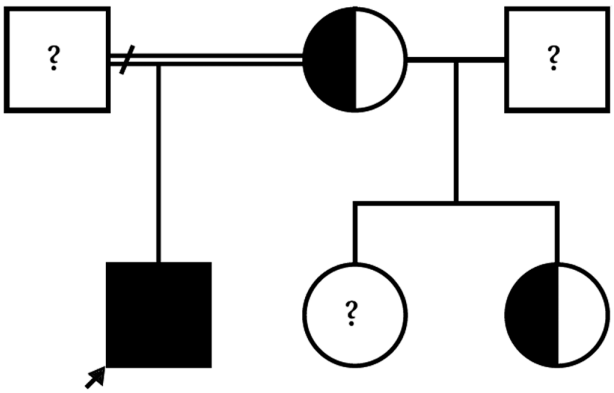

Figure 1. Pedigree of Family 1 carrying the D223N mutation in the HSD11B2 gene. The index case is indicated with a black arrow. Potential consanguinity in index case parents is indicated by a double line. Heterozygous individuals (mother and sister) are represented by half-shaded symbols. No information or nonstudied subjects are indicated with a question mark (?).

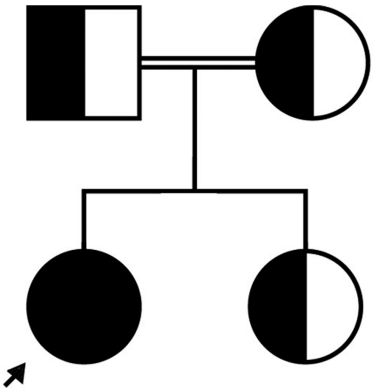

Figure 2. Pedigree of Family 2 carrying the R213C mutation in the HSD11B2 gene. The index case is indicated by a black arrow. Consanguinity of the index case's parents is indicated by a double line. The parents and sibling are heterozygous for R213C (half-shaded symbols). No information or nonstudied subjects are indicated with a question mark (?).

sister (8 years old). The index case was previously diagnosed in 2003 with AME syndrome (D223N mutation)[2] (Figure 1). The second family comprises a 2-year-old female (Index case 2), her father (30 years old), her mother (30 years old) and her sister (6 years old). Parents of the index case are first-degree cousins, both without any previous report of AHT (Figure 2). All their clinical details are presented in the Supplementary File.

Biochemical methods

All subjects and controls enrolled in this study were subjected to specific clinical, biochemical, and genetic analyses. Briefly, after an overnight fast, basal blood samples were withdrawn between 08:00 AM and 10:00 AM and also 24-hour urine samples were obtained from each subject. In blood, we measured aldosterone,[25] plasma renin activity (PRA),[26] electrolytes (Na+, K+), cortisol, and cortisone. In urine, we measured electrolytes, cortisol, cortisone, tetrahydrocortisol (THF), allo-tetrahydrocortisol (allo-THF or aTHF), and tetrahydrocortisone (THE). We also calculated the 24-hour sodium excretion (mEq/24h) (as measurement of sodium intake), fractional excretion of sodium (FENa, %), fractional excretion of potassium (FEK, %), and the Na+/K+ ratio ($U_{Na:K}$). We also calculated the transtubular transport of potassium gradient (TTKG) from urinary and serum electrolyte concentrations, and osmolality (Osmometer from Advanced Instruments® 3300, Waters), since TTKG has been associated to mineralocorticoid activity.

We measured serum and urinary cortisol, cortisone, tetrahydrocortisol (THF), allo-tetrahydrocortisol (aTHF), and tetrahydrocortisone (THE) by liquid chromatography associated with tandem mass spectrometry (LC-MS/MS) in a ABSciex 4500-QTrap (Framingham, MA) with deuterated standards. Details are in the Supplementary File.

