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Recent advances in diagnosis and treatment of congenital adrenal hyperplasia due to 21-hydroxylase deficiency

Edited by
Serkan Yener and Semra Çaglar Çetinkaya

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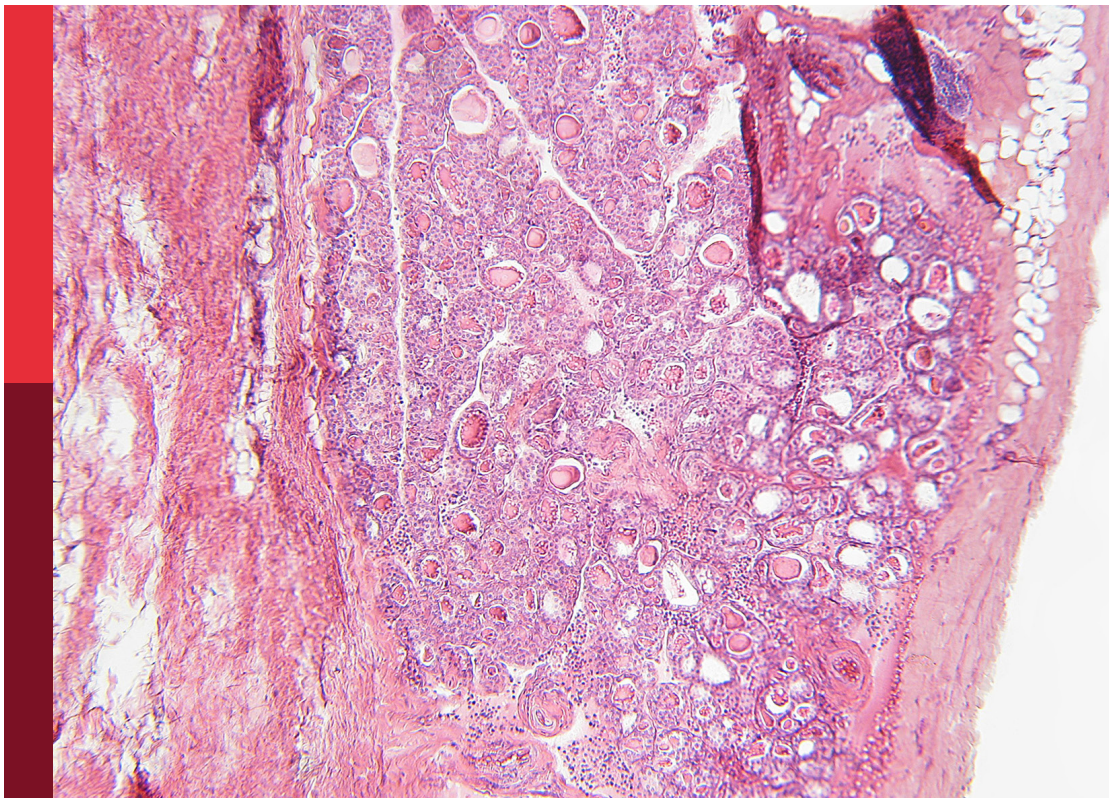

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Recent advances in diagnosis and treatment of congenital adrenal hyperplasia due to 21-hydroxylase deficiency

Topic editors

Serkan Yener — Dokuz Eylül University, Türkiye

Semra Çaglar Çetinkaya — University of Health Sciences, Türkiye

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Henrik Falhammar,
Karolinska Institutet (KI), Sweden

*CORRESPONDENCE
Semra Çaglar Çetinkaya
semcetinkaya@gmail.com

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Editorial: Recent advances in diagnosis and treatment of congenital adrenal hyperplasia due to 21-hydroxylase deficiency

Semra Çaglar Çetinkaya[1,2]*

[1]University of Health Sciences (Turkey), Istanbul, Türkiye, [2]Dr Sami Ulus Child Health and Diseases Training and Research Hospital, Ankara, Türkiye

KEYWORDS

21 hydroxylase deficiency, follow-up, treatment, new therapies, comorbidities

Editorial on the Research Topic
Recent advances in diagnosis and treatment of congenital adrenal hyperplasia due to 21-hydroxylase deficiency

This special edition Research Topic was designed to evaluate all aspects of congenital adrenal hyperplasia due to 21-hydroxylase deficiency (21OHD). Congenital adrenal hyperplasia (CAH) due to 21OHD is the most common type of CAH (90-99% frequency) (1). Depending on the degree of enzyme deficiency, signs of glucocorticoid and/or mineralocorticoid deficiency are observed. The insufficient glucocorticoid and mineralocorticoid hormones are replaced in the treatment. The dose adjustment is important in the treatment. Excess doses cause hypercortisolism, while insufficient doses cause hyperandrogenism. Both hypercortisolism and hyperandrogenism can result in negative cardiometabolic outcomes, for example, and thus, CAH is associated with long-term health risks (2).

The correlation of genotype and phenotype in CAH

Tang et al. investigated the relationship between genotype and phenotype in 15 individuals diagnosed with CAH due to 21OHD from three unrelated families and showed dramatically different phenotypes in the three probands with different compound heterozygous pathological variants in *CYP21A2*. The authors stated that genetic analysis could help the etiologic diagnosis, especially for atypical 21OHD patients, because of wide-spectrum residual enzyme activity.

Zhao et al. focused on another aspect of the genotype and phenotype relationship in CAH cases with 21OHD. Observing that the genotype harboring the P31L promoter variant in *CYP21A2* has a simple virilization phenotype at a rate of 57.1%, this study drew attention to the importance of elaborating the phenotype–genotype relationship in CAH cases.

The diagnostic and monitoring methods of CAH

There have been changes in the diagnostic process of CAH in the last 15 years. Nakhleh et al. investigated the value of 17 hydroxyprogesterone (17OHP) measured by ELISA and RIA in diagnosing non-classical CAH with the cosyntropin test in their nearly 20-year-old archives. Although 17OHP has been a marker used in the diagnosis, screening, and monitoring of CAH for 40 years, its reliability is poor. Therefore, there is a need to develop new techniques that can measure adrenal androgens and their precursors. In their mini-review, de Hora et al. emphasized the advantages of the liquid chromatography–tandem mass spectrometry method for this purpose. In particular, that the adrenal steroidogenesis backdoor pathway is more active in newborns diagnosed with CAH and that oxygenated androgens are high in untreated CAH cases have made it important to analyze androgenic steroids and their precursors by the liquid chromatography–tandem mass spectrometry method. Especially, 21-deoxycortisol has emerged as a marker of 21OHD.

The follow-up of CAH

Itonaga et al. compared the first morning urinary pregnanetriol with 17OHP values at various times during the day for the biochemical monitoring of 21OHD. They showed that the first morning pregnanetriol before morning medication correlated well with 17OHP and could be a good marker (more practical and useful) for monitoring 21OHD.

The comorbidities of CAH

Auchus et al. conducted a survey study in which they investigated experts' opinions on treatment practices and unmet needs in adults with classical CAH (a modified Delphi consensus study). In this study, all panelists stated that glucocorticoid-related comorbidities are difficult to treat in CAH cases and that new treatments are important to prevent them.

Lim et al. compared Korean adults with CAH (71 men-mean age: 27 years, 93 women-mean age: 28 years) with age- and sex-matched healthy controls in terms of some long-term health risks and comorbidities [obesity, testicular adrenal rest tumors (TARTs), menstrual irregularity]. In this study, the researchers found high waist circumference and blood pressure in CAH adults according to the control group. They reported that the TARTs frequency in men was 58.1%, and the irregular menstruations' frequency in women was 57.1% (both genders had the same treatment regimens and hormonal status). They found a 2.7-fold increased risk for hypertension in men with CAH and a 2.0-fold increased risk for women with obesity and CAH. They showed higher adrenal limb thicknesses (men) and 17OHP and dehydroepiandrosterone sulfate levels (women) in obese CAH cases. The authors pointed out that poor metabolic control in CAH patients increases the risk of metabolic morbidity.

In the study of Saho et al., the prevalence of TART (all salt-wasting form and poor metabolic control) in Slovakian patients with CAH was reported to be 29%. Harasymiw et al. investigated the prevalence of depressive and anxiety disorders in CAH individuals (4-25 y) and non-CAH individuals in the United States. They found a higher adjusted prevalence ratio (aPR) for depressive disorders, anxiety disorders, and antidepressant prescriptions in patients with CAH compared to the controls. The metabolic risks and comorbidities should be investigated in CAH patients. Checklists can be created to facilitate the routine monitoring of patients with CAH.

Shafaay et al. assessed the clinical characteristics and quality of life (QoL) (main evaluation domains: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression) of pediatric and adult CAH patients. They found that CAH patients had lower QoL scores in all domains compared to the healthy control group (in particular, approximately half of those patients the in pain/discomfort and anxiety/depression domains). In addition, this study also stated that decreased mobility is the most important risk factor for lower QoL in obese CAH patients. They recommended a multidisciplinary team approach, pre-marital screening, and the implementation of awareness programs for CAH cases, especially in highly consanguineous communities.

The new treatment strategies in CAH

In their study, Khattab and Charlton showed that testicular testosterone levels increased with tildacerfont treatment, an oral active corticotrophin-releasing factor type 1 (CRF1) receptor antagonist, at different doses in patients with uncontrolled CAH. They emphasized that this drug is a new treatment option for cases developing TART and azoospermia and that further studies are needed.

In this Research Topic, the diagnosis, follow-up and treatment processes, and possible comorbidities of CAH cases with different levels of enzyme activities due to 21OHD were evaluated. New methods such as the liquid chromatography–tandem mass spectrometry method for hormonal profile analysis of CAH cases, new monitoring markers such as first monitoring urinary pregnanetriol, neglected problems such as pain/discomfort and anxiety/depression, and new treatment options such as CRF1 receptor antagonist have been presented. As a Research Topic, CAH is a broad and complex field on which more study needs to be performed.

Author contributions

SÇÇ: Writing – original draft.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

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ORIGINAL RESEARCH
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Long-Term Health Outcomes of Korean Adults With Classic Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency

Seung Gyun Lim[1,2], *Young Ah Lee*[3]*, *Han Na Jang*[1,2], *Sung Hye Kong*[2,4], *Chang Ho Ahn*[2,4], *Sang Wan Kim*[2,5], *Choong Ho Shin*[3] and *Jung Hee Kim*[1,2]*

[1] Department of Internal Medicine, Seoul National University Hospital, Seoul, South Korea, [2] Department of Internal Medicine, Seoul National University College of Medicine, Seoul, South Korea, [3] Department of Pediatrics, Seoul National University Children's Hospital, Seoul National University College of Medicine, Seoul, South Korea, [4] Department of Internal Medicine, Seoul National University Bundang Hospital, Seongnam, South Korea, [5] Department of Internal Medicine, Seoul Metropolitan Government Seoul National University Boramae Medical Center, Seoul, South Korea

There is a lack of studies regarding the long-term outcomes of Asian adults with classic congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency. We hypothesized that adults with CAH are at higher metabolic risk than their age-, and sex-matched controls. We further investigated the long-term health outcome-related factors in adults with CAH. We compared metabolic risk between adults with CAH (71 men, 93 women) and age-, and sex-matched controls (190 men, 261 women) from the Korean National Health and Nutrition Examination Survey data. The presence of obesity, testicular adrenal rest tumors (TARTs), and menstrual irregularity was assessed. Hormone status and treatment regimens were compared according to the presence of adverse outcomes. The median age was 27.0 y and 28.0 y for men and women, respectively. Adults with CAH had a higher waist circumference (88.0 vs. 82.3 cm in men, and 83.5 *vs*. 72.3 cm in women), and blood pressure (125.0 vs. 113.0 mmHg in men, and 120.0 *vs*. 104.0 mmHg in women) than age- and sex-matched controls ($P<0.05$ for all). The 2.7-fold increased risk for hypertension (men) and 2.0-fold increased risk for obesity (women) was significant in patients with CAH ($P<0.05$ for both). Obese adults with CAH showed significantly higher adrenal limb thicknesses (men) and 17-hydroxyprogesterone and dehydroepiandrosterone sulfate levels (women) ($P<0.05$ for both). TARTs occurred in 58.1% of men and did not differ by hormone or treatment regimen. Irregular menstruation was observed in 57.1% of women, with higher dehydroepiandrosterone sulfate levels in

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Henrik Falhammar, Karolinska Institutet (KI), Sweden
Miguel Debono, Royal Hallamshire Hospital, United Kingdom

***Correspondence:**
Jung Hee Kim
jhee1@snu.ac.kr;
jhkxingfu@gmail.com
Young Ah Lee
nina337@snu.ac.kr

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those with irregular periods. Adults with CAH had a higher metabolic risk than the general population. Poor disease control may increase their risk of metabolic morbidity and menstrual irregularity.

Keywords: adrenal hyperplasia, congenital, dyslipidemia, hyperglycemia, hypertension, obesity

INTRODUCTION

Congenital adrenal hyperplasia (CAH) refers to a group of genetic disorders characterized by defective steroidogenesis due to enzyme deficiency. The most common form of CAH, 21-hydroxylase deficiency, affects approximately 1:15,000 live births (1, 2). A 21-hydroxylase deficiency results in glucocorticoid deficiency and an increase in pituitary adrenocorticotropin (ACTH) secretion, leading to adrenal androgen excess and adrenal hyperplasia. Depending on disease severity, CAH due to 21-hydroxylase deficiency is classified into classic (salt-wasting and simple virilizing) and non-classic forms (1). The early diagnosis of 21-hydroxylase deficiency through newborn screening test using 17-hydroxyprogesterone (17-OHP) has decreased mortality and morbidity rates, leading to increased interest in improving long-term health outcomes in adulthood (1, 2).

Several studies have reported adverse outcomes in adults with CAH. In the United Kingdom Congenital Adrenal Hyperplasia Adult Study Executive (CaHASE), 203 adults with CAH were significantly shorter and had a higher body mass index (BMI) compared to the health survey data (3). In a Swedish study, based on disease codes, 360 adults with CAH had an approximately four-fold higher risk of having any cardiovascular and metabolic disorders than matched controls (4). In a cross-sectional study of the National Institutes of Health (NIH) and French cohort, the prevalence of obesity was common, ranging from 35% to 44% (5, 6), although the latter two studies did not compare the patients' metabolic risk with that of healthy controls. In a European multicenter study, 226 adults with CAH were a higher risk for hypertension, dyslipidemia, and cardiovascular disease but not type 2 diabetes (7). A recent meta-analysis including children and adults with CAH demonstrated that these patients had a high prevalence of cardiovascular and metabolic risk factors (8).

The two main goals for CAH management are to replace the deficient hormones for adrenal insufficiency and control androgen excess. However, it is challenging to balance glucocorticoid doses because supraphysiologic doses of glucocorticoids are usually required to suppress androgen excess. It remains controversial whether over- or undertreatment is more harmful to the metabolic and cardiovascular health of patients with CAH (3, 5, 6, 9–11). Moreover, the relationship between testicular adrenal rest tumors (TARTs) and disease control remains unclear (5, 6, 12).

Most related studies have not performed a sex-stratified analysis and rarely evaluated adrenal morphology such as hyperplasia or thinning (6, 13). In Asia, few studies have examined the determining factors for adverse outcomes in adults with CAH. In this context, we hypothesized that adults with CAH are at a higher risk of metabolic morbidity than their age- and sex-matched controls in the Asian general population. Furthermore, we aimed to investigate sex-specific indicators related to adverse health outcomes by focusing on metabolic morbidity, TARTs, and menstrual irregularity in adults with CAH.

MATERIALS AND METHODS

Study Subjects

Among the 233 adults with CAH aged over 20 years at the last follow-up visit between 2000 and 2020 at Seoul National University Hospital, those with non-classic CAH (n = 19) or for whom laboratory findings or an appropriate medical history was lacking (n = 50) were excluded. Finally, we included 164 adults with classic 21-hydroxylase deficiency. Of the 71 men and 93 women included in this study 42 (59.2%) men and 34 (36.6%) women had salt-wasting form. We diagnosed patients with CAH based on clinical and biochemical data since the genetic testing for CYP21A2 mutation was only done in 34 of 164 patients. All patients were diagnosed clinically since neonatal screening was first introduced in 2006 in Korea. For comparison, age- and sex-matched controls (190 men, 261 women) were included in this study, with a 1:3 case to control ratio from the nationwide representative survey database (Korean National Health and Nutrition Examination Survey [KNHANES] 2015) (14).

Clinical, Imaging, and Biochemical Data

We retrospectively reviewed the patients' electronic medical records and retrieved the data from the last follow-up visit. BMI was calculated as body weight divided by height squared (kg/m^2). Waist circumference was measured at the level of the umbilicus with the subject standing and breathing normally while wearing light clothing. Blood pressure was measured twice on different days using an automated technique while the subjects were in a seated position after a 20-min rest. Body composition data, such as lean mass and fat mass, were obtained from a bioimpedance analyzer (Inbody720®; Inbody Co. Korea).

Obesity was defined as a BMI > 25 kg/m^2 (15). Dyslipidemia was defined as the use of lipid-lowering agents or having an abnormal lipid panel (total cholesterol ≥ 240 mg/dL, LDL cholesterol ≥ 160 mg/dL, triglycerides ≥ 200 mg/dL, or HDL cholesterol < 40 mg/dL). The presence of hyperglycemia included diabetes mellitus and prediabetes. Diabetes mellitus was defined as an HbA1c ≥ 6.5% or the use of any oral anti-diabetic drugs or insulin therapy. Prediabetes was determined as an HbA1c value between 5.7% and 6.4% or a fasting plasma glucose ≥100 mg/dL. Subjects taking any antihypertensive medications or systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg in repeated measurements

were considered to have hypertension (16). In men, TARTs were identified using testicular sonography. In women, the regularity of menstruation was classified into two categories: regular (21–35 days) or irregular (oligomenorrhea/amenorrhea).

Computed tomography was performed to evaluate the morphology of the adrenal glands. The examiner was blinded to the patients' history. The widths of the medial and lateral adrenal limbs were measured as the maximum width of the limbs perpendicular to the long axis (17). The thickness of the adrenal limb was defined as the mean medial and lateral limb widths. The current glucocorticoid and mineralocorticoid (fludrocortisone) regimen was identified for each patient. The daily glucocorticoid dose was calculated based on glucocorticoid type and daily dose. Glucocorticoid doses were calculated based on the anti-inflammatory equivalent dose compared to hydrocortisone (30 mg hydrocortisone = 7.5 mg prednisolone).

Morning fasting blood samples were taken before steroid medications were administered. Laboratory tests included hemoglobin A1c (HbA1c), plasma glucose, total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, 17-hydroxyprogesterone (17-OHP), total testosterone, dehydroepiandrosterone sulfate (DHEAS), plasma renin activity, and adrenocorticotropin (ACTH).

Biochemical assays

Serum 17-OHP and DHEAS levels were measured using a radioimmunoassay (RIA) CT kit (Asbach Medical Products GmbH, Germany), with intra- and inter-assay CVs of 4.6–6.8% and 7.7–8.8% for 17-OHP (reference range, 0.11-5.00 ng/mL) and 3.6–5.9% and 6.5% for DHEAS (reference range, 1187-4289 ng/mL), respectively. Serum total testosterone was measured using a TESTO-CT2 kit (Cisbio Bioassays, Saclay, France) with intra- and inter-assay CVs of 3.1–8.9% and 5.2–11.6%, respectively (reference range, 2.7-10.7 ng/mL in men, and 0-1.0 ng/mL in women). Plasma renin activity was measured using a PRA RIA kit (TFB, Inc.), with intra- and inter-assay CVs of 3.8% and 6.7%, respectively (reference range, 0.32-1.84 ng/mL/hr). Plasma ACTH was measured using an immunoradiometric assay (Cisbio Bioassays) with a reference range of 10.0–60.0 pg/mL. The intra- and inter-assay CVs of the ACTH used were 3.7% and 3.8%, respectively.

Statistical Analysis

Data are shown as number (percentage) for categorical variables and mean ± standard deviation or median (interquartile range) for continuous variables based on the results of the normality test. The normality test was performed using the Shapiro-Wilk test. Categorical variables were analyzed using the chi-squared test. Continuous variables were compared using Student's t-test or the Mann-Whitney U test according to the distribution of normality. Logistic regression models were constructed to assess the metabolic risk of patients with CAH by sex. To adjust for the effect of age and BMI, we performed a multivariate logistic regression analysis, and age and BMI-adjusted odds ratio (OR) and 95% confidence interval (95% CI) are presented. Statistical significance was set at $P < 0.05$. All statistical analyses were performed using R Statistical Software (version 4.0.3).

RESULTS

Comparison of Metabolic Risk Between Patients with CAH and Healthy Controls

Table 1 compares the clinical and biochemical characteristics of patients with CAH and age- and sex-matched controls by sex. The median age for men and women was 27.0 and 28.0 years, respectively. The men with CAH (n = 71) exhibited a shorter stature ($P < 0.001$), higher weight, and greater waist circumference ($P = 0.005$ for both) than the control men (n = 190) without a BMI difference. Fasting plasma glucose and blood pressure were higher in the men with CAH ($P < 0.05$), but HbA1c was similar between the two groups. Although total cholesterol, triglyceride, and LDL cholesterol levels were similar between the two groups, HDL cholesterol was higher in the men with CAH than in the control men ($P < 0.001$).

The women with CAH (n = 93) showed a shorter stature, higher BMI, and higher waist circumference ($P < 0.05$) than the

TABLE 1 | Comparison of clinical characteristics between CAH patients and age- and sex-matched control.

Variables	CAH men (N = 71)	Control men (N = 190)	*P*	CAH women (N = 93)	Control women (N = 261)	P
Age, years	27.0 [23.0;33.0]	27.0 [23.0;33.0]	0.918	28.0 [23.0;36.0]	28.0 [23.0;36.0]	0.937
Height, cm	163.8 [157.6;167.9]	174.7 [170.5;177.8]	<0.001	152.8 [147.6;157.6]	160.3 [157.0;164.6]	<0.001
Weight, kg	66.3 [57.0;76.9]	71.5 [65.0;79.7]	0.005	54.0 [46.2;59.3]	55.7 [50.6;62.8]	0.020
Body mass index, kg/m^2*	23.8 [21.2;30.0]	23.3 [21.6;26.0]	0.111	23.2 [20.2;25.7]	21.4 [19.7;23.6]	0.006
Waist circumference, cm*	88.0 [78.0;97.5]	82.3 [77.2;89.4]	0.005	83.5 [77.0;92.0]	72.3 [67.4;79.3]	<0.001
Systolic blood pressure, mmHg	125.0 [120.5;135.0]	113.0 [106.0;122.0]	<0.001	120.0 [112.0;130.0]	104.0 [99.0;110.0]	<0.001
Diastolic blood pressure, mmHg	77.0 [73.0;85.0]	73.0 [68.0;81.0]	0.007	75.0 [67.0;82.0]	70.0 [64.0;75.0]	<0.001
Fasting plasma glucose, mg/dL	94.0 [89.0;99.0]	91.0 [87.0;96.0]	0.030	89.0 [86.0;96.0]	88.0 [84.0;93.0]	0.234
HbA1c, %	5.2 [5.1; 5.4]	5.3 [5.1; 5.5]	0.067	5.2 [5.0; 5.4]	5.3 [5.1; 5.5]	0.013
Total cholesterol, mg/dL	188.9 ± 34.2	180.7 ± 31.2	0.070	190.0 [167.0;217.0]	180.0 [158.0;202.0]	0.007
Triglyceride, mg/dL	114.0 [85.0;151.0]	104.0 [74.5;175.0]	0.503	108.0 [83.0;139.0]	71.0 [55.0;100.0]	<0.001
HDL cholesterol, mg/dL	55.0 [49.0;69.0]	47.3 [40.8;55.4]	<0.001	65.1 ± 16.1	58.7 ± 12.6	0.001
LDL cholesterol, mg/dL	109.0 [93.0;128.5]	109.0 [91.0;126.5]	0.832	113.0 [95.5;134.0]	103.0 [84.0;124.0]	0.058

*Data are shown as mean ± standard deviation, median (interquartile range), or numbers (percentages). CAH, congenital adrenal hyperplasia; HDL, high-density lipoprotein; LDL, low-density lipoprotein; *Body mass index and waist circumference were not available in 12 and 54 patients with CAH.*

TABLE 2 | Metabolic risks in CAH adults compared with age- and sex-matched controls.

	Men	Women
Obesity*	1.58 (0.89-2.80)	2.04 (1.18-3.50)
Dyslipidemia	0.81 (0.44-1.44)	1.17 (0.61-2.14)
Hyperglycemia	0.81 (0.42-1.51)	1.25 (0.70-2.17)
Hypertension	2.67 (1.22-5.82)	1.84 (0.78-4.17)

*Data are shown as odds ratios (95% confidence interval). CAH, congenital adrenal hyperplasia; OR, odds ratio. *BMI was not available in 12 patients.*

control women (n = 261). Blood pressure was also higher in the women with CAH than in the control women (P < 0.001). Fasting plasma glucose levels were similar between the two groups, but HbA1c was lower in women with CAH (P < 0.013). Total cholesterol, triglyceride, and HDL cholesterol levels were higher in the women with CAH than in the control women (P < 0.05).

We analyzed the metabolic outcomes of the CAH group versus the control group (**Table 2**). The CAH group had a two-fold higher risk of obesity than the control group among the women (OR [95% CI], 2.04 [1.18-3.50]), but the difference was not significant in the men. The increased risk of hypertension was found in CAH men (OR [95% CI], 2.67 [1.22-5.82]) but not in women. The risk of dyslipidemia and hyperglycemia did not differ between the two groups in either sex.

Comparison According to Whether CAH Patients Have Metabolic Comorbidities

Figure 1 shows the prevalence of comorbidities in adults with CAH. The prevalence of any metabolic comorbidity was significantly higher in men than in women (70.4% *vs.* 52.7%, P = 0.032) without sex differences for each component. The prevalence of obesity, dyslipidemia, hyperglycemia, and hypertension was 43.9%, 29.6%, 23.9%, 19.7% in men and 33.7%, 18.3%, 23.7%, 10.8% in women, respectively. The number of patients with diabetes was only 6 in women and 2 in men with CAH. There was no history of gestational diabetes, cardiovascular or cerebrovascular disease.

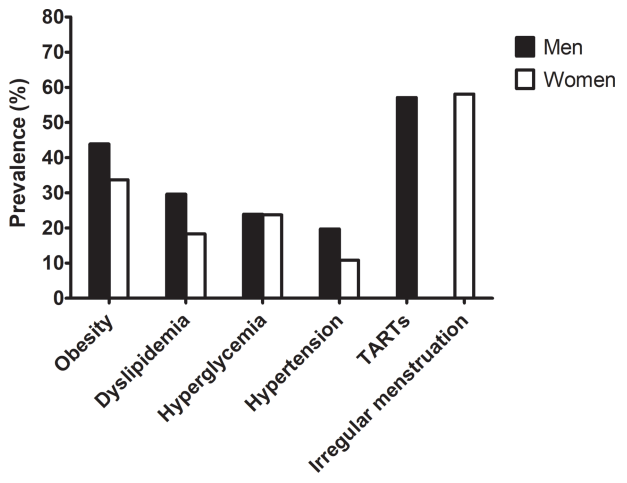

FIGURE 1 | Prevalence of metabolic risk and gonadal dysfunction in the adult CAH patients according to sex.

We further analyzed the differences in hormone status and treatment regimen according to whether CAH patients were obese (**Table 3**). Neither age at diagnosis nor proportion of subtypes differed between obese and non-obese patients. Obese adults with CAH had a higher waist circumference and percentage of fat mass in both sexes but a lower percentage of lean mass than those without (P < 0.05 for all for both sexes). Adrenal limb thickness was significantly higher in obese men compared to non-obese men (P< 0.001). Although the difference of adrenal limb thickness was marginally significant between obese and non-obese women, adrenal tumors was more frequently found in obese women compared to non-obese women (P=0.019). Among the hormones, 17-OHP and DHEAS levels were significantly higher in obese women than in non-obese ones (P< 0.001, and P=0.009, respectively). All obese women had a 17-OHP level ≥ of 10 ng/mL. However, the glucocorticoid regimen and dose were not related to obesity in either sex. The number of patients with 0, 1, and ≥2 metabolic comorbidities was 29.6% (n=21), 35.2% (n=25), and 35.2% (n=25) in men with CAH, and 47.3% (n=44), 26.8% (n=25), and 25.8% (n=24) in women with CAH, respectively. No significant relationships of age at diagnosis, proportion of subtypes, and glucocorticoid doses with number of metabolic comorbidities were found in both sex (data not shown).

