Evaluation of molecular characteristics and steroid metabolomics in a large cohort of children with 3β-hydroxysteroid dehydrogenase 2 deficiency

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Tulay Guran[1], Cengiz Kara[2], Melek Yildiz[3], Eda C. Bitkin[2], Goncagul Haklar[4], Jen-Chieh Lin[5], Lorna C. Gilligan[6], Lise Barnard[7], Mehmet Keskin[8], Ahmet Anik[9], Gonul Catli[10], Ayla Guven[11], Birgul Kirel[12], Filiz Tutunculer[13], Hasan Onal[3], Serap Turan[1], Teoman Akcay[3], Zeynep Atay[1], Elizabeth S. Baranowski[6], Gulay C. Yilmaz[2], Jamala Mamadova[2], Azad Akbarzade[1], Onder Sirikci[4], AghaRza Aghayev[14], Afra Alkan[15], Cedric H.L. Shackleton[6], Karl H. Storbeck[7], Tugba Baris[16], Wiebke Arlt[6,17], Bon-Chu Chung[5], Abdullah Bereket[1].

1.Marmara University, School of Medicine, Department of Pediatric Endocrinology and Diabetes, Istanbul, Turkey; 2. Ondokuz Mayis University, Department of Pediatric Endocrinology and Diabetes, Samsun, Turkey; 3. Kanuni Sultan Suleyman Training and Research Hospital, Department of Pediatric Endocrinology and Diabetes, Istanbul, Turkey; 4.Marmara University, School of Medicine, Department of Biochemistry, Istanbul, Turkey; 5. Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan; 6. Institute of Metabolism and Systems Research (IMSR), College of Medical & Dental Sciences, University of Birmingham, UK; 7.Stellenbosch University, Department of Biochemistry, Western Cape, South Africa; 8. Gaziantep University, Department of Pediatric Endocrinology and Diabetes, Gaziantep, Turkey; 9. Adnan Menderes University, School of Medicine, Department of Pediatric Endocrinology and Diabetes, Aydin, Turkey; 10. Katip Celebi University, School of Medicine, Department of Pediatric Endocrinology and Diabetes, Izmir, Turkey; 11. Health Science University, Faculty of Medicine, Zeynep Kamil Women and Children Diseases Education and Research Hospital, Pediatric Endocrinology, Istanbul, Turkey; 12. Eskisehir Osmangazi University, Department of Pediatric Endocrinology, Eskisehir, Turkey; 13. Department of Pediatrics, Division of Pediatric Endocrinology, Trakya University School of Medicine, Edirne, Turkey; 14. Istanbul Faculty of Medicine, Department of Medical Genetics, Istanbul University, Istanbul, Turkey; 15. Yildirim Beyazit University, School of Medicine, Department of Medical Bioinformatics and Biostatistics, Bilkent, Ankara, Turkey; 16. Gelisim Genetik Tani Merkezi, Istanbul, Turkey; 17.Centre for Endocrinology, Diabetes and Metabolism, Birmingham Health Partners, Birmingham, UK

Context: Deficiency of 3β-hydroxysteroid dehydrogenase 2 (3βHSD2) causes a very rare form of congenital adrenal hyperplasia (CAH) known as 3βHSD2 deficiency, which is a consequence of biallelic *HSD3B2* gene defects. The estimated prevalence is less than 1/1,000,000 live births. Knowledge of comprehensive steroid metabolome patterns in 3βHSD2 deficiency is scarce.

Objective: We aimed to investigate phenotypical, molecular, and biochemical characteristics, as well as the genotype-phenotype relationship in patients with 3βHSD2 deficiency. We evaluated steroid hormone profiles in individuals with homozygous and heterozygous *HSD3B2* gene defects, mutation-negative "functional 3βHSD2 deficiency", and patients with 21-hydroxylase deficiency (21-OHD).

Setting: Multi-centre, cross-sectional study in nine tertiary pediatric endocrinology clinics in Turkey

Patients or Other Participants: Children with homozygous 3βHSD2 deficiency (n=31), individuals with heterozygous 3βHSD2 deficiency (n=31), children with classical 21-OHD (n=57), functional 3βHSD2 deficiency (n=18), and healthy controls (n=172).

Main Outcome Measures: A structured questionnaire was used to assess clinical and biochemical phenotype data. Genetic analysis of *HSD3B2* was performed using Sanger sequencing. We measured Δ5-to-Δ4 steroids and 11-oxygenated C19 androgens in serum and urine by mass spectrometry. Novel *HSD3B2* mutations were studied *in silico* and by *in vitro* enzyme kinetic assays **(Fig 1)**.

