

Steroid Hormone Profiles and Molecular Diagnostic Tools in Pediatric Patients With non-CAH Primary Adrenal Insufficiency

Tuba Seven Menevse,^{1, ID} Yasemin Kendir Demirkol,^{2, ID} Busra Gurpinar Tosun,^{1, ID}
Elvan Bayramoglu,^{3, ID} Melek Yildiz,^{4, ID} Sezer Acar,^{5, ID} Seda Erisen Karaca,^{6, ID} Zerrin Orbak,^{7, ID}
Asan Onder,^{8, ID} Elif Sobu,^{9, ID} Ahmet Anik,^{10, ID} Zeynep Atay,^{11, ID} Fuat Bugrul,^{12, ID}
Ayse Derya Bulus,^{13, ID} Korcan Demir,^{14, ID} Durmus Dogan,^{15, ID} Hamdi Cihan Emeksiz,^{8, ID}
Heves Kirmizibekmez,^{16, ID} Nurhan Ozcan Murat,^{17, ID} Akan Yaman,^{18, ID} Serap Turan,^{1, ID}
Abdullah Bereket,^{1, ID} and Tulay Guran^{1, ID}

¹Department of Pediatric Endocrinology and Diabetes, Marmara University, School of Medicine, 34899, Ustkaynarca/Pendik Istanbul, Turkey

²Department of Pediatric Genetics, Umraniye Research and Training Hospital, University of Health Sciences, 34764 Istanbul, Turkey

³Department of Pediatric Endocrinology, Haseki Training and Research Hospital, 34096 Istanbul, Turkey

⁴Department of Pediatric Endocrinology and Diabetes, Istanbul University, School of Medicine, 34093 Istanbul, Turkey

⁵Department of Pediatric Endocrinology and Diabetes, Behcet Uz Education and Research Hospital, 35210 Izmir, Turkey

⁶Department of Pediatric Pediatrics, Duzce University, School of Medicine, 81620 Bolu, Turkey

⁷Department of Pediatric Endocrinology and Diabetes, Ataturk University, School of Medicine, 25030 Erzurum, Turkey

⁸Department of Pediatric Endocrinology and Diabetes, Medeniyet University, School of Medicine, 34722 Istanbul, Turkey

⁹Department of Pediatric Endocrinology, Kartal Training and Research Hospital, 34865 Istanbul, Turkey

¹⁰Department of Pediatric Endocrinology, Aydin Adnan Menderes University, School of Medicine, 09010 Aydin, Turkey

¹¹Department of Pediatric Endocrinology and Diabetes, Istanbul Medipol University, School of Medicine, 34810 Istanbul, Turkey

¹²Department of Pediatric Endocrinology and Diabetes, Selcuk University, School of Medicine, 42250 Konya, Turkey

¹³Department of Pediatric Endocrinology and Diabetes, Ankara Kecioren Research and Training Hospital, University of Health Sciences, 06000 Ankara, Turkey

¹⁴Department of Pediatric Endocrinology and Diabetes, Dokuz Eylul University, School of Medicine, 35340 Izmir, Turkey

¹⁵Department of Pediatric Endocrinology and Diabetes, Onsekiz Mart University, School of Medicine, 17110 Canakkale, Turkey

¹⁶Department of Pediatric Endocrinology and Diabetes, Umraniye Research and Training Hospital, University of Health Sciences, 34764 Istanbul, Turkey

¹⁷Department of Pediatric Endocrinology and Diabetes, Derince Research and Training Hospital, 41900 Kocaeli, Turkey

¹⁸Department of Pediatrics, Gungoren Hospital, 34164 Istanbul, Turkey

Correspondence: Tulay Guran, MD, Marmara University School of Medicine, Department of Pediatric Endocrinology and Diabetes, Fevzi Cakmak Mh. Mimar Sinan Cd. No 41, 34899, Ustkaynarca/Pendik, Istanbul, Turkey. Email: tulayguran@yahoo.com; tulayguran@marmara.edu.tr.

Abstract

Context: There is a significant challenge of attributing specific diagnoses to patients with primary adrenal insufficiency of unknown etiology other than congenital adrenal hyperplasia (non-CAH PAI). Specific diagnoses per se may guide personalized treatment or may illuminate pathophysiology.

Objective: This work aimed to investigate the efficacy of steroid hormone profiles and high-throughput sequencing methods in establishing the etiology in non-CAH PAI of unknown origin.

Methods: Pediatric patients with non-CAH PAI whose etiology could not be established by clinical and biochemical characteristics were enrolled. Genetic analysis was performed using targeted-gene panel sequencing (TPS) and whole-exome sequencing (WES). Plasma adrenal steroids were quantified by liquid chromatography–mass spectrometry and compared to that of controls. This study comprised 18 pediatric endocrinology clinics with 41 patients (17 girls, median age: 3 mo, range: 0–8 y) with non-CAH PAI of unknown etiology.

Results: A genetic diagnosis was obtained in 29 (70.7%) patients by TPS. Further molecular diagnosis could not be achieved by WES. Compared to a healthy control group, patients showed lower steroid concentrations, most statistically significantly in cortisone, cortisol, and corticosterone ($P < .0001$, area under the receiver operating characteristic curve: .96, .88, and .87, respectively). Plasma cortisol of less than 4 ng/mL, cortisone of less than 11 ng/mL, and corticosterone of less than 0.11 ng/mL had a greater than 95% specificity to ensure the diagnosis of non-CAH PAI of unknown etiology.

Conclusion: Steroid hormone profiles are highly sensitive for the diagnosis of non-CAH PAI of unknown etiology, but they are unlikely to point to a specific molecular diagnosis. TPS is an optimal approach in the molecular diagnosis of these patients with high efficacy, whereas little additional benefit is expected from WES.