Comparison of Whether CAH Patients Had TARTs or Menstrual Irregularities

The prevalence of TARTs in men was 58.1% (**Figure 1** and **Table 4**). The presence of TARTs was not related to disease control, hormone status, glucocorticoid regimen, or adrenal limb thickness in men. Among the women, 57.1% exhibited menstrual irregularities (**Figure 1** and **Table 4**). Women with irregular periods showed a shorter stature and a higher DHEAS level than those with regular periods (P< 0.05 for both). Other hormones, steroid regimens, and adrenal limb thickness did not differ according to menstrual regularity.

DISCUSSION

The present study suggested that waist circumference and blood pressure were higher in adults with CAH compared with age- and BMI-matched controls from the KNHANES database. Moreover, there was an increased risk of hypertension in men and obesity in women with CAH than controls. Obese men with

TABLE 3 | Hormone status and treatment regimens in CAH men and women according to the presence of obesity.

Variables	Men			Women		
	Obesity (-) (N = 37)	Obesity (+) (N = 29)	*P*	Obesity (-) (N = 57)	Obesity (+) (N = 29)	*P*
Age at evaluation, years	26.0 [23.0;31.0]	28.0 [24.0;37.0]	0.164	28.0 [22.0;35.0]	32.0 [24.0;41.0]	0.177
Age at diagnosis, years	1.0 [0.0; 7.0]	1.0 [0.0; 8.0]	0.520	2.0 [0.0; 10.0]	5.0 [1.0; 17.0]	0.149
Salt-wasting form, n (%)	21 (56.8%)	18 (62.1%)	0.854	23 (40.4%)	10 (34.5%)	0.768
Height, cm	163.9 [157.6;168.3]	163.2 [158.9;167.8]	0.846	152.5 [147.2;157.0]	155.0 [148.8;158.6]	0.419
Weight, kg	57.4 [53.2;61.0]	79.8 [73.5;87.1]	<0.001	50.2 [43.1;54.6]	63.9 [58.4;70.6]	<0.001
Body mass index, kg/m^2	21.7 [20.6;23.4]	30.4 [28.1;31.5]	<0.001	21.0 ± 2.6	28.5 ± 3.5	<0.001
Waist circumference (cm)	80.0 ± 5.6	98.8 ± 6.6	<0.001	79.0 [75.0;87.0]	90.0 [84.0;105.0]	<0.001
Lean mass (kg)	24.7 [23.6;26.5]	28.7 [26.7;31.3]	0.001	18.4 [16.7;21.0]	23.3 [20.8;29.4]	<0.001
Percentage lean mass (%)	42.6 [40.3;46.4]	36.4 [34.3;38.4]	<0.001	36.9 [34.2;39.9]	33.0 [29.8;57.3]	0.054
Fat mass (kg)	15.4 ± 4.6	29.1 ± 6.5	<0.001	19.5 [16.2;22.2]	25.0 [22.5;35.5]	<0.001
Percentage fat mass (%)	25.0 ± 6.1	36.5 ± 5.8	<0.001	36.0 ± 6.2	41.1 ± 6.9	0.006
17-OHP, ng/mL	60.0 [31.1;83.9]	81.6 [56.2;105.3]	0.055	38.3 [20.3;60.7]	71.8 [44.3;109.0]	<0.001
17-OHP <10 ng/mL (%)	2 (5.4%)	1 (3.5%)	1.000	9 (15.8%)	0 (0.0%)	0.026
Total testosterone, ng/mL	4.3 [3.5; 6.5]	4.7 [3.7; 5.4]	0.838	0.5 [0.1; 0.9]	0.7 [0.5; 1.8]	0.138
DHEAS, ng/mL	736.0 [277.0;1351.0]	686.5 [295.5;1435.0]	0.639	292.0 [105.5;743.5]	632.0 [306.0;1690.0]	0.009
Plasma renin activity, ng/mL/hr	7.4 [3.4;10.4]	10.7 [5.4;21.4]	0.077	8.4 [4.2;11.9]	8.2 [4.8;15.4]	0.428
Plasma renin activity <3 ng/mL/hr	7 (20.0%)	7 (7.1%)	0.277	8 (14.3%)	2 (8.3%)	0.715
ACTH, pg/mL	117.2 [40.5;281.3]	232.6 [81.9;681.0]	0.164	33.1 [14.9;232.7]	70.2 [37.4;188.0]	0.228
Adrenal thickness on CT, mm	5.5 [4.1; 6.3]	7.4 [6.1; 8.9]	<0.001	5.8 [4.5; 7.3]	6.7 [5.3; 9.2]	0.064
Adrenal tumors, n (%)	3 (12.0%)	5 (26.3%)	0.262	1 (2.9%)	4 (28.6%)	0.019
Glucocorticoid regimen, n (%)			1.000			1.000
Hydrocortisone	1 (2.7%)	0 (0.0%)		5 (8.8%)	3 (10.3%)	
Prednisolone	36 (97.3%)	29 (100.0%)	0.850	52 (91.2%)	26 (89.7%)	0.400
Glucocorticoid dose, mg/day	30.0 [20.0;30.0]	30.0 [20.0;30.0]	0.850	30.0 [20.0;30.0]	25.0 [20.0;30.0]	0.400
Fludrocortisone use, n (%)	31 (83.8%)	22 (75.9%)	0.623	43 (75.4%)	14 (48.3%)	0.023

Data are shown as mean ± standard deviation, median [interquantile range], or numbers (percentages). BMI and adrenal thickness were not available in 12 and 75 patients. Body composition data were not available in 20 men and 38 women. 17-OHP, 17-hydroxyprogesterone; DHEAS, dehydroepiandrosterone sulfate.

CAH showed higher adrenal limb thicknesses than non-obese ones, while obese women had higher 17-OHP and DHEAS levels than non-obese ones. In addition, more than half of the adults with CAH exhibited TARTs or menstrual irregularities. The presence of TARTs in men with CAH was not related to disease control, while women with CAH who had irregular periods were shorter and had higher DHEAS levels than those with regular periods.

The prevalence of obesity or central obesity in adults with classic CAH is reportedly 20–50%, higher than that in healthy controls (3–6). In the present study, obesity was more prevalent in women with CAH, and waist circumference was obviously higher in both sexes than in healthy controls, consistent with the increased abdominal obesity and higher visceral to subcutaneous fat ratio linked to insulin resistance and inflammation, suggesting an unhealthy metabolic phenotype among adults with CAH (18).

It remains controversial whether hypertension, dyslipidemia, and hyperglycemia are more prevalent in adults with CAH than in healthy controls. The prevalence of hypertension was higher in the NIH and Swedish nationwide CAH cohorts (4, 5, 8) and similar or lower in the CaHASE and French cohorts (3, 19). In our study, men with CAH but not women with CAH showed a higher prevalence of hypertension than the controls, which can be related to the relatively high proportion of fludrocortisone use and central obesity among men with CAH and the protective effect of estrogen on blood pressure among women with CAH. The prevalence of dyslipidemia and hyperglycemia was similar between adults with CAH and controls in this study. However, fasting glucose and HDL cholesterol levels in men with CAH and total cholesterol, triglyceride, and HDL cholesterol levels in women with CAH were higher than the values in controls, although a recent meta-analysis including 300 pediatric and 137 adults showed no difference in glucose and lipid levels (8). The result of higher HDL-cholesterol levels in our patients with CAH remains to be reproduced in further studies, and the underlying mechanism also remains to be determined. However, Falhammar et al. suggested that older women with CAH had also higher lean mass which may explain the favorable lipid profile (20). Our study population mainly consisted of young adult patients who showed elevated androgen levels, suggesting poor disease control. Inconsistency in reporting the prevalence of metabolic comorbidities may have resulted from differences in patient age, glucocorticoid and mineralocorticoid doses, treatment target goals, disease control status, and 21-hydroxylase activity.

We further analyzed factors that differed according to whether patients with CAH had more metabolic risk factors. Previous studies identified that adults with CAH are at risk for hyperandrogenism and iatrogenic hypercortisolism, both of which lead to obesity, insulin resistance, and cardiometabolic comorbidities (5, 6, 9, 10). In addition to glucocorticoid overtreatment, suppressed 17-OHP levels were related to obesity or metabolic morbidity (3, 11) as well as androgen excess, suggesting that poor disease control contributed to higher metabolic risk factors as shown in our study.

TABLE 4 | Hormone status and treatment regimen in men with CAH according to the presence of testicular adrenal rest tumors (n = 70) and women with CAH according to menstruation (n = 86).

Variables	Men			Women		
	TART (-) (N = 30)	TART (+) (N = 40)	*P*	Irregular (n = 50)	Regular (n = 36)	*P*
Age at evaluation, years	27.0 [22.0;35.0]	27.0 [24.0;31.0]	0.957	29.5 [22.0;39.0]	27.0 [22.5;34.5]	0.277
Height, cm	164.6 ± 8.2	162.2 ± 6.1	0.196	151.0 [146.4;156.5]	155.3 [151.6;159.7]	0.011
Weight, kg	66.0 [56.8;76.4]	67.4 [57.4;79.8]	0.822	54.4 [45.6;58.6]	52.0 [44.7;59.8]	0.601
Body mass index, kg/m^2	24.0 [21.4;28.1]	23.8 [21.7;30.6]	0.474	23.2 [20.2;25.6]	22.5 [20.1;25.8]	0.768
Waist circumference (cm)	89.9 ± 11.1	88.5 ± 11.5	0.669	87.0 [78.0;93.0]	80.5 [75.0;90.0]	0.136
Lean mass (kg)	26.9 [24.0;30.5]	26.2 [24.5;29.1]	0.702	20.7 [17.9;23.1]	19.5 [17.4;21.9]	0.788
Percentage lean mass (%)	39.8 [37.6;45.5]	39.6 [34.8;42.8]	0.371	35.1 [32.2;38.3]	36.5 [33.0;41.6]	0.345
Fat mass (kg)	22.3 ± 7.9	21.9 ± 9.7	0.865	21.1 [18.8;25.5]	21.6 [16.2;24.9]	0.570
Percentage fat mass (%)	31.3 ± 7.4	30.2 ± 8.9	0.657	39.0 ± 6.9	37.0 ± 5.9	0.270
17-OHP, ng/mL	58.2 [20.7;85.9]	72.5 [48.6;100.9]	0.098	49.5 [28.5;97.4]	45.3 [27.1;68.0]	0.203
17-OHP <10 ng/mL	3 (10.0%)	1 (2.5%)	0.307	4 (8.0%)	5 (13.9%)	0.482
Total testosterone, ng/mL	4.2 [3.3; 5.4]	4.8 [3.7; 6.2]	0.314	0.7 [0.3; 1.6]	0.5 [0.1; 0.7]	0.086
DHEAS, ng/mL	885.5 [285.0;1396.0]	584.0 [302.0;1348.0]	0.963	462.5 [199.0;1559.0]	251.0 [102.5;573.5]	0.013
Plasma renin activity, ng/mL/hr	8.1 [4.2;12.3]	7.3 [3.8;19.1]	0.748	8.6 [4.6;14.2]	7.4 [4.7;11.2]	0.458
Plasma renin activity <3 ng/mL/hr	3 (10.0%)	7 (18.9%)	0.493	5 (10.9%)	4 (12.5%)	1.000
ACTH, pg/mL	82.5 [42.4;699.2]	187.0 [46.9;318.0]	0.948	41.0 [16.0;125.8]	58.9 [35.5;241.6]	0.188
Adrenal thickness on CT, mm*	6.2 [5.4; 7.4]	6.1 [4.1; 7.2]	0.512	6.7 [5.2; 8.4]	6.0 [4.5; 6.9]	0.202
Adrenal tumors, n (%)	4 (20.0%)	4 (16.7%)	1.000	4 (14.8%)	1 (5.3%)	0.387
Glucocorticoid regimen, n (%)			1.000			0.649
Hydrocortisone	1 (3.3%)	2 (5.0%)		3 (6.0%)	4 (11.1%)	
Prednisolone	29 (96.7%)	38 (95.0%)		47 (94.0%)	32 (88.9%)	
Glucocorticoid dose, mg/day	30.0 [20.0;30.0]	30.0 [20.0;30.0]	0.736	30.0 [20.0;30.0]	27.5 [20.0;30.0]	0.379
Fludrocortisone use, n (%)	24 (80.0%)	32 (80.0%)	1.000	29 (58.0%)	27 (75.0%)	0.161

*Data are shown as mean ± standard deviation, median (interquartile range), or numbers (percentages). 17-OHP, 17-hydroxyprogesterone; DHEAS, dehydroepiandrosterone sulfate. *Adrenal thickness was available in 43 men and 50 women.*

Intriguingly, obese men with CAH presented with thick adrenal limbs, while obese women had high 17-OHP and DHEAS levels. Few studies have examined the adrenal morphology of adults with CAH (6, 21). Adrenal nodules were more frequently detected in poorly controlled patients with CAH, and regressed in size after high-dose steroids (13). Adrenal hyperplasia and/or nodules have been reported in 29.3– 45% of patients with CAH (22), with significant correlation with high levels of ACTH, 17-OHP, and plasma renin activity (6). Adrenal tumors were detected in 15% (14/93) among our adult patients evaluated by CT. Despite the limitation of a single hormonal measurement in the morning due to the cross-sectional nature of this study, adrenal limb thickness or the presence of adrenal tumors could be a long-term disease control marker in adults with CAH.

Obese women with CAH showed higher 17-OHP levels than non-obese ones, suggesting the contribution of androgen excess. A similar phenomenon was demonstrated in polycystic ovary syndrome in postmenopausal women (23–25). Androgen excess drives visceral fat accumulation and induces the android phenotype in women with CAH (26, 27). Even with continued treatment, patients may respond poorly to glucocorticoid treatment (6, 28) related to glucocorticoid receptor gene polymorphism in addition to low adherence to glucocorticoid therapy regimens (29). Further studies are warranted to determine optimal targets and androgen metabolites that reflect adequate hormonal control and ideal glucocorticoid regimens to minimize cardiometabolic comorbidities.

The presence of TART is the main factor of male infertility since it blocks the seminiferous tubules and leads to Leydig cell failure, which has a reported prevalence of around 37% (14–86%) depending on age and genotype (5, 6, 19, 30). In our study, TARTs were found in 57.1% of men with CAH who underwent testicular sonography. Since TARTs express adrenal-specific enzymes and ACTH and angiotensin II receptors (31), poor disease control and high ACTH or renin activity can lead to adrenocortical hyperplasia and TARTs (5, 32, 33), although the lack of a correlation between 17-OHP levels and TARTs has been reported (6, 12, 34). Although the present study failed to demonstrate a relationship between disease control and the presence of TARTs, in our previous study, TART size was related to the undertreatment percentage (35).

The prevalence of menstrual irregularity was up to 58.1% in the women with CAH in our study, which is consistent with that of a previous report of 30–75% depending on genotype (3). Women with CAH and irregular periods exhibited shorter stature and higher DHEAS levels, reflecting hyperandrogenism. Patients with CAH exposed to high androgens in childhood can experience earlier epiphyseal closure and short stature. Hyperandrogenism suppresses serum progesterone, which reduces luteinizing hormone pulsatility during the follicular phase and induces endometrial thinning, leading to oligo- or anovulation and oligo- or amenorrhea (36–38).

This cross-sectional study had several limitations. There were several missing values due to the retrospective study design. We could not assess the total cumulative doses of glucocorticoid or

perform a longitudinal hormone assessment to determine disease control due to a lack of childhood data before the electronic medical record era. Since most patients were diagnosed before the genetic testing era, we could not analyze the patients' clinical or hormone characteristics according to genotype. When we assessed single hormone measurements in the morning at the last visit, only 7.3% of patients had well-controlled disease based on the early-morning 17-OHP levels. Since undertreatment still affected most of our patients compared to those in previous reports (3), we could not compare the effect of undertreatment versus overtreatment on long-term outcomes. Androstenedione, which was known to be the reliable marker for disease control (1), was not checked due to the cost and unavailability. Although this cross-sectional design could not prove the causal relationship of disease itself or treatment effect with metabolic complications, our study had several strengths. First, it is the largest single-center Asian study of adults with CAH. We also compared age-, sex-, and ethnicity-matched controls to assess metabolic risk. We separately analyzed sex-specific indicators according to health outcomes and obtained different findings by sex. Adrenal thickness assessed by CT was incorporated into the variables to reflect long-term glucocorticoid exposure. Most patients had taken the same dosage of prednisolone in adulthood, thereby allowing us to compare their hormonal effects in patients with CAH without glucocorticoid dose interference.

CONCLUSION

The present study confirmed the higher prevalence of metabolic morbidities in young adults with CAH than in healthy controls. Moreover, adverse metabolic outcomes and menstrual irregularity were associated with poor disease control in our study subjects. Considering the harmful effects of poor disease control on long-term outcomes, compliance should be guaranteed. Early attention should be paid to identifying at-risk patients and regular follow-up provided to minimize cardiovascular risk in patients with CAH. Further studies are needed to elucidate the mechanisms leading to metabolic morbidity and the respective roles of androgen excess and glucocorticoid exposure.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary files. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Seoul National University Hospital Institutional Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SL and JK contributed in the conception of the work, participated in the study design, and wrote the manuscript. YL contributed in the conception of the work, participated in the study design, and critically revised the manuscript. HJ, SK, CA, SK, and CS contributed in the conception of the work, performed statistical analyses, and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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First Morning Pregnanetriol and 17-Hydroxyprogesterone Correlated Significantly in 21-Hydroxylase Deficiency

Tomoyo Itonaga[1,2], *Masako Izawa*[3], *Takashi Hamajima*[3] *and Yukihiro Hasegawa*[1*]

[1] Division of Endocrinology and Metabolism, Tokyo Metropolitan Children's Medical Center, Tokyo, Japan, [2] Department of Pediatrics, Oita University Faculty of Medicine, Oita, Japan, [3] Department of Pediatric Endocrinology and Metabolism, Aichi Children's Health and Medical Center, Aichi, Japan

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***Correspondence:**
Yukihiro Hasegawa
yhaset@gmail.com

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Background: Biochemically monitoring 21-hydroxylase deficiency (21-OHD) is challenging. Serum/blood 17-hydroxyprogesterone (17OHP) measurements are normally used for this purpose. Urinary pregnanetriol (PT), a urinary metabolite of 17OHP, may also be used. Based on auxological data, we previously reported that the optimal first morning PT value fell in the range of 2.2–3.3 mg/gCr (95% confidence interval of the mean) and 0.59-6.0 mg/gCr (10^{th} – 90^{th} percentile) for monitoring 21-OHD treatment. No report thus far has directly compared the first morning urinary PT value with the 17OHP value at various times during the day.

Objective: To explore the correlation between the first morning urinary PT value before glucocorticoid administration and the serum/blood 17OHP value at three time points, namely, before and two and four hours after glucocorticoid administration.

Design: This was a prospective study done at two children's hospitals.

Methods: In total, 25 patients with 21-OHD aged 3-25 years were recruited. Their urinary PT levels and 17OHP levels were measured for three days within a total period of one week. The first morning PT value was collected on all three days. Dried blood spots and serum were used to measure 17OHP.

Results: The range for the first morning PT value for all the samples (n=69) was 0.10-56.1 mg/gCr. A significant, positive correlation was found between the first morning PT and 17OHP values before medication ($r=0.87$, $p<0.01$), and weaker correlation was observed between the first morning PT and 17OHP values after medication.

Conclusions: The first morning PT correlated more significantly with 17OHP before the morning medication. Measuring the first morning PT value may be more practical and useful for monitoring 21-OHD biochemically.

Keywords: Urinary pregnanetiol, 17-hydroxyprogesterone, 21-hydroxylase deficiency, congenital adrenal hyperplasia, first morning urine sample, therapy monitoring, glucocorticoid

INTRODUCTION

The most common form of congenital adrenal hyperplasia, 21-hydroxylase deficiency (21-OHD), is an autosomal recessive disease caused by mutations in *CYP21A2* and has an incidence of 1:15,000-18,000 births (1, 2). Insufficient cortisol synthesis in patients with 21-OHD leads to an impaired negative feedback drive and increased ACTH secretion, resulting in excess 17OHP and adrenal androgens. Glucocorticoid (GC) therapy for children with 21-OHD aims to compensate for the cortisol deficiency and suppress excess adrenal androgen production (3, 4). In childhood, excess androgens lead to masculinization in females. Increased height velocity and acceleration of bone maturation are observed regardless of sex. The abnormal rate of bone maturation results in loss of growth potential and short stature.

Calibrating the medications for 21-OHD is difficult. The gold standard of monitoring of 21-OHD is auxological data, such as height, body weight, and bone age, which require a long time period (months to a year) to evaluate (5–7). Theoretically, biochemical monitoring using serum 17OHP, a substrate of 21-OH, and pregnanetriol (PT), its urinary metabolite, allows a much shorter monitoring period (hours to days). However, in the clinical setting, monitoring with 17OHP is challenging because the target range for disease control is not well-defined (3, 4, 8). The target for 17OHP is reportedly 4-12 ng/mL before early morning medication, but this information is not based on auxological data (9, 10). Our previous studies demonstrated that morning urine PT can be used as an index of control in prepubertal patients with 21-OHD based on height velocity, body weight, and bone age (11, 12). According to these studies, 2.2-3.3 mg/gCr (95% confidence interval (CI) for the mean) and 0.59-6.0 mg/gCr (10th - 90th percentile) were proposed as the optimal range for the first morning urinary PT value (12).

Measuring urinary steroid metabolites, including PT, is noninvasive and is more useful for periodic, repetitive measurements than measuring serum/blood 17OHP, which is invasive and varies highly depending on when the samples are taken (10). Furthermore, 17OHP measurements vary depending on the method used. Commonly used immunological assays are known to have cross-reactivity for other steroids, such as 17-hydroxypregnenolone; thus, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is recommended internationally (3).

The present study aimed to explore the correlation between the first morning urinary PT value before glucocorticoid administration (0h-PT) and the serum and blood 17OHP values at three time points, namely, before (0h-17OHP) and two and four hours after medication (2h-17OHP, 4h-17OHP), with 17OHP used as a reference for the LC-MS/MS value.

PATIENTS AND METHODS

Patients

In total, 25 patients with 21-OHD aged 3-33 years who were followed up at Tokyo Metropolitan Children's Medical Center and Aichi Children's Health and Medical Center were recruited after obtaining their informed consent (**Supplementary Table 1**). The inclusion criteria were as follows:

1. Well-defined classic and non-classic 21-OHD (3, 4); 21-OHD was diagnosed on the basis of elevated 17OHP and the patient's urinary steroid metabolic profile as determined by gas chromatography-mass spectrometry (GC-MS) analysis.
2. Oral GCs, including hydrocortisone (HDC), dexamethasone (DEX), and prednisolone, administered as treatment for 21-OHD only.
3. No change in GC administration for one month before study commencement and during the study.
4. Availability of early morning, first urine sample

Of the 25 patients enrolled, 24 had the classic phenotype. The one, remaining patient had the non-classic phenotype. As replacement therapy, 17 and 8 patients received HDC in three divided doses and DEX once daily, respectively. In addition to GCs, fludrocortisone was administered to 23 of the 24 patients with the classical phenotype.

Protocol

Morning urine samples for measuring PT and serum/blood samples for measuring 17OHP taken at the three times points described above were collected for three days within a period of one week (**Table 1**). On day 1, morning urine sampling (0h-PT) was done at home, and dried blood spots were collected at the hospital using filter paper (DBS) at 2 and 4 hours after GC administration (2h-17OHP, 4h-17OHP). On days 2-3, morning urine sampling (0h-PT) and DBS before GC administration (0h-

TABLE 1 | Protocol.

Day	Day 1			Day 2	Day 3
	Before morning administration	2 hours after administration	4 hours after administration	Before morning administration	Before morning administration
Time	7:00-8:00 a.m.	9:00-10:00 a.m.	11:00-12:00 a.m.	7:00-8:00 a.m.	7:00-8:00 a.m.
Sampling place	Home	Hospital	Hospital	Home	Home
Sample types					
Urine	(1)	–	–	(2)	(3)
Dried blood spot	–	(4)	(5)	(6)	(7)
Serum	–	(8)	–	–	–

17OHP) were done at home. Thus, the 0h-PT values were taken on all three days. DBS was collected for 2h-17OHP and 4h-17OHP on day 1 and for 0h-17OHP on days 2 and 3. In addition, serum sampling at two hours after GC administration (2h-17OHP) for measuring 17OHP was performed at the hospital to compare the LC-MS/MS and immunoassay results on day 1 (**Table 2**).

The patients or their guardians were instructed in how to perform DBS sampling on day 1. The instructions were as follows: 1) Disinfect fingertip. 2) Prick the disinfected finger with a clean puncture needle. 3) Use filter paper to absorb the blood, completely filling the two, 1 cm-diameter circles. 4) Dry the filter paper at room temperature, place it in a plastic bag, and send it to the hospital.

Samples were not taken for two weeks following any experience of stress requiring additional GC administration.

Sample Analysis

Urinary PT (0h-PT) was measured by GC-MS using an QP2010 mass selective detector (Shimadzu, Kyoto, Japan) with an Ultra ALLOY-5 stainless steel capillary column (Frontier Laboratories, Fukushima, Japan). The sensitivity was 0.01 mg/L. Blood 17OHP in the DBS (0h-, 2h- and 4h-17OHP) was measured by enzyme linked immunosorbent assay (ELISA) (Siemens Healthcare Diagnostics, Tokyo, Japan) after steroid extraction. The 17OHP values were measured twice in both spots, and the average of the two values was calculated. Serum 17OHP (2h-17OHP) was measured using ELISA (IBL International GmbH, Hamburg, Germany) and LC-MS/MS (ASKA Pharmaceutical Medical Corporation, Kanagawa, Japan). Approximately 20 μL of each sample after extraction and derivatization, as described previously (13), was analyzed for LC-MS/MS using a Nexera LC system (Shimadzu, Kyoto, Japan) equipped with an API 4000 triple quadrupole mass spectrometer (Sciex, Framingham, MA, USA) on positive ion mode. The separation of the steroids in the samples was carried out using a Kinetex C18 column (1.7 μm, 2.1 × 150 mm i.d.; Phenomenex, CA, USA) with a flow rate of 0.5 mL/min at 50°C. The selected reaction monitoring (SRM) method was used to detect 17OHP. The lower limit of quantification was 50 pg/mL.

The 2h-17OHP value on day 1 was measured using all the methods, namely DBS 17OHP with ELISA and serum 17OHP with ELISA and LC-MS/MS. Next, the correlation between the DBS 17OHP and serum 17OHP values (using by ELISA and LC-MS/MS) was analyzed. Based on the results, the DBS 17OHP values were converted into serum 17OHP values (by ELISA and LC-MS/MS).

TABLE 2 | PT and 17OHP values.

	Range (mean)
First morning PT (0h-PT), mg/gCr	
Day 1 (n=25)	0.12-56.1 (9.14)
Day 2 (n=24)	0.10-32.7 (6.95)
Day 3 (n=23)	0.12-41.3 (7.56)
Additional day* (n=2)	1.96-7.80
17OHP before oral GC (DBS 0h-PT), ng/mL	
Day 2 (n=24)	0.28-98.1 (28.4)
Day 3 (n=22)	0.63-99.0 (29.4)
Additional day* (n=2)	1.73-34.0
17OHP 2 hours after oral GC (2h-17OHP), ng/mL	
DBS 17OHP (n=23)	0.44-77.1 (12.9)
Serum 17OHP by ELISA (n=24)	0.30-126 (23.6)
Serum 17OHP by LC-MS/MS (n=23)	0.14-71.6 (13.1)
17OHP 4 hours after oral GC (4h-17OHP), ng/mL	
DBS 17OHP (n=23)	0.30-87.2 (12.8)
Other data from venous blood at 2 hours after oral GC	
Cortisol†, μg/dL (n=15)	4.3-43.5 (18.5)
ACTH, pg/mL (n=23)	<2.0-466 (106)
Plasma renin activity, ng/mL/hr (n=24)	0.2-11 (4.1)

*Two patients agreed to provide samples for one more day.
†Only patients receiving hydrocortisone (HDC).