Results: Eleven homozygous (6 novel) in 31 children from 24 families (19 male/12 female; mean age: 6.6±5,1 yrs) were identified **(Fig 2A)**. The missense variants >5% of wild-type 3βHSD2 activity *in vitro* were associated with non-salt losing clinical phenotype **(Table 1, Fig 2B)**. There was a significant genotype-phenotype-steroid metabolome correlation in patients with 3βHSD2 deficiency **(Fig 3)**. The plasma ratio of (17OH-Pregnenolone + Pregnenolone + DHEA)/(17OHProgesterone + Progesterone + Androstenedione + Cortisol) was superior to (17OH-Pregnenolone/Cortisol) to discriminate 3βHSD2 deficiency from the other groups. Heterozygote carriers and functional 3βHSD2 deficiency patients showed higher Δ5-to-Δ4 steroids than controls **(Fig 4A, 4B, 5A)**. 11-oxygenated androgens were significantly lower in patients with 3βHSD2 deficiency **(Fig 5B)**.

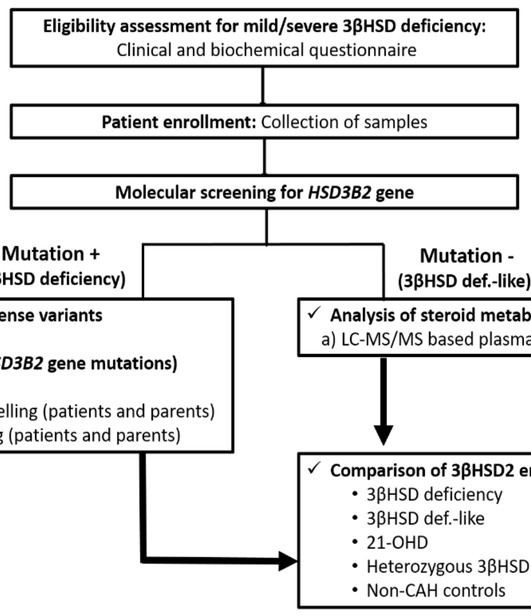

Figure 1. Overview of the study design and recruitment of patients and samples.

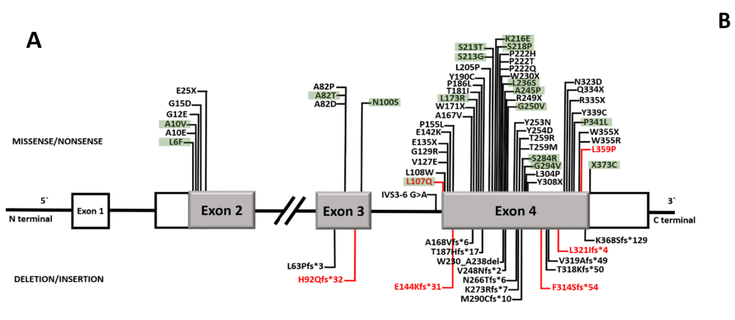

Figure 2. Molecular characteristics of *HSD3B2* mutations. **(A)** Schematic presentation of *HSD3B2* with all known mutations and novel mutations detected in this study. Non-coding exonic segments are indicated as white boxes. Most of the previously reported mutations are located in exon four. Pathogenic missense/nonsense mutations are shown in the upper panel, while insertion/deletions are indicated in the lower panel. Novel mutations reported in this study are shown in red. Mutations with *in vitro* >5% residual 3βHSD2 activity are highlighted in green boxes. **(B)** The activities of wildtype and mutant HSD3B2 enzymes. The graph shows the relative activities of wildtype and mutant HSD3B2 expressed in COS-1 cells following incubating with pregnenolone for 1 h. The Western blot on the top shows the levels of HSD3B2 in the cells, and the numbers on top of the protein bands are the relative protein levels after quantitation of the images.