Analysis of genomic DNA

Genomic DNA of index cases, their family members, and control subjects were isolated from peripheral blood mononuclear cells (PBMC). PCR amplification of proximal promoter, 5UTR, and five exons of the HSD11B2 gene (NG_016549.1) was performed in a T100 thermocycler (Bio-Rad, Hercules, CA). The oligonucleotides were designed and generated with IDTDNA or Primer3 online tools, and edited manually (Supplementary File; Supplementary Tables 1S and 2S). The HSD11B2 promoter was amplified with a specific primer located in areas potentially unmethylated using a GC-rich enzyme (Roche Diagnostics GmbH, Basel, Switzerland) (Supplementary Table 2S). The PCR analysis was developed in a final volume of 25 µl, with 1 µl of genomic DNA, 5 pmol of each primer (Integrated DNA Technologies, Inc., San Diego, CA), 2.5 nmol of each dNTP (Invitrogen, Carlsbad, CA). PCR amplification was performed with Taq enzyme (GoTaq® Colorless Master Mix, Promega, Madison, WI) for exons 2–5 of HSD11B2 gene, and for exon 1 and HSD11B2 promoter we used 1.0 U of FastStart Taq DNA Polymerase, 19.5 nmol of $MgCl_2$, 2.5 µl reaction buffer and 5.2 µl GC-rich reaction buffer (Roche Diagnostics GmbH, Basel, Switzerland). PCR amplification was developed with initial denaturation at 94 °C for 4 minutes, 35 cycles of 94 °C for 35 seconds, the annealing temperature was adjusted and optimized at 56 °C for 35 seconds for all HSD11B2 PCRs (Supplementary Tables 1S and 2S); the extension was at 72 °C for 20–45 seconds. Amplified products were visualized on a 1.5% agarose gel and documented in a photo-documentation system (Bio-Rad, Hercules, CA). Sequencing was performed by the fluorescent-dideoxy chain terminator method with an ABI Prism 377 DNA sequencer (Macrogen, Seoul, South Korea). Sequences were matched with published HSD11B2 gene sequences by using BLAST and aligned by using Clustal Omega.

Data and statistical analyses

The results are expressed as the median and interquartile range. Reference ranges are defined as the range between the percentiles 2.5th and 97.5th. Group comparisons were performed by using unpaired rank-sum tests and Spearman correlation analysis with Prism 5 (GraphPad) and SPSS 21 (IBM). Differences were considered significant at P <0.05.

RESULTS

Genetic studies

None of the control subjects had any functional genetic variants in the promoter, 5UTR or gene of HSD11B2. We identified and confirmed the presence of two different coding mutations in the HSD11B2 gene in both of the index cases. In the index case of Family 1, we confirmed the homozygous presence of the D223N pathogenic variant (rs121917833) of the HSD11B2 gene, which has been reported previously (NG_016549.1: g.10119G>A; NM_000196.3:c.667G>A; NP_000187.3:p.Asp223Asn), and the noncoding pathogenic variant rs376023420 located in intron 3 (NG_016549.1:g.10024C>T).[2] In his mother and sister, we identified heterozygous genotypes involving the D223N variant (rs121917833) and the non-coding pathogenic variant in intron 3 (rs376023420) of the HSD11B2 gene.

In Family 2, we found the female index case to be homozygous for the R213C pathogenic variant (rs28934591) of the HSD11B2 gene (NG_016549.1:g.9983C>T; NM_000196.3:c.637C>T; NP_000187.3:p.Arg213Cys). Her parents and her sister exhibited the heterozygous genotype of the R213C variant at the HSD11B2 locus. No other pathogenic variations were found in the coding regions of the studied subjects.

Biochemical studies and serum cortisol and cortisone determinations

Normal values were obtained from the healthy control subjects (median [interquartile range]) and used also to establish the reference ranges (RR) [percentiles 2.5th–97.5th] for serum and urinary F, E, the F/E ratio, and the (THF+aTHF)/THE ratio for normotensive groups of children and adults (Table 1).

Family 1. The index case for D223N mutation in HSD11B2, showed very low plasma potassium (2.1 mEq/L) and plasma renin activity (<0.2 ng/ml × h), and aldosterone

Table 2. Clinical and biochemical characteristics of Family 1 carrying the pathogenic variant D223N