Received: 21 September 2021. Editorial Decision: 8 January 2022. Corrected and Typeset: 28 January 2022

© The Author(s) 2022. Published by Oxford University Press on behalf of the Endocrine Society. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Key Words: adrenal insufficiency, children, LC-MS/MS, steroid profile, non-CAH primary adrenal insufficiency

Abbreviations: ACTH, adrenocorticotropin; CAH, congenital adrenal hyperplasia; LC-MS/MS, liquid chromatography–tandem mass spectrometry; PAI, primary adrenal insufficiency; TPS, targeted-gene panel sequencing; WES, whole-exome sequencing.

Primary adrenal insufficiency (PAI) is associated with decreased cortisol production due to impaired function of the adrenal cortex, which is related to an increase in morbidity and mortality and a reduction in quality of life (1). Inherited PAI develops because of 2 major groups of disorders: congenital adrenal hyperplasia (CAH) and non-CAH PAI (1–4). CAH is caused by reduced activity of an enzyme required for cortisol production. However, non-CAH PAI develops due to variations in the genes that are involved in diverse pathways in adrenocortical development, adrenocorticotropin (ACTH) action, redox homeostasis, metabolism, autoimmunity, and others (2–4). Although CAH is far more common than non-CAH PAI in children, the group of non-CAH PAI disorders is equally serious and life-threatening if the diagnosis is overlooked or delayed. Furthermore, reaching a specific diagnosis of non-CAH PAI can have important implications for the child and his or her family in terms of treatment, long-term outcome, possible associated features that might need to be considered, and counseling the family about recurrence risk and identifying other family members who might be in a presymptomatic stage of the disease (5, 6).

The specific diagnosis of non-CAH PAI can be approached depending on which steroid system is affected (mineralocorticoids, glucocorticoids, androgens), age of presentation, or associated features. Nevertheless, it has become evident that there is a much greater overlap between etiologic diagnoses than previously thought. Genetic analysis is playing an increasingly important role in reaching a specific diagnosis.

Several genes have been implicated in the etiology of non-CAH PAI cases, including homozygous or compound heterozygous inactivating mutations of *MC2R*, *MRAP*, *CYP11A1*, *StAR*, *NR5A1*, *AAAS*, *NNT*, *SGPL1*, *AIRE*, *TXNRD2*, *MCM4*, and *POLE1*; or heterozygous activating mutations of *SAMD9* and *CDKN1C*. *NR0B1/DAX1* and *ABCD1* mutations are inherited in an X-linked manner in the great majority of cases. There are a few other gene defects responsible for very rare and syndromic non-CAH PAI, although the underlying etiology remains unknown in many cases (2, 7–12).

While CAH is easily identified and subtyped biochemically by quantification of accumulated or deficient steroid precursors/hormones (13–16), detailed steroid hormone profiles have not been evaluated in patients with non-CAH PAI. In this study, we have investigated adrenal steroid hormone profiles of a multicenter cohort of pediatric patients with the clinical diagnosis of non-CAH PAI whose etiology could not be established by clinical and biochemical characteristics. We aimed to assess the efficacy of steroid hormone profiles in the diagnosis and in relation with the underlying molecular diagnosis of non-CAH PAI. We have also tested the efficiency of high-throughput sequencing methods including targeted-gene panel sequencing (TPS) and whole-exome sequencing (WES) to achieve a molecular diagnosis of non-CAH PAI.

Materials and Methods

A total of 41 patients with a clinical diagnosis of inherited non-CAH PAI of unknown molecular etiology from 40 unrelated families followed up in 18 pediatric endocrinology centers in Turkey between 2016 and 2021 were included. Inclusion criteria of a PAI phenotype was defined as the presence of signs and symptoms of adrenal insufficiency together with high plasma ACTH and low serum cortisol and intermediary glucocorticoid metabolites at initial presentation. Exclusion criteria were the following: 1) CAH (21 α -hydroxylase, 11 β -hydroxylase, 3 β -hydroxysteroid dehydrogenase 2, 17 α -hydroxylase or cytochrome P450 reductase deficiencies) diagnosed by available serum steroid hormones; 2) X-linked adrenoleukodystrophy (ALD) in boys with neurological findings and elevated very long-chain fatty acids, or a family history of affected male relatives with ALD; 3) clinical and biochemical evidence of autoimmune adrenal failure; and 4) known syndromic causes of PAI (specifically, classic triple A syndrome or Xp deletion syndrome involving *NR0B1/DAX1* with Duchenne muscular dystrophy). All patients were assessed by a pediatric endocrinologist. A structured questionnaire was used to systematically evaluate all available clinical, biochemical, and imaging data related to the diagnosis and treatment of PAI, and all other relevant medical and family history. One patient with additional autoimmune findings and another male adolescent patient with hypogonadotropic hypogonadism were thought to have *AIRE* and *NR0B1* mutations respectively by clinical characteristics, but they were not excluded from further studies as positive controls for genetic tests. Studies were performed with the approval of the ethics committee of the Marmara University Faculty of Medicine, Istanbul, Turkey (09.2021.895). Patients and/or parents provided written informed consent, and all studies were conducted in accordance with the principles of the Declaration of Helsinki.