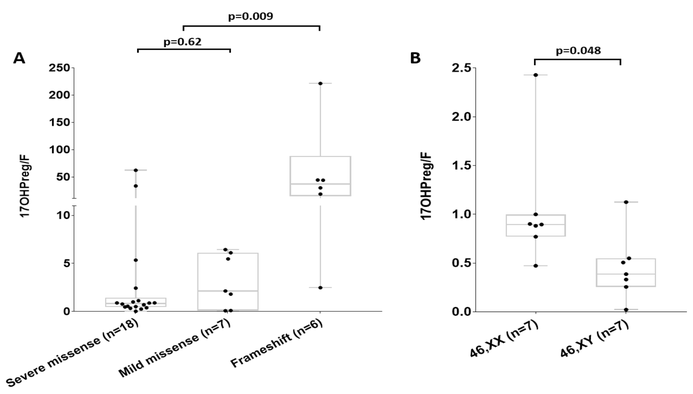

Figure 3. The effect of the severity of *HSD3B2* mutation and karyotype on 3βHSD2 enzyme activity index. The effect of the severity of *HSD3B2* mutation **(A)** and karyotype **(B)** on 3βHSD2 enzyme activity index that is represented by simultaneously measured Δ5 (pregnenolone, 17α-hydroxypregnenolone, DHEA) to Δ4 (progesterone, 17α-hydroxyprogesterone, androstenedione and cortisol) steroids ratio. *HSD3B2* mutations causing a frameshift of gene sequence (p.H92Qfs*32, p.E144Kfs*31, p.F314Sfs*54, p.L321Ifs*4) result in a severely impaired 3βHSD2 enzyme activity index compared to missense mutations (p.L107Q, p.S218P, p.A245P, p.L304P, p.N323D, p.W355R, p.L359P). The difference of this ratio in patients with mild missense (p.L107Q, p.S218P, p.A245P; HSD3B2 activity ~5-10% of wild type) and severe missense (p.L304P, p.N323D, p.W355R and p.L359P; HSD3B2 activity <5% of wild type) mutations was not statistically significant (p=0.62) **(A)**. 3βHSD2 enzyme activity index was similar in 7 girls and 7 boys with classical 3βHSD2 deficiency due to homozygous p.N323D mutation (p=0.048) **(B)**.

Table 1. Sequence variations and genotype-phenotype relationships in 31 children with 3βHSD2 deficiency

	Sequence Variation			Variant Effect Predictor Software								Phenotype		
Number of cases/families (n/n)	Genomic co-ordinates and nucleotide change (Genome assembly GRCh37.p13)	Protein (prediction)	cDNA position and nucleotide change (transcript NM_001166120, ENST00000543831)	PROVEAN	SIFT	PolyPhen2 (HumVar)	Mutation taster	Case report (DOI)	First functional characterization report (DOI)	Apparent activity in intact cells	Karyotype	Age(s) at diagnosis	Clinical type	Salt-wasting	DSD
1/1	**g.4619_4620delCA chr1:119962172_119962173delCA**	**p.H92Qfs*32**	**c.274_275delCA cDNA.523_524delCA**	ND	ND	ND	Disease causing	Present study	Present study	ND	46, XX	Newborn	Classical	+	+
1/1	**g.6891T>A chr1:119964444T>A**	**p.L107Q**	**c.320T>A cDNA.569T>A**	-5.39	0.000	1	Disease causing	Present study	Present study	~12% of wild-type enzyme activity	46, XY	Newborn	Classical	+ (presentation and stress), normal potassium	+
2/1	**g.7000_7001insAA chr1:119964553_119964554insAA**	**p.E144Kfs*31**	**c.429_430insAA cDNA.678_679insAA**	ND	ND	ND	Disease causing	Present study	Present study	ND	46, XY (n=1) 46, XX (n=1)	Newborn-2 months	Classical	+	+ (46, XY) - (46, XX)
4/4	g.7223T>C chr1:119964776T>C	p.S218P	c.652T>C cDNA.901T>C	-1.93	0.101	0.117	Polymorphism	Present study	10.1111/cen.12394	<1-10% of wild-type enzyme activity[#]	46, XY (n=3) 46, XX (n=1)	Newborn-7 months	Classical	+ (presentation and stress), normal potassium	+ (46, XY) - (46, XX)
2/1	g.7304G>C chr1:119964857G>C	p.A245P	c.733G>C cDNA.982G>C	-1.57	0.196	0.979	Polymorphism	Present study	10.1210/mend.7.5.8316254[‡] 10.1210/jcem.84.12.6288[©]	~10%[‡] and ~35%[©] of wild-type enzyme activity	46, XY (n=2)	~2 years	Classical	+ (presentation and stress), normal potassium	+
1/1	g.7482T>C chr1:119965035T>C	p.L304P	c.911T>C cDNA.1160T>C	-5.66	0.009	0.783	Disease causing	Present study	10.1007/s11033-019-04809-4	ND	46, XY	Newborn	Classical	+	+
1/1	**g.7505delC chr1:119965058delC**	**p.F314Sfs*54**	**c.934delC cDNA.1183delC**	ND	ND	ND	Disease causing	Present study	Present study	ND	46, XY	Newborn	Classical	+	+
2/1	**g.7530_7531insC chr1:119965083_119965084insC**	**p.L321Ifs*4**	**c. 959_960insC c.DNA.1208_1209insC**	ND	ND	ND	Disease causing	Present study	Present study	ND	46, XY (n=1) 46, XX (n=1)	Newborn	Classical	+	+ (46, XY) - (46, XX)
14/11	g.7538A>G chr1:119965091A>G	p.N323D	c.967A>G cDNA.1216A>G	-4.09	0.006	0.999	Disease causing	Present study	10.1007/s11033-019-04809-4	<5% of wild-type enzyme activity	46, XY (n=7) 46, XX (n=7)	Newborn-8 months	Classical	+	+ (46, XY) - (46, XX)
2/1	g.7634T>C chr1:119965187T>C	p.W355R	c.1063T>C cDNA.1312T>C	-12.43	0.000	0.996	Disease causing	10.4274/jcrpe.3306	Present study	<5% of wild-type enzyme activity	46, XY (n=2)	Newborn	Classical	+	+
1/1	**g.7647T>C chr1:119965200T>C**	**p.L 359P**	**c.1076T>C cDNA.1325T>C**	-3.12	0.004	0.913	Disease causing	Present study	Present study	<5% of wild-type enzyme activity	46, XX (n=1) 46, XY (n=1)	1-2 months	Classical	+	-