	Index case (D223N)	Mother (D223N heterozygote)	Sister (D223N heterozygote)	Reference value or range
Age (years)	17	33	9	
SBP (mm Hg)	165	110	90	<120
SBPi	1.42		0.9	1 [1–1.05]
DBP (mm Hg)	110	70	60	<80
DBPi	1.64		0.98	1 [1–1.15]
BMI (kg/m^2)	22.6 (69th percentile)	29.9	18.1 (84th percentile)	20–25
K+ (mEq/L)	2.1	3.7	3.8	[3.5–5]
Aldosterone (ng/dl)	1.0	3.8	13.9	[1.8–23.2]
PRA (ng/mL × h)	<0.2	2.6	4,6	[1.3–4]
Cortisol (μg/dl)	13.3	26.3[a]	13.3	[4.1–11.8][b]
				[4.2–21.2][c]
Cortisone (μg/dl)	0.46	2.0 (29th percentile)	2.2 (18th percentile)	[1.74–3.8][b]
				[1.38–3.33][c]
Serum F/E ratio	28.8 (>>99th percentile)	13.2 (>97th percentile)	6.0 (>97th percentile)	[1.6–5.1][b]
				[2.6–7.8][c]
Urinary F/E ratio	2	1.11	0.57	[0.18–0.47][b]
				[0.24–0.73][c]
(THF + alloTHF)/THE	10.15	2.19	1.20	[0.28–1.05][b]
				[0.43–2.01][c]

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; DBPi, index DBP; E, cortisone; F, cortisol; PRA, plasma renin activity; SBP, systolic blood pressure; SBPi, index SBP; THE, tetrahydrocortisone; THF, tetrahydrocortisol.

[a]Use of oral contraceptives was reported. References ranges are defined as the range between the percentiles 2.5th and 97.5th.

[b]Children range.

[c]Adult range.