Steroid Hormone Analysis

Blood samples were collected either at pretreatment or at between 8 and 9 am after 48 hours in an off-treatment state in a hospital setting with continuous observation for general well-being, blood pressure, and other vital signs. A liquid chromatography–tandem mass spectrometry (LC-MS/MS)–based panel of plasma adrenal steroids was evaluated using a Eureka kit (Eureka Lab Division) as previously described (17). A Poroshell 120 EC-C18 (50 $\times$ 2.1 mm, 2.7 μ m; Agilent Technologies) column was used. Analyses were performed on a Shimadzu LCMS 8050 MS/MS equipped with a Nexera XR LC-20AD HPLC system (Shimadzu Corp) that was operated using an electrospray ionization source in positive and multiple reactions monitoring mode. Method validation was performed according to the Clinical and Laboratory Standards Institute C62-A guidelines. The steroids simultaneously assessed by the LC-MS/MS method were aldosterone, corticosterone, 11-deoxycorticosterone, pregnenolone, 17OH-pregnenolone, progesterone, 17OH-progesterone, 21-deoxycortisol, 11-deoxycortisol, cortisol, cortisone, dehydroepiandrosterone, Δ 4-androstenedione, and androsterone (14). Adrenal steroids measured in the patient group

were compared to those of sex- and age-matched controls. Control plasma samples were obtained from children examined for other conditions, including well-controlled type 1 diabetes, euthyroid hypothyroidism on treatment, or simple growth retardation, but without an adrenal or pubertal problem. We have also used control steroid hormone profile data from healthy infants younger than age 6 months from another study (18).

Genetic Analysis

Genomic DNA was extracted from EDTA-anticoagulated peripheral blood by using a semiautomated robot as recommended by the manufacturer (Qiagen). The library preparation for next-generation sequencing was performed using a capture-based Clinical Exome Solution Kit by Sophia Genetics, including a gene panel for PAI (*CYP11A1*, *CYP11B1*, *CYP11B2*, *CYP21A2*, *HSD3B2*, *CYP17A1*, *POR*, *StAR*, *AAAS*, *ABCD1*, *MC2R*, *MRAP1*, *NR0B1/DAX1*, *NR5A1*, *NNT*, *TXNRD2*, *MCM4*, *AIRE*, *SGPL1*, *SAMD9*, *CDKN1C*, *POLE1*) (19).

NextSeq 500 (Illumina) was used as the sequencing platform. Data quality control, alignment, variant calling, and variant annotations were performed by using the Sophia DDM analysis tool, version 5.2 (SOPHiA Genetics). Coverage was 99.43% at a minimum depth of 25 reads for 4493 genes. To call variants, sequencing data were aligned to the human reference genome, hg19. All variations in exons and exon-intron boundaries with a variant fraction over 0.20 and having a minor allele frequency under 0.01 in population frequency databases were evaluated. The 1000 Genome Project, dbSNP ExAC, and GnomAD population frequency databases were used as the control population. The interpretations of the variants were performed according to the 2015 American College of Medical Genetics and Genomics standards and guidelines (20). The possible effects of the variants on protein function were determined by using pathogenicity prediction tools such as SIFT (21), Polyphen (22), MutationTaster (23), and VarSome (24). Confirmation of the variation and segregation analysis in patients and parents were performed via Sanger sequencing. Briefly, genomic DNA was amplified by polymerase chain reaction and amplicons were sequenced using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems; Life Technologies) on an Applied Biosystems 3130 Genetic Analyzer. The primers were designed using Primer3 software (primer3.ut.ee).

WES was undertaken for the patients in whom a molecular diagnosis could not be achieved by evaluation of TPS by next-generation sequencing.

For library preparation and sequencing for WES, a capture-based Illumina Nextera Exome Oligo kit was used. Sequencing was performed on a NovaSeq (Illumina). Data quality control, alignment, variant calling, and variant annotations were performed by using the Seq by Genomize analysis tool, version 16.7. The NCBI Build37 (hg19) version of the human genome was used as a reference. As a primary variant-filtering strategy, variants located within the ± 10 base pairs boundary of targeted exons with a minimum read depth of 20 $\times$ were selected.

Statistical Analysis

Statistical evaluation was performed using GraphPad Prism version 5.0 software (GraphPad Software Inc). Pairwise comparisons were performed using the *t* test. The nonparametric

Mann-Whitney *U* test was used when the assumptions of the *t* test could not be respected. Data were expressed as median and range, or mean $\pm$ SD. Statistical significance was set at *P* less than .05.

Results

Clinical and Molecular Characteristics

A total of 41 PAI patients from 40 families were included in the study (17 girls and 24 boys, median age: 3 mo, range: 0-8 y). Twelve patients had 46,XX and 29 had 46,XY karyotypes. There was consanguinity in 25 (60%) 40 families. Common presenting features of the patients were hyperpigmentation (78%), failure to thrive or weight loss (63%), salt loss/electrolyte imbalance (58%), fatigue (18%), convulsive/nonconvulsive hypoglycemia (19%), prolonged jaundice (14%), and frequent infections (13%).

Molecular diagnosis was found in 11 genes in 29 (70.7%) cases. Twenty-four mutations were identified (8 novel), including missense (*n* = 11, 4 novel), nonsense mutations (*n* = 7, 1 novel), deletions (*n* = 4, 2 novel), splice-site mutations (*n* = 2, 1 novel) (Table 1). Twenty-seven patients were homozygous; 2 were compound heterozygous carriers of the variants in the identified gene. The range of genetic etiologies found in this cohort were *StAR* (*n* = 6; 20%), *MC2R* (*n* = 6; 20%), *NNT* (*n* = 3; 10%), *NR0B1/DAX1* (*n* = 3; 10%), *CYP11A1* (*n* = 2; 7%), *MRAP* (*n* = 2; 7%), *SGPL1* (*n* = 2; 7%), *ABCD1* (*n* = 1; 3%), *AIRE* (*n* = 1; 3%), *AAAS* (*n* = 1; 3%), and *HSD3B2* (*n* = 2; 7%). Other clinical characteristics of the patients are shown in Table 1 and the supplementary material (case summaries according to molecular etiologies and Supplementary Table 1) (25).

WES was performed on 12 patients whose genetic etiology could not be found by TPS. No further etiologic diagnosis could be achieved by WES in these patients. Compared to the ones with identified molecular etiology, these patients were older at the time of diagnosis, had a lower rate of parental consanguinity, and a higher rate of male sex as shown in Supplementary Tables 2 and 3 (25).