Statistical Analysis

Pearson or Spearman correlation and regression analysis was used to assess for a relationship between each of the following pairs: 1) 0h-PT and DBS 17OHP; 2) 0h-PT and DBS 2h-17OHP; 3) 0h-PT and DBS 4h-17OHP; 4) DBS 17OHP (ELISA) and serum 17OHP (ELISA); and 5) DBS 17OHP (ELISA) and serum 17OHP (LC-MS/MS). The value for each pair was measured on the same day. $P < 0.05$ indicated statistical significance. All statistical analyses were performed using Easy R version 3.4.1 (14). Finally, the optimal range was calculated based on past reports (9, 10, 12) using the regression formula described above.

Ethics Statement

The present study was conducted in accordance with the 1964 Helsinki Declaration and its later amendments (in 2013) or comparable ethical standards. This study was approved by the Institutional Ethics Committee of Tokyo Metropolitan Children's Medical Center (No. H29b-176). Written informed consent for participation was obtained from the patients and/or their legal guardians.

RESULTS

Regression Analysis of the Measurement Methods

There was a significant correlation between the DBS 17OHP by ELISA and serum 17OHP by ELISA ($p < 0.0001$; $r = 0.95$; serum 17OHP by ELISA = 1.70 × DBS 17OHP + 3.35); and the DBS 17OHP and serum 17OHP by LC-MS/MS ($p < 0.0001$; $r = 0.95$; serum 17OHP by LC-MS/MS = 1.31 × DBS 17OHP + 1.17).

Morning PT and Morning 17OHP on Days 2 and 3

The first morning urinary PT value (0h-PT) ranged from 0.10 to 41.3 mg/gCr (n=49; mean: 7.15 mg/gCr). Twenty-two of the 49 samples fell within the 10^{th} - 90^{th} percentile of the previous studies at 0.56-6.0 mg/gCr. The morning DBS 17OHP value (0h-17OHP) ranged from 0.28 to 99.0 ng/mL (n=48; mean: 28.4 ng/mL). There was a positive correlation between the 0h-PT and 0h-17OHP values ($n = 46$; $p < 0.0001$; $r = 0.87$; **Figure 1A**). By log transformation, morning PT values were normally distributed but not 17OHP values were. The regression formula after log transformation was shown in **Figure 1B**. Extremely high PT

values (37.2 and 41.3 mg/gCr) were obtained from one patient, whose 0h-17OHP values were 15.5 and 82.7 ng/mL for the respective time points. This patient's condition was poorly controlled during the study period.

Morning PT and 17OHP After Oral GC Administration (2h- and 4h-17OHP) on Day 1

The first morning urinary PT value (0h-PT) ranged from 0.12 to 56.1 mg/gCr (n=27; mean: 8.82 mg/gCr). The DBS 2h- and 4h-17OHP values ranged from 0.44 to 77.1 ng/mL (n=23; mean: 12.9 ng/mL) and from 0.30 to 87.2 ng/mL (n=23; mean: 12.8 ng/mL), respectively. There was a correlation between the 0h-PT and DBS 2h or 4h 17OHP values (**Figure 1C**; r =0.78, p <0.0001 and **Figure 1D**; r =0.76, p <0.0001).

Optimal Range of 0h-17OHP Value

Based on the data of **Figure 1B**, morning DBS 17OHP, serum 17OHP by ELIZA, and LC-MS/MS value of 2.79-23.2 ng/mL, 7.94-42.8 ng/mL, and 4.71-31.6 ng/mL, respectively, was found to correspond to the 10^{th}-90^{th} percentile of the first morning urine PT value (0.56-6.0 mg/gCr) demonstrated in our previous

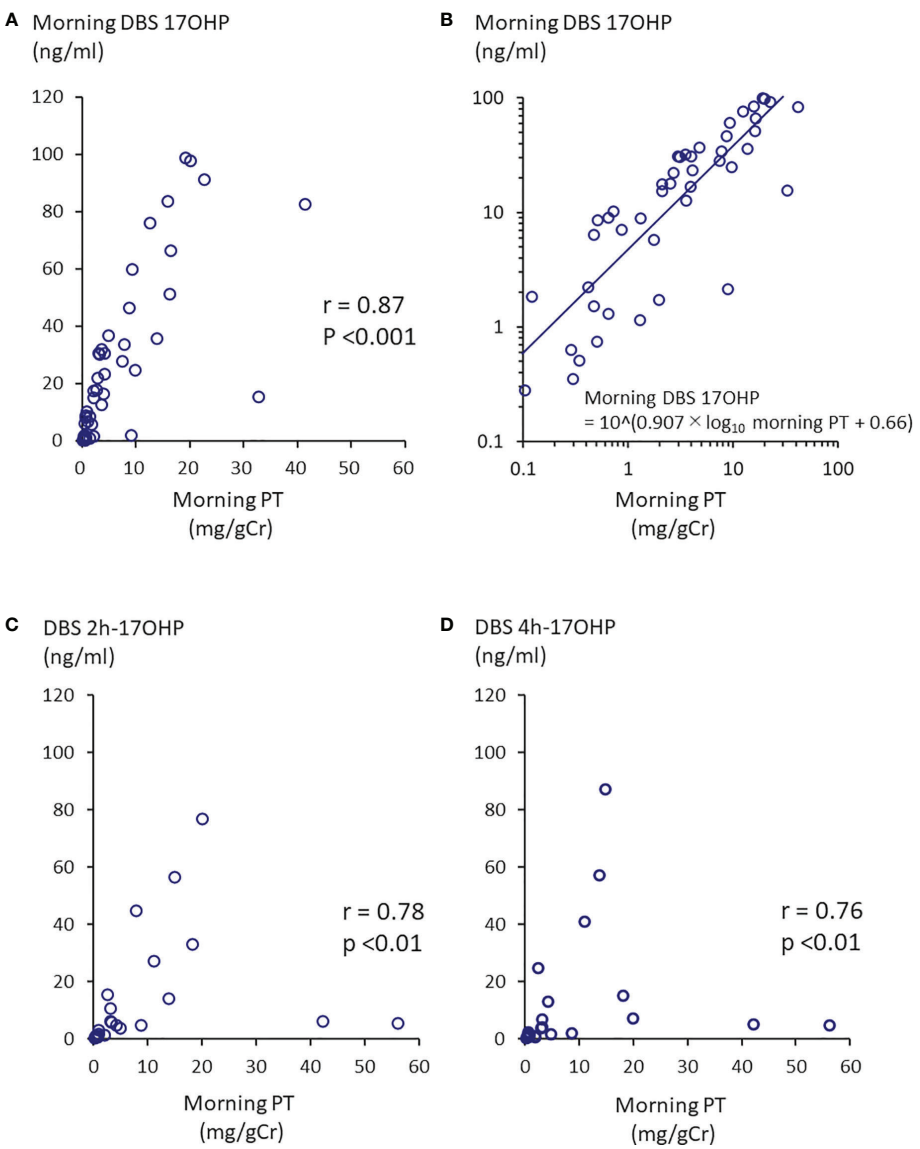

FIGURE 1 | Correlation between morning PT and 17OHP. **(A)** Morning PT and morning DBS 17OHP showed a significant correlation (n =46, p <0.0001, r =0.87). **(B)** Regression formula between morning PT and morning DBS 17OHP. By log transformation, morning PT values were normally distributed. There were weaker correlations between **(C)** morning PT and 17OHP at 2 hours after morning GC administration (DBS 2h-17OHP) and **(D)** morning PT and 17OHP at 4 hours after morning GC administration (DBS 4h-17OHP).

study (12) to be indicative of well-controlled disease. **Table 3** shows the range for each these 17OHP values corresponding to the 95% confidence interval of the mean first morning urine PT value (2.2-3.3 mg/gCr) in the well-controlled group.

The optimal range equivalent to 4.0-12.0 ng/mL for morning serum 17OHP levels reported in previous studies (9, 10) was unable to be calculated because it was obtained using older, radioimmune assays, which were unavailable for use in the present study.

DISCUSSION

The present study is the first to compare morning urinary PT and blood 17OHP values. The first morning PT and 17OHP could be equivalent for biochemical monitoring because of the significant, positive correlation. On the other hand, it may be difficult to demonstrate the optimal 17OHP range after GC administration because weaker correlation between the 0h-PT and 2h- or 4h-17OHP values was found. **Table 3** shows the converted, optimal 0h-17OHP range, which is more reliable because the present study was designed specifically to assess the correlation between 0h-PT and 0h-17OHP and because the original, optimal morning urinary PT range was based on auxological data (11, 12).

A few studies have reported a correlation between blood 17OHP and urinary steroid metabolites (15, 16). Twenty-four-hour urinary steroid metabolites showing a significant correlation with 17OHP values were the PT/tetrahydrocortisone ratio, three 17OHP metabolites/three cortisol-cortisone metabolites ratio, 5α-pregnane-3α, 17α-diol-20-one (a backdoor pathway metabolite), among others. However, unlike our study, which used first morning urine samples, these previous studies used 24-hour urine samples. In most recently, Pussard et al. developed a novel LC-MS/MS method for urine measurement of 23-urinary steroids to show morning plasma and urinary 17-OHP were closely correlated (17).

Studies of the optimal range for biomarkers using auxological data, the gold standard index for 21-OHD, are limited in number (11, 12, 18–21). Most recently, Kamrath *et al.* suggested target values for urinary steroid metabolite excretions in children with 21-OHD based on their growth rate (21). They reported that the target range for androgen metabolite z-scores and hydrocortisone metabolite tetrahydrocortisol was 0.164 - 0.512 and <1480 μg/m^2 body surface area/day, respectively. As described above, we previously demonstrated the optimal range for first morning urinary PT levels based on prepubertal auxological data (11, 12). Early morning urine collection is easier and more suitable for repetitive measurements than 24-hour urine collection. We have already shown a significant correlation in terms of PT between 24-hour urine and first morning urine samples (11). As far as cost is concerned, measuring PT alone than steroid metabolites in urine is more cost-effective.

The optimal range of 0h-17OHP in **Table 3** seems higher than the previously reported target range of 4-12 ng/mL (9, 10). The difference could be explained by differences in measurement assays and by the fact that the conventional optimal range were not based on auxological data.

In the clinical setting, measuring the first morning urinary PT value is more feasible and useful than measuring the morning blood 17OHP level for the obvious reason that urine collection is non-invasive and easier to perform than DBS. Robinson *et al.* (22) encountered difficulty in taking DBS samples from infants and young children. Further, performing an early morning blood collection before morning medication at a hospital is difficult; blood collection at the hospital outpatient clinic typically begins at 8:30AM whereas morning medication, in particular HDC, is ideally taken immediately after waking, given that 17OHP peaks from 4:00AM due to the circadian rhythm of ACTH (10). In fact, the blood collection time for 17OHP was not fixed at the outpatient department of most institutions (4) while no optimal 17OHP range was determined for the various times at which collection occurred. Finally, the first morning urine PT value can aid in monitoring disease control in a more integrated manner than the blood 17OHP value.

The present study also investigated the 17OHP level after each GC administration and revealed correlation with urinary PT. However, regression analysis was not investigated because following two reasons. First, the subjects included patients receiving two kinds of GC with differing pharmacokinetics, such as time to max. Second, even if only a single kind of GC, for example HDC, were administered, the peak plasma cortisol concentration after administration and the half-life of the GC would vary among individuals (23). In fact, the serum cortisol level at two hours after HDC administration in our subjects varied from 4.3 to 43.5 μg/dL (see **Table 1**).

A potential limitation of our study is that the DBS samples were collected by the patients themselves. As discussed above, DBS sampling at home may be difficult for non-medically-trained individuals, especially young pediatric patients, to perform (22). This difficulty may have affected the accuracy of our DBS 17OHP measurements. Therefore, the 17OHP in the DBS samples was measured twice using different spots, and the

TABLE 3 | Optimal range of first morning PT and 0h-17OHP based on regression analysis.

	95% confidence interval	10th-90th percentile
Morning PT in previous study (mg/gCr)[#]	2.2-3.3	0.56-6.0
Calculated		
DBS 17OHP with ELISA (ng/mL)	9.34-13.5	2.70-23.2
Serum 17OHP with ELISA (ng/mL)	19.2-26.3	7.94-42.8
Serum 17OHP with LC-MS/MS (ng/mL)	13.4-18.9	4.71-31.6

[#]*Ref. (11, 12).*

average value was used. In addition, the heterogeneity of the cohort may be another limitation. The present study contained various backgrounds including age, kind of GCs, and phenotype. If the purpose of study had been the utility of 17OHP and PT in monitoring of 21OHD, this would have been certainly a major problem. However, the aim of the present study was to explore the correlation between 17OHP and PT.

In conclusion, the first morning PT correlated more significantly with DBS 17OHP before morning medication. Measuring the first morning PT value may be more practical and useful to biochemically monitor 21-OHD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Ethics Committee of Tokyo Metropolitan Children's Medical Center. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

TI and YH designed the study. TI, MI, and TH collected the samples and data. TI analysed the data and wrote the first draft of the manuscript. YH reviewed/edited the manuscript and was responsible for all aspects of the research design and manuscript as the guarantor. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.808254/full#supplementary-material

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EDITED BY
Valentina Morelli,
Istituto Auxologico Italiano, Italy

REVIEWED BY
Oksana Lekarev,
NewYork-Presbyterian, United States
Ahmed Khattab,
Rutgers, The State University of New Jersey, United States

*CORRESPONDENCE
Richard J. Auchus
rauchus@med.umich.edu

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Treatment patterns and unmet needs in adults with classic congenital adrenal hyperplasia: A modified Delphi consensus study

Richard J. Auchus[1*], Carine Courtillot[2], Adrian Dobs[3], Diala El-Maouche[4], Henrik Falhammar[5,6], Andre Lacroix[7], Mallory Farrar[8], Conor O'Donoghue[9], Milena Anatchkova[10], Katelyn Cutts[10], Natalie Taylor[10], Chuck Yonan[8], Mark Lamotte[11] and Philippe Touraine[2]

[1]Departments of Pharmacology and Internal Medicine, Division of Metabolism, Endocrinology and Diabetes, University of Michigan Medical School, Ann Arbor, MI, United States, [2]Department of Endocrinology and Reproductive Medicine, Center for Rare Endocrine and Gynecological Disorders, Groupe Hospitalier Pitié-Salpêtrière, Sorbonne Université, Paris, France, [3]Department of Medicine, Division of Endocrinology and Metabolism, The Johns Hopkins University School of Medicine, Baltimore, MD, United States, [4]Division of Endocrinology and Metabolism, George Washington University, Washington, DC, United States, [5]Department of Endocrinology, Karolinska University Hospital, Stockholm, Sweden, [6]Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden, [7]Division of Endocrinology, Department of Medicine and Research Center, Centre hospitalier de l'Université de Montréal (CHUM), Montréal, QC, Canada, [8]Neurocrine Biosciences, Inc., Health Economics and Outcomes Research, San Diego, CA, United States, [9]Neurocrine Biosciences, Inc., New Product Commercialization, San Diego, CA, United States, [10]Evidera, Patient-Centered Research, Bethesda, MD, United States, [11]IQVIA, Global Health Economics and Outcomes Research (HEOR), Zaventem, Belgium

Background: Classic congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is a rare autosomal recessive condition characterized by cortisol deficiency and excess androgen production. The current standard of care is glucocorticoid (GC) therapy, and sometimes mineralocorticoids, to replace endogenous cortisol deficiency; however, supraphysiologic GC doses are usually needed to reduce excess androgen production. Monitoring/titrating GC treatment remains a major challenge, and there is no agreement on assessment of treatment adequacy. This study surveyed expert opinions on current treatment practices and unmet needs in adults with classic CAH.

Methods: A modified two-round Delphi process with adult endocrinologists was conducted *via* online questionnaire. Survey questions were organized into three categories: practice characteristics/CAH experience, GC management, and unmet needs/complications. Anonymized aggregate data from Round 1 were provided as feedback for Round 2. Responses from both rounds were analyzed using descriptive statistics. Consensus was defined *a priori* as: full consensus (100%, n=9/9); near consensus (78% to <100%, n=7/9 or 8/9); no consensus (<78%, n<7/9).

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Results: The same nine panelists participated in both survey rounds; five (56%) were based in North America and four (44%) in Europe. Most panelists (78%) used hydrocortisone in the majority of patients, but two (22%) preferred prednisone/prednisolone. Panelists agreed (89%) that adequate control is best evaluated using a balance of clinical presentation and androgen/precursor laboratory values; no consensus was reached on optimal timing of collecting samples for androgen testing or laboratory values indicating good control. Despite lack of consensus on many aspects of CAH management, panelists agreed on the importance of many disease- and GC-related complications, and that there is a large unmet need for new treatments. With currently available treatments, panelists reported that 46% of classic CAH patients did not have optimized androgen levels, regardless of GC dose.

Conclusions: The limited areas of consensus obtained in this study reflect the variability in treatment practices for adults with classic CAH, even among clinicians with expertise in treating this population. However, all panelists agreed on the need for new treatments for classic CAH and the importance of many disease- and GC-related complications, which are difficult to manage with currently available treatments.

KEYWORDS

classic CAH, classic congenital adrenal hyperplasia, glucocorticoid management, treatment complication, unmet needs

1 Introduction

Congenital adrenal hyperplasia (CAH) refers to a group of rare autosomal recessive disorders that result in disordered adrenal steroidogenesis, including impaired cortisol and aldosterone synthesis (1–5). Approximately 95-99% of CAH cases are the result of mutations in the *CYP21A2* gene encoding for the adrenal steroidogenic enzyme, 21-hydroxylase, which is required for the production of cortisol and aldosterone in the adrenal cortex (1, 6). Severe blockage of cortisol synthesis reduces normal negative feedback inhibition on the hypothalamus and the pituitary gland, leading to increased secretion of adrenocorticotropic hormone (ACTH) and excess production of adrenal androgens and their precursors (1–5).

The "classic" form of CAH is associated with severe 21-hydroxylase deficiency and occurs in approximately 1:14,000 to 1:18,000 births (1). High intrauterine androgen concentrations are clinically evident in newborn females, whose external genitalia are virilized to varying degrees, while males with classic CAH usually have typical male genitalia (7, 8).

Androgen excess during childhood and adolescence raises the risk for precocious puberty or pseudopuberty, as well as accelerated somatic growth with advanced bone age, which results in below-predicted adult height (2, 7). During adulthood, females can develop hirsutism, acne, and irregular menses; males are at risk of developing testicular adrenal rest tumors (TARTs). Both males and females are at risk for long-term problems with bone health, cardiovascular and metabolic comorbidities, fertility, and psychosocial health and well-being, due to the disease and/or its conventional treatments (2, 7, 9–11). Patients of all ages are at risk of adrenal gland nodular enlargement and adrenal crisis, which is potentially life-threatening if untreated (2, 7, 9, 12).

Management of classic CAH presents unique challenges distinct from other forms of adrenal insufficiency (1–5, 8). The current standard of care is glucocorticoid (GC) therapy, with or without mineralocorticoid treatment, to replace the endogenous cortisol deficiency and reduce excess androgen production. However, unlike acquired primary adrenal insufficiency, supraphysiologic GC doses are usually needed to simultaneously reduce the elevated ACTH secretion and excess androgen production (3, 4). Chronic exposure to supraphysiologic GC doses can lead to a number of potentially serious health complications, including growth suppression and decreased bone density with increased fracture risk, as well as metabolic complications such as obesity, insulin resistance, hypertension, and diabetes, which can increase cardiovascular risk (9, 13–20). Thus, the need for adequate androgen control should be balanced with the risks of prolonged supraphysiologic GC exposure, as both under- and overtreatment with GCs can cause complications. In

addition to these challenges, there is a lack of consensus among practitioners on optimal GC regimens in adult patients (4, 8, 21). Although hydrocortisone in divided doses is a common treatment option for adults, once- or twice-daily preparations of synthetic long-acting GCs such as prednisone, prednisolone, and dexamethasone are also used; modified-release hydrocortisone has recently gained approval from the European Medicines Agency (1, 3, 4, 22, 23). In addition, monitoring and titrating GC treatments remains a major clinical challenge, and there is no agreement on the assessment of treatment adequacy (1, 4, 8).

The purpose of this study was to survey expert opinions on current GC treatment practices and unmet needs in adult patients with classic CAH. The Delphi method, a systematic group communication process, was developed by the RAND Corporation in the 1950s to forecast the impact of technology on warfare and is well suited to assist in decision-making when evidence is incomplete, unclear, or unavailable (24–28). The iterative and anonymous nature of the traditional Delphi questioning process, with analysis and feedback provided after survey rounds, represents a structured process to collect knowledge from a panel of experts, with the capability of achieving consensus when uncertainty may exist due to lack of definitive evidence (24–28). Panelists' anonymity during the survey process can reduce the effects of dominant individuals or pressure to conform, which often is a concern when using group-based processes to collect and synthesize information. Controlled feedback in the form of a well-organized summary of the prior iteration allows each participant an opportunity to generate additional insights, clarify the information developed in previous iterations, and minimize the effects of noise. The Delphi method has been used successfully for various medical applications, from the assessment of knowledge gaps to the development of treatment guidelines (29–33). This study utilizes a modified Delphi method to survey expert opinions on the management of adult patients with classic CAH, as well as unmet needs in this patient population.

2 Methods

2.1 Expert panel

Survey panelists from the US, Canada, and Europe were recruited by Evidera upon recommendation by Neurocrine Biosciences, Inc., the study sponsor. Participation was voluntary, but respondents were compensated for their time in completing the survey. Recruitment efforts for the survey panel focused on adult endocrinologists who frequently managed patients with classic CAH (i.e., currently seeing at least 10-20 adults with classic CAH every quarter). Additional criteria for recruitment included involvement in publications on CAH, participation in CAH clinical trials, or participation in the development of CAH guidelines. Of the 21 panelists invited to participate in the survey panel, nine agreed to participate (Figure 1). All nine panelists completed both rounds of the survey, and seven participated in the development of this paper (two panelists did not participate in manuscript preparation and have elected to remain anonymous).

2.2 Modified Delphi procedure

Two internet-based survey rounds were conducted from September 2020 to April 2021 (Figure 1). The questions were organized into three categories: 1) panel members' practice characteristics and CAH experience (Round 1 only), 2) GC

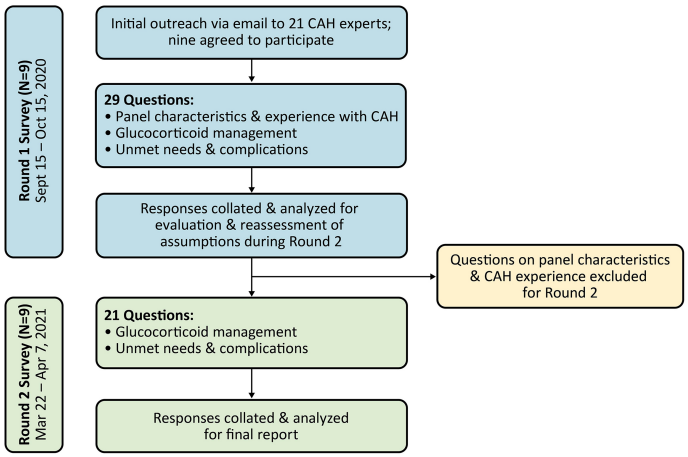

FIGURE 1
Flowchart of modified Delphi panel process. CAH, congenital adrenal hyperplasia.

management (including GC treatment patterns, hydrocortisone equivalency ratios, target androgen laboratory values and indicators of good control, and treatment optimization), and 3) unmet needs and complications.

The Delphi process traditionally begins with an open-ended (free-response) questionnaire in Round 1, but a common and acceptable modification is to use more structured questions if basic information on the target issue is available (27). In this study, most Round 1 questions were free-response, but some closed-ended questions (e.g., 5-point Likert scale [select rating of 1 "not important" to 5 "very important"] or multiple choice [select 1 or select any]) were used based on input from several prior virtual Advisory Boards with CAH experts on current treatment practices and unmet needs, as well as analysis by Neurocrine to identify potential gaps in research, published literature, and treatment guidelines for classic CAH (survey questionnaires are shown in Supplementary Materials). Responses were collected and collated by Evidera and analyzed by Evidera, Neurocrine, and IQVIA.

For Round 2, panelists were provided anonymous aggregate data from Round 1 (and their individual responses from Round 1 where applicable) as feedback. Questions for Round 2 were refined as follows: 1) If there was general agreement in Round 1 responses, panelists were asked if they agreed or disagreed with the conclusion to establish consensus; 2) If there was variability in Round 1 responses, the question was re-circulated (and in some cases, modified for clarity) for a second round of input from panelists.

2.3 Analysis

Quantitative responses from both survey rounds were analyzed using descriptive statistics, including mean, standard deviation, range, and frequency. Qualitative data from the free-response questions were analyzed using key codes developed with clinical input from Neurocrine. For Round 2 survey results, consensus was defined *a priori* as follows: full consensus (100%, n=9/9); near consensus (78% to <100%, n=8/9 or 7/9); no consensus (<78%, n<7/9).

3 Results

3.1 Panel characteristics and experience with CAH

Of the nine total panel members, five (56%) were based in North America, and four (44%) were based in Europe (Supplementary Table 1). All nine panelists were adult endocrinologists, with the majority working in an academic or university hospital setting. Most of the panelists had ≥15 years of experience treating adults with CAH and were currently treating ≥10 adult patients with classic CAH. In an average month, the panel members reported spending a median of 4% of their time treating adults with classic CAH.

3.2 Glucocorticoid management

Survey results on GC treatment patterns, hydrocortisone equivalency ratios, target androgen laboratory values, and treatment optimization are presented in the following sections. Key findings are summarized in Box 1.

3.2.1 Glucocorticoid treatment patterns

In Round 1, all nine panelists reported prescribing hydrocortisone in an average of 62% of patients, with individual responses ranging from 10% to 96% of patients (Table 1). Most panelists (78%) prescribed hydrocortisone in more than half of their patients; the other two (22%) panelists used prednisone or prednisolone in 80% of patients. Six panelists reported prescribing dexamethasone in an average of 8% of their patients. When asked to select up to three different GC combinations they typically used in their practice, a total of five panelists reported using at least one GC combination: three used only hydrocortisone and dexamethasone; one used hydrocortisone with dexamethasone, prednisone, or prednisolone; and one used hydrocortisone with prednisolone or methylprednisolone.

When asked in Round 2 to provide the average daily GC dose and/or average dose range used globally to treat patients with classic CAH, the mean hydrocortisone dose was 27.2 mg/day, and the mean hydrocortisone dose range was 14.2 to 40.8 mg/day (Table 1). For dexamethasone, the panelists reported an average daily dose of 0.6 mg/day and a range of 0.4 to 1.5 mg/day. When asked about the typical timing of GC doses, nine (100%) panelists prescribed the first dose of hydrocortisone in the morning; 89% also prescribed it in the afternoon, 78% in the evening, and 22% at bedtime (Table 1). The timing and frequency of dosing for prednisone, prednisolone, or methylprednisolone varied among panelists, but the first dose was usually in the morning. Dexamethasone was usually dosed at bedtime. Two (33%) panelists reported using reverse circadian dosing.

In Round 2, near consensus was reached that hydrocortisone is the most widely used GC globally (89%), and that dexamethasone should be prescribed at bedtime if given once daily (78%) (Box 1).

3.2.2 Physiologic hydrocortisone dose

When asked in Round 1 to provide what they considered to be the upper end of a physiologic GC dose with hydrocortisone, panelists reported a mean dose of 27.2 mg/day, with individual responses ranging from 15 to 40 mg/day – which was consistent

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Box 1

Key Findings: Glucocorticoid Management in Adults with Classic CAH. ✓ indicates full consensus (100%, 9/9 respondents); ✓ indicates near consensus (78% to <100%, 8/9 or 7/9 respondents); ✗ indicates no consensus (<78%, <7/9 respondents). 17-OHP, 17-hydroxyprogesterone; A4, androstenedione; CAH, congenital adrenal hyperplasia; GC, glucocorticoid; ULN, upper limit of normal.