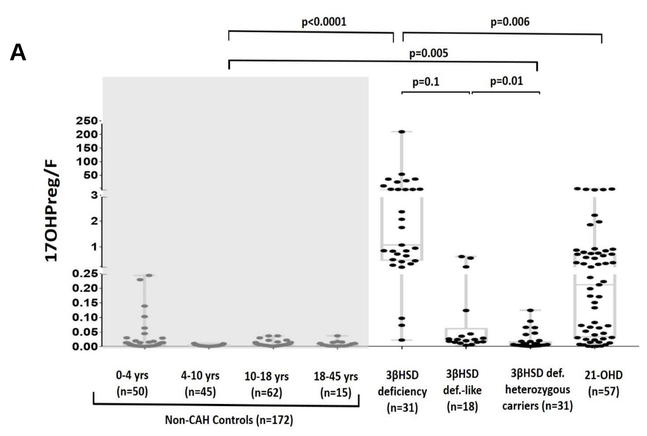

Figure 4. Assessment of two baseline Δ5-to-Δ4 steroid ratios in the diagnosis and differential diagnosis of 3βHSD2 deficiency. Two baseline plasma adrenal Δ5-to-Δ4 adrenal steroid ratios were compared between the individuals with 3βHSD2 deficiency, 3βHSD2 deficiency-like conditions, heterozygous 3βHSD2 deficiency, 21-OHD and non-CAH control groups. Grey areas show control groups stratified according to age. Both of these ratios were very efficient to diagnose and to *differentiate* 3βHSD2 deficiency from the other groups. (Preg+17OHPreg+DHEA)/(Prog+17OHP+A4+F) ratio **(B)** was superior to 17OHPreg/F **(A)** to differentiate 3βHSD2 deficiency from 21-OHD (p<0.001).