Adrenocortical Steroids

All patients had severe cortisol deficiency and significantly elevated ACTH concentrations. Thirty-nine patients (95%) had adrenal androgen, 24 (58.5%) had mineralocorticoid, and 13 (31.7%) had gonadal sex steroid (testosterone/estrogen) deficiencies. To assess the severity of reduced steroid hormone production, also considering that the median age of the patient group was 3 months, we compared the adrenocortical steroids of the PAI patients with an identified molecular etiology with those of a healthy control group of infants aged 3 days to 6 months (*n* = 324) (Table 2). Patients with identified molecular etiologies had lower concentrations of steroids than healthy infants, who has physiologically low steroid concentrations. The difference was most statistically significant for aldosterone, cortisol, cortisone, corticosterone, pregnenolone, 17OH-pregnenolone, 11-deoxycortisol, and 21-deoxycortisol (see Table 2). Plasma cortisol of less than 4 ng/mL, cortisone of less than 11 ng/mL, and corticosterone of less than 0.11 ng/mL had a greater than 95% specificity to differentiate the non-CAH PAI patients from the controls (*P* < .0001, area under the receiver operating characteristic curve: .96, .88, .87, respectively) (Supplemental Fig. 1) (26). Steroid hormone profiles were compared between the subgroup of non-CAH PAI

Table 1. Clinical, molecular, and hormonal characteristics of our patients with rare causes of primary adrenal insufficiency according to underlying molecular etiology

Gene (chromosome locus)	Variant	No.	Age of diagnosis	GC deficiency	MC deficiency	Sex steroid deficiency		Elevated TSH	Other features	IUGR/ Short stature
						Gonadal	Adrenal			
MC2R (18p11.21)	c0.455C > A (p.T152K)	2	6.25 y 4 d	6/6	0/6	0/2	2/2	1/2	1 ^a /2	0/1
	c0.560del (p. V187fs)	2	4 d 3 mo			0/2	2/2	1/2	0/2	0/0
	c. 476C > A (p.T159K)	2	2.48 y 14 d			0 ^g /2	2/2	2/2	1 ^b /2	0/0
StAR (8p11.23)	c0.505G > A (p.E169K)	3	3 d 5 d 5 mo	6/6	6/6	3/3	3/3	1/3	0/3	1/0
	c0.470T > C (p.L157P)	3	1 mo 2 mo 2 mo			3/3	3/3	0/3	1 ^c /3	0/0
NNT (5p12)	Exon 12-14 deletion	1	1.5 y	3/3	0/1	0/1	1/1	0/1	0/1	0/0
	c0.259C > T (p.Q87*)	1	6 mo		1/1	0/1	1/1	0/1	0/1	1/0
	c0.2507G > A (p.G836D)	1	1.48 y		0/1	0/1	1/1	0/1	0/1	0/0
NR0B1/DAX1 (Xp21.2)	c0.1075C > T (p.Q359*)	1	1.5 y	3/3	3/3	1/1	1/1	0/1	0/1	0/0
	c0.1210C > T (p. Q404*)	1	2.5 y			1/1	1/1	0/1	0/1	1/0
	Exon 1-2 deletion	1	1.5 y			1/1	1/1	0/1	0/1	0/0
CYP11A1 (15q24.1)	c0.461T > C (p.L154P)	1	2 d	2/2	2/2	1/1	1/1	0/1	0/1	0/0
	c0.1351C > T (p.R451W)	1	1.66 y			0/1	1/1	0/1	0/1	1/0
MRAP (21q22.11)	c0.106 + 1del	1	2 d	2/2	0/2	0/1	1/1	0/1	0/0	0/0
	c0.106 + 2dupT	1	1.4 y			8/1	1/1	1/1	0/0	0/0
SGPL1 (10q22.1)	c0.1018C > T (p.R340W)	1	5 mo	2/2	2/2	1/1	1/1	1/1	1 ^d /1	0/1 ^b
	c0.518T > A (p.L173Q)	1	2 mo			0/1	1/1	1/1	1 ^d /1	0/1 ^b
ABCD1 (Xq28)	c0.1772G > T (p.R591L)	1	8 y	1	0	0/1	1/1	0/0	0/0	0/0
AIRE (21q22.3)	c0.415C > T (p.R139*)/ c0.260T > C (p.L87P)	1	4.5 y	1	1	1/1	1/1	1/1	1 ^e /1	0/1
AAAS (12q13.13)	c0.1333C > T (p.R478*)	1	4.5 y	1	0	*1	1/1	0/1	1 ^f /1	0/0
HSD3B2 (1p12)	c0.1003C > T (p.R335*)	1	1 mo	2/2	2/2	0/1	0/1	0/1	0/1	0/0
	c0.939del (p.F314Sfs*54)/ c0.745C > T (p.R249*)	1	1 mo			0/1	0/1	1/1	0/1	1/0

Novel variants are marked in bold.

Abbreviations: GC, glucocorticoid; IUGR, intrauterine growth retardation; MC, mineralocorticoid; TSH, thyrotropin.

^aGalactose intolerance, precocious puberty.^bDilated cardiomyopathy.^cNeurodevelopmental delay.^dNephrotic syndrome and renal failure.^eAutoimmune hepatitis, optic neuritis, Hashimoto thyroiditis, delayed puberty due to low gonadotropins.^fAlacrimia.^gThe patient could not be evaluated because he or she was not in minipuberty or puberty.^hGrowth retardation occurred after development of kidney failure.