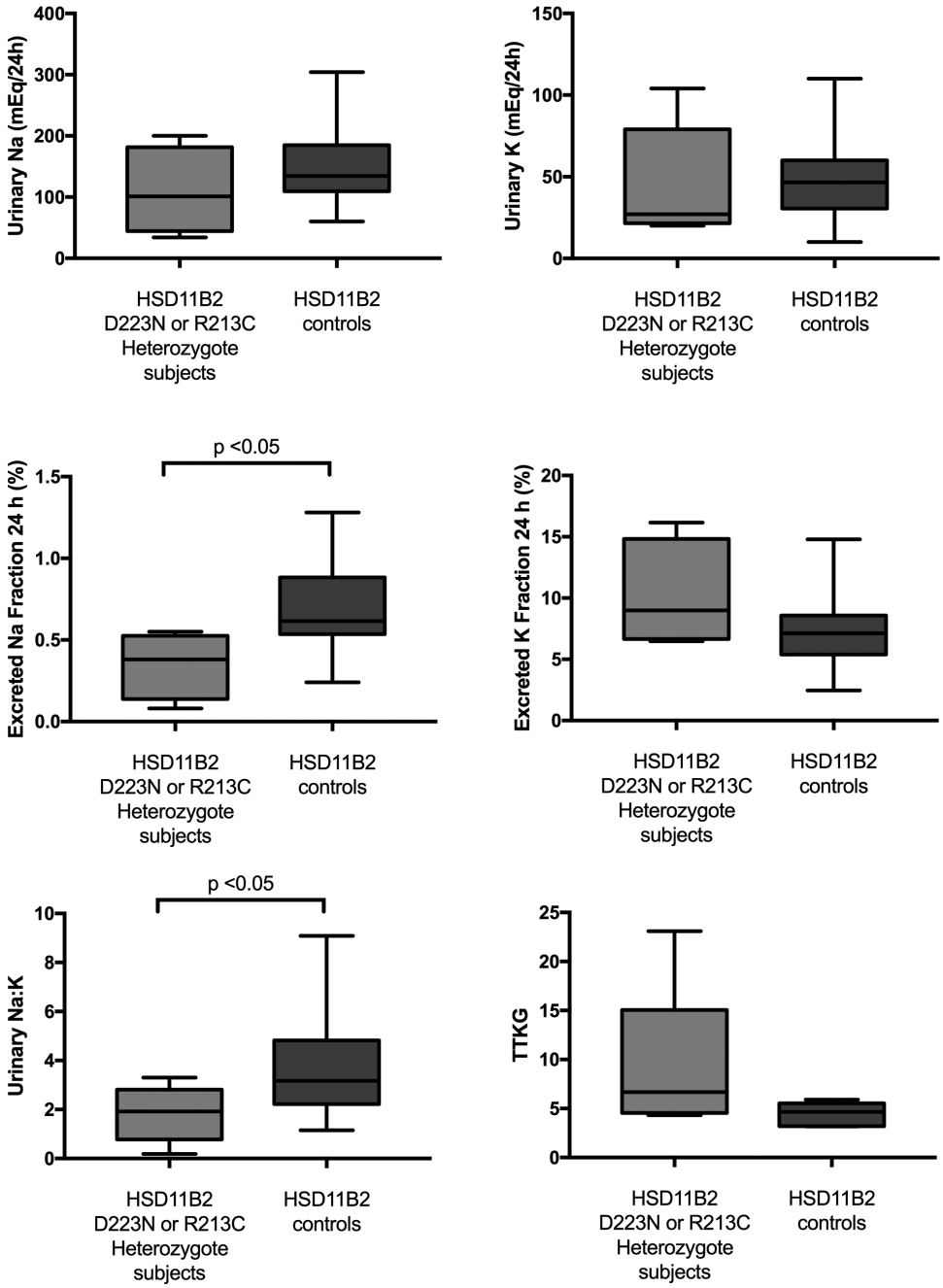

Figure 3. Natriuretic effect of HSD11B2 mutations in heterozygous HSD11B2–D223N and HSD11B2–R213C subjects. Urinary sodium excretion (24 h), urinary potassium, fractional excretion of sodium and potassium, and the urinary Na/K ratio ($U_{Na:K}$) were measured in heterozygous subjects having D223N and R213C mutations, compared with 32 healthy control subjects. We found similar urinary sodium excretion (Na mEq/24 h) and lower fractional excretion of sodium and $U_{Na:K,}$ in heterozygote subjects vs. controls. The nonparametric Mann–Whitney *t*-test was performed for all comparisons. A *P* value lower than 0.05 was considered significant ($P < 0.05$).

was undetectable (<1 ng/dl). These findings are compatible with classical AME features, including a high serum F/E ratio (28.8 (>99th percentile)) and very low cortisone (0.46 µg/dl) (Table 2). Additionally, we observed a high urinary F/E ratio (2.0), high (THF+aTHF)/THE ratio (10.2), low Na/K ratio ($U_{Na:K}$ 1.37 [1.63–6.85]),) and high TTKG value (11.0 [3.17–5.83]) (Table 2 and Figure 3). His mother and sister were normotensive and heterozygous for the D223N mutation without clinical and biochemical signs of hypertensive disease; however, they had high serum F/E ratio (13.1 (97th percentile) and 7.4 (97th percentile) and very low cortisone concentrations (below the first quartile). They also displayed high urinary F/E and high (THF+aTHF)/THE ratios (Table 2).

Family 2. The AME R213C index case displayed typical features of AME, including a history of being small

Table 3. Clinical and biochemical characteristics of Family 2 carrying the pathogenic variant R213C

	Index case (R213C)	Mother (R213C heterozygote)	Father (R213C heterozygote)	Sister (R213C heterozygote)	Reference values
Age (years)	2	34	36	8	
SBP (mm Hg)	197	124	150	110	<120
SBPi	1.24			1.14	1 [1–1.05]
DBP (mm Hg)	133	68	75	60	<80
DBPi	1.22			1.05	1 [1–1.15]
BMI (kg/m^2)	13.5 (3rd percentile)	31	28.6	16.9 (78th percentile)	20–25
K+ (mEq/L)	4.7	4.2	3.7	4.4	[3.5–5]
Aldosterone (ng/dl)	1.0	4.8	8.5	8.0	[1.8–23.2]
PRA (ng/ml × h)	<0.2	2.04	12.8	1.85	[1.3–4]
Cortisol (μg/dl)	19.7	13.7[a]	13.9	7.3	[4.1–11.8][b] [4.2–21.2][c]
Cortisone (μg/dl)	0.11	1.96 (25th percentile)	2.13 (30th percentile)	1.71 (2.5th percentile)	[1.74–3.8][b] [1.38–3.33][c]
Serum F/E ratio	175 (>>99th percentile)	6.99 (93th percentile)	6.55 (92th percentile)	4.29 (85th percentile)	[1.6–5.1][b] [2.6–7.8][c]
Urinary F/E ratio	29.9	0.46	0.46	0.14	[0.18–0.47][b] [0.24–0.73][c]
(THF + alloTHF)/THE	3.16	0.83	0.95	0.45	[0.28–1.05][b] [0.43–2.01][c]

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; DBPi, index DBP; E, cortisone; F, cortisol; PRA, plasma renin activity; SBP, systolic blood pressure; SBPi, index SBP; THE, tetrahydrocortisone; THF, tetrahydrocortisol.