GC Treatment Patterns

- Hydrocortisone is the most widely used GC globally ✓
- The average daily dose of hydrocortisone used globally was 27.2 mg/day, with a range of 14.2 to 40.8 mg/day
- Hydrocortisone, prednisone, prednisolone, and methylprednisolone were typically given in the morning; the frequency and timing of subsequent doses varied
- Dexamethasone should be given at bedtime if given once daily ✓
- No consensus was reached on the upper end for a physiologic hydrocortisone dose, but six (67%) panelists agreed that 25-30 mg/day was appropriate ✗

Hydrocortisone Equivalency Ratios

- The appropriate equivalency ratio is 4.4 mg/day for prednisone and prednisolone and 4.8 mg/day for methylprednisolone ✓
- No consensus was reached on the appropriate equivalency ratio for dexamethasone, but six (67%) panelists agreed that 43.1 mg/day was appropriate ✗

Androgen Laboratory Values & Indicators of Control

- The balance of clinical presentation and laboratory values is the leading indicator of control ✓
- No consensus was reached for 17-OHP or A4 laboratory values indicating good control ✗
- Testosterone laboratory values within ULN indicate good control in females ✓
- No consensus was reached on optimal timing of androgen laboratory testing relative to morning GC dose, but morning laboratory testing prior to GC administration was preferred by six (67%) clinicians ✗

GC Treatment Optimization

- Almost half of adult classic CAH patients (46%) did not have optimized androgen levels, regardless of GC dose
- Just 25% of adult classic CAH patients received physiologic GC doses and had optimized androgen levels

TABLE 1 Glucocorticoid treatment patterns in adults with classic CAH.

	Hydrocortisone	Dexamethasone	Prednisone	Prednisolone	Methylprednisolone
% of patients taking GC					
Number of respondents who used GC in >0% of patients	9	6	5	3	1
Mean (SD)	62 (33)	8 (7)	26 (32)	32 (42)	25
Median (range)	65 (10-96)	3 (2-20)	10 (5-80)	10 (5-80)	25
Daily GC dose, mg/d[a]					
Number of respondents	8	7	6	5	5
Mean (SD)	27.2 (3.6)	0.6 (0.3)	4.9 (1.5)	4.4 (0.9)	5.4 (1.3)
Median (range)	25.0 (25.0-35.0)	0.5 (0.4-1.0)	5.0 (3.0-7.5)	5.0 (3.0-5.0)	6.0 (3.0-6.0)
Daily GC dose range, mg/d[a]					
Number of respondents	6	5	5	4	4
Lower range, mean (SD)	14.2 (3.8)	0.4 (0.1)	3.8 (1.8)	3.3 (1.7)	3.8 (2.1)
Upper range, mean (SD)	40.8 (10.2)	1.5 (0.6)	7.5 (1.8)	7.1 (2.2)	6.5 (1.9)
Timing of GC dosing, n (%) respondents					
Number of respondents	9	6	5	3	1
Morning	9 (100)	1 (17)	4 (80)	3 (100)	1 (100)
Afternoon	8 (89)	0 (0)	1 (20)	1 (33)	0 (0)
Evening	7 (78)	1 (17)	0 (0)	1 (33)	0 (0)
Bedtime	2 (22)	5 (83)	3 (60)	1 (33)	1 (100)
Uses reverse circadian dosing	1 (11)	2 (33)	1 (20)	0 (0)	0 (0)

[a]Some panelists provided both a number and a range for the average daily GC dose.
GC, glucocorticoid; SD, standard deviation.

with hydrocortisone dosing that the panelists reported using in clinical practice (see previous section). All but one panelist felt that the dose they indicated was reliable (some risk of being wrong, seven [78%]) or certain (low risk of being wrong, one [11%]). In Round 2, consensus agreement was not reached, but six (67%) panelists agreed that 25-30 mg/day was the upper end for a physiologic hydrocortisone dose (Box 1). The three panelists who disagreed with this dose range provided doses of 15, 20, and 40 mg/day.

3.2.3 Hydrocortisone equivalency ratios

In Round 1, panelists were asked to provide an appropriate hydrocortisone equivalency ratio to use when summarizing a data set (i.e., not in clinical practice, but when reading a peer-reviewed article). The mean (SD, range) hydrocortisone equivalency ratios reported by panelists were as follows: dexamethasone, 43.1 (25.4, 25.0-80.0); prednisone, 4.4 (0.5, 4.0-5.0); prednisolone, 4.4 (0.5, 4.0-5.0); methylprednisolone, 4.8 (0.4, 4.0-5.0). In Round 2, all but one panelist (89%) agreed that the Round 1 equivalency ratios for prednisone, prednisolone, and methylprednisolone were appropriate to use when summarizing a dataset (Box 1). Consensus was not reached for dexamethasone, but six (67%) panelists agreed with the appropriateness of the Round 1 equivalency ratio (Box 1). The three panelists who disagreed reported that the equivalency ratio for dexamethasone should be 25.0, 26.7, or 80.0 mg/day.

3.2.4 Androgen laboratory values and indicators of control

Figure 2 summarizes responses to questions in Round 1 and Round 2 on what laboratory values for 17-hydroxyprogesterone (17-OHP), androstenedione (A4), and testosterone are considered appropriate indicators of good control in three patient subgroups: males with TARTs, males without TARTs, and females. For 17-OHP, consensus was not reached in any subgroup on an appropriate laboratory value to indicate good control. In males with TARTs, a total of six (67%) panelists agreed in Round 2 that within 2X the upper limit of normal (ULN) was appropriate, but this did not meet the threshold for near consensus (Figure 2A and Box 1). Of the three panelists who responded in Round 1 that they did not treat to a specific 17-OHP range, two panelists modified their response in Round 2 to 2X ULN. For males without TARTs, six (67%) panelists agreed in Round 2 that they did not have a 17-OHP laboratory range that they treated to. For females, most panelists reported that within 2X ULN (33%) or 3X ULN (44%) was appropriate for 17-OHP.

For A4, consensus was not reached in any subgroup on an appropriate laboratory value to indicate good control, but six (67%) panelists agreed in Round 2 that within ULN was appropriate in males with TARTs (Figure 2B and Box 1). There was no agreement on an appropriate A4 laboratory value in males without TARTs. For females, most panelists reported that within ULN (33%) or 2X ULN (44%) was appropriate for A4. For testosterone, near consensus (89%) was reached in Round 2 that within ULN was an appropriate testosterone laboratory value to indicate good control in females (Figure 2C and Box 1).

In Round 2, near consensus (89%) was reached that the leading indicator of control is the balance of clinical presentation and laboratory values (Box 1). Based on comments received in Round 1 that the expectations for appropriate target laboratory values would vary depending on the timing of the laboratory testing relative to the administration of the GC dose, a question was added to Round 2 to indicate the optimal timing of laboratory testing. Consensus was not reached by the panelists, but morning laboratory testing prior to GC administration was preferred by six (67%) panelists (Box 1).

3.2.5 Glucocorticoid treatment optimization

Based on their own definitions for what they considered to be "optimized" androgen levels and "physiologic" GCs, panelists reported that almost half of their adult patients with classic CAH (46%) did not have optimized androgen levels, regardless of GC doses (Figure 3A). A total of 29% of patients had androgens optimized but were receiving supraphysiologic GC doses. Just 25% of patients received physiologic GC doses and had optimized androgen levels.

Panelists reported the most frequent GC treatment regimen changes in patients whose androgens were not optimized, with seven (78%) panelists reporting making changes at least twice a year in these patients (Figure 3B). There was a lack of agreement on the frequency of changes for patients whose androgens are optimized with supraphysiologic GC doses, with four (44%) panelists reporting changes at least twice a year and five (56%) panelists reporting changes every one to five years. Panelists reported the least frequent changes in patients with androgens optimized and physiologic GC doses, with eight (89%) panelists reporting changes every two to five or more years. When asked to provide reasons for changing a patient's GC regimen (free-response question), six (67%) panelists reported factors related to good androgen control/hyperandrogenism, with five (56%) panelists specifically mentioning fertility. A total of four (44%) panelists reported factors related to managing supraphysiologic GC doses.

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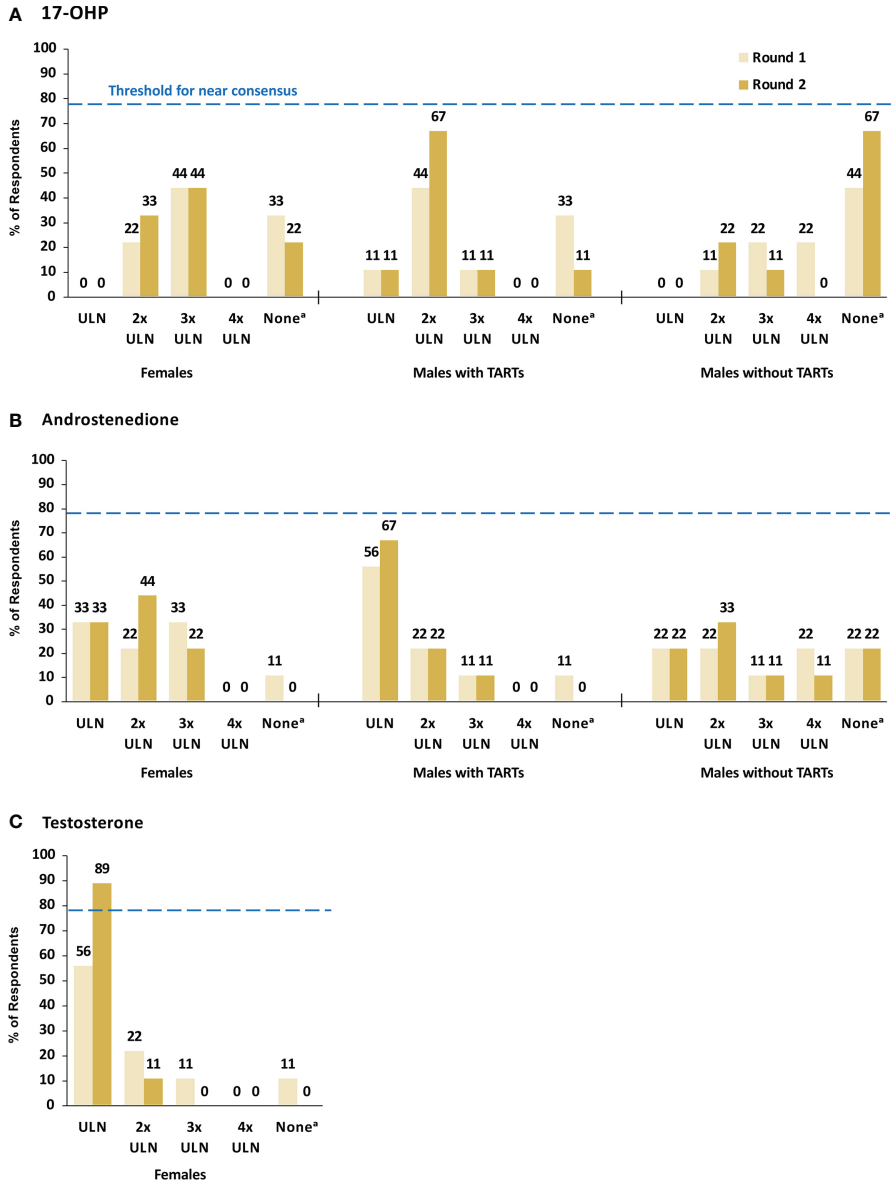

FIGURE 2
17-OHP **(A)**, Androstenedione **(B)**, and Testosterone **(C)** Laboratory Values Indicating Good Control. Dashed line indicates minimum threshold for near consensus (78%, or 7/9 respondents). [a] The survey response option for this category was "I do not have a lab range I treat to". 17-OHP, 17-hydroxyprogesterone; TARTs, testicular adrenal rest tumors; ULN, upper limit of normal.

3.3 Exploring unmet needs and complications in adults with classic CAH

Survey results on unmet needs and the relative importance of disease- and GC-related complications are presented in the following sections, and key findings are summarized in Box 2.

3.3.1 Unmet needs

In Round 1, the panelists were asked free-response questions to provide the most important short- and long-term unmet needs, as well as the most important unmet needs for classic CAH patients categorized by sex, age, and GC treatment optimization. Based on responses in Round 1, the unmet needs were categorized into three groups for Round 2 questioning:

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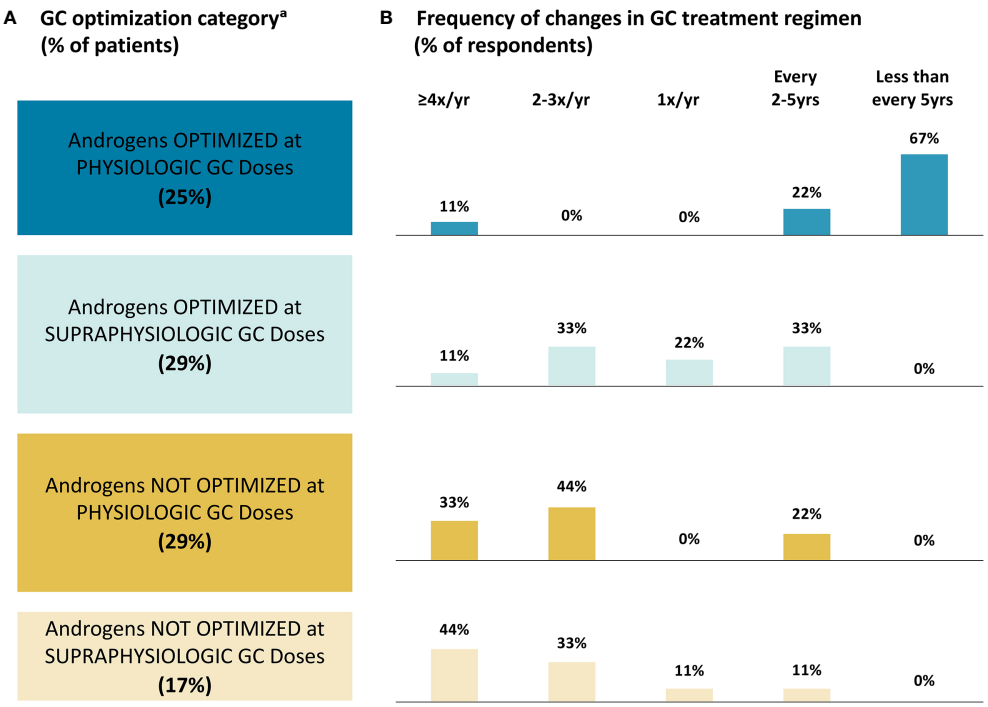

FIGURE 3
Glucocorticoid Treatment Optimization. **(A)** Patient Category; **(B)** Frequency of Changes. [a] The survey questionnaires did not define "optimized" androgen levels or "physiologic" GC doses; therefore, panelists reported percentages of patients in each category based on their own definitions of optimized androgens and physiologic GCs. GC, glucocorticoid; yr, year.

- o Good androgen control/avoidance of hyperandrogenism (including fertility, androgen replacement therapy, and hirsutism)
- o Managing/reducing supraphysiologic GC doses (including consequences related to cardiovascular, bone, and metabolic health)
- o Treatment-related needs (including simplified dosing and affordability)

In Round 2, the panel ranked the importance of the three unmet needs categories from 1 "not at all important" to 5 "very important". Near consensus (78%) was reached that good androgen control was very important for short- and long-term treatment, as well as for females, younger patients (age 18 to ≤55 years), and patients whose androgens were not optimized (Supplementary Table 2 and Box 2). Consensus or near consensus was reached that managing supraphysiologic GC doses was very important for short- and long-term, females, males, younger patients, older patients (age >55 years), and patients with supraphysiologic GC doses. Consensus (100%) was also reached that there is an important unmet need for new treatments across all adult CAH subgroups (Box 2).

3.3.2 Disease- and GC-related complications

In Round 1, panelists were asked to rank the importance of several disease- or GC-related complications from 1 "not at all important" to 5 "very important". Based on the mean response from Round 1, panelists were asked in Round 2 if they agreed (yes/no) that the complication was "important/very important" (mean ≥4, Round 1) or "moderately important" (mean <4, Round 1). In Round 2, consensus (100%) or near consensus (78%) was reached that all of the complications related to cardiovascular and metabolic health, bone health, female health, and male health were important or very important, except dyslipidemia, which the panel agreed was moderately important (78%) (Table 2 and Box 2). No consensus was reached for most of the complications related to psychosocial health and well-being, except for depression and decreased sexual satisfaction, which panelists agreed were important or very important, and anxiety, which panelists agreed was moderately

Box 2
Key Findings: Unmet Needs and Complications in Adults with Classic CAH. ✓ indicates full consensus (100%, 9/9 respondents); ✓ indicates near consensus (78% to <100%, 8/9 or 7/9 respondents); ✗ indicates no consensus (<78%, <7/9 respondents). CAH, congenital adrenal hyperplasia; GC, glucocorticoid; TARTs, testicular adrenal rest tumors.

Unmet Needs

- There is an important unmet need for new treatments across all adult CAH subgroups ✓
- Good androgen control is very important for short- and long-term treatment, as well as for females, younger patients, and patients whose androgens were not optimized ✓
- Managing supraphysiologic GC doses was very important for short- and long-term treatment, as well as females, males, younger patients, older patients, and patients with supraphysiologic doses ✓ ✓

Disease- or GC-Related Complications

- The following complications are important or very important ✓ ✓
  - For cardiovascular and metabolic health: pre-diabetes, type 2 diabetes, hypertension, overweight/obesity, cardiovascular disease, and change in body composition
  - For bone health: osteopenia/osteoporosis and fragility fracture
  - For female health: irregular menses, hirsutism/acne, virilization, and infertility
  - For male health: TARTs and infertility
  - For psychosocial health: depression and decreased sexual satisfaction
- No consensus was reached on the importance of the following complications: reduced vitality, impaired cognition, insomnia/poor sleep quality, fatigue, increased infections, skin bruising, myopathy, or ocular complications ✗

important. No consensus was reached on "other" complications, such as increased infections, skin bruising, myopathy, and ocular complications.

4 Discussion

Despite the advances in the past several decades in the understanding of the genetics, pathophysiology, and treatment of classic CAH, many challenges remain in managing the condition (1, 4, 5, 8, 21). The lifelong supraphysiologic GC doses that are often needed to attenuate the excess adrenal-derived androgen production are associated with a high burden of comorbidities and reduced quality of life in adult patients with classic CAH (9, 17–20). Thus, clinicians must balance the need for adequate androgen control with the risks of health problems from prolonged supraphysiologic GC exposure. Adding to this challenge is the limited evidence from randomized trial data comparing long-term outcomes of different GCs and GC regimens in adults, leading to a lack of consensus on how to optimize GC therapy (1, 4, 34).

This study aimed to provide a view of expert opinions on current practices and unmet needs in the management of adult patients with classic CAH. The survey results showed some areas of agreement in GC management, including near consensus that hydrocortisone was the most widely used GC. Most panelists reported using hydrocortisone in the majority of their patients, but two panelists preferred the long-acting GCs, prednisone or prednisolone. These findings align with recent published reviews, which describe hydrocortisone split in two to three doses as the most common treatment option for adult patients due to its lower risk of adverse effects on metabolic, cardiovascular, and bone health (1, 3, 7). Long-acting synthetic GCs were often used for regulation of menstrual cycles, fertility induction, TART treatment, or patients who have difficulty adhering to a three-times daily regimen, but their longer duration and higher potency may increase the risk of metabolic comorbidities (1, 7).

A potential limitation of this study is the small number of panel participants, whose opinions might not reflect those of other endocrinologists who treat adults with CAH. In addition, the small number of panelists meant that consensus or near consensus would not be reached if only one or three panelists gave dissenting opinions, respectively, which could skew results. To ensure a representative sample of expert opinions, panelists from different institutional and clinical settings throughout the US and Europe were recruited who met academic and clinical criteria. However, classic CAH is a rare disorder; thus, there were few clinicians who met the study inclusion criteria of seeing at least 10-20 adults with classic CAH every quarter. Larger studies surveying a broader geographical range of expert opinions (beyond the US and Europe) may expand our findings and help to provide a more comprehensive, global view of adult CAH care. Financial support for the study was provided by Neurocrine, who is investigating crinecerfont, a corticotropin-releasing factor type 1 receptor (CRF1R) antagonist, for potential use in CAH. To mitigate potential bias introduced by the commercial sponsor, the survey questions were specifically designed to address a broad and comprehensive clinical approach to the management of classic CAH.

Typical daily GC doses reported by the panelists in this study generally aligned with the dose ranges suggested in the 2018

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TABLE 2 Importance of disease- or GC-related complications in adults with classic CAH.

Complication, n (%) of respondents	Round 1					Round 2	
	Not at all important	Somewhat important	Moderately important	Important	Very Important	Do you agree that the complication is important/very important or moderately important? Yes	No
Cardiovascular and metabolic health						**Important or Very Important?**	
Pre-diabetes	0 (0)	1 (11)	1 (11)	4 (44)	3 (33)	9 (100)	0 (0)
Type 2 diabetes	0 (0)	2 (22)	1 (11)	1 (11)	5 (56)	9 (100)	0 (0)
Hypertension	0 (0)	2 (22)	0 (0)	3 (33)	4 (44)	9 (100)	0 (0)
Overweight/obesity	0 (0)	0 (0)	0 (0)	4 (44)	5 (56)	9 (100)	0 (0)
Cardiovascular disease	0 (0)	1 (11)	0 (0)	2 (22)	6 (67)	9 (100)	0 (0)
Other: change in body composition	0 (0)	0 (0)	0 (0)	1 (100)[a]	0 (0)	7 (78)	2 (22)
						Moderately Important?	
Dyslipidemia	0 (0)	1 (11)	2 (22)	3 (33)	3 (33)	7 (78)	2 (22)
Bone health						**Important or Very Important?**	
Osteopenia/osteoporosis	0 (0)	0 (0)	1 (11)	4 (44)	4 (44)	9 (100)	0 (0)
Fragility fracture	0 (0)	0 (0)	1 (11)	2 (22)	6 (67)	9 (100)	0 (0)
Female health						**Important or Very Important?**	
Irregular menses/ anovulation/amenorrhea	0 (0)	0 (0)	1 (11)	6 (67)	2 (22)	9 (100)	0 (0)
Hirsutism/acne	0 (0)	0 (0)	1 (11)	6 (67)	2 (22)	9 (100)	0 (0)
Virilization	1 (11)	0 (0)	0 (0)	2 (22)	6 (67)	9 (100)	0 (0)
Infertility	0 (0)	0 (0)	0 (0)	2 (22)	7 (78)	Not asked (consensus reached in R1)	
Male health						**Important or Very Important?**	
TARTs	0 (0)	0 (0)	0 (0)	3 (33)	6 (67)	9 (100)	0 (0)
Infertility	0 (0)	0 (0)	0 (0)	2 (22)	7 (78)	Not asked (consensus reached in R1)	
Psychosocial health and well-being						**Important or Very Important?**	
Depression	0 (0)	1 (11)	0 (0)	5 (56)	3 (33)	9 (100)	0 (0)
Decreased sexual satisfaction	0 (0)	1 (11)	0 (0)	6 (67)	2 (22)	9 (100)	0 (0)
						Moderately Important?	
Anxiety	0 (0)	1 (11)	1 (11)	5 (56)	2 (22)	7 (78)	2 (22)
Reduced vitality	0 (0)	0 (0)	4 (44)	3 (33)	2 (22)	5 (56)	4 (44)
Impaired cognition	0 (0)	3 (33)	1 (11)	2 (22)	3 (33)	3 (33)	6 (67)
Insomnia/poor sleep quality	0 (0)	2 (22)	2 (22)	3 (33)	2 (22)	5 (56)	4 (44)
Fatigue	0 (0)	1 (11)	3 (33)	3 (33)	2 (22)	5 (56)	4 (44)
Other complications						**Moderately Important?**	
Increased infections	0 (0)	2 (22)	2 (22)	3 (33)	2 (22)	6 (67)	3 (33)
Skin bruising/thinning/ fragility	0 (0)	1 (11)	2 (22)	4 (44)	2 (22)	6 (67)	3 (33)
Myopathy	0 (0)	1 (11)	2 (22)	3 (33)	3 (33)	6 (67)	3 (33)
Ocular (glaucoma, cataracts)	0 (0)	1 (11)	4 (44)	2 (22)	2 (22)	6 (67)	3 (33)

Green indicates full consensus (100%, 9/9 respondents), blue indicates near consensus (78% to <100%, 8/9 or 7/9 respondents), and red indicates no consensus (<78%, <7/9 respondents).
[a]One respondent listed "change in body composition" under "Other" in Round 1.
R1, round 1; TARTs, testicular adrenal rest tumors.

Endocrine Society guidelines and in recent literature, although the panelists reported higher upper ranges for hydrocortisone (40.8 vs 25 mg) and dexamethasone (1.5 vs 0.5 mg) (1, 3, 7). These findings are in alignment with cross-sectional studies of adults with classic CAH in the UK (20) and the US (35), which found a wide variation of GC regimens among clinical practice settings in both countries. A recent retrospective study of children with classic CAH in the International-CAH registry (www.i-cah.org) also revealed large variations in GC treatments and doses (36).

There was a lack of consensus among panelists on what they considered to be a physiologic hydrocortisone dose for adults, but the majority agreed that 25-30 mg was the appropriate upper end for a physiologic hydrocortisone dose range. This lack of consensus is reflected in published estimates of physiologic hydrocortisone dose, which ranged from 7.5-15 mg/m^2/day, or approximately 15-25 mg/day of hydrocortisone (37, 38). However, prior studies in children with classic CAH have shown that a hydrocortisone dose of 8 mg/m^2/day was not associated with clinical manifestations of GC insufficiency, and these data suggest that 8 mg/m^2/day (or approximately 15 mg/day in adults) might be an adequate physiologic dose (39, 40).

In terms of the timing of GC dose administration, consensus was reached that once-daily dexamethasone should be administered at bedtime. The panelists typically prescribed hydrocortisone three times daily, starting in the morning. There was less agreement on the timing and frequency of dosing for prednisone, prednisolone, and methylprednisolone, but the first dose was usually given in the morning.

When asked about best practices for patient monitoring, panelists agreed that adequate control is best evaluated using the balance of clinical presentation and androgen/precursor laboratory values, but there was a lack of consensus on optimal timing for androgen/precursor laboratory testing and 17-OHP and A4 laboratory values indicating good control. The Endocrine Society recommends monitoring treatment through annual physical examinations and consistently timed biochemical measurements to assess the adequacy of GC treatment; however, the guidelines do not include specific recommendations on how to time the measurements or what the target levels should be (3). More recently, it has been suggested that the use of biomarkers such as 21-deoxycortisol and 11-oxysteriods may provide more direct evidence of adrenal androgen precursor production and thereby improve monitoring and titrating of current GC regimens; however, the use of these biomarkers has not been established in clinical care (1, 3). In addition, Saevik et al. recently proposed the use of circulating mRNA from GC-responsive genes, such as *DSIPI*, *DDIT4*, and *FKBP5*, as potential biomarkers in patients with autoimmune Addison's disease; however, further research is needed to explore the potential and validity of transcriptional biomarkers for GC replacement therapy (41).

The lack of agreement among panelists in most areas of GC management reflects the difficulties in using a "population" level approach for treating patients with classic CAH and suggests the need for a patient-specific approach in this population. A "treat-to-target" approach, as used in diabetes and dyslipidemia, is generally not appropriate; rather, treatment decisions should include careful consideration of the individual characteristics of each patient, including age, gender, genetic background (e.g., GC receptor polymorphisms), treatment goals, and side effects to guide shared decision making. This need for individualized treatment is also reflected in the relatively broad Endocrine Society treatment guidelines, which recommend the use of daily hydrocortisone and/or long-acting GCs plus mineralocorticoids for adults with classic CAH "as clinically indicated", with limited guidance on treatment optimization or patient monitoring (3).

Despite the lack of consensus on many aspects of CAH management, there was consensus agreement on the importance of many disease- and GC-related complications. In addition, all panelists agreed that there is a large unmet need for new treatments. With the currently available treatment options, panelists reported that almost half of their patients with classic CAH did not have optimized androgen levels, and another 29% had androgens optimized but were receiving supraphysiologic GC doses. Just 25% of patients were receiving physiologic GC doses and were perceived to have optimized androgen levels. These findings are in agreement with the previously mentioned cross-sectional studies in the UK and US, in which only 36% and 40% of adults with classic CAH, respectively, had normal serum A4 levels (20, 35).

Newer therapies, such as modified release hydrocortisone preparations and alternative hydrocortisone delivery systems (continuous subcutaneous infusion), have been developed as alternatives to long-acting synthetic GCs (42–44). Studies of these therapies indicated improved biomarker control, but GC exposure remained >20 mg/day (42–44). Bilateral adrenalectomy has been attempted as a strategy for management of classic CAH with lower (physiologic) GC dosing similar to the approach used for acquired primary adrenal insufficiency, but this approach is associated with a risk of short- and long-term adverse outcomes, including development of adrenal rest tumors (even in women) and an increased risk of adrenal crisis (45, 46). A promising strategy is the development of adjunctive therapies to reduce androgen production without the need for supraphysiologic GC dosing. Abiraterone acetate for six days added to 20 mg/day hydrocortisone normalized A4 in six adult women with classic CAH (47), but longer studies have not been performed. Crinecerfont, a CRF1R antagonist, was shown in a phase 2 trial to lower ACTH and afford clinically meaningful

reductions of elevated 17-OHP, A4, testosterone (women), or A4/testosterone ratio (men) (48). Phase 3 trials of crinecerfont are currently ongoing. Another CRF1R antagonist, tildacerfont, was shown in 14-day and three-month phase 2 trials to reduce ACTH, 17-OHP and A4 levels (testosterone levels were not reported in this study) (49). These potential treatments and others are discussed in more detail elsewhere (1, 2).