patients with identified molecular etiologies causing isolated glucocorticoid deficiency (*MC2R*, *MRAP*, and *NNT*) and the ones with glucocorticoid and mineralocorticoid with/without sex steroid deficiencies (*StAR*, *CYP11A1*, *SGPL1*, *ABCD1*, *AAAS*, *NR0B1/DAX1*, and *AIRE*). There was no statistically significant difference in any of metabolites between the groups (Table 3). Adrenal androgens were low in all non-CAH PAI patients irrespective of molecular etiologies except 2 46,XX patients with CAH due to homozygous *HSD3B2* mutations who had high $\Delta 5$ steroids (Table 4). Pretreatment plasma cortisol was higher than 50 ng/mL in only 3 patients. One of them had nonclassical *CYP11A1* deficiency due to a homozygous c0.1351C > T (p.Arg451Trp) variant in *CYP11A1*. The pretreatment plasma cortisol concentration of this patient was 77.50 ng/mL with a simultaneous ACTH greater than 1250 pg/mL (N = 10-60). This patient was diagnosed at the presymptomatic stage by hyperpigmentation and a medical history of a deceased sibling with a similar clinical presentation. The second patient had a homozygous c0.518T > A (p.L173Q) change in *SGPL1*. This patient was followed up for adrenal calcification detected bilaterally on prenatal ultrasonography. ACTH concentrations gradually increased and glucocorticoid therapy was initiated at age 2 months. Her basal cortisol was 52.2 ng/mL during the presymptomatic stage. This patient developed proteinuria and renal failure at age 3 months. Mineralocorticoid treatment was added to her treatment at the eighth month. The third patient presented with a very recent onset of fatigue and hyperpigmentation. His basal cortisol was 71.5 ng/mL and simultaneous ACTH was 638 pg/mL, suggesting recent-onset, compensated adrenal failure (see the supplementary material, case summaries according to molecular etiologies) (25).

We performed an additional comparison of steroid hormone profiles between the subgroup of non-CAH PAI patients with identified vs unidentified molecular etiologies. Cortisol, cortisone, corticosterone, and 11-deoxycorticosterone

concentrations were statistically significantly lower in the patients with identified molecular etiologies than those of unidentified ones (see Supplementary Table 3) (25).

Discussion

Non-CAH PAI designates a heterogeneous group of congenital conditions that are characterized by a severe deficiency of cortisol with or without other steroid hormones and intermediate precursors. The present study provides, for the first time, a comprehensive steroid profiling of patients with non-CAH PAI. Our results revealed a profound and generalized reduction of adrenocortical steroid production in these patients, which is evident even in early infancy.

Achieving a specific molecular diagnosis in PAI has is important for counseling families, presymptomatic diagnosis, personalized treatment (eg, mineralocorticoid replacement), and predicting comorbidities (eg, neurological, puberty/fertility). In this study, we have identified an underlying molecular etiology by TPS in 70% of patients with a clinical diagnosis of non-CAH PAI whose etiology could not be identified by clinical and biochemical characteristics. In this highly selected cohort, molecular etiologies were distributed in 11 genes, including *MC2R*, *StAR*, *NNT*, *NR0B/DAX1*, *CYP11A1*, *MRAP*, *SGPL1*, *ABCD1*, *AIRE*, *AAAS*, and *HSD3B2*. In a recent 25-year large cohort study in the United Kingdom, Buonocore et al (8) identified a genetic diagnosis in 75 of 155 non-CAH PAI patients (66.5%) using similar enrollment criteria and TPS method. The genetic causes of their cohort included *MC2R*, *NR0B/DAX1*, *CYP11A1*, *AAAS*, *NNT*, *MRAP*, *TXNRD2*, *StAR*, *SAMD9*, *CDKN1C*, and *NR5A1/SF1* in decreasing order. Using the same inclusion criteria and TPS method, in 2016, we reported a genetic etiology in 81% of 95 pediatric patients with non-CAH PAI of unknown etiology in Turkey, where genetic enrichment due to a high rate of consanguineous marriages exists. The range of

Table 2. Adrenal steroid hormones of primary adrenal insufficiency patients with identified molecular etiologies compared to the control group

ng/mL	Patients (n = 29)		Control (n = 324)		P
	Median	Range	Median	Range	
Aldosterone	0.06	0.007-0.70	0.198	0.002-0.982	< .001
Cortisol	0.891	0.021-77.5	31.08	1.681-248.8	< .001
DHEA-S	4.22	0.308-2757	132.1	5.609-978.1	.53
Corticosterone	0.057	0.002-5.12	0.926	0.025-19.62	.006
11-Deoxycortisol	0.029	0.001-2.03	0.231	0.028-2.094	.002
Androstenedione	0.013	0.001-0.42	0.073	0.001-0.824	.28
11-Deoxycorticosterone	0.017	0.001-0.55	0.035	0.002-3.085	.44
DHEA	0.112	0.01-35.9	0.626	0.012-28.07	.51
17OH-Pregnenolone	0.105	0.034-46.55	0.506	0.033-9.487	.004
17OH-Progesterone	0.013	0.002-1.934	0.214	0.001-2.728	.09
Progesterone	0.021	0.003-0.174	0.027	0.001-1.684	.29
Androsterone	0.44	0.004-10.01	0.97	0.004-24.56	.94
Pregnenolone	0.126	0.017-51.97	0.421	0.042-10.19	.002
Cortisone	0.426	0.026-23.09	29.3	1.376-165.9	< .001
21-Deoxycortisol	0.031	0.003-0.211	0.086	0.001-0.944	.02

To convert ng/mL to nmol/L, multiply by 2.77 for aldosterone; 2.89 for corticosterone; 3.03 for 11-deoxycorticosterone; 3.10 for pregnenolone; 3.02 for 17 α -hydroxyprogesterone; 3.18 for progesterone; 3.01 for 17 α -hydroxypregnenolone; 2.88 for 21-deoxycortisol and 11-deoxycortisol; 2.75 for cortisol; 2.77 for cortisone; 3.47 for DHEA; 2.71 for DHEA-S; 3.49 for androstenedione and androsterone. P values reaching statistical significance were indicated in bold characters.

Abbreviation: DHEA-S, dehydroepiandrosterone sulfate.