[a]Use of monthly contraceptive was reported. References values are defined as the range between the percentiles 2.5th and 97.5th.

[b]Children range.

[c]Adult range.

for gestational age (SGA) in the neonatal period, low renin hypertension, hypokalemia, nephrocalcinosis, and parent consanguinity. Her mother and sister are heterozygous and normotensives without clinical signs of cardiovascular or renal diseases (Table 3). Her father occasionally displays "white-coat" hypertension, but no other pathologies were informed or detected by clinical-biochemical examination. She also exhibited a very high serum F/E ratio (175 (>99th percentile) and very low cortisone (0.11 ng/dl). In the analysis of 24 hour urine, we found a high F/E ratio (29.9), high (THF+aTHF)/THE ratio (3.16), low Na/K ratio ($U_{Na:K}$ 0.56 [1.63–6.85]), and high TTKG value (8.4 [3.17–5.83]) (Table 3 and Figure 3). Her parents and sister were clinically and biochemically normal except for high serum F/E ratios (7.0 (92nd percentile), 6.6 (93rd percentile), and 4.3 (85th percentile), respectively) and low cortisone levels (30th percentile or lower). They displayed normal urinary F/E and (THF+aTHF)/THE ratios (Table 3).

Natriuretic effects of D223N and R213C mutations in heterozygous subjects

The 24 hour sodium excretion, plasma renin activity (PRA), urinary fractional excretion of sodium, urinary fractional excretion of potassium, TTKG, and the urinary sodium to potassium ratio ($U_{Na:K}$) was evaluated to measure of natriuretic effects of HSD11B2 pathogenic variants in heterozygous subjects for D223N and R213C. We compared these variables between heterozygous and healthy control subjects, and we found similar levels in 24 hour sodium excretion (101 [45–182] vs. 134 [109–185] mEq/24 h; P = non-significant), PRA (2.6 [1.9–8.7] vs. 1.71 [1.2–2.78] ng/ml × h; P = non-significant) and fractional excretion of potassium (8.9 [6.66–14.82] vs. 7.13 [5.40–8.57]%; P = non-significant). We also found lower values in heterozygous subjects in the fractional excretion of sodium (0.38 [0.14–0.53] vs. 0.61 [0.54–0.88] %; $P = 0.007$) and $U_{Na:K}$ (1.92 [0.94–2.81] vs. 3.17 [2.22–4.82]; $P = 0.027$) (Figure 3).

DISCUSSION

In the current report, subjects with AME syndrome having either the D223N or R213C pathogenic variants in the HSD11B2 gene displayed the classical AME phenotype, including low-renin hypertension, low serum K+, and a high serum cortisol/cortisone ratio. Both mutations show high pathogenic potential as demonstrated previously by the very low activity of mutants *in vitro* and *in silico*.[2,14,27] New *in silico* models suggest that both mutations induce a structural disruption of the 11BHSD2 protein, impairing its catalytic