5 Conclusions

The limited areas of consensus obtained in this study reflect the variability in treatment practices for adults with classic CAH, even among clinicians with expertise in treating this population. The management of classic CAH is heterogeneous and varies widely by patient and provider; there is no single agreed-upon way to treat or manage classic CAH. However, this study found full consensus on the need for new treatments for classic CAH and the importance of many disease- and GC-related complications, which are difficult to manage with currently available therapeutic options.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

MF, CO'D, CY, and ML contributed to the conception and design of the Delphi methodology. RA, CC, AD, DE-M, HF, AL, and PT were expert panel members and survey participants. MA, KC, and NT collected and collated the survey responses. MA, KC, NT, and ML contributed to the analysis and interpretation of the results. All authors contributed to the drafting and critical revision of this manuscript and approved the submitted version.

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Conflict of interest

MF, CO'D and CY were employed by Neurocrine Biosciences, Inc. MA, KC, and NT were employed by Evidera. ML, full-time employee of IQVIA. IQVIA received consulting fees for the advice of ML on this research. RA received research funding from Neurocrine Biosciences, Inc., Diurnal, LTD, and Spruce Biosciences; RA served as consultant for Neurocrine Biosciences, Inc., Crinetics Pharmaceuticals, OMass Therapeutics, H Lundbeck A/S, and Adrenas Therapeutics. HF served as consultant for Neurocrine Biosciences, Inc., Diurnal Ltd., Roche Diagnostics International Ltd., H Lundbeck A/S, and Adrenas Therapeutics. AL served as editor Adrenal Section, UpToDate.

The authors declare that this study received funding from Neurocrine Biosciences, Inc. The funder had the following involvement in the study: Contracted with Evidera and IQVIA to conduct the Delphi surveys, collate data, perform statistical analyses, and assist with interpretation of data and preparation of the manuscript. Contracted with Prescott Medical Communications Group to assist with preparation of the manuscript.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1005963/full#supplementary-material

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EDITED BY
Semra Çaglar Çetinkaya,
University of Health Sciences, Turkey

REVIEWED BY
Dominika Janus,
Jagiellonian University Medical College, Poland
Wen Hsuan Tsai,
Mackay Memorial Hospital, Taiwan

*CORRESPONDENCE
Afif Nakhleh
anakhleh@gmail.com

†These authors have contributed equally to this work

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Screening for non-classic congenital adrenal hyperplasia in women: New insights using different immunoassays

Afif Nakhleh[1,2*†], Leonard Saiegh[3,4†], Naim Shehadeh[1,2,3], Naomi Weintrob[5,6], Mohammad Sheikh-Ahmad[4], Lia Supino-Rosin[7], Sandra Alboim[7], Raya Gendelman[8] and Moshe Zloczower[1]

[1]Institute of Endocrinology, Diabetes and Metabolism, Rambam Health Care Campus, Haifa, Israel, [2]Diabetes and Endocrinology Clinic, Maccabi Healthcare Services, Haifa, Israel, [3]Ruth & Bruce Rappaport Faculty of Medicine, Technion, Israel Institute of Technology, Haifa, Israel, [4]Department of Endocrinology, Bnai Zion Medical Center, Haifa, Israel, [5]Department of Pediatrics, Sackler Faculty of Medicine, Tel Aviv University, Tel-Aviv, Israel, [6]Pediatric Endocrinology and Diabetes Unit, Dana-Dwek Children's Hospital, Tel-Aviv Medical Center, Tel-Aviv, Israel, [7]Central Laboratory, Maccabi Healthcare Services, Rehovot, Israel, [8]The Endocrine Laboratory, Rambam Health Care Campus, Haifa, Israel

Context: The 250µg-cosyntropin stimulation test (CST) is used to diagnose non-classic congenital adrenal hyperplasia (NCCAH). The current recommendation is to perform CST when follicular 17-hydroxyprogesterone (17OHP) is 6-30 nmol/L, a cutoff derived from radioimmunoassay (RIA). Recently, enzyme-linked immunosorbent assay (ELISA) has replaced RIA.

Objectives: We aimed to (1) determine the RIA and ELISA-based 17OHP cutoffs at which CST should be performed, (2) identify predictors of NCCAH.

Methods: A retrospective study at an Israeli Health Maintenance Organization. Data were retrieved from women with suspected NCCAH, referred for CST during 2001–2020. NCCAH was defined as a stimulated 17OHP >30 nmol/L. Serum 17OHP levels were assayed by RIA from 1/2000-3/2015, and by ELISA from 4/2015-12/2020. ROC curves were generated and optimal 17OHP thresholds were determined. Multivariate analysis was performed.

Results: CST was performed in 2409 women (1564 in RIA, 845 in ELISA). NCCAH was diagnosed in 4.7% of the RIA group and 7.5% of the ELISA group. The optimal basal 17OHP cutoff values predicting NCCAH were 6.1 nmol/L in RIA (sensitivity=93.2%, specificity=91.7%) and 8.2 nmol/L in ELISA (sensitivity=93.7%, specificity=92.3%). In multivariate analysis, higher basal 17OHP, lower LH: FSH ratio, and oligomenorrhea were predictors of NCCAH in RIA. Higher basal 17OHP, androstenedione, and total testosterone were predictors of NCCAH in ELISA. A lower LH: FSH ratio showed similar trend in ELISA.

Conclusions: Optimal RIA-based basal 17OHP cutoff was comparable with that recommended in guidelines. The results suggest adopting a higher 17OHP cutoff when using ELISA. LH : FSH ratio improves the negative predictive value of basal 17OHP.

KEYWORDS

non-classic congenital adrenal hyperplasia, radioimmunoassay, enzyme-linked immunosorbent assay, 17-hydroxyprogesterone, cosyntropin stimulation test, LH : FSH ratio

1 Introduction

The 250 μg cosyntropin stimulation test (CST) is used to diagnose non-classic congenital adrenal hyperplasia (NCCAH) due to 21-hydroxylase deficiency. The current recommendation is to perform CST when basal serum 17-hydroxyprogesterone (17OHP) levels (performed in the early follicular phase in females) are 6-30 nmol/L, and the test is considered positive for NCCAH diagnosis when the 60-minutes post-CST 17OHP level is > 30 nmol/L (1). These 17OHP cutoffs were mainly derived from radioimmunoassay (RIA) data (2). The Endocrine Society recommends screening with an early morning (before 8 AM) basal serum 17OHP measurement by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (1). However, because of the limited availability of LC-MS/MS, immunoassays remain the assays most frequently used.

There are several disadvantages to using RIA including reagent instability, radioactive waste management, and the need for manual handling. Recently, a validated enzyme-linked immunosorbent assay (ELISA) has widely replaced RIA in the measurement of serum 17OHP (3). ELISA is simple, easy to perform, and uses commercially available reagents (4). A validation study by an Australian laboratory showed that IBL's 17OHP ELISA assay provides an acceptable alternative for the Siemens Healthcare Diagnostics 17OHP RIA (3, 5).

The 17OHP cutoff of 6 nmol/L for NCCAH screening has been questioned by several studies utilizing RIA assays that suggested lower cutoffs (6–9). Several studies showed that between 2% and 11% of adult patients with NCCAH might be missed using this approach (10). Implementation of a new assay justifies re-evaluation of the basal 17OHP threshold level for performing CST, especially in the era of cost-effective medicine.

Since most of the patients diagnosed with NCCAH are females (11, 12), we studied only females aged >16 years with clinical suspicion of NCCAH. The current study aimed to determine the best cutoff for performing CST using ELISA compared to RIA. In addition, we aimed to identify clinical and laboratory factors that could predict the diagnosis of NCCAH.

2 Materials and methods

This non-interventional, retrospective, cohort study was conducted using the electronic medical database of Maccabi Healthcare Services (MHS), a large health maintenance organization (HMO) in Israel serving over 2 million patients. All medical data were obtained from the MHS automated database. The retrieval of patients' records was performed using MDClone, a query tool that provides comprehensive patient-level data for a wide range of variables in a defined time frame around an index event (13, 14). This platform was used to elicit information on demographic, clinical, and biochemical data, and dispensed community prescriptions. Approval was obtained from MHS institutional review board (IRB) and ethics committee to access and analyze data. Individual patient informed consent was not required because of the anonymized nature of patient records.

2.1 Study subjects and definitions

We retrieved data on consecutive women over 16 years of age with suspected NCCAH, referred for 250 μg CST from January 2001 – December 2020. As it is a real-life study, all CSTs for suspected NCCAH were included regardless of pretest 17OHP levels. The time of the CST was set as the index date. Subjects meeting inclusion criteria had clinical data available for at least 12 months before the index date.

Subjects using estrogen-containing oral contraceptives (OC), systemic or topical hormone replacement therapy (HRT), or systemic glucocorticoids (GC) were excluded (n=141). Subjects were considered as users of OC (n=109), HRT (n=3), or GC (n=29) if their prescription was filled within three months before the index date.

Clinical presentations that led to 17OHP measurements were hirsutism, oligomenorrhea, amenorrhea, acne, alopecia, or infertility that were identified within 12 months before the index date, based on the International Classification of Diseases-9 (ICD-9) or MHS internal diagnostic codes—called "Y" codes.

Demographic variables included age and last body mass index (BMI) at the index date. Laboratory values were defined as the last available value during the six months before or at the index date. Laboratory values included pre- and post-CST (basal and stimulated) serum 17OHP and cortisol, serum TSH, prolactin, total testosterone, dehydroepiandrosterone sulfate (DHEAS), androstenedione, LH, and FSH; LH to FSH ratio was also calculated.

NCCAH was defined as a 60-minute post-CST 17OHP serum level >30 nmol/L.

2.2 Biochemical analyses

Serum cortisol, TSH, prolactin, total testosterone, LH, and FSH levels were measured by chemiluminescent immunoassays (CLIAs) (Advia Centaur or Centaur XP, Siemens). Serum DHEAS levels were measured by CLIA (Immulite 2000, Siemens) from January 2000 through May 2014, and by CLIA (Centaur XP, Siemens) from June 2014 to December 2020. Serum androstenedione levels were measured using RIA (DSL) from January 2000 through December 2011, and by CLIA (Immulite 2000, Siemens) from January 2012 through December 2020.

From January 2000 through March 2015, serum 17OHP levels were assayed by direct RIA using Wizard gamma counter, Perkin-Elmer (OHP-CT: Cis Bio, Gif-sur-Yvette, France), and from April 2015 to December 2020 by ELISA (MG12181: Tecan IBL GmbH, Hamburg, Germany). The intra-assay coefficient of variation was 12.3-8.3% (range 0.345-13.21 nmol/L) for RIA, and 2.8-4.9% (range 7.39-34.57 nmol/L) for ELISA. The inter-assay coefficient of variation was 12-12.8% (range 2.94-22.81 nmol/L) for RIA, and 5.8-9.2% (range 0.78-17.39 nmol/L) for ELISA. The limit of detection was 0.09 nmol/L in both methods. MHS central laboratory performed validation for 17OHP assays, comparing 48 samples from their daily routine. Passing-Bablok regression revealed a good correlation between ELISA and RIA assays (r=0.97; range 0-25 nmol/L). The absolute 17OHP values measured by ELISA were slightly (34% on average) higher than by RIA (ELISA = 1.3462 RIA - 0.04); however, they were still within the allowable limits in accordance with the manufacturer's package insert reference range.

2.3 Statistical analysis

Categorical variables were described as numbers and frequencies. Continuous variables were described as means and standard deviations (SD), or medians and ranges. The Chi-square test or Fisher's exact test was used to compare categorical variables, and the student's t-test was used to compare continuous variables, respectively. We allocated the individuals into two groups according to the 17OHP assay method used (RIA vs ELISA). For each group, a receiver-operating characteristic (ROC) curve was generated and an optimal basal 17OHP threshold with the highest sensitivity and specificity was determined. Positive (PPV) and negative (NPV) predictive values were calculated. We compared the sensitivity and specificity between the RIA and the ELISA groups when using the optimal RIA-based basal 17OHP cutoff. For each 17OHP assay group, univariate and multivariate logistic regression analyses were performed to identify variables that predict NCCAH, defined as a post-CST 17OHP serum level of >30 nmol/L. Variables with a p-value <0.1 on the univariate analysis were included in the multivariate analysis, which aimed to determine any independent predictors of NCCAH.

A two-sided P-value < 0.05 was considered statistically significant. All analyses were conducted with the statistical software SPSS, version 25 (IBM Corporation, Armonk, NY, USA).

3 Results

CST was performed in 2409 women who satisfied the inclusion criteria (1564 in the RIA group and 845 in the ELISA group). The mean (± SD) age was 24.1 ± 7 years. Symptoms that prompted cosyntropin testing were hirsutism in 45% of subjects, oligomenorrhea in 40%, amenorrhea in 25%, acne in 39.4%, infertility in 13.5%, and alopecia in 12.5%. The two groups were comparable in terms of the indication for testing, except for hirsutism which was more common in the RIA group (p<0.001) (Table 1).

The mean basal and stimulated 17OHP levels were lower in the RIA group as compared to the ELISA group (4.1 ± 6.4 vs. 5.9 ± 8.9 and 9.9 ± 15.3 vs. 12.3 ± 17.3, respectively, p<0.001 for both comparisons). Basal serum cortisol, total testosterone, and LH : FSH ratio were higher in the RIA group. Serum androstenedione levels were higher in the ELISA group (Table 1). The same pattern of differences was also observed between the two groups when comparing subjects without NCCAH (Table 2).

NCCAH was diagnosed in 74 (4.7%) subjects in the RIA group and 63 (7.5%) in the ELISA group (p=0.008) (Table 1). NCCAH subjects in the two groups had comparable parameters, except for higher total testosterone levels in the RIA group and higher androstenedione levels in the ELISA group (Table 3).

Using ROC analysis, the optimal basal 17OHP cutoff values predicting NCCAH were 6.1 nmol/L in the RIA group (sensitivity 93.2%, specificity 91.7%, NPV 99.6%, and PPV 35.8%) (Figure 1) and 8.2 nmol/L in the ELISA group

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TABLE 1 Clinical and biochemical characteristics of women in the RIA and the ELISA groups.

	RIA group (n=1564)	ELISA group (n=845)	P-value
Age, y			
Mean ± SD	24.1 ± 7.0	24.1 ± 7.4	0.83
Median (range)	22.4 (16–54)	22.2 (16-53.8)	
(n)	1564	845	
BMI, kg/m^2			
Mean ± SD	25.5 ± 6.2	24.8 ± 5.6	0.52
Median (range)	24.2 (16.0-56.9)	23.6 (12.3-48.5)	
(n)	681	602	
Hirsutism			
n (%)	804 (51.4)	291 (34.4)	<0.001
Oligomenorrhea			
n (%)	629 (40.2)	335 (39.7)	0.83
Amenorrhea			
n (%)	393 (25.1)	211 (25)	
Acne			
n (%)	615 (39.3)	336 (39.8)	0.87
Infertility			
n (%)	200 (12.8)	125 (14.8)	0.15
Alopecia			
n (%)	199 (12.7)	102 (12.1)	0.66
Basal 17OHP level*, nmol/L			
Mean ± SD	4.1 ± 6.4	5.9 ± 8.9	<0.001
Median (range)	2.6 (0.3-82.5)	3.6 (0.5-66.0)	
(n)	1564	845	
Stimulated 17OHP level, nmol/L			
Mean ± SD	9.9 ± 15.3	12.3 ± 17.3	<0.001
Median (range)	6.3 (0.4-82.5)	7.3 (0.5-66.0)	
(n)	1564	845	
NCCAH (stimulated 17OHP level>30nmol/L)			
n (%)	74 (4.7%)	63 (7.5%)	0.008
Basal cortisol level, nmol/L			
Mean ± SD	480.6 ± 196.6	430.4 ± 173.8	<0.001
Median (range)	453 (114.0-1487.0)	409 (67.0-1275.0)	
(n)	1365	765	

(Continued)

TABLE 1 Continued

	RIA group (n=1564)	ELISA group (n=845)	P-value
Stimulated cortisol level, nmol/L			
Mean ± SD	819.8 ± 209.8	807.2 ± 170.5	0.16
Median (range)	791 (143.0-2275.90)	795 (73.0-1594.0)	
(n)	1365	765	
Total testosterone, nmol/L			
Mean ± SD	2.2 ± 0.9	1.7 ± 0.7	<0.001
Median (range)	2.0 (0.6-9.70)	1.4 (0.4-8.6)	
(n)	1253	866	
Androstenedione, ng/ml			
Mean ± SD	2.9 ± 1.9	3.7 ± 1.8	<0.001
Median (range)	2.5 (0.3-16.9)	3.5 (0.3-11.0)	
(n)	1028	491	
DHEAS, µmol/L			
Mean ± SD	6.9 ± 3.6	6.8 ± 3.4	0.57
Median (range)	6.3 (0.4-22.8)	6.3 (0.4-19.5)	
(n)	1376	801	
LH, IU/L			
Mean ± SD	8.7 ± 7.9	8.7 ± 8.3	0.89
Median (range)	6.50 (0.5-76.9)	6.30 (0.5-68.0)	
(n)	1305	815	
FSH, IU/L			
Mean ± SD	5.5 ± 5.5	7.0 ± 7.4	<0.001
Median (range)	5.0 (0.7-111.8)	6.2 (0.7-116.7)	
(n)	1318	813	
LH to FSH ratio			
Mean ± SD	1.7 ± 1.5	1.4 ± 1.1	<0.001
(n)	1295	811	
Prolactin, mIU/L			
Mean ± SD	310.0 ± 247.4	280.5 ± 183.6	0.005
Median (range)	251.0 (18.0-3578.0)	228.0 (16.0-1999.0)	
	1198	731	
TSH, mIU/L			
Mean ± SD	2.4 ± 1.7	2.2 ± 1.4	0.013
Median (range)	2.1 (0.03-31.9)	2.0 (0.03-23.1)	
(n)	1412	845	

*For 17OHP, the intra-assay coefficient of variation was 9-22% for RIA and 13-25% for ELISA. The inter-assay coefficient of variation was 16%.
Continuous parameters are shown as mean ± SD and median (range), and categorical variables as n (%). 17OHP, 17-hydroxyprogesterone; BMI, body mass index; CST, cosyntropin stimulation test; DHEAS, dehydroepiandrosterone sulfate; FSH, follicle stimulating hormone; LH, luteinizing hormone; NCCAH, non-classic congenital adrenal hyperplasia; TSH, thyroid stimulating hormone.

TABLE 2 Comparison of clinical and biochemical characteristics among women without NCCAH from the RIA and the ELISA groups.

	RIA group (n=1564) Subjects without NCCAH (n=1492)	ELISA group (n=845) Subjects without NCCAH (n=782)	P-value
Age, y			
Mean ± SD	24.0 ± 7.0	24.0 ± 7.3	1
Median (range)	22.3 (16–54)	22.0 (16-53.8)	
(n)	1492	782	
BMI, kg/m^2			
Mean ± SD	25.4 ± 6.1	24.8 ± 5.6	0.08
Median (range)	24.1 (16.0-56.9)	23.6 (12.3-48.5)	
(n)	647	566	
Hirsutism			
n (%)	768 (51.5)	268 (34.3)	<0.001
Oligomenorrhea			
n (%)	614 (40.1)	314 (40.2)	0.67
Amenorrhea			
n (%)	376 (25.2)	201 (25.6)	0.83
Acne			
n (%)	590 (39.5)	309 (39.5)	0.99
Infertility			
n (%)	185 (12.4)	109 (13.9)	0.33
Alopecia			
n (%)	191 (12.8)	98 (12.5)	0.91
Basal 17OHP level*, nmol/L			
Mean ± SD	3.2 ± 2.5	4.0 ± 2.7	<0.001
Median (range)	2.5 (0.3-20.1)	3.4 (0.5-23.6)	
(n)	1492	782	
Stimulated 17OHP level, nmol/L			
Mean ± SD	6.8 ± 3.4	7.9 ± 3.9	<0.001
Median (range)	6.1 (0.4-29.3)	7.0 (0.5-29.1)	
(n)	1492	782	
Basal cortisol level, nmol/L			
Mean ± SD	480.7 ± 197.4	432.4 ± 172.3	<0.001
Median (range)	453 (114.0-1487.0)	408 (96.0-1275.0)	
(n)	1302	710	

(Continued)

TABLE 2 Continued

	RIA group (n=1564) Subjects without NCCAH (n=1492)	ELISA group (n=845) Subjects without NCCAH (n=782)	P-value
Stimulated cortisol level, nmol/L			
Mean ± SD	829.4 ± 206.6	821.6 ± 162.4	0.39
Median (range)	796.0 (196.0-2275.9)	803.5 (240.0-1594.0)	
(n)	1302	710	
Total testosterone, nmol/L			
Mean ± SD	2.2 ± 0.9	1.6 ± 0.6	<0.001
Median (range)	2.0 (0.6-9.7)	1.4 (0.4-8.6)	
(n)	1194	807	
Androstenedione, ng/ml			
Mean ± SD	2.9 ± 1.9	3.6 ± 1.7	<0.001
Median (range)	2.5 (0.3-16.9)	3.45 (0.3-11.0)	
(n)	985	458	
DHEAS, µmol/L			
Mean ± SD	6.9 ± 3.6	6.8 ± 3.4	0.49
Median (range)	6.3 (0.4-22.8)	6.2 (0.4-19.5)	
(n)	1322	747	
LH, IU/L			
Mean ± SD	8.8 ± 8.0	8.6 ± 7.7	0.62
Median (range)	6.6 (0.5-76.9)	6.3 (0.5-68.0)	
(n)	1244	763	
FSH, IU/L			
Mean ± SD	5.4 ± 5.2	6.8 ± 5.7	<0.001
Median (range)	4.9 (0.7-111.8)	6.1 (0.7-104.0)	
(n)	1256	761	
LH to FSH ratio			
Mean ± SD	1.8 ± 1.6	1.4 ± 1.1	<0.001
(n)	1234	759	
Prolactin, mIU/L			
Mean ± SD	312.0 ± 251.4	281.3 ± 186.0	0.006
Median (range)	251.0 (18.0-3578.0)	228.0 (16.0-1999.0)	
(n)	1143	687	

(Continued)

TABLE 2 Continued

	RIA group (n=1564) Subjects without NCCAH (n=1492)	ELISA group (n=845) Subjects without NCCAH (n=782)	P-value
TSH, mIU/L			
Mean ± SD	2.4 ± 1.7	2.2 ± 1.4	0.02
Median (range)	2.1 (0.03-31.9)	2.0 (0.03-23.1)	
(n)	1347	814	

*For 17OHP, the intra-assay coefficient of variation was 9% for RIA and 13% for ELISA. The inter-assay coefficient of variation was 18.5%.
Continuous parameters are shown as mean ± SD and median (range), and categorical variables as n (%). 17OHP, 17-hydroxyprogesterone; BMI, body mass index; CST, cosyntropin stimulation test; DHEAS, dehydroepiandrosterone sulfate; FSH, follicle stimulating hormone; LH, luteinizing hormone; NCCAH, non-classic congenital adrenal hyperplasia; TSH, thyroid stimulating hormone.

(sensitivity 93.7%, specificity 92.3%, NPV 99.5%, PPV 49.6%) (Figure 2). When basal 17OHP cutoff value of 6.1 nmol/L was used in the ELISA group, sensitivity was 93.7% (p=1 for comparison with RIA), but specificity decreased to 84.5% (p<0.001 for comparison with RIA).

When applying a post-CST 17OHP diagnostic cutoff of > 40 nmol/L, NCCAH was diagnosed in 69 (4.4%) subjects in the RIA group and 60 (7.1%) in the ELISA group (p=0.007). Using ROC analysis, the optimal basal 17OHP cutoff values predicting NCCAH were 6.1 nmol/L in the RIA group (sensitivity 92.8%, specificity 91.4%, NPV 99.6%, PPV 33.2%) (Figure 3) and 8.2 nmol/L in the ELISA group (sensitivity 95%, specificity 92%, NPV 99.6%, PPV 47.5%) (Figure 4).

Table 4 compares the clinical and biochemical characteristics of women with and without NCCAH (defined as a post-CST 17OHP serum level of >30 nmol/L) in the RIA and the ELISA groups. In both groups, the following variables met the criteria for inclusion in the multivariate logistic regression analysis: age, basal serum 17OHP, total testosterone, androstenedione, and LH : FSH ratio. A history of oligomenorrhea and a history of infertility were included in the analyses of the RIA and the ELISA groups, respectively.

In the multivariate analysis, higher basal 17OHP level (p<0.001), lower LH: FSH ratio (p=0.005), and oligomenorrhea (p=0.021) emerged as independent predictors of NCCAH in the RIA group. Higher basal 17OHP (p<0.001), androstenedione (p=0.004), and total testosterone (p=0.012) levels were identified as independent predictors of NCCAH in the ELISA group. Of note, a lower LH : FSH ratio showed a similar trend in ELISA but did not reach significance (p=0.054). The results of the multivariate analyses are shown in Tables 5 and 6.

Using ROC analysis, the optimal LH : FSH ratio cutoff values for predicting NCCAH were 1.4 in the RIA group (sensitivity 85.3%, specificity 46.8%, NPV 98.5%, PPV 7.3%) and 1.2 in the ELISA group (sensitivity 71.2%, specificity 43.4%, NPV 95.6%, PPV 7.9%).

In the RIA group, the combination of a serum basal 17OHP value below 6.1 and an LH : FSH ratio above 1.4 increased the NPV to 100%. In the ELISA group, the combination of a serum basal 17OHP value below 8.2 and LH : FSH ratio above 1.2 increased the NPV to 99.7%. Combining basal serum 17OHP values below the optimal cutoffs with an LH : FSH ratio > 2, yielded an increase in the NPV to 100% in both groups.

4 Discussion

This study showed that the optimal RIA-based basal 17OHP cutoff at which to refer a woman suspected of NCCAH to CST was 6.1 nmol/L, comparable with that recommended in the guidelines (6 nmol/L) (1). When using ELISA, we observed an upward shift in the basal 17OHP cutoff to 8.2 nmol/L. This result is in accordance with a recent report by Domagala et al. (15) who examined the validity of the currently accepted 17OHP threshold at which CST should be performed in 343 polish individuals with suspected NCCAH. They showed that the basal ELISA-based 17OHP value that best qualified patients for testing was 2.8 ng/mL (8.4 nmol/L), with a sensitivity and a specificity of 77.2% and 91.3%, respectively.

While LC-MS/MS-based 17OHP measurement has been recommended by the Endocrine Society for the screening of NCCAH due to 21-hydroxylase deficiency (1), the use of LC-MS/MS is expensive, labor consuming and therefore not widespread, especially in adult outpatient clinics. In addition, standard cutoffs for this technique are still evolving (10).

Immunoassays are still the assays most frequently used in clinical settings and present good performance in NCCAH diagnosis. The classical basal 17OHP cutoff value of 6 nmol/L for performing CST was established based on immunoassays, mainly RIA (1). This cutoff has been called into question by

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TABLE 3 Comparison of clinical and biochemical characteristics among women with NCCAH from the RIA and the ELISA groups.