Table 3. Comparison of adrenal steroids of non-congenital adrenal hyperplasia primary adrenal insufficiency patients with identified molecular etiologies causing isolated glucocorticoid (GC; patients with *MC2R*, *MRAP*, and *NNT* mutations) vs GC + mineralocorticoid ± sex steroid deficiency (patients with *StAR*, *CYP11A1*, *SGPL1*, *ABCD1*, *AAAS*, *NR0B1/DAX1*, and *AIRE* mutations)

ng/mL	Isolated GC deficient (n = 11)		GC + MC ± sex steroid deficient (n = 16)		P
	Median	Range	Median	Range	
Aldosterone	0.08	0.016-0.3	0.06	0.007-0.706	.93
Cortisol	1.78	0.053-12.24	5.70	0.021-77.49	.45
DHEA-S	5.71	1.991-213.7	4.47	0.308-36.56	.30
Corticosterone	0.10	0.002-1.353	0.09	0.015-5.129	.36
11-Deoxycortisol	0.04	0.002-0.286	0.06	0.001-2.037	.27
Androstenedione	0.02	0.001- 0.128	0.02	0.001-0.419	.34
11-Deoxycorticosterone	0.03	0.006-0.222	0.01	0.001-0.569	.67
DHEA	0.08	0.01-1.16	0.13	0.032-2.384	.30
17OH-Pregnenolone	0.11	0.049-2.866	0.12	0.027-0.751	.28
17OH-Progesterone	0.02	0.006-0.755	0.01	0.002-0.448	.75
Progesterone	0.03	0.004-0.148	0.02	0.003-0.221	.61
Androsterone	0.99	0.004-9.461	0.30	0.027-4.279	.18
Pregnenolone	0.07	0.008-0.638	0.13	0.017-1.299	.53
Cortisone	0.77	0.056-23.09	3.99	0.026-23.11	.64
21-Deoxycortisol	0.02	0.003-0.211	0.07	0.004-0.109	.71

To convert ng/mL to nmol/L, multiply by 2.77 for aldosterone; 2.89 for corticosterone; 3.03 for 11-deoxycorticosterone; 3.10 for pregnenolone; 3.02 for 17 α -hydroxypregnenolone; 3.18 for progesterone; 3.01 for 17 α -hydroxypregnenolone; 2.88 for 21-deoxycortisol and 11-deoxycortisol; 2.75 for cortisol; 2.77 for cortisone; 3.47 for DHEA; 2.71 for DHEA-S; 3.49 for androstenedione and androsterone. Abbreviations: DHEA-S, dehydroepiandrosterone sulfate; GC, glucocorticoid; MC, mineralocorticoid.

Table 4. Adrenal steroid hormones of non-congenital adrenal hyperplasia primary adrenal insufficiency patients with identified molecular etiologies compared to control group

Median (IQR) ng/mL	Patients (n = 27)	Control (n = 324)	P
DHEA	0.0955 (0.05175-0.6933)	0.626 (0.214-1.935)	.06
DHEA-S	3.84 (2.15-9.08)	132.1(70.44-268.5)	< .001
Androstenedione	0.0115 (0.00275-0.03525)	0.073 (0.0315-0.178)	.01
Pregnenolone	0.1205 (0.03275-0.276)	0.421 (0.3175-2.096)	.01
17OH-Pregnenolone	0.105 (0.056-0.43)	0.506 (0.2225-1.205)	.03

To convert ng/mL to nmol/L, multiply by 3.10 for pregnenolone; 3.01 for 17 α -hydroxypregnenolone; 3.47 for DHEA; 2.71 for DHEA-S; 3.49 for androstenedione. Abbreviations: DHEA-S, dehydroepiandrosterone sulfate; IQR, interquartile range.

genetic etiologies found in that national cohort of 95 patients were *MC2R*, *NR0B/DAX1*, *StAR*, *CYP11A1*, *MRAP*, *NNT*, *ABCD1*, *NR5A1*, and *AAAS*. Because approximately 20 genes provide a molecular diagnosis for 65% to 80% cases of non-CAH PAI, TPS is the easiest and the most cost-effective first-line approach to perform the genetic analysis as employed in the present study. However, further molecular studies did not improve the rate of genetic diagnosis statistically significantly in this and some other previous studies (7). An international and multicenter network and collaboration for patients with

unidentified genetic diagnoses using advanced bioinformatic tools are proposed to discover further etiologies and pathways in the pathogenesis of PAI (27, 28). It is important to note that our results indicate that identification of a molecular diagnosis is less likely in cases with a lower ACTH, absent parental consanguinity, late-onset of clinical presentation, and a less severe deficiency of steroid hormones.

In regard to the molecular etiologies of non-CAH PAI, *MCM4*, *TXNRD2*, *SAMD9*, *CDKN1C*, or *POLE1* mutations have not yet been described in any patient of Turkish origin. These conditions may show a specific geographical distribution, may go unrecognized, or the patients may die of other comorbidities pertaining to these disorders (29-31). According to our observations, ALD and X-linked adrenal hypoplasia congenital (*NR0B/DAX1* mutations) are more common causes for non-CAH PAI than reported in general, and triple A is also more common in Turkey because of geographical location and some founder mutations (9-12, 32). Therefore, it is of paramount importance to assess any male patient with non-CAH PAI for ALD and X-linked adrenal hypoplasia for appropriate and timely treatment, long-term monitoring, and genetic counseling (33, 34). Because of obvious clinical and biochemical features in ALD (eg, associated neurological symptoms, positive family history for X-linked genetic transmission), triple A syndrome (alacrimia, achalasia, growth retardation), and X-linked adrenal hypoplasia congenita (hypogonadism, positive family history for X-linked genetic transmission), we have excluded these patients from the present study and prefer single-gene screening for further studies (6). The patients diagnosed with these conditions in the present cohort did not have any of the aforementioned typical characteristics of those disorders.