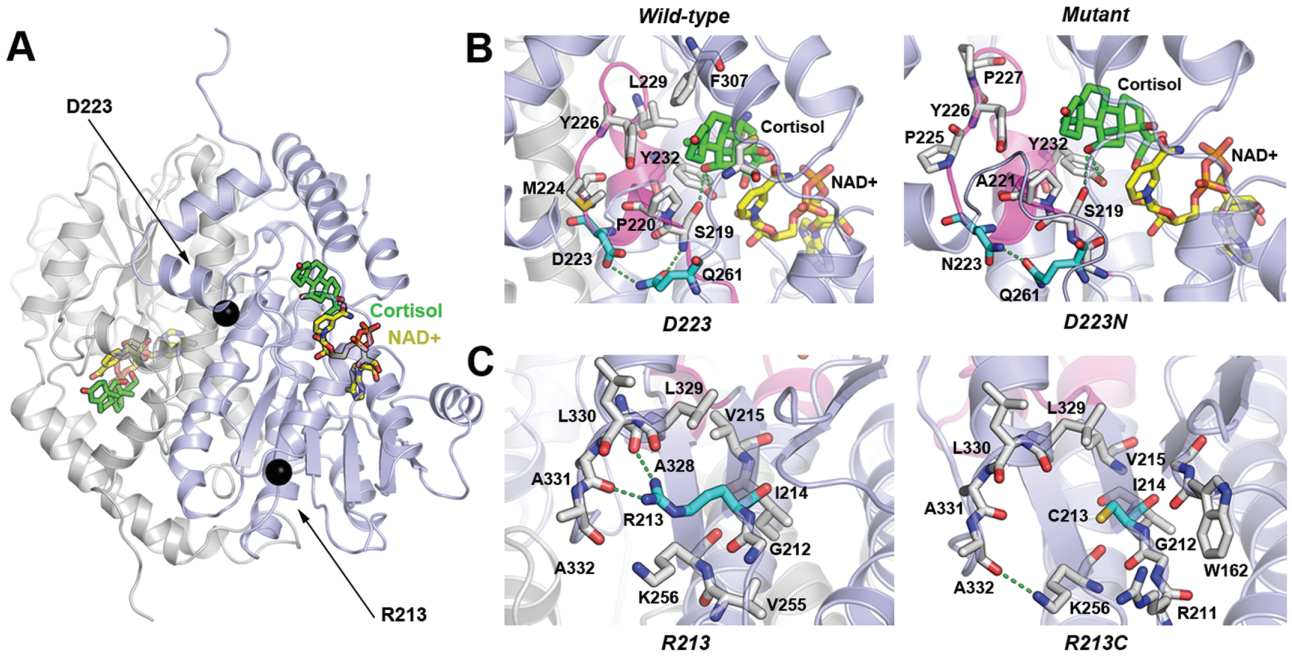

Figure 4. (**A**) *In silico* model of 11β-HSD2 (residues 81–375). Cortisol and NAD+ are shown in stick model. Black spheres represent localization of residues Asp-223 (D223) and Arg-213 (R213). (**B**) Asp-223-Asn (D223N) mutation locates nearby the cortisol entry tunnel, and is proposed to interact by a hydrogen bond with the Q261 side-chain. It may affect the overall catalytic activity of 11BHSD2 by affecting the plasticity of the binding site, reducing the ability of the charge relay system or altering interaction with the key catalytic amino-acid S219. (**C**) Arg-213-Cys (R213C) mutation locates near 25 Å distance from the catalytic center. R213 binds by a hydrogen-bond with the main chain carbonyl of A331 and A328. R213C mutation affects the critical hydrogen bonds located in the central sheet of the 11BHSD2 structure, which probably will modify the activity and protein folding.

activity and dimerization[27–29] (See Supplementary File for details). The D223N mutation may alter the plasticity of the binding site or reduce the ability of the charge relay system associated to the Q261 side-chain, reducing enzyme activity. The R213C mutation disrupts critical hydrogen bonds in the central β-sheet of the structure that will probably changes the protein folding and impairs the catalytic activity[28,29] (Figure 4).

In the first-degree relatives of AME subjects, all heterozygous carriers of either mutation, we observed normal clinical and biochemical features (e.g., blood pressure, K+, and ARP in the normal ranges) but high serum F/E ratios (highest quartile) and low cortisone levels (lowest quartile). These phenotypes are consistent with HSD11B2 haploinsufficiency,[23] in which the maintenance of normal 11BHSD2 protein levels and further 11BHSD2 activity are impaired. Since it is well known that 11BHSD2 activity decreases with age,[30,31] our study supports the concept that heterozygous subjects with known mutations for AME are at risk of displaying mild cases of AME, also named "nonclassical" AME.

In our study, high serum F/E ratios with concomitant low cortisone levels were more accurate biomarker than was the urinary F/E ratio[32,33] and (THF+aTHF)/THE ratio to biochemically identify HSD11B2 heterozygous subjects. A high serum F/E ratio and low E identified 100% of the heterozygous subjects, whereas urinary F/E and (THF+aTHF)/THE ratios allowed detection of only 2/5 (40%) of the heterozygous subjects studied here (Tables 2 and 3). These results might be partially explained by the fact that the urinary F/E and (THF+aTHF)/THE ratios correspond to 24-hour urinary collection and include the nadir of cortisol in the evening. In contrast, serum determinations of morning cortisol and cortisone are associated with the morning adrenocorticotrophic hormone peak due to circadian rhythm. High morning cortisol is a natural endogenous challenge for the 11βHSD2 enzyme in mineralocorticoid-sensitive tissues, where impaired 11βHSD2 should result in a decreased rate of conversion of active cortisol to cortisone.