	RIA group (n=1564) Subjects with NCCAH (n=74)	ELISA group (n=845) Subjects with NCCAH (n=63)	P-value
Age, y			
Mean ± SD	26.5 ± 8.1	26.1 ± 8.5	0.78
Median (range)	25.6 (16-52.7)	24.4 (16-50.3)	
(n)	74	63	
BMI, kg/m^2			
Mean ± SD	26.2 ± 7.5	25.4 ± 5.6	0.61
Median (range)	25.1 (17.2-47.8)	24.9 (17-42.3)	
(n)	34	36	
Hirsutism			
n (%)	36 (48.6)	23 (36.5)	0.21
Oligomenorrhea			
n (%)	15 (20.3)	21 (33.3)	0.12
Amenorrhea			
n (%)	17 (23.0)	10 (15.9)	0.41
Acne			
n (%)	25 (33.8)	27 (42.9)	0.36
Infertility			
n (%)	15 (20.3)	16 (25.4)	0.26
Alopecia			
n (%)	8 (10.8)	4 (6.3)	0.54
Basal 17OHP level*, nmol/L			
Mean ± SD	23.5 ± 18.8	29.2 ± 20.2	0.09
Median (range)	16.6 (4.1-82.5)	20.6 (2.1-66.0)	
(n)	74	63	
Stimulated 17OHP level, nmol/L			
Mean ± SD	67.1 ± 16.1	63.5 ± 7.8	0.21
Median (range)	71.6 (30.9-82.5)	66.0 (32.8-66.0)	
(n)	74	63	
Basal cortisol level, nmol/L			
Mean ± SD	477.9 ± 179.6	434.3 ± 163.7	0.17
Median (range)	459.0 (155–949)	459 (67.0-948)	
(n)	63	55	

(Continued)

TABLE 3 Continued

	RIA group (n=1564) Subjects with NCCAH (n=74)	ELISA group (n=845) Subjects with NCCAH (n=63)	P-value
Stimulated cortisol level, nmol/L			
Mean ± SD	634.5 ± 160.5	641.5 ± 158.1	0.81
Median (range)	606.0 (143.0-1083.0)	637.0 (73.0-1092.0)	
(n)	63	55	
Total testosterone, nmol/L			
Mean ± SD	2.9 ± 0.9	2.0 ± 0.9	<0.001
Median (range)	2.9 (1.4-5.4)	1.7 (0.6-5.0)	
(n)	59	59	
Androstenedione, ng/ml			
Mean ± SD	4.2 ± 2.6	5.6 ± 2.4	0.04
Median (range)	3.3 (0.6-11.0)	5.9 (1.3-11.0)	
(n)	43	33	
DHEAS, µmol/L			
Mean ± SD	7.3 ± 3.5	7.3 ± 3.5	0.96
Median (range)	7.0 (1.2-18.2)	6.9 (0.7-16.9)	
(n)	54	54	
LH, IU/L			
Mean ± SD	5.9 ± 5.7	8.3 ± 8.2	0.08
Median (range)	4.2 (0.5-31.4)	5.6 (0.5-50.1)	
(n)	61	52	
FSH, IU/L			
Mean ± SD	6.7 ± 9.0	11.0 ± 18.9	0.12
Median (range)	5.6 (0.7-74.0)	6.30 (1.1-116.7)	
(n)	62	52	
LH to FSH ratio			
Mean ± SD	1.0 ± 0.7	1.1 ± 0.9	0.39
(n)	61	52	
Prolactin, mIU/L			
Mean ± SD	265.9 ± 134.6	268.1 ± 141.0	0.94
Median (range)	252.0 (58.0-584.0)	226.0 (119.0-792.0)	
(n)	55	44	

(Continued)

TABLE 3 Continued

	RIA group (n=1564) Subjects with NCCAH (n=74)	ELISA group (n=845) Subjects with NCCAH (n=63)	P-value
TSH, mIU/L			
Mean ± SD	2.4 ± 1.3	2.6 ± 1.6	0.97
Median (range)	2.1 (0.5-6.9)	2.0 (0.7-11.2)	
(n)	65	57	

*For 17OHP, the intra-assay coefficient of variation was 22% for RIA and 25% for ELISA. The inter-assay coefficient of variation was 13.5%.
Continuous parameters are shown as mean ± SD and median (range), and categorical variables as n (%).
17OHP, 17-hydroxyprogesterone; BMI, body mass index; CST, cosyntropin stimulation test; DHEAS, dehydroepiandrosterone sulfate; FSH, follicle stimulating hormone; LH, luteinizing hormone; NCCAH, non-classic congenital adrenal hyperplasia; TSH, thyroid stimulating hormone.

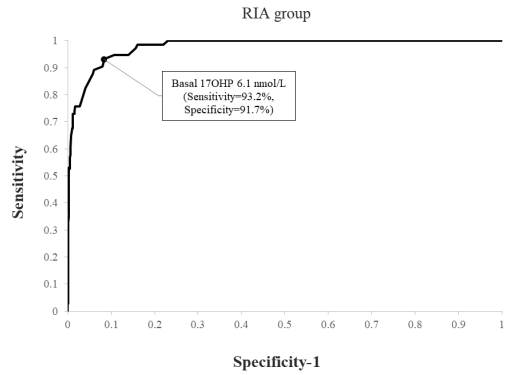

FIGURE 1
ROC curve analysis for determination of optimal basal 17OHP threshold in RIA (using a post-CST 17OHP cutoff > 30 nmol/L for the diagnosis of NCCAH).

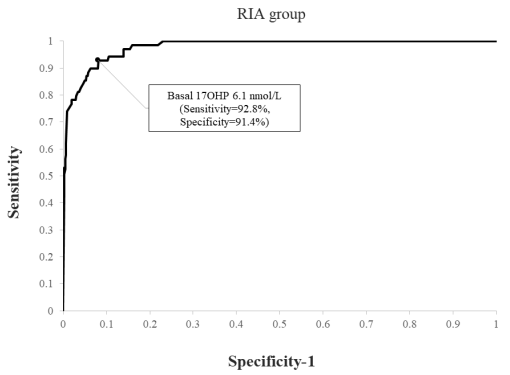

FIGURE 3
ROC curve analysis for determination of optimal basal 17OHP threshold in RIA (using a post-CST 17OHP cutoff > 40 nmol/L for the diagnosis of NCCAH).

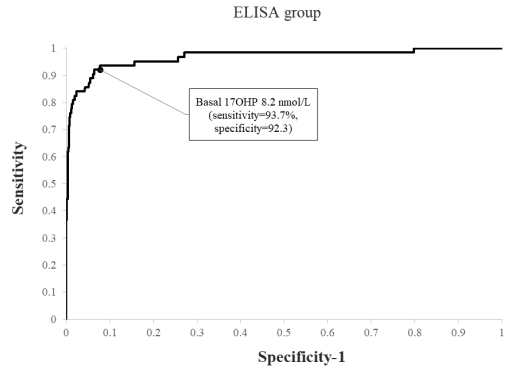

FIGURE 2
ROC curve analysis for determination of optimal basal 17OHP threshold in ELISA (using a post-CST 17OHP cutoff > 30 nmol/L for the diagnosis of NCCAH).

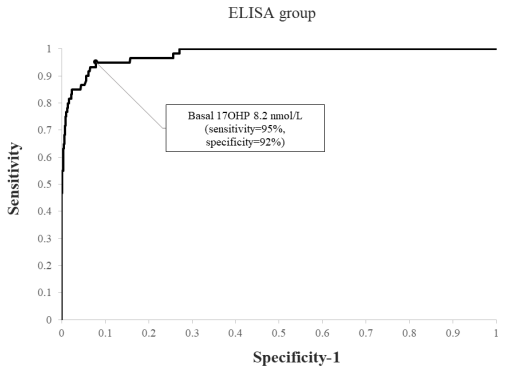

FIGURE 4
ROC curve analysis for determination of optimal basal 17OHP threshold in ELISA (using a post-CST 17OHP cutoff > 40 nmol/L for the diagnosis of NCCAH).

TABLE 4 Comparison of clinical and biochemical characteristics between women with and without NCCAH in the RIA and the ELISA groups.

	RIA group (n=1564)		P-value	ELISA group (n=845)		P-value
	Subjects without NCCAH (n=1492)	Subjects with NCCAH (n=74)		Subjects without NCCAH (n=782)	Subjects with NCCAH (n=63)	
Age, y						
Mean ± SD	24.0 ± 7.0	26.5 ± 8.1	0.002	24.0 ± 7.3	26.1 ± 8.5	0.03
Median (range)	22.3 (16–54)	25.6 (16-52.7)		22.0 (16-53.8)	24.4 (16-50.3)	
(n)	1492	74		782	63	
BMI, kg/m^2						
Mean ± SD	25.4 ± 6.1	26.2 ± 7.5	0.49	24.8 ± 5.6	25.4 ± 5.6	0.57
Median (range)	24.1 (16.0-56.9)	25.1 (17.2-47.8)		23.6 (12.3-48.5)	24.9 (17-42.3)	
(n)	647	34		566	36	
Hirsutism						
n (%)	768 (51.5)	36 (48.6)	0.72	268 (34.3)	23 (36.5)	0.82
Oligomenorrhea						
n (%)	614 (40.1)	15 (20.3)	<0.001	314 (40.2)	21 (33.3)	0.35
Amenorrhea						
n (%)	376 (25.2)	17 (23.0)	0.79	201 (25.6)	10 (15.9)	0.11
Acne						
n (%)	590 (39.5)	25 (33.8)	0.39	309 (39.5)	27 (42.9)	0.69
Infertility						
n (%)	185 (12.4)	15 (20.3)	0.07	109 (13.9)	16 (25.4)	0.02
Alopecia						
n (%)	191 (12.8)	8 (10.8)	0.69	98 (12.5)	4 (6.3)	0.21
Basal 17OHP level, nmol/L						
Mean ± SD	3.2 ± 2.5	23.5 ± 18.8	<0.001	4.0 ± 2.7	29.2 ± 20.2	<0.001
Median (range)	2.5 (0.3-20.1)	16.6 (4.1-82.5)		3.4 (0.5-23.6)	20.6 (2.1-66.0)	
(n)	1492	74		782	63	
Stimulated 17OHP level, nmol/L						
Mean ± SD	6.8 ± 3.4	67.1 ± 16.1	<0.001	7.9 ± 3.9	63.5 ± 7.8	<0.001
Median (range)	6.1 (0.4-29.3)	71.6 (30.9-82.5)		7.0 (0.5-29.1)	66.0 (32.8-66.0)	
(n)	1492	74		782	63	
Basal cortisol level, nmol/L						
Mean ± SD	480.7 ± 197.4	477.8 ± 179.6	0.91	432.4 ± 172.3	434.3 ± 163.7	0.94

(Continued)

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TABLE 4 Continued

	RIA group (n=1564)		P-value	ELISA group (n=845)		P-value
	Subjects without NCCAH (n=1492)	Subjects with NCCAH (n=74)		Subjects without NCCAH (n=782)	Subjects with NCCAH (n=63)	
Median (range)	453 (114.0-1487.0)	459.0 (155–949)		408 (96.0-1275.0)	459 (67.0-948)	
(n)	1302	63		710	55	
Stimulated cortisol level, nmol/L						
Mean ± SD	829.4 ± 206.7	634.5 ± 160.5	<0.001	821.6 ± 162.4	641.6 ± 158.1	<0.001
Median (range)	796.0 (196.0-2275.90)	606.0 (143.0-1083.0)		803.5 (240.0-1594.0)	637.0 (73.0-1092.0)	
(n)	1302	63		710	55	
Total testosterone, nmol/L						
Mean ± SD	2.2 ± 0.9	2.9 ± 0.9	<0.001	1.6 ± 0.7	2.0 ± 0.9	<0.001
Median (range)	2.0 (0.6-9.7)	2.9 (1.4-5.4)		1.35 (0.4-8.6)	1.7 (0.6-5.0)	
(n)	1194	59		807	59	
Androstenedione, ng/ml						
Mean ± SD	2.9 ± 1.9	4.2 ± 2.6	<0.001	3.6 ± 1.7	5.5 ± 2.5	<0.001
Median (range)	2.5 (0.3-16.9)	3.3 (0.6-11.0)		3.45 (0.3-11.0)	5.9 (1.3-11.0)	
(n)	985	43		458	33	
DHEAS, µmol/L						
Mean ± SD	6.9 ± 3.6	7.3 ± 3.5	0.41	6.8 ± 3.4	7.3 ± 3.5	0.31
Median (range)	6.3 (0.3-22.8)	7.0 (1.2-18.2)		6.2 (0.4-19.5)	6.9 (0.7-16.9)	
(n)	1322	54		747	54	
LH, IU/L						
Mean ± SD	8.8 ± 8.0	5.9 ± 5.7	0.007	8.6 ± 7.7	8.3 ± 8.2	0.79
Median (range)	6.6 (0.5-76.9)	4.2 (0.5-31.4)		6.3 (0.5-68.0)	5.6 (0.5-50.1)	
(n)	1244	61		763	52	
FSH, IU/L						
Mean ± SD	5.4 ± 5.2	6.7 ± 9.0	0.07	6.8 ± 5.7	11.0 ± 18.9	<0.001
Median (range)	4.9 (0.7-111.8)	5.6 (0.7-74.0)		6.1 (0.7-104)	6.30 (1.1-116.7)	
(n)	1256	62		761	52	

(Continued)

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TABLE 4 Continued

	RIA group (n=1564)		P-value	ELISA group (n=845)		P-value
	Subjects without NCCAH (n=1492)	Subjects with NCCAH (n=74)		Subjects without NCCAH (n=782)	Subjects with NCCAH (n=63)	
LH to FSH ratio						
Mean ± SD	1.8 ± 1.6	1.0 ± 0.70	<0.001	1.4 ± 1.1	1.1 ± 0.9	0.09
(n)	1234	61		759	52	
Prolactin, mIU/L						
Mean ± SD	312.0 ± 251.4	265.9 ± 134.6	0.18	281.3 ± 186.0	268.1 ± 141.0	0.64
Median (range)	251.0 (18.0-3578.0)	252.0 (58.0-584.0)		228.0 (16.0-1999.0)	226.0 (119.0-792.0)	
(n)	1143	55		687	44	
TSH, mIU/L						
Mean ± SD	2.4 ± 1.7	2.4 ± 1.3	0.89	2.2 ± 1.4	2.5 ± 1.6	0.25
Median (range)	2.0 (0.03-31.88)	2.1 (0.5-6.9)		2.0 (0.03-23.1)	2.0 (0.7-11.2)	
(n)	1347	65		814	57	

Continuous parameters are shown as mean ± SD and median (range), and categorical variables as n (%).
17OHP, 17-hydroxyprogesterone; BMI, body mass index; CST, cosyntropin stimulation test; DHEAS, dehydroepiandrosterone sulfate; FSH, follicle stimulating hormone; LH, luteinizing hormone; NCCAH, non-classic congenital adrenal hyperplasia; TSH, thyroid stimulating hormone.

several studies, most of which used RIA assays (2). Maffazoli et al. showed that 6.2% of women with NCCAH could have been missed using the classical threshold and Bidet et al. reported that 8% of NCCAH women had basal 17OHP levels < 6 nmol/L (6, 7). Escobar-Morreale et al. suggested a lower basal 17OHP level (5.1 nmol/L) to improve the screening sensitivity for NCCAH diagnosis in a cohort of women with hyperandrogenism (9).

Nonetheless, in light of the widespread use of immunoassays and the replacement of RIA by the ELISA 17OHP assay, it is pertinent to check whether the guideline-recommended basal 17OHP cutoff applies. This is the first sizable study gleaning data from a large cohort of women with hyperandrogenism and comparing data between the old RIA and the new ELISA method.

In the present study, 137/2409 (5.7%) women were diagnosed with NCCAH, defined as a stimulated 17OHP level >30 nmol/L. This figure is similar to previous studies of hyperandrogenic females (16, 17). This implies a significant number of unnecessary and costly CSTs in a real-world setting. Thus, adopting a higher 17OHP cutoff in ELISA assays might reduce the number of unnecessary tests.

TABLE 5 Multivariate analysis for predictors of NCCAH among the RIA group.

Variable	Odds ratio	95% confidence interval	P-value
Age, y	1.02	0.96-1.08	0.61
History of oligomenorrhea	0.08	0.01-0.69	0.021
Basal 17OHP level*, nmol/L	1.45	1.29-1.63	<0.001
Total testosterone, nmol/L	1.09	0.72-1.63	0.69
Androstenedione, ng/ml	1.06	0.82-1.38	0.65
LH to FSH ratio	0.22	0.08-0.63	0.005

*For 17OHP, the intra-assay coefficient of variation was 9-22% for RIA. The inter-assay coefficient of variation was 16%.
17OHP, 17-hydroxyprogesterone; CST, cosyntropin stimulation test; FSH, follicle stimulating hormone; LH, luteinizing hormone.

TABLE 6 Multivariate analysis for predictors of NCCAH among the ELISA group.

Variable	Odds ratio	95% confidence interval	P-value
Age, y	0.95	0.82-1.10	0.53
History of infertility	3.46	0.36-33.57	0.29
Basal 17OHP level*, nmol/L	1.74	1.41-2.14	<0.001
Total testosterone, nmol/L	2.41	1.21-4.79	0.012
Androstenedione, ng/ml	1.78	1.21-2.62	0.004
LH to FSH ratio	.38	0.14-1.01	0.054

*For 17OHP, the intra-assay coefficient of variation was 13-25% for ELISA. The inter-assay coefficient of variation was 16%.
17OHP, 17-hydroxyprogesterone; CST, cosyntropin stimulation test; FSH, follicle stimulating hormone; LH, luteinizing hormone.

Noteworthy, NCCAH was diagnosed more often in the ELISA (7.5%) than the RIA (4.7%) group. This difference could be explained by the upward shift in 17OHP levels in the ELISA group (Tables 1, 2) which might have led to more false-positive tests, although among subjects diagnosed with NCCAH the basal 17OHP levels did not differ significantly between assays (Table 3). This hypothesis is supported by the Ambroziak et al. study that used an ELISA-based 17OHP assay for the diagnosis of NCCAH among women with hyperandrogenism and observed a considerable probability of false-positive tests when based only on a stimulated 17OHP cutoff value of ≥30 nmol/l (18). They showed that among 21 women with pre- or post-CST 17OHP ≥30 nmol/l, NCCAH was confirmed by genetic testing only in five women, of whom four subjects were heterozygous carriers. They also showed that post-CST ELISA-based 17OHP <30 nmol/l excludes NCCAH with a high degree of confidence (18). In addition, Azziz et al. showed that when post-CST 17OHP values were within the range of 30–45 nmol/l, most patients were heterozygous carriers (19). In the absence of genetic data, we cannot rule out the possibility of more heterozygous carriers (of CYP21A2 monoallelic mutations) in the ELISA group.

The validation study, performed by MHS central laboratory, established that when using ELISA, the absolute 17OHP values are higher (34% on average) than that achieved if the same samples were analyzed using RIA. This raises a question of what effect would employing a 34% higher diagnostic 17OHP cutoff (~ 40 nmol/L) have on our results. Interestingly, applying a higher post-CST 17OHP cutoff of > 40 nmol/L did not affect the observed discordance in the diagnosis rates of NCCAH nor the optimal basal 17OHP thresholds in the RIA and the ELISA groups.

NCCAH diagnosis might be truly more prevalent in the ELISA group. This might be explained by the introduction of guidelines in recent years that led to more precise referrals and testing. The higher androstenedione levels in the ELISA group may remark a higher degree of clinical suspicion that led to more positive CSTs. However, further studies that incorporate genetic data are needed.

We observed that androstenedione levels were higher in the ELISA than the RIA group, while testosterone levels were higher in the RIA. We do not have a clear explanation for these differences; however, as the androstenedione assay was changed in 2012, assays were not identical in the two groups. Consequently, the difference in assays could explain these discrepancies, and the fact that we still have these differences between RIA and ELISA groups in both women with and without NCCAH (Tables 2, 3), supports this assumption. Basal cortisol, TSH and prolactin levels were higher in the RIA than in the ELISA group. However, they did not differ in NCCAH subjects between the two groups (Table 3) and did not differ within each group of RIA and ELISA, between women with and without NCCAH (Table 4). We do not have a decent explanation for that. However, as it is a real-life and not a randomized controlled trial, we cannot exclude the possibility that other unknown factors may have played a role in these differences.

Remarkably, in the RIA group, lower LH: FSH ratio and oligomenorrhea emerged as independent predictors of NCCAH. In the ELISA group, higher androstenedione and total testosterone levels were independent predictors, while lower LH: FSH ratio showed a marginal trend of significance.

Polycystic ovary syndrome (PCOS) is an important differential diagnosis of NCCAH in women and is much more frequent (20). NCCAH and PCOS share several clinical and biochemical features. Basal 17OHP, androstenedione, and testosterone levels might be increased in both NCCAH and PCOS female patients (6). Thus, it is imperative to investigate new biochemical markers rather than 17OHP alone, to better differentiate between these entities. Although elevated LH : FSH ratio (usually >2) is much more prevalent in polycystic ovary syndrome (PCOS) than in NCCAH (21), its discriminatory utility has not been thoroughly addressed.

In our study, a low LH: FSH ratio emerged as an independent predictor of NCCAH in the RIA group and showed a similar trend in the ELISA group. We found that combining basal serum 17OHP below the suggested cutoff with an LH: FSH ratio > 2 increases the NPV for the diagnosis of NCCAH to 100% in both RIA and ELISA groups. To our knowledge, this is the first study to show that the LH : FSH ratio improves the NPV for the diagnosis of NCCAH.

Our study's main strength is the large number of subjects in each group, which enabled several analyses and allowed drawing important implications for clinical practice. We studied females older than 16 years to avoid 17OHP level differences derived from different pubertal development (22).

This study has some limitations which stem from its retrospective design. An unknown confounding may have influenced our results. The results of this study may not be generalizable to users of other assays. We cannot rule out the inclusion of peri- or post-menopausal women in the study population that may have affected the results, although the median age was 22 years. The lack of genetic profiling is another key limitation, as it may have helped better define the NCCAH populations and draw further distinctions between the 17OHP-assay groups. Further studies that incorporate genetic data are mandatory.

In conclusion, our results suggest adopting a higher ELISA-based 17OHP cutoff (8.2 nmol/L) at which patients suspected of NCCAH should be referred for further evaluation. This may help eliminate the number of unnecessary tests. When using RIA, the classical 17OHP cutoff level of 6 nmol/L seems to apply. Utilization of LH : FSH ratio can improve the negative predictive value of the basal 17OHP levels.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Maccabi Healthcare Services (MHS) institutional review board (IRB) and ethics committee. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

AN, LS, and MZ were responsible for the design of the study and data acquisition. AN, LS, and MZ contributed to the analysis and the interpretation of data, drafted the manuscript and revised it critically for important intellectual content. NS, NW, and MS-A contributed to the analysis and the interpretation of data and revised the manuscript critically for important intellectual content. LS-R, SA, and RG contributed to the interpretation of data. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Henrik Falhammar,
Karolinska Institutet (KI), Sweden

REVIEWED BY
Tony Yuen,
Icahn School of Medicine at Mount Sinai, United States
Avinaash Vickram Maharaj,
Queen Mary University of London, United Kingdom

*CORRESPONDENCE
Min Nie
nm_pumch@aliyun.com

†These authors have contributed equally to this work

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The underlying cause of the simple virilizing phenotype in patients with 21-hydroxylase deficiency harboring P31L variant

Zhiyuan Zhao[1†], Yinjie Gao[1†], Lin Lu[1], Anli Tong[1], Shi Chen[1], Wei Zhang[1], Xiaoxia Zhang[1], Bang Sun[1], Xueyan Wu[1], Jiangfeng Mao[1], Xi Wang[1] and Min Nie[1,2*]

[1]Department of Endocrinology, National Health Commission (NHC) Key Laboratory of Endocrinology (Peking Union Medical College Hospital), Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, China, [2]State Key Laboratory of Complex, Severe and Rare Diseases, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, China

Objective: To analyze the relationship between genotype and phenotype in 21-Hydroxylase deficiency patients harboring P31L variant and the underlying mechanism.

Methods: A total of 29 Chinese patients with 21-OHD harboring P31L variant were recruited, and the detailed clinical features of the patients were extracted and analyzed retrospectively. The TA clone combined with sequencing of the region containing the promotor and exon1 of *CYP21A2* was performed to determine whether the variants in promotor and P31L aligned in cis. We further compared the clinical characteristics of 21-OHD patients between the promoter variant group and no promoter variant group.

Results: Among the 29 patients diagnosed with 21-OHD harboring P31L variant, the incidence of classical simple virilizing form was 62.1%. Thirteen patients owned promoter variants (1 homozygote and 12 heterozygote) and all exhibited SV form. The promoter variants and the P31L variant were located in the same mutant allele as validated by TA cloning and sequencing. There were statistically significant differences in clinical phenotype and 17-OHP level between the patients with and without promoter region variations (P<0.05).

Conclusion: There exists high incidence (57.4%) of SV form among the 21-OHD patients harboring P31L variant, and the underlying mechanism is partially due to both the promoter variants and P31L aligning in cis on one allele. Further sequencing of promoter region will provide important hints for the explanation of phenotype in patients harboring P31L.

KEYWORDS

classical simple virilizing, promoter variation, 21- hydroxylase deficiency, P31L, congenital adrenal hyperplasia

Introduction

21-hydroxylase deficiency (21-OHD), ranks among one of the most frequent inborn errors of the adrenal endocrine metabolism following an autosomal recessive trait (1). It is characterized by the impairment of cortisol synthesis with or without aldosterone deficiency, and increased androgen synthesis (1, 2). Based on the clinical manifestations, 21-OHD can be classified into three types: the classical salt-wasting (SW) form (approximately 75% of the classic 21-OHD), classical simple virilizing (SV) form (about 25% of the classic 21-OHD) and nonclassical form (NC-21OHD). The SW form is characterized by life-threatening adrenal crises in the neonatal period accompanied with hyperandrogenemia causing sexual ambiguity in affected females (3). The classical SV form usually exhibited precocious puberty combining with accelerated linear growth velocity, the affected females present virilization of external genitalia (i.e., clitoromegaly) or urogenital sinus in the early postnatal period (4). The NC-21OHD may be asymptomatic or clinically mild in the early stages and tend to display signs or symptoms of androgen excess until preadolescent, adolescent or young adult period, characterized by hirsutism, acne, menstrual disorders, subfertility and recurrent miscarriage (5–8).

The original cause of 21-OHD can be ascribed to the decrease or abrogation of P450C21 enzyme activity, which is encoded by the *CYP21A2* gene. *CYP21A2* is located on chromosome 6p21.3 adjacent to a nonfunctional pseudogene *CYP21A1P*. The *CYP21A2* and *CYP21A1P* genes show a high homology, with a nucleotide identity of 98% in their exon and 96% in their intron sequences (9–11). Approximately 95% of *CYP21A2* pathogenic variants are *CYP21A1P*-derived or large deletions due to non-homologous recombination events in meiotic (3, 12–14).

The clinical phenotype of 21-OHD is usually well correlated with the residual enzyme activity of mutant P450C21 resulted from *CYP21A2* variant (1, 15). Variants leading to 0 to 1% enzymatic activity remaining of mutant P450C21 typically correspond to classical SW 21-OHD, such as 30KB deletions, L308Ffs*6(F308 +T), R357W, E6 Cluster (I237D/V238E/M240K), 8bp deletion (E3Δ8bp, c.332_339del GAGACTAC, p.G111Vfs∗21), Q319* and c.293-13A/C>G(i2g). Variants resulting in nearly 1% to 2% enzyme activity retaining of mutant P450C21 frequently cause the classical SV 21-OHD, as demonstrated by I173N. Mutant P450C21 preserving 20% to 60% enzyme activity (e.g.,P31L, V282L and P454S) usually bring about the NC-21OHD (16). About 65–70% of patients with 21-OHD are compound heterozygous, while the clinical phenotype is generally considered to be determined by the less severely affected allele (4, 14).

However, some discrepancies between genotype/phenotype correlation had been found in 21-OHD patients. For example, P31L mutant P450C21 usually lead to NC-21OHD phenotype due to its retention of more than 50% residual enzyme activity (17), while the patients with P31L variant might present SV phenotype (8, 18). Some investigators showed that it could be the result of the occurrence of both promoter region variants and P31L in the same allele (19). In this study, we mainly focused on the relationship between genotype and phenotype in 21-OHD patients harboring P31L variant and its mechanism to augment our understanding of 21-OHD.

Subjects and methods

Subjects

A total of 29 Chinese patients with 21-OHD harboring P31L variant identified by genetic testing were recruited for this study, who presented to Peking Union Medical College Hospital (Beijing) between 2003 and 2021.

The study was approved by the ethics committee of Peking Union Medical College Hospital (No.JS-2111).

Study design

This was a retrospective study. Detailed medical data pertaining to age, sex, previous medical history (clinical diagnosis and history of vulvar surgery), clinical presentations (hirsutism, acne, menstrual abnormalities, clitoromegaly or labial fusion and precocious pubarche), laboratory data including cortisol(F), testosterone(T), 17α-hydroxyprogesterone (17- OHP) and plasma adrenocorticotropic hormone (ACTH) at baseline (without treatment) or after discontinuation of treatment were extracted and analyzed. The variants of *CYP21A2* were identified.