In this cohort, we have demonstrated that steroid profiling is a very sensitive tool for the biochemical diagnosis of non-CAH PAI. The patients showed statistically significantly low cortisol concentrations with a median of 0.8 ng/mL. This was lower than the median cortisol of 31 ng/mL in healthy infants younger than age 6 months. More than half ($n = 22$, 53.5%) of the patients with non-CAH PAI in the present cohort were diagnosed before age 6 months, when plasma steroid concentrations are physiologically low even in healthy babies. Therefore, it is important to have a diagnostic tool that can differentiate the abnormally low steroid concentrations of the patients from healthy young infants. We have found that plasma cortisol of less than 4 ng/mL, cortisone of less than 11 ng/mL, and corticosterone of less than 0.11 ng/mL had a greater than 95% specificity to differentiate the non-CAH PAI patients from healthy infants younger than 6 months. In addition, mineralocorticoids and adrenal androgens are lower than that of controls in all patients with non-CAH PAI irrespective of the underlying genetic etiology. Low adrenal androgens may cause subtle clinical presentations and maybe underrated (33, 35). However, the gene mutations affecting gonadal androgen synthesis, including *StAR*, *NROB/DAX1*, *CYP11A1*, and *SGPL1* were associated with a differences/disorders of sex development phenotype and gonadal failure as in our cohort.

Unlike CAH, there is no mineralocorticoid or androgen excess in any form of non-CAH PAI. However, 2 46,XX female patients in our cohort were diagnosed with 3 β -hydroxysteroid dehydrogenase 2 (3 β HSD2) deficiency CAH due to homozygous *HSD3B2* mutations. Biochemical findings of adrenal insufficiency, lack of genital virilization, low 17OH-progesterone and androstenedione, and unavailability of $\Delta 5$ steroid measurements in the referring clinic caused a misdiagnosis of non-CAH PAI in these 2 girls. Steroid profiles including dehydroepiandrosterone, pregnenolone, and 17OH-pregnenolone could easily identify the diagnosis of 3 β HSD2 deficiency, which was subsequently confirmed genetically. Indeed, we have recently found that one-third of patients (girls) with 3 β HSD2 deficiency were initially misdiagnosed as non-CAH PAI before their condition was genetically documented (14). Based on these observations we suggest considering 3 β HSD2 deficiency in a 46,XX nonvirilized patient in the differential diagnosis of non-CAH PAI of unknown etiology.

In conclusion, adrenocortical hormone profiles determined by LC-MS/MS demonstrate statistically significantly low glucocorticoids, mineralocorticoids, and adrenal androgens in non-CAH PAI patients. Adrenal steroid profiling was found to be highly sensitive for the recognition of non-CAH PAI even at early infancy, but the specificity to indicate a definitive molecular etiology is low. TPS is a first-line approach in the molecular diagnosis of non-CAH PAI with high efficacy. Nevertheless, lower ACTH, absent parental consanguinity, late-onset of clinical presentation, and a less severe deficiency profile of steroid hormones decreases the probability of identifying a mutation in any of the currently known non-CAH PAI genes.

Acknowledgments

We are deeply grateful to the patients and families, without whom this study could not have been performed.

Financial Support

This work was supported by the Medical Research Council of Marmara University (project grant No. SAG-A-120418-0152).

Author Contributions

T.S.M., Y.K.D., A.B., and T.G. designed the study. B.G.T., E.B., M.Y., S.A., S.E.K., Z.O., A.O., E.S., A.A., Z.A., F.B., K.D., D.D., H.C.E., H.K., N.O.M., and A.Y. recruited and clinically characterized the patients. T.G., T.S.M., and B.G.T. conducted and analyzed the biochemical and LC-MS/MS measurements. Y.K.D. performed and analyzed the sequencing data. T.S.M., B.G.T., S.T., A.B., and T.G. prepared the draft manuscript. All authors contributed to the discussion of results, and edited and approved the final manuscript.

Disclosures

The authors have nothing to disclose.

Data Availability

Some data sets analyzed during the present study are included in the data repositories listed in "References."

References

- Husebye ES, Pearce SH, Krone NP, Kämpe O. Adrenal insufficiency. *Lancet* 2021;397(10274):613-629.
- Buonocore F, Achermann JC. Primary adrenal insufficiency: new genetic causes and their long-term consequences. *Clin Endocrinol (Oxf)* 2020;92(1):11-20.
- Kirkgoz T, Guran T. Primary adrenal insufficiency in children: diagnosis and management. *Best Pract Res Clin Endocrinol Metab*. 2018;32(4):397-424.
- Malikova J, Flück CE. Novel insight into etiology, diagnosis and management of primary adrenal insufficiency. *Horm Res Paediatr*. 2014;82(3):145-157.
- Buonocore F, McGlacken-Byrne SM, Del Valle I, Achermann JC. Current insights into adrenal insufficiency in the newborn and young infant. *Front Pediatr*. 2020;8:619041.
- Güran T. Latest insights on the etiology and management of primary adrenal insufficiency in children. *J Clin Res Pediatr Endocrinol* 2017;9(suppl 2):9-22.
- Guran T, Buonocore F, Saka N, et al. Rare causes of primary adrenal insufficiency: genetic and clinical characterization of a large nationwide cohort. *J Clin Endocrinol Metab*. 2016;101(1):284-292.
- Buonocore F, Maharaj A, Qamar Y, et al. Genetic analysis of pediatric primary adrenal insufficiency of unknown etiology: 25 years' experience in the UK. *J Endocr Soc*. 2021;5(8):bvab086.
- Capalbo D, Moracas C, Cappa M, et al. Primary adrenal insufficiency in childhood: data from a large nationwide cohort. *J Clin Endocrinol Metab*. 2021;106(3):762-773.
- Wijaya M, Huamei M, Jun Z, et al. Etiology of primary adrenal insufficiency in children: a 29-year single-center experience. *J Pediatr Endocrinol Metab*. 2019;32(6):615-622.
- Amano N, Narumi S, Hayashi M, et al. Genetic defects in pediatric-onset adrenal insufficiency in Japan. *Eur J Endocrinol*. 2017;177(2):187-194.
- Perry R, Kecha O, Paquette J, Huot C, Van Vliet G, Deal C. Primary adrenal insufficiency in children: twenty years experience at the Sainte-Justine Hospital, Montreal. *J Clin Endocrinol Metab*. 2005;90(6):3243-3250.
- Claahsen-van der Grinten HL, Speiser PW, Ahmed SF, et al. Congenital adrenal hyperplasia—current insights in