We suggest that phenotypic expression in heterozygous subjects is also influenced by other factors, such as BMI, age,[30] sodium intake,[34] and epigenetic factors,[22,35] which play roles in blood pressure regulation. A previous study by our group demonstrated that the activity of 11βHSD2 decreases with age, which at least partially explains the high prevalence of low renin hypertension observed in older subjects.[30,31] Epigenetic modifications such as local CpG methylation and specific miRNA expression are currently being evaluated as early, complementary, and dynamic biomarkers of MR activation by a partial deficiency of 11βHSD2.

In the current available literature, information and long-term studies regarding human HSD11B2 heterozygotes are scarce. However, evidence suggests that these individuals have abnormal steroid metabolism and excretion, as evidenced by abnormal (THF+allo-THF)/THE ratio, a propensity toward low-renin hypertension in later life and a conditional salt sensitivity.[21,36,37] In 1997, Li et al.[37] showed that the presence of AME was associated with the A328V mutation in the HSD11B2 gene in a Brazilian family. The index case's father was heterozygous

for the A328V mutation and developed hypertension at 38 years of age, with a suppressed PRA and high (THF+allo-THF)/THE ratio. A recent study by Pizzolo et al.[21] of an Italian family carrying the A221G missense mutation reported a late onset of hypertension associated to high (THF+allo-THF)/THE ratios in probands and normal-high (THF+allo-THF)/THE ratio detected in some elderly heterozygous relatives.

In 2011, Bailey et al.[23] reported that Hsd11b2 heterozygote mice showed inverse relationships among 11βHSD2 activity, heart weight and blood pressure in a clinically relevant context. Moreover, these Hsd11b2 heterozygote mice displayed salt-sensitive AHT with normal clinical and biochemical profiles.[23] On a control diet, heterozygote mice displayed subtle signs of mineralocorticoid excess but had no derangements in blood pressure or plasma electrolytes. Another study by Evans *et al.*[38] showed that the conditional deletion of Hsd11b2 in mice brains (Hsd11b2.BKO) increased the appetite for salt and turned subjects from salt resistant to salt sensitive. Thus, global missense R213C or D223N mutations and other known pathogenic variants of the HSD11B2 gene appear to doubly impact in AHT, by impairing the ability of the kidney to excrete sodium and increasing the behavioral drive to consume sodium salt. In fact, this study identifies a lower fractional excretion of sodium and urinary Na/K ratio in heterozygotes compared to control subjects, which highlight the impaired sodium excretion in these subjects (Figure 3).

A strength of this study is to our knowledge, one of the first studies measuring serum and urinary cortisol and cortisone by the same method and simultaneous determinations of cortisol, cortisone, and tetrahydrometabolites by LC-MS/MS technology, which constitutes the gold standard for this type of measurements.[39,40] This technology shows that serum F/E ratio and cortisone concentration are better tools than are urinary F/E and (THF+allo-THF)/THE ratios for detecting subtle changes in 11βHSD2 activity in either hypertensive patients or normotensive subjects. Further rational design and validation of a diagnostic algorithm based in serum F/E and cortisone would support the etiologic diagnosis of AHT, and the subsequent application of specific treatments, and genetic and nutritional counseling of affected subjects. Preliminary results from a Chilean cohort show that partial 11βHSD2 deficiency also called non-classic AME could be present in around 7% of general population, and these subjects had higher systolic BP and lower PRA than control subjects (data not shown).

One limitation of our report is a cross-sectional study. We are planning to perform a longitudinal study with each AME subject and a collaborative study with other AME subjects and families, to identify BP changes and also assay biomarkers of endothelial damage, inflammation, and oxidative stress, which can be used to help identify early signs of enhanced mineralocorticoid activity associated to a partial 11βHSD2 deficiency. Our study also reveals the urgent need for new sensitive and reliable biomarkers of MR activity.

In summary, a high serum F/E ratio in combination with low serum cortisone is suggestive of a partial 11βHSD2 deficiency, also called a "nonclassic" AME. Identification of subjects with partial 11βHSD2 deficiency should encourage further clinical, genetic and nutritional advice, since a second hit can induce AHT.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *American Journal of Hypertension* online.

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DISCLOSURE

The authors declared no conflict of interest.

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