Laboratory test

Plasma adrenocorticotropic hormone (ACTH) and serum cortisol(F) at 8:00 AM were measured by chemiluminescence immunoassay (Advia Centaur XP, Bayer). Serum testosterone (T) was measured with chemiluminescence (ACS:180; Automatic Chemiluminescence Systems, Bayer). 17α-hydroxyprogesterone (17-OHP) concentrations was determined by radioimmunoassay (Active 17α-OHP Progesterone DSL-5000, DSL). The intra and inter assay coefficients of variation were 5.6% and 6.6% for T, 6.7% and 8.2% for ACTH,5.3% and 5.7% for serum cortisol, 3.9% and 5.6% for 17-OHP, respectively.

Variant analysis of the *CYP21A2* gene

Genomic deoxyribonucleic acid (DNA) from the peripheral blood leukocytes were obtained from all patients using a standard procedure (Omega Blood DNA Midi Kit, Omega Bio-Tek, USA). Multiplex Ligation-dependent probe amplification (MLPA) and PCR combined with sequencing were employed to detect the variants in the region between 700 bp upstream from the start codon ATG of *CYP21A2* gene and the entire *CYP21A2*. The specific primer sequences and PCR amplification methods were described previously (20). The sequencing results were compared with the reference sequence NM_000500.9 of the *CYP21A2* through the NCBI website to determine the variants. MLPA (P050-C1 CAH Kit, MRC Holland) was performed according to the manufacturer's instructions.

TA cloning

The subjects harboring variants both in promoter region and P31L were further sequenced. The fragments containing 5' UTR and exon1 of *CYP21A2* were cloned into pMD19T vector by TA cloning kit (Takara, Bio, Inc.) according to the instruction to determine whether the variants in promoter region were aligned with P31L in cis.

Statistical analysis

All statistical analyses were performed using SPSS (version 26, SPSS Inc., IBM). Shapiro-Wilk test was performed to determine whether the continuous variables conform to normal distribution. The normally distributed quantitative variables were represented as mean ± standard deviation ($\bar{x}$ ± s), and the non-normally quantitative distributed variables were expressed as median (upper and lower quartiles) [M (Q1, Q3)]. Comparison between two groups was performed using independent t-test or Mann–Whitney U test, as appropriate. Categorical variables were expressed as cases (n) and percentages and were compared by the Chi-square test or Fisher exact test. *P* value less than 0.05 was considered statistically significant.

Results

Clinical characteristics of 21-OHD patients harboring P31L

Among the 29 patients diagnosed with 21-OHD harboring P31L variant, 18 patients presented classical SV form, and the remaining 11 patients manifested as nonclassical form. The patients in the SV group were younger than NC group (18.72 ± 6.96 years v.s. 25.55 ± 6.49years, respectively) and with higher serum 17-OHP (58.95 ± 26.20ng/ml v.s. 19.91 ± 16.94ng/ml, respectively). No significant differences were found in other laboratory results (Table 1).

Variants in the promoter of *CYP21A2* in the 21-OHD patients harboring P31L

A total of 13 patients have the variants in promoter region, and 12 patients were compound heterozygote, while one patient was homozygote (Table 2). TA cloning and sequencing showed that the

TABLE 1 Comparison of clinical characteristics of 21-OHD patients harboring P31L between SV and NC group.

Clinical characteristics	SV (n=18)	NC (n=11)	*P*
Age	18.72 ± 6.96	25.55 ± 6.49	0.014
17-OHP	58.95 ± 26.20	19.91 ± 16.94	<0.001
T	2.15 (0.98,3.51)	1.79 (1.27,2.34)	0.637
F	10.22 (5.93,14,64)	10.04 (13.07,17.08)	0.239
ACTH	159.40 (58.80,233.00)	87.20 (49.00,177.00)	0.384

SV, simple virilizing form; NC, non classical form.
17-OHP, 17-Hydroxyprogesterone; ACTH, Adrenocorticotropic hormone; T, Testosterone; F, cortisol.
Normal reference interval:17-OHP: 0.31-2.17(M); 0.10-0.80ng/ml(F); T: 1.75-7.81ng/ml (M), 0.10-0.75ng/ml(F); ACTH: 0-46pg/ml, F:4-22.3ug/dl.

TABLE 2 Genotypes and phenotypes of 29 patients with 21-OHD harboring P31L.

Patients No.	Clinical category	Clinical characteristics	Allele 1	Allele 2
21OHD01	SV	H,M,C	promoter variant, P31L	del
21OHD02	SV	C, CP	promoter variant, P31L	exon1-3 del
21OHD03	SV	H, M, C, A, CP	promoter variant, P31L	Del
21OHD04	SV	H, C, CP	promoter variant, P31L	E3Δ8bp
21OHD05	SV	H, M, C, A	promoter variant, P31L	promoter variant, P31L
21OHD06	SV	C	promoter variant, P31L	I173N
21OHD07	SV	H, C, PP, CP	promoter variant, P31L	I173N
21OHD08	SV	H, M, C, A, CP	promoter variant, P31L	Q319X
21OHD09	SV	C, CP	promoter variant, P31L	I173N
21OHD10	SV	H, M, C, A	promoter variant, P31L	i2g
21OHD11	SV	H, C, CP	promoter variant, P31L	i2g
21OHD12	SV	C, CP	promoter variant, P31L	I173N
21OHD13	SV	H, M, A, CP	promoter variant, P31L	I173N
21OHD14	SV	H, C, PP, CP	P31L	exon4-8 deletion
21OHD15	SV	C	P31L	Del
21OHD16	NC	H, M	P31L	I173N
21OHD17	NC	H, M	P31L	Del
21OHD18	NC	H, M, A	P31L	Del
21OHD19	NC	H, M, A	P31L	i2g
21OHD20	NC	H, M, A	P31L	Del
21OHD21	NC	H, M	P31L	I173N
21OHD22	NC	H, M	P31L	I173N
21OHD23	NC	H, M, A	P31L	I173N
21OHD24	NC	H, M, A	P31L	V282L
21OHD25	NC	H, M, A	P31L	i2g
21OHD26	SV	H, M, C, A	P31L	Del
21OHD27	SV	H, M, C	P31L	I173N
21OHD28	NC	H, M	P31L	I173N
21OHD29	SV	H, M, C	P31L	P31L

SV, the simple virilizing form; H, hirsutism; M, menstrual abnormalities; C, clitoromegaly or labial fusion; A, acne; PP, precocious pubarche; CP, clitoroplasty.
Promoter variants:-4C→T,-103A→G,-110T→C,-113G→A, -126C→T, -198InsT,-201C→T, -212T→C,-284T→G,-286A→G,-297A→C,-298T→C,-310G→C,C.-448InsA.

promoter variants and the P31L variant were located in the same allele, and the detailed promoter variants are listed as follows: -4C→T,-103A→G,-110T→C,-113G→A,-126C→T,-198InsT,-201C→T,-212T→C,-284T→G,-286A→G,-297A→C,-298T→C,-310G→C,-448InsA. The above variants are consistent with the corresponding locus in the *CYP21A1P* gene (Supplementary Figure S1).

Comparison of clinical characteristics of 21-OHD patients between promoter variant group and no promoter variant group

The 29 patients were divided into promoter variant (PV) group and no promoter variant(NPV) group according to whether the promoter variations exist or not.17-OHP level were higher in the PV group than the NPV group. The clinical phenotype of 21-OHD patients in the PV group were quite different from the NPV group ($P<0.05$). All the 13 patients in the PV group were SV form, and 9 of them had undergone clitoroplasty. While most (11/16,68.8%) 21-OHD patients in the NPV group showed NC-21OHD form and the 5 other patients SV form. No statistical difference was noted in the other laboratory results and residual enzyme activity of the variant on the other allele (other than P31L located allele) (Table 3).

Discussion

This study investigated the relation of genotype and phenotype and the occurrence of promoter variants among 21-OHD patients harboring P31L variant in a single medical center. Our findings demonstrated that the incidence of SV was 57.4% in 21-OHD patients harboring P31L variant, of which 84.6% patients were

TABLE 3 Comparison of clinical characteristics of 21-OHD patients with both promoter variation and P31L variation versus P31L variation alone.

Clinical characteristics		PV (n=13)	NPV (n=16)	*P*
Age		23.20 ± 5.81	25.92 ± 5.55	0.570
17OHP		64.63 (52.99,72.86)	18.71 (8.72,53.35)	0.014
T		1.01 (0.85,2.74)	1.99 (1.40,3.37)	0.079
F		10.11 ± 3.06	13.87 ± 4.85	0.244
ACTH		177.00 (48.23,240.00)	101.60 (56.63,175.75)	0.865
Cases of patients undergoing clitoroplasty(%)		9 (69.23%)	1 (6.25%)	0.001
clinical phenotype	NC	0	11	<0.001
	SV	13	5	
residual enzyme activity of the mutation on the other alelle (other than P31L)	0-1%	7	8	1.000
	1%-2%	5	6	
	20%-60%	1	2	

PV, promoter variant group; NPV, no promoter variant group.
SV, simple virilizing form; NC, non classical form; 17-OHP, 17-Hydroxyprogesterone; ACTH, Adrenocorticotropic hormone; T, Testosterone; F, cortisol.
Normal reference interval:17-OHP: 0.31-2.17(M);0.10-0.80ng/ml(F);T:1.75-7.81ng/ml(M), 0.10-0.75ng/ml(F);ACTH: 0-46pg/ml, F:4-22.3ug/dl.

caused by promoter variants besides P31L on the same allele of *CYP21A2*.

In recessive disorders, the clinical phenotype of 21-OHD is usually determined by the activity of the milder variant in compound heterozygotes. According to the *in vitro* studies, residual enzyme activity of the P31L mutant usually leads to a relatively mild NC-21OHD (21, 22). In this study, the ratio of the classical SV to the NC-21OHD female patients harboring P31L variants was 18:11. Another study from Argentina found that the ratio of the SV form to the NC-21OHD patients with P31L variants was 1:1 (23). Other studies have found that in different 21-OHD patient series harboring P31L variants, the incidence of SV clinical phenotype ranged from 20% to 100% (4, 22, 24–29). As described in previous literature (30), comparing with other variants causing NC-21OHD, the patients affected by P31L tended to have more severe clinical phenotype and even exhibit the SV form. Our study showed the similar results, which strongly indicates that there also existed the genotype/phenotype discrepancies among the Chinese 21-OHD patients harboring P31L. For 21-OHD patients harboring P31L variants, additional attention should be paid to whether more severe clinical phenotype exists or not, so as to provide important basis for precise clinical intervention.

In this study, we found that all the 21-OHD patients with promoter variants in cis with the P31L variant presented SV phenotype. These results suggest that, in addition to the impairment of P450C21 protein activity by the P31L variant, promoter variants may further affect the function of the enzyme, and some evidence supported it. It was reported that c.-113G>A variant of *CYP21A2* could reduce the basal transcriptional activity to 20% of *CYP21A2* (31), and the c.-126C>T could decrease the transcriptional activity of *CYP21A2* to 52% (32). The transcriptional activity of variants c.-126C > T, c.-113G > A, c.-110T > C and c.-103T > C in the promoter is reduced to 20% of the wild type and correlated with the SV 21-OHD (20, 33, 34). Our TA clone sequencing results demonstrated that *CYP21A2* promoter variants within c.-500bp to c.-1bp upstream of the ATG occurred in all thirteen patients, including c.-448InsA, c.-310G>C, c.-298T>C, c.-297A>C, c.-286A>G, c.-284T>G, c.-212T>C, c.-201C>T, c.-198InsT, c.-126C>T, c.-113G>A, c.-110T>C, c.-103A>G, c.-4C>T, along with c.91C>T (P31L), are consistent with the corresponding locus of *CYP21A1P*, implying that the gene conversion from the *CYP21A1P* to the *CYP21A2*. For 21-OHD patients tested out P31L variant, further sequencing of genetic locus in the promoter region are needed to obtain the comprehensive and complete molecular diagnosis results, thus providing important basis for subsequent precise clinical treatment and reliable genetic risk assessment.

In our study, the promoter region variation might account for 72.2% of all the classical SV form 21-OHD patients harboring P31L, which is similar to the previous results (19, 23, 34) (Table 4), and their general occurrence of promoter variant in SV 21-OHD patients harboring P31L were 84.6%. Our study demonstrated that 5 patients in the NPV group also showed the clinical phenotype of SV form. This implied that other reasons (besides promoter variants) could account for the more severe clinical phenotype of 21-OHD patients haboring P31L. The following three mechanisms were reported. First, *CYP3A7* gene and its transcriptional regulator constitutive androstane receptor (CAR) might be involved in fetal virilization in female 21-OHD. Specifically, the CAR variant could account for a higher degree of external genitalia virilization (35). Second, CAG repetition in the exon 1 of androgen receptor gene matters and SV 21-OHD patients tended to have fewer CAG repeats (36). Third, the alternative pathway of androgen biosynthesis during embryonic development and corresponding proteins (SRD5A1, AKR1C1/3, HSD17B6, etc.) may be involved in fetal virilization in females, through which the 17-OHP accumulated in the 21-OHD may be

TABLE 4 The summary of incidence of SV phenotype and promoter variants in 21-OHD patients harboring P31L.

The number of patients	SV incidence (No.)	Detection rate* (No.)	Reference
15	40% (6)	100% (6)	(23)
15	80% (12)	100% (12)	(34)
9	33.3% (3)	66.7% (2)	(19)
29	62.1% (18)	72.2% (13)	Present study
Total (68) #	57.4%(39)	84.6% (33)	–

*Detection rate of promoter region in SV patients.
The total number of 21-OHD patients harboring P31L undergoing sequence of promoter region.

converted to the androgen and thus aggravate female virilization (37).

Limitations

There are some limitations to this research. This was a retrospective study, hence, the medical information, including the neonatal virilization of external genitalia, might have been affected by recall bias. This might have resulted in an underestimation of the incidence of SV form among the 21-OHD patients harboring P31L variant.

Conclusion

There exists high incidence (57.4%) of SV form among the 21-OHD patients harboring P31L variant, and the underlying mechanism is partially due to both the promoter variants and P31L aligning in cis on one allele. Further sequencing of promoter region will provide important hints for the explanation of phenotype in patients harboring P31L.

Data availability statement

The original contributions presented in the study are included in the article/supplementary files, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by ethics committee of Peking Union Medical College Hospital (No.JS-2111). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

MN conceived the project, designed the experiments. LL, AT, SC, XiW, JM and XuW collected the clinical data, YG, WZ, BS and ZZ performed the experiments. MN and ZZ analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1015773/full#supplementary-material

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EDITED BY
Semra Çaglar Çetinkaya,
University of Health Sciences, Türkiye

REVIEWED BY
Sifeng Qu,
Shandong University, China
Tony Yuen,
Icahn School of Medicine at Mount Sinai, United States

*CORRESPONDENCE
Jun Jiang
jiangjun_64@163.com
Weihua Lan
doclan@yeah.net

†These authors have contributed equally to this work and share first authorship

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Genotype-phenotype correlation in patients with 21-hydroxylase deficiency

Peng Tang[1†], Jun Zhang[1†], Song Peng[1], Yapeng Wang[1], Haoyang Li[2], Ze Wang[1], Yao Zhang[1], Yiqiang Huang[1], Jing Xu[1], Dianzheng Zhang[3], Qiuli Liu[1], Luofu Wang[1], Weihua Lan[1*] and Jun Jiang[1*]

[1]Department of Urology, Daping Hospital, Army Medical University, Chongqing, China, [2]Fifteen Squadron Five Brigade, School of Basic Medical Science, Army Medical University, Chongqing, China, [3]Department of Bio-Medical Sciences, Philadelphia College of Osteopathic Medicine, Philadelphia, PA, United States

Introduction: 21-hydroxylase deficiency (21OHD) is the most common cause of congenital adrenal hyperplasia (CAH). However, patients with 21OHD manifest various phenotypes due to a wide-spectrum residual enzyme activity of different CYP21A2 mutations.

Methods: A total of 15 individuals from three unrelated families were included in this study. Target Capture-Based Deep Sequencing and Restriction Fragment Length Polymorphism was conducted on peripheral blood DNA of the three probands to identify potential mutations/deletions in CYP21A2; Sanger sequencing was conducted with the DNA from the family members of the probands.

Results: Dramatically different phenotypes were seen in the three probands of CAH with different compound heterozygous mutations in CYP21A2. Proband 1 manifested simple virilizing with mutations of 30-kb deletion/c.[188A>T;518T>A], the latter is a novel double mutants classified as SV associated mutation. Although both probands carry the same compound mutations [293-13C>G]:[518T>A], gonadal dysfunction and giant bilateral adrenal myelolipoma were diagnosed for proband 2 and proband 3, respectively.

Conclusion: Both gender and mutations contribute to the phenotypes, and patients with the same compound mutations and gender could present with different phenotypes. Genetic analysis could help the etiologic diagnosis, especially for atypical 21OHD patients.

KEYWORDS

congenital adrenal hyperplasia, 21OHD, genotype, phenotype, CYP21A2

Abbreviations: CAH, congenital adrenal hyperplasia; CYP, cytochrome P450; 21OH, 21-hydroxylase; SW, salt-wasting; SV simple virilizing; NC, non-classic; DHEA, dehydroepiandrosterone; IHH, idiopathic hypogonadotropic hypogonadism; 17-OHP, 17-hydroxyprogesterone; GnRH, Gonadotropin-releasing hormone; DHT, dihydrotestosterone; 17-OH, 17-hydroxycorticosteroid; 17-KS, 17-ketosteroid; HPG, hypothalamic pituitary gonadal; TART, testicular adrenal rest tumor; DSD, differences of sex development.

1 Introduction

Most congenital adrenal hyperplasia (CAH) resulted from deficiencies of the enzymes, encoded by different cytochrome P450 (CYP) genes, in adrenal steroidogenesis (1). In humans, six CYPs including *CYP11A1*, *CYP11B1*, *CYP11B2*, *CYP17A1*, *CYP19A1*, and *CYP21A2* are involved in the synthesis of steroid hormones and deficiencies in any of them can cause CAH. Of note, ~95% of CAH patients have a deficient 21-hydroxylase (21OH) due to the mutation of *CYP21A2*. Since adrenocorticotropic hormone (ACTH) is partially downregulated by glucocorticoid and mineralocorticoid through a feed-back mechanism, reduced levels of glucocorticoid and mineralocorticoid are accompanied by elevated level of ACTH, which in turn results in not only accumulation of precursors in adrenal androgenesis but also exacerbates CAH (2). However, a wide spectrum of clinical manifestations was observed in patients with different mutations of their CYPs.

Patients with 21OHD can be categorized into three subgroups based on their clinical phenotypes: classic salt-wasting (SW), classic simple virilizing (SV), and the non-classic (NC) forms (3). SW is most severe and could be life-threatening if occurring in early infancy. Classic SV is characterized by prenatal virilization in females and virilization in both sexes without salt wasting (4). NC usually shows mild symptoms of androgen excess and sometimes is even asymptomatic (5). The diverse phenotypes of 21OH-caused CAH are the results of varying degrees of enzymatic deficiency. For instance, Wilson RC et al. found that patients with mutations causing complete loss of enzymatic activity presented with more severe phenotype, whereas patients with partial loss of enzymatic activity exhibited non-classical CAH (6). Genotype-phenotype correlations in CAH have been reported previously (7, 8). In this study, we found that some phenotype is associated certain *CYP21A2* mutation, and sometimes the same compound heterozygous mutation of *CYP21A2*, can lead to different phenotypes. These specific cases add to our knowledge of genotype-phenotype correlations of *CYP21A2*.

2 Materials and methods

2.1 Patients

This study design was reviewed and approved by the ethics committee in the Daping Hospital of Army Medical University. A total of 13 individuals from three unrelated families with their medical records including computed tomography (CT) or magnetic resonance imaging (MRI) scan, and laboratory examinations as well as their family history were included in this study. All procedures were carried out in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written informed consent for the use of medical records and related images was obtained from each patient.

2.2 PCR-based restriction fragment length polymorphism

Total DNA isolated from the peripheral blood leukocytes were obtained from the 15 individuals and used for sequencing to identify potential mutations and large deletions in CYP21A2 gene. To identify large gene deletions, primers CYP779f and Tena32F (available upon request) were used to amplify a fragment of 8515-bp followed the manufacturer's recommendations. The 8.5-kb PCR product was digested with TaqI restriction endonuclease and the digested products were analyzed by electrophoresis in agarose gels.

2.3 DNA sequencing and analyses

Target Capture-Based Deep Sequencing (AmCare Genomic Laboratory, Guangzhou, Guangdong, China) was conducted with the PCR product amplified by CYP779f and Tena32F. The sequencing was carried out on NGS platform (HiSeq X system; Illumina) with PEx150 read length according to the manufactory's instructions. Bioinformatics analyses were performed with in-house pipeline to identify rare or novel variants of CYP21A2 with Gnomad, HMGD, ClinVar, dbSNP for filtering and computational prediction algorithms. Software PolyPhen-2, SIFT, and MutationTaster were used for pathogenesis evaluation (Supplementary Table 1).

After the identification of the variants in the probands, Sanger sequencing were conducted to determine family co-segregation. The corresponding primers were designed by Primer 3.0 and available upon request. The final pathogenicity of the variants was estimated using the American College of Medical Genetics and Genomics guidelines.

3 Results

3.1 The probands

Proband 1 was an 8-year-old girl who was diagnosed as congenital adrenal hyperplasia when she was born with an atypical genitalia but no salt-wasting symptom in another hospital. She underwent clitoroplasty in that hospital at the age of 8 months and glucocorticoid and mineralocorticoid replacement therapy was prescribed. Laboratory investigations revealed elevated levels 17-hydroxyprogesterone (17-OHP, >75.75 nmol/L, normal 0.2–3.0 nmol/L) androgens, testosterone, and ACTH as shown in Table 1. Her karyotype is 46, XX.

She was referred to our hospital due to virilization and presented with high stature (140 cm tall, >97th percentile of girls of Han nationality in the same age group in China) and deep voice. Physical examination identified clitoral enlargement, partially fused labia majora, and a urogenital sinus in place of separate urethral and vaginal openings. She was taking hydrocortisone (20 mg per two days) before admission to our hospital. We noticed that based on her weight of 41 kg, she should be treated with 12.7-19.1 mg in a day

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TABLE 1 Laboratory parameters of the probands.

	Reference range	Proband 1 (female)		Proband 2 (male)		Proband 3 (male)
		After birth	During readmission	On admission	After hormone supplement	
17-OHP (nmol/L)	0.2–3.0	>75.75	–	485.20	–	–
DHEA (ng/mL)	1.70-6.10	–	–	7.99	–	–
Testosterone (ng/mL)	male:1.75-7.81 female: 0-0.75	3.41	7.60	4.05	1.86	–
DHT (pg/mL)	250.0-990.0	–	–	1777.99	–	–
Androstenedione (ng/mL)	0.25–1.21	>10	–	–	–	–
ACTH (pg/mL)	4.7-48.8	–	1131.00	136.0	–	–
Cortisol (nmol/L)	185-624	–	199.6	212	–	–
Aldosterone (ng/dL)	Supine:3.0–23.6/ Standing:3.0–35.3	–	3.45/2.95	14.9/11.4	–	–
FSH (mIU/mL)	1-8	3.17	0.23	0.25	6.40	–
LH (mIU/mL)	1.24-8.62	0.25	<0.20	<0.20	3.18	–
Na^+ (mmol/L)	137.0-147.0	–	140.9	140.3	–	141.2
K^+ (mmol/L)	3.5-5.3	–	4.08	3.98	–	4.52
17-OH (umol/24H)	8.3-33.2	–	–	–	–	38.5
17-KS (umol/24H)	20.8-76.3	–	–	–	–	98.8

17-OHP, 17-hydroxyprogesterone; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; ACTH, adrenocorticotropic hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; 17-OH, 17-hydroxycorticosteroid; 17-KS, 17-ketosteroid.

according to the guideline (9). Laboratory examination revealed a markedly elevated testosterone level of 7.60 ng/mL and CT identified hyperplasia of bilateral adrenal gland, and the cavernous structure in the perineum as shown in Figure 1. She was advised to maintain her regular replacement therapy for now and will have genitoplastic surgery and vaginoplasty when she becomes an adult. None of her parents has any related symptom.

Proband 2 was a 37-year-old male referred to our hospital due to the azoospermia, without other symptoms. He had been diagnosed as idiopathic hypogonadotropic hypogonadism (IHH) based on small testes and significantly decreased serum levels of FSH (0.07 mIU/mL) and LH (0.00 mIU/mL, Table 1) but without salt wasting. The patient had sex premature with masculinization and precocious pubarche when he was only 7 years old. He had a short stature (149 cm in height), small testis (left: 11 mL; right: 12 mL), and mild hypospadias. CT scan revealed left adrenal hyperplasia and right adrenal adenomatous hyperplasia (4.3*3.7 cm, Figure 2). Scrotal ultrasound detected an 8 mm*6mm mass in his left testis, likely to be testicular adrenal rest tumors (TARTs). Laboratory tests showed elevated serum 17OHP (485.20 nmol/L, normal 0.2–3.0 nmol/L),

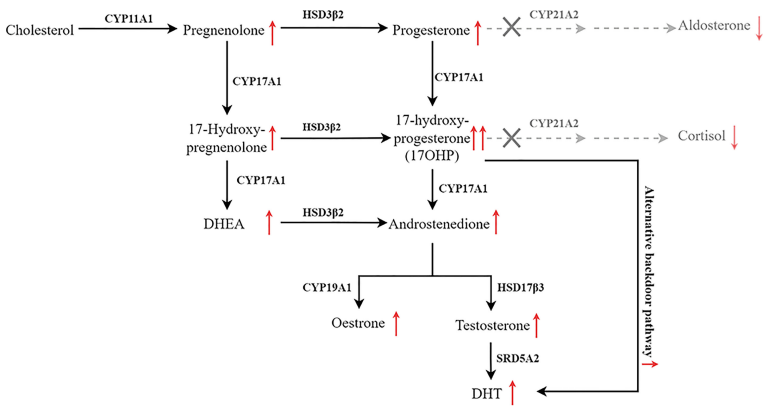

FIGURE 1
Pathway of steroid biosynthesis in the adrenal cortex. 21-hydroxylase deficiency results in decreased cortisol and aldosterone, and elevated cortisol precursors and adrenal androgens. The grey denotes deficient pathways.

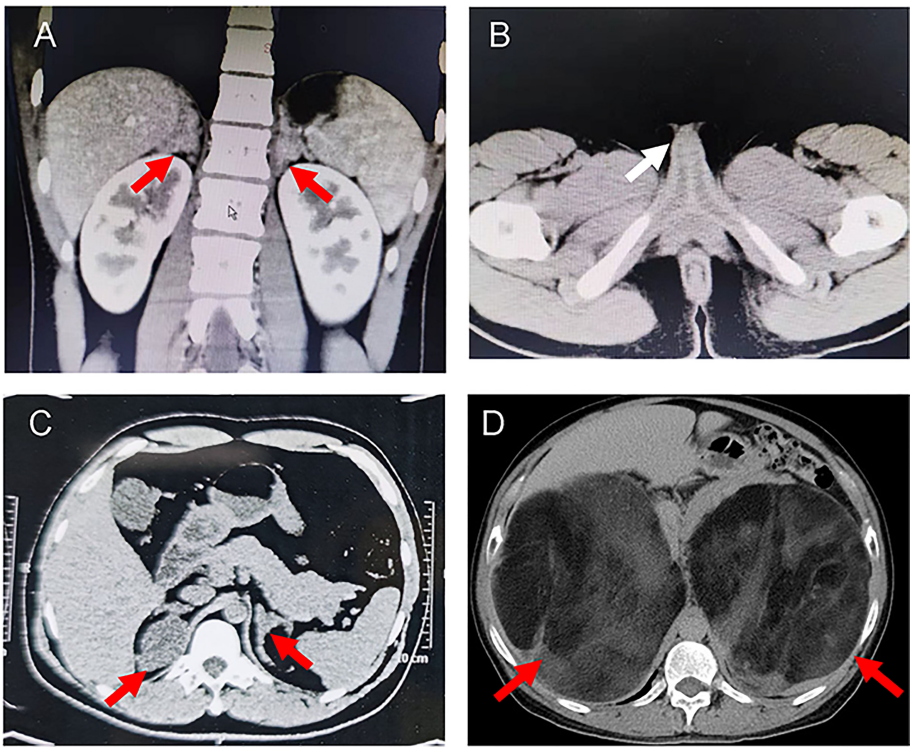

FIGURE 2
Representative computed tomography (CT) scans of the probands. **(A)** CT images showing bilateral adrenal hyperplasia in the proband 1. **(B)** Pelvic CT image of proband 1 indicated the cavernous structure in the perineum (white arrow). **(C)** CT images showing left adrenal hyperplasia and right adrenal adenomatous hyperplasia (4.3*3.7 cm) in the proband 2. **(D)** CT image showing giant bilateral adrenal masses (left: 20*25 cm; right: 30*40 cm) in the proband 3. The red arrows indicate the adrenal lesions.

dehydroepiandrosterone (DHEA) (7.99ng/mL, normal 1.70-6.10), and dihydrotestosterone (DHT) (1777.99 pg/mL, normal 250.0-990.0, Table 1). Gonadotropin-releasing hormone (GnRH) stimulation test was negative (Supplementary Table 2). We concluded that he has congenital adrenal hyperplasia and secondary hypogonadotropic hypogonadism. After eight months of replacement therapy, his semen count was relatively normal (Supplementary Table 3). His parents were normal.