- pathophysiology, diagnostics and management. *Endocr Rev*. Published online May 7, 2021. doi:10.1210/edrv/bnab016
14. Guran T, Kara C, Yildiz M, *et al*. Revisiting classical 3 β -hydroxysteroid dehydrogenase 2 deficiency: lessons from 31 pediatric cases. *J Clin Endocrinol Metab*. 2020;105(4):e1718-e1728.
 15. Yildiz M, Isik E, Abali ZY, *et al*. Clinical and hormonal profiles correlate with molecular characteristics in patients with 11 β -hydroxylase deficiency. *J Clin Endocrinol Metab*. 2021;106(9):e3714-e3724.
 16. Kurnaz E, Kartal Baykan E, Türkylmaz A, *et al*. Genotypic sex and severity of the disease determine the time of clinical presentation in steroid 17 α -hydroxylase/17,20-lyase deficiency. *Horm Res Paediatr*. 2020;93(9-10):558-566.
 17. Eureka Lab Division. Steroid hormones in serum/plasma by LC/MS. Updated March, 2021. Accessed January 2022. https://www.eurekakit.com/wp-content/uploads/2021/03/LC72310_STEROID-HORMONES-100-TEST_010.pdf
 18. Enver EO, Vatansever P, Guran O, *et al*. Adrenal steroids reference ranges in infancy determined by LC-MS/MS. *Pediatr Res*. Published online September 23, 2021. doi:10.1038/s41390-021-01739-5
 19. Gurpinar Tosun B, Kendir Demirkol Y, Seven Menevse T, *et al*. Catch-up growth and discontinuation of fludrocortisone treatment in aldosterone synthase deficiency. *J Clin Endocrinol Metab*. 2021;107(1):e106-e117.
 20. Richards S, Aziz N, Bale S, *et al*. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17(5):405-424.
 21. Sim NL, Kumar P, Hu J, Henikoff S, Schneider G, Ng PC. SIFT web server: predicting effects of amino acid substitutions on proteins. *Nucleic Acids Res*. 2012;40(Web Server issue):W452-W457.
 22. Adzhubei IA, Schmidt S, Peshkin L, *et al*. A method and server for predicting damaging missense mutations. *Nat Methods*. 2010;7(4):248-249.
 23. Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. *Nat Methods*. 2014;11(4):361-362.
 24. Kopanos C, Tsiolkas V, Kouris A, *et al*. VarSome: the human genomic variant search engine. *Bioinformatics*. 2019;35(11):1978-1980.
 25. Menevse TS, Demirkol YK, Tosun BG, *et al*. Supplementary data for "Adrenocortical hormone profiles in non-CAH primary adrenal insufficiency." Deposited December 26, 2021. <https://figshare.com/s/5e79cbdec3508dfdd929>
 26. Menevse TS, Demirkol YK, Tosun BG, *et al*. Supplementary data for "Adrenocortical hormone profiles in non-CAH primary adrenal insufficiency." Deposited December 26, 2021. <https://figshare.com/s/28d1699f7f14a99b4e1a>
 27. Prasad R, Hadjimetriou I, Maharaj A, *et al*. Sphingosine-1-phosphate lyase mutations cause primary adrenal insufficiency and steroid-resistant nephrotic syndrome. *J Clin Invest*. 2017;127(3):942-953.
 28. Maharaj A, Buonocore F, Meimaridou E, *et al*. Predicted benign and synonymous variants in CYP11A1 cause primary adrenal insufficiency through missplicing. *J Endocr Soc*. 2019;3(1):201-221.
 29. Hughes CR, Guasti L, Meimaridou E, *et al*. MCM4 mutation causes adrenal failure, short stature, and natural killer cell deficiency in humans. *J Clin Invest*. 2012;122(3):814-820.
 30. Prasad R, Chan LF, Hughes CR, *et al*. Thioredoxin reductase 2 (TXNRD2) mutation associated with familial glucocorticoid deficiency (FGD). *J Clin Endocrinol Metab*. 2014;99(8):E1556-E1563.
 31. Narumi S, Amano N, Ishii T, *et al*. SAMD9 mutations cause a novel multisystem disorder, MIRAGE syndrome, and are associated with loss of chromosome 7. *Nat Genet*. 2016;48(7):792-797.
 32. Polat R, Ustyol A, Tuncez E, Guran T. A broad range of symptoms in Allgrove syndrome: single center experience in Southeast Anatolia. *J Endocrinol Invest*. 2020;43(2):185-196.
 33. Suntharalingham JP, Buonocore F, Duncan AJ, Achermann JC. DAX-1 (NR0B1) and steroidogenic factor-1 (SF-1, NR5A1) in human disease. *Best Pract Res Clin Endocrinol Metab*. 2015;29(4):607-619.
 34. Engelen M, Kemp S, Eichler F. Endocrine dysfunction in adrenoleukodystrophy. *Handb Clin Neurol*. 2021;182:257-267.
 35. Roucher-Boulez F, Brac de la Perriere A, Jacquez A, *et al*. Triple-A syndrome: a wide spectrum of adrenal dysfunction. *Eur J Endocrinol*. 2018;178(3):199-207.