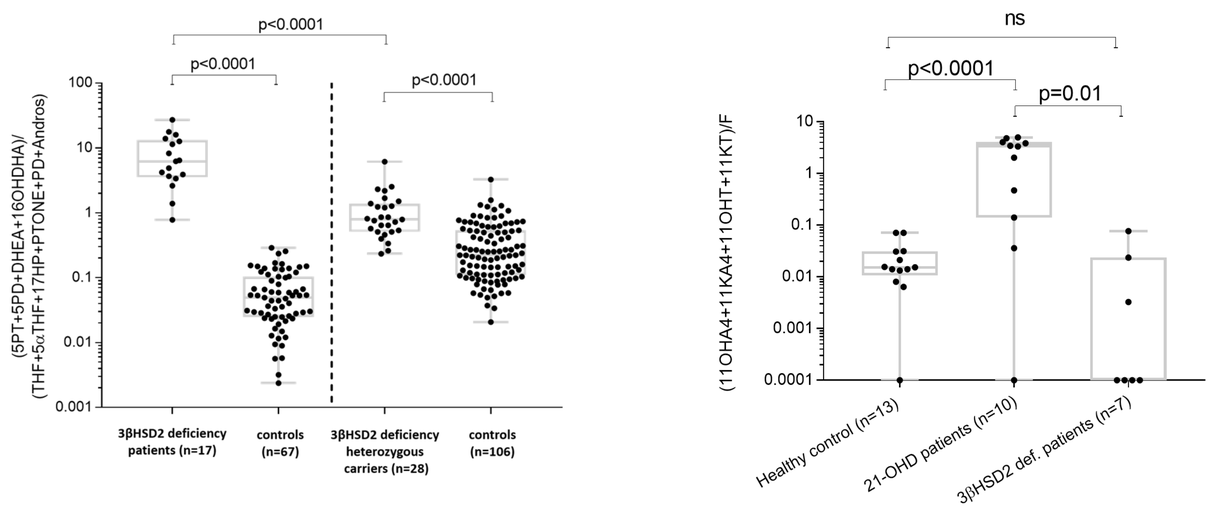

Figure 5. The effect of the severity of *HSD3B2* mutation and karyotype on 3βHSD2 enzyme activity index. The effect of the severity of *HSD3B2* mutation **(A)** and karyotype **(B)** on 3βHSD2 enzyme activity index that is represented by simultaneously measured 17α-hydroxypregnenolone/cortisol ratio. *HSD3B2* mutations causing a frameshift of gene sequence (p.H92Qfs*32, p.E144Kfs*31, p.F314Sfs*54, p.L321Ifs*4) result in a severely impaired 3βHSD2 enzyme activity index compared to missense mutations (p.L107Q, p.S218P, p.A245P, p.L304P, p.N323D, p.W355R, p.L359P). The difference of this ratio in patients with mild missense (p.L107Q, p.S218P, p.A245P; HSD3B2 activity ~5-10% of wild type) and severe missense (p.L304P, p.N323D, p.W355R and p.L359P; HSD3B2 activity <5% of wild type) mutations was not statistically significant (p=0.62) **(A)**. 3βHSD2 enzyme activity index was slightly higher in girls than boys with classical 3βHSD2 deficiency due to homozygous p.N323D mutation (p=0.048) **(B)**. The *box and whiskers plots* represent the mean, 25%, 75% and minimum and maximum of the measurements. Each *black dot* represents an individual case-specific measurement. Abbreviations: Preg, pregnenolone; 17OHPreg, 17α-hydroxypregnenolone; DHEA, dehydroepiandrosterone; Prog, progesterone; 17OHP, 17α-hydroxyprogesterone; A4, androstenedione; F, cortisol.

Conclusions:

According to our results obtained from this large cohort,

- **There is a good correlation between glucocorticoid and mineralocorticoid functions with *in vitro* and biochemical enzyme activity in 3βHSD2 deficiency, whereas genital and gonadal phenotype and behaviour are more complex and variable.**
- **In contrast to common knowledge, mineralocorticoid deficiency is not apparent in 1/3 of the cases.**
- **This 46, XY DSD is a "*sine qua non* " in affected males whereas ambiguous genitalia is only rarely seen in affected 46, XX individuals due to decreased production of potent androgens via classical or alternative pathways.**
- **On the other hand, premature pubarche is very common on either sex in 3βHSD2 deficiency.**
- **Spared mineralocorticoid function and unvirilized genitalia in females may lead to misdiagnosis and underestimation of the frequency of 3βHSD2 deficiency.**
- **Mass spectrometry-based measurements of Δ5-to-Δ4 steroids are very sensitive and specific to diagnose and differentiate 3βHSD2 deficiency from clinically look-alike conditions.**
- **Heterozygous 3βHSD2 deficiency impairs biochemical 3βHSD2 enzyme activity but does not cause a clinically significant phenotype.**
- **The term "non-classic or late-onset" form of 3βHSD2 deficiency, if it is existent, should only be used following genetic confirmation. The role of the regulators of 3βHSD2 in the pathogenesis of adrenal androgen excess needs to be elucidated.**
- **The correct diagnosis of 3βHSD2 deficiency is not only essential for the proper clinical management in infancy and childhood but also for the surveillance of gonadal functions and fertility of the patients in later life.**