Proband 3 was a 59-year-old male admitted to our hospital with marked abdominal distension. CT scan revealed giant bilateral myelolipoma (left: 20*25 cm; right: 30*40 cm; Figure 2). His 24-hour urine 17-hydroxycorticosteroid (17-OH) and 17-ketosteroid (17-KS) were a bit higher (Table 1). The proband underwent resection of his myelolipoma accompanied with glucocorticoid and mineralocorticoid replacement therapy. Patient history indicates that he had a short stature at birth but no salt wasting. Both his parents died with unknown causes. His brother also underwent resection of bilateral giant myelolipomas at age of 50. But his three sisters and three daughters appear to be normal.

3.2 Genetic mutations

Proband 1 inherited a 30kb deletion (a classic category of chimeric *CYP21A1P*/*CYP21A2* genes: CH-2, which carries common mutations including: c.92C>T, p.P31L; c.188A>T, p.H63L; c.293-13C>G; c.332_339del, p.G111Vfs*21; c.518T>A, p.I173N) (10) and a double mutants *in cis* (c.[188A>T;518T>A], p.[H63L;(I173N)]) (Figure 3). Both proband 2 and 3 have a compound heterozygous mutation of *CYP21A2* (c.293-13C>G and c.518T>A, p.I173N, Figure 3), the detailed pedigree for family of proband 3 has been previously reported (11). We found that proband 2 inherited c.293-13C>G from his mother and c.518T>A, p.I173N from his father. However, we cannot determine his genetic heritage since both his parents are dead. His brother harbored the same compound mutations. Each of the other family members of the three probands harbored a monoallelic *CYP21A2* variant. The genotypes and phenotypes of the individuals were summarized in Table 2 and Supplementary Table 4.

4 Discussion

CAH is an autosomal recessive disease caused by deficient enzymes including 21OH, 11β-hydroxylase, 17α-hydroxylase, 3β-hydroxysteroid dehydrogenase type 2, steroidogenic acute regulatory protein, and P450 oxidoreductase (12). 21OHD-caused CAH is found 1 in every 10 000 to 20 000 new born (13) with diverse phenotype. Complete inactive *CYP21A2* results in deficiencies of both glucocorticoid and mineralocorticoid, and severe adrenal-derived androgen excess. In newborn females, excess androgen is clinically evident due to virilization of their external genitalia. However, males with excess androgen appear to be normal at birth but with premature growth of pubic and axillary hair, oily skin, rapid somatic and skeletal

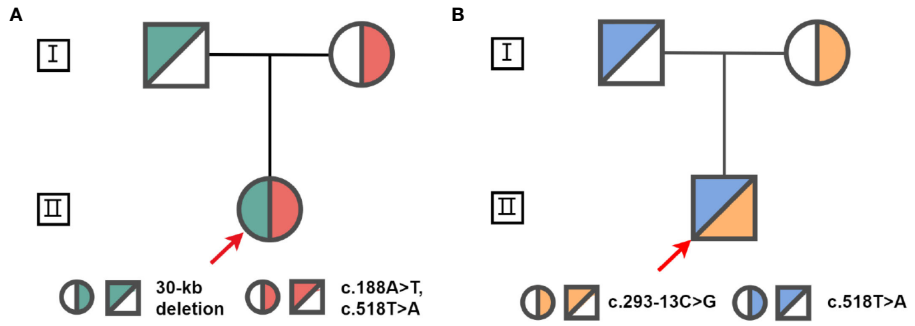

FIGURE 3
Pedigrees for families of proband 1 **(A)** and s proband 2 **(B)**. The red arrow indicates the probands.

maturation. Proband 1 described in this study is a girl and diagnosed after birth, who carried compound mutations with the 30-kb deletion inherited from her father and c.[188A>T;518T>A], a novel double mutants *in cis* (14), from her mother. The c.188A>T mutation has a mild effect on the enzyme; whilst the c.518T>A affects the enzymatic activity more dramatically (15, 16). The double mutants in the young girl (proband 1) appears to be associated with simple virilization according to her clinical presentations. On the other hand, both probands 2 and 3 in this study are male and their azoospermia and giant bilateral adrenal myelolipomas were not recognized until much later. Thus, the gender could be one of the main factor that contributes to the diverse phenotype (1, 17).

Findings from previous studies indicate that phenotypes of 21OHD-caused CAH maybe mutation site-specific. When the genotype-phenotype correlation was examined with more than 230 mutations (18), 70–75% of the CAH cases were caused by the ten most common variants (7, 19, 20). The p.V282L, one of the ten most common variants, accounts for 0.9% cases in Chinese people, compared with 26.2% and 23.9% in Argentinian and other heterogeneous western population respectively (21). In this study, we found that proband 2 and 3 have the same compound mutations [293-13C>G]:[518T>A] but totally different clinical manifestations. Previous study reported patients with this compound heterozygous mutation shows phenotypic variability (NC : SV:SW = 1:36:13) (7). In our report, proband 2 presented with azoospermia mainly due to secondary gonadal dysfunction. The proband has been initially misdiagnosed as IHH, a rare type of congenital disease characterized with dysfunction in the secretion of hypothalamic GnRH and reduced serum levels of sex steroids (22). In fact, the lower normal range of testosterone with suppressed gonadotropins is also the typical profile of secondary hypogonadotropic hypogonadism (23). Previous studies have reported that the accumulation of excessive progesterone, 17OHP, estrogen, and androgens jointly contribute to hypothalamic-pituitary -gonadal (HPG) axis suppression and the secondary gonadal failure (23–25). Based on the elevated levels of 17OHP, DHT, and DHEA, proband 2 is

TABLE 2 The genotype-phenotype of the probands.

Patients	1	2	3
Genotype	c.[188A>T;518T>A], 30-kb deletion	c.293-13C>G, c.518T>A	c.293-13C>G, c.518T>A
Sex	Female	Male	Male
Age (year)	8	37	59
Virilization	+	–	–
Dehydration	–	–	–
Short stature	–	+	+
Premature growth of pubic hair	–	+	–
Fertility	N/A	–	+
Appearance of genital	Clitoral hypertrophy	Small testes	Almost normal
TART or OART	–	+	–
Adrenal hyperplasia	Bilateral hyperplasia	Bilateral hyperplasia	Bilateral myelolipomas
17-OHP (nmol/L)	>75.75	485.20	N/A

17-OHP, 17-hydroxyprogesterone; TART, testicular adrenal rest tumor; OART, ovarian adrenal rest tumor; N/A, not applicable. +, with the symptom; -, without the symptom.

diagnosed as CAH and nearly full recover of semen count after glucocorticoid supplementary therapy further confirmed his gonadal dysfunction is secondary to hypogonadotropic hypogonadism. In addition, the testicular mass might be TART, since it is well known that upregulated ACTH secretion causes TARTs (25). Previous study has shown that hypogonadism and azoospermia could be associated with bilateral TARTs (26). Noteworthy, proband 2 had relatively high levels of DHT, which might be due to the activation of alternative backdoor pathway as described previously (27). Although carrying the same compound mutations, proband 3 presented with giant bilateral adrenal myelolipomas, which are caused by chronic ACTH hyperstimulation (28). One of the potential explanations is that different c.293-13C>G were transcribed in these patients (29).

Glucocorticoid replacement is a life-long treatment and the options for glucocorticoid are hydrocortisone or long-acting synthetic glucocorticoid (2). To avoid suppressive effect of growth and chronic cushingoid complication, hydrocortisone is usually prescribed for children (30). The long-acting synthetic glucocorticoid is generally recommended for adolescents and adults (31). Although hydrocortisone was prescribed for proband 1, virilization has developed progressively. This could be the result of insufficient dosage and/or unresponsive surveillance (9). Intensified glucocorticoid treatment is the first choice for gonadal dysfunction (24). For proband 2, normal level of FSH after one month dexamethasone supplement suggests the rehabilitation of HPG axis. After eight months of treatment his sperm production became relatively normal. In addition, differences of sex development (DSD) often occur in females with classic 21OHD. Decision for sex mainly depends on karyotype and the degree of virilization. Timely diagnosis is the key for early treatment of CAH and the ratio between the intermediates and the hormones is the mainstay for the diagnosis. Since elevated 17OHP is a typical indicator of 21OHD-associated CAH (32), the levels of 17OHP should be monitored closely for individuals showing evidence of androgen excess.

In conclusion, we described three probands with diverse phenotypes of CAH carrying different compound heterozygous mutations of *CYP21A2*. The novel double mutants (c.[188A>T;518T>A]) is a SV subtype. In addition to be consistent with that genders and different mutations contribute to various phenotypes, we found that diverse phenotypes could be presented in patients with identical compound mutations in the same gender. Moreover, genetic analysis could help the etiologic diagnosis, especially for atypical 21OHD patients. These specific cases add to our knowledge of genotype-phenotype correlations of *CYP21A2*.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee in the Daping Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Study design were conducted by JJ, WL, and JZ. Samples and clinical data were collected by YH, LW, YZ, JX, and YW. Analysis of data were performed by PT, SP, ZW, YZ, and HL. DZ, QL, JZ and PT wrote the manuscript which was revised and approved by all authors.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1095719/full#supplementary-material

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Semra Çaglar Çetinkaya,
University of Health Sciences, Türkiye

REVIEWED BY
Domenico Corica,
University of Messina, Italy
Marek Niedziela,
Poznan University of Medical Sciences, Poland

*CORRESPONDENCE
Urh Groselj
urh.groselj@kclj.si
Zuzana Pribilincova
zpribilincova@gmail.com

†These authors share senior authorship

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Genetic and clinical characteristics including occurrence of testicular adrenal rest tumors in Slovak and Slovenian patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency

Robert Saho[1,2], Vita Dolzan[3], Mojca Zerjav Tansek[4,5], Andrea Pastorakova[6], Robert Petrovic[6], Maria Knapkova[7], Katarina Trebusak Podkrajsek[3,8], Jasna Suput Omladic[4,5], Sara Bertok[5], Magdalena Avbelj Stefanija[4,5], Primoz Kotnik[4,5], Tadej Battelino[4,5], Zuzana Pribilincova[9*†] and Urh Groselj[4,5*†]

[1]Department of Pediatrics and Inherited Metabolic Disorders, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czechia, [2]Faculty of Medicine, Comenius University, Bratislava, Slovakia, [3]Faculty of Medicine, Institute of Biochemistry and Molecular Genetics, University of Ljubljana, Ljubljana, Slovenia, [4]Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia, [5]Department of Endocrinology, Diabetes, and Metabolic Diseases, University Children's Hospital, University Medical Centre Ljubljana, Ljubljana, Slovenia, [6]Genetics and Clinical Genetics, Faculty of Medicine, Institute of Medical Biology, Comenius University, Bratislava, Slovakia, [7]Neonatal Screening Centre (NSC) of SR Banská Bystrica, Children University Hospital (CHUH), Banská Bystrica, Slovakia, [8]Clinical Institute for Special Laboratory Diagnostics, University Children's Hospital, University Medical Centre Ljubljana, Ljubljana, Slovenia, [9]Department of Pediatrics, Faculty of Medicine, National Institute of Children's Diseases, Comenius University in Bratislava, Bratislava, Slovakia

Objective: To analyze the mutational spectrum, clinical characteristics, genotype–phenotype correlations, testicular adrenal rests tumor prevalence, and role of neonatal screening in congenital adrenal hyperplasia (CAH) patients from Slovakia and Slovenia.

Design and methods: Data were obtained from 104 patients with CAH registered in Slovak and Slovenian databases. Low-resolution genotyping was performed to detect the most common point mutations. To detect deletions, conversions, point mutations, or other sequence changes in the *CYP21A2* gene, high-resolution genotyping was performed. Genotypes were classified according to residual 21-hydroxylase activity (null, A, B, C).

Results: 64% of the individuals had the salt-wasting form (SW-CAH), 15% the simple virilizing form (SV-CAH), and 21% the non-classic (NC-CAH). *CYP21A2* gene deletion/conversion and c.293-13A/C>G pathogenic variant accounted together for 55.5% of the affected alleles. In SV-CAH p.Ile172Asn was the most common pathogenic variant (28.13%), while in NC-CAH p.Val282Leu (33.33%), *CYP21A2* gene deletion/conversion (21.43%), c.293-13A/C>G (14.29%), Pro30Leu (11.90%). The frequency of alleles with multiple pathogenic variants was higher in

Slovenian patients (15.83% of all alleles). Severe genotypes (0 and A) correlated well with the expected phenotype (SW in 94.74% and 97.3%), while less severe genotypes (B and C) correlated weaklier (SV in 50% and NC in 70.8%). The median age of SW-CAH patients at the time of diagnosis was 6 days in Slovakia vs. 28.5 days in Slovenia (p=0.01). Most of the Slovak patients in the cohort were detected by NBS. (24 out of 29). TARTs were identified in 7 out of 24 male patients, of whom all (100%) had SW-CAH and all had poor hormonal control. The median age at the diagnosis of TARTs was 13 years.

Conclusion: The study confirmed the importance of neonatal screening, especially in the speed of diagnosis of severe forms of CAH. The prediction of the 21-OH deficiency phenotype was reasonably good in the case of severe pathogenic variants, but less reliable in the case of milder pathogenic variants, which is consistent compared to data from other populations. Screening for TARTs should be realized in all male patients with CAH, since there is possible remission when identified early.

KEYWORDS

congenital adrenal hyperplasia, CAH, CYP21A2, genotype-phenotype, 21 hydroxylase deficiency, 21-OH deficiency, newborn screening, testicular adrenal rest tumors (TART)

1 Introduction

Congenital adrenal hyperplasia (CAH; incidence 1:14000–18000) is an autosomal recessive disorder, mostly caused by a 21-hydroxylase deficiency (21-OH) (1, 2). A complete loss of 21-OH function results in the most severe salt-wasting (SW-CAH) phenotypes, whereas a minimal residual 21-OH production is sufficient to maintain aldosterone homeostasis, resulting in moderate simple virilizing phenotypes (SV-CAH) or mild less-symptomatic nonclassical CAH phenotypes (NC-CAH) (1, 2). Numerous studies have examined large nation-based or population-based cohorts of patients around the world to establish genotype-phenotype associations (3–9). *CYP21A2* is located near its pseudogene (*CYP21A2P*), which is 96% homologous to it. Up to 75% of CAH pathogenic variants result from gene recombination and gene conversion events in this region. The majority of pathogenic variants are inherited, while only a small fraction are *de novo* mutations (10).

It is important to study *CYP21A2* molecular genetics and the genotype-phenotype correlation of pathogenic variants to develop relevant and effective newborn screening (NBS) programs that may prevent neonatal salt crisis consequently reducing morbidity and mortality (2, 11). Nationwide NBS for CAH has been performed in Slovakia since 2003. In 2020, 56,756 neonates were screened and 4 cases of CAH were detected. Since 2003 951,200 newborns have been screened and 80 cases of CAH have been detected in Slovakia. (incidence 1:11 890). In Slovenia NBS for CAH is planned to be introduced in 2023 (12).

Testicular adrenal rest tumors (TARTs) are a common complication typically in classic CAH male patients with a prevalence from 14% to 86%, with an average of 25% in adolescents, and 46% in men, they also occur in children, with an increase in prevalence during puberty (1, 13). TART tissue has adrenal as well as testicular characteristics and can produce steroids. Poor hormonal control with elevated ACTH, 17-hydroxyprogesterone (17-OHP), and androstenedione concentrations seems to be associated with TARTs. However, TARTs also occur in well-controlled patients and only a few studies have found a clear association between hormonal control and TARTs. TARTs can cause severe pain complaints and irreversible damage to the testicular parenchyma, leading to infertility (13).

The study aimed to systematically analyze the mutational spectrum, clinical characteristics, genotype–phenotype correlations, and TARTs prevalence in 104 CAH patients from Slovakia and Slovenia. Furthermore, we compared the clinical characteristics of screened and unscreened patients, as one country performs NBS for CAH, while the other does not.

2 Materials and methods

The studied population included individuals followed up with a diagnosis of CAH at the Department of Paediatrics, Comenius University, Bratislava, Slovakia (n=40) and the Department of Pediatric Endocrinology, Diabetes and Metabolic Diseases (DPEDMD), University Children's Hospital, Ljubljana, Slovenia (n=60). Patients from the Slovak cohort originated from the capital, Bratislava, and the western region of Slovakia which has a total population of 2,496,000 people (Figure 1). The DPEDMD serves as the nationwide reference center for CAH in Slovenia which has a total population of 2,109,000 (Figure 1). Individuals included in the cohort were followed from 1984 in Slovenia and

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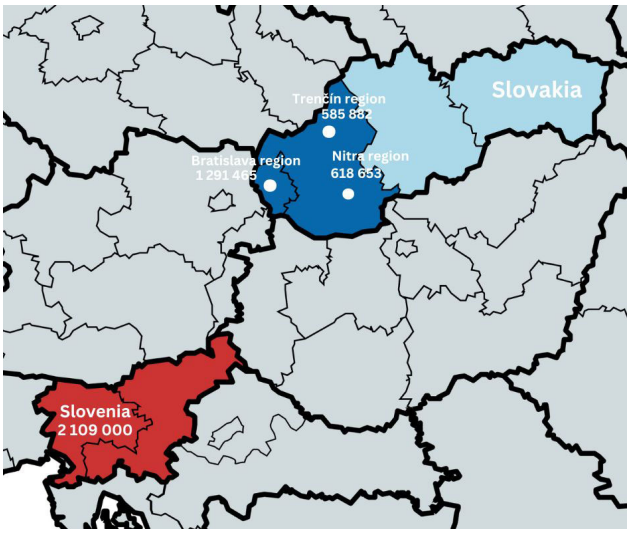

FIGURE 1
The map showing the origin of Slovak and Slovenian cohorts.

from 1996 in Slovakia until 2021 when the cohort data were evaluated. Clinical and laboratory data on the patients were obtained by retrospective data collection from the medical records in both countries. The study protocol was approved by the National Medical Ethics Committee of Slovenia (No.: 0120-290/2021/3).

2.1 Clinical classification/phenotyping

In all patients, the diagnosis of CAH was made or verified by a pediatric endocrinologist, based on the history, physical examination, electrolyte, and hormonal data. Serum 17-OHP values were available for Slovenian and Slovak patients before the introduction of NBS. Slovak patients born after the introduction of NBS for CAH had SW-CAH assigned based on high concentrations of 17-OHP measured from a dry drop of capillary blood on filter paper using an immunofluorescence method, supplemented with a mineralogram, serum steroid values, and in some patients renin.

In NBS for CAH, the screening marker is 17-OHP, which can detect 21-hydroxylase deficiency early and reliably. 17-OHP as a precursor of stress hormones is often elevated in newborns with other perinatal complications than CAH. Its concentration also depends on the maturity and birth weight of the newborn. Because of the potential for false positivity, a method was introduced in a pilot study to divide neonates according to maturity and weight into 4 categories with respect to the cut-off limit for 17-OHP. The reference values determined in this way will allow capturing all positive cases of 21-hydroxylase deficiency with a low percentage of false positive cases. In addition to correctly defined cut-off limits, the category of rescreenings - repeated screenings - is important. The selected group of newborns has been evaluated for NBS only after two samples. The first collection of a dry blood sample on the 4th day of life, the second collection on the 10th-14th day of life (12).

The criteria for SW-CAH in addition to basal 17-OHP levels were: either salt crisis in the neonatal period or/and elevated plasma renin activity (PRA), hyponatremia and hyperkalemia, virilized genitalia in girls. Treatment for the SW-CAH consisted of hydrocortisone (HC), fludrocortisone (FC), and salt replacement. For SV-CAH the criteria were: absence of salt wasting disorder, genital virilization in girls, gonadotropin-independent precocious puberty, accelerated growth and advanced bone age, and high 17-OHP levels on repeated sampling or after ACTH stimulation. Treatment was mostly limited to hydrocortisone replacement and low doses of mineralocorticoids. In girls, NC-CAH was diagnosed when they had no or minimal genital virilization, further had precocious pubarche, hirsutism or other signs of hyperandrogenemia and high 17-OHP levels after ACTH stimulation. In boys, NC-CAH was diagnosed if precocious puberty/virilization occurred after 4th year of life along with high levels of 17-OHP after ACTH stimulation (3, 7, 14).

In boys diagnosed with CAH, TARTs were diagnosed by ultrasonographic (USG) evaluation performed by paediatric radiologists with experience in scrotal ultrasound. Male patients were approximately once every 2 years subjected to scrotal ultrasound for TARTs screening. Longitudinal and transverse axes of the testes and TARTs, when visible, were measured.

2.2 *CYP21A2* gene analysis

A two-step genotyping approach was used for *CYP21A2* gene analysis. Low-resolution genotyping (qPCR, MLPA, and SnaPshot analysis) was performed to detect the most common pathogenic variants. High-resolution genotyping including Southern blotting, allele-specific long-range PCR, SNPCheck, and sequencing was performed to detect gene deletions, conversions, point mutations, or other sequence changes in the *CYP21A2* gene. The genotyping approach is described in detail elsewhere (7, 12, 15). CAH

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genotype-phenotype correlations were predicted based on Speiser´s classification of pathogenic variants into four mutation groups: null (SW-CAH), A (SW-CAH/SV-CAH), B (SV-CAH), C (NC-CAH) (16). In addition, the genotype of a novel pathogenic variant with no information about impairment of 21-OH function on at least one allele was assigned as D and excluded from the genotype-phenotype correlation analysis. As CAH is caused by homozygous or compound heterozygous pathogenic variants, the allele coding for the protein with greater residual 21-OH activity usually determines the severity of the overall genotype. Therefore, the haplotypes containing multiple pathogenic variants were grouped based on the most severe pathogenic variant.

2.3 Statistical analysis

Continuous data with normal distribution are presented as mean or median value and standard deviation (SD). For categorical data, percentages are reported. T-tests were used to compare means. Categorical variables were compared by Fisher's exact test. A *P*-value below 0.05 was considered statistically significant.

3 Results

Our database included 104 patients. As genotype and phenotype information was complete for 100 patients (96.15%), all the analyses were performed in a cohort consisting of 100 patients. The genotype-phenotype correlations were analyzed in 98 patients, as we excluded 2 patients with group D genotypes. The main characteristics of the cohort are summarized in Table 1.

In our cohort, there were a total of 57 female patients, of which 32 (56.14%) had SW-CAH, 18 (31.58%) NC-CAH, and 7 (12.28%) SV-CAH. There were a total of 43 male patients in the cohort, of which 31 (72.09%) had SW-CAH, 9 SV-CAH, and 3 (6.98%) NC-CAH.

The median age of SW-CAH patients at the time of diagnosis was 6 days in Slovakia vs. 28.5 days in Slovenia (p=0.01). Most of the Slovak patients in the cohort were detected by NBS. (24 out of 29). The median age at diagnosis of Slovak SV-CAH patients was 0.74 years, and in Slovenian 1.65 years.

Complete genotypes and clinical phenotypes of the cohort are shown in Supplementary Table 1. Among 100 patients 63% presented with SW-CAH (31 boys and 32 girls), 16% had SV-

TABLE 1 The main characteristics of the cohort (n = 100).

	Slovakia (Western region)	Slovenia	p-value
Probands in total (n)	40 (25 girls, 15 boys)	60 (32 girls, 28 boys)	0.414
Mean current age (years)	11.5	19.5	0.000001
SW-CAH in total (n)	29 (18 girls, 11 boys)	34 (14 girls, 20 boys)	0.134
SW-CAH - median age at dg.	6 days (3 to 30 days)	28.5 days (0 to 120 days)	0.011
SW-CAH diagnosed by screening (n)	24 (14 girls, 10 boys)	NA	NA
SW-CAH screened - median age at dg.	5 days	NA	NA
SW-CAH average 17-OHP at dg. + SD (ng/ml)	563.9 ± 1001.2(dry blood)	671.7± 1041.5 (venous blood)	0.356
SV-CAH in total	5 (1 girl, 4 boys)	11 (6 girls, 5 boys)	0.58
SV-CAH - median age at dg.	0.74 years (4 days -10.5 years)	1.65 years (2.79 – 7 years)	0.498
SV-CAH diagnosed by screening	2 (1 girl, 1 boy)	NA	NA
SV-CAH screened - median age at dg.	6.5 days	NA	NA
SV-CAH average 17-OHP at dg. + SD (ng/ml)	51.8 ± 14 (dry blood)	1388.8 ± 1959.7 (venous blood)	0.057
NC-CAH	6 (6 girls)	15 (12 girls, 3 boys)	0.318
NC-CAH - median age at dg.	7.19 years	7.2 years	0.445
NC-CAH average 17-OHP at dg. + SD (ng/ml)	38 ± 22.7(venous blood)	116 ± 117.8 (venous blood)	0.012
TARTS in male patients (%)*	33.3% (2 out of 6)	27.8% (5 out of 18)	1
SW-CAH in patients with TARTs*	2 (100%)	5 (100%)	1
Median age at dg. of CAH in patients with TARTs*	16.5 days	15 days	0,44
Median age at dg. of TARTs*	13 years		
Avare 17-OHP at dg. of TARTs*	217.88 nmol/l		

* information on scrotal ultrasound available from 24 male probands.
CAH, congenital adrenal hyperplasia; SW-CAH, salt-wasting form; SV-CAH, simple virilizing form; NC-CAH, non-classic form; dg, diagnosis; 17-OHP, 17-hydroxyprogesterone; SD, standard deviation; TART, testicular adrenal rest tumour; NA, not applicable.

CAH (9 boys and 7 girls) and 21% had NC-CAH (3 boys and 18 girls). Most patients in our cohort were hemizygous (35%), with a complete deletion of the *CYP21A2* gene or a large deletion/conversion on one allele, 34% were compound heterozygotes and 31% were homozygous. The most common pathogenic variant in SW-CAH were c.293-13A/C>G (44.53%) and *CYP21A2* gene deletion/conversion (32.03%). In SV-CAH, the most common pathogenic variant was p.Ile172Asn (28.13%), but it was also observed in 3.91% of SW-CAH alleles as well as in 7.14% of NC-CAH patients' alleles. Other common pathogenic variants found in SV-CAH were c.293-13A/C>G in 21.88% of alleles, *CYP21A2* gene deletion/conversion in 15.63% of alleles, and Pro30Leu in 12.5% of alleles of SV-CAH patients. Together with p.Ile172Asn, these pathogenic variants accounted for 78.13% of SV-CAH. The most common pathogenic variants in NC-CAH patients were p.Val282Leu (33.33%), *CYP21A2* gene deletion/conversion (21.43%), c.293-13A/C>G (14.29%) Pro30Leu (11.90%), which together accounted for more 80% of NC-CAH. p.Val282Leu pathogenic variant also represented the least severe pathogenic variant in 6.25% of SV-CAH patients.

As summarized in Supplementary Table 2, a good correlation between genotype and phenotype was observed in patients with severe pathogenic variants assigned to mutation groups 0 and A (94.74% for mutation group 0 and 97.92% for mutation group A). Greater variability in clinical phenotypes was observed in patients with less severe pathogenic variants, particularly in carriers of p.Ile172Asn and Pro30Leu, assigned to mutation groups B and C, respectively. (62.5% of relative frequency of the predicted phenotype for mutation group B and 75.28% for mutation group C). In most patients, the observed phenotype corresponded to the severity of the less severely affected of the two alleles; however, the presence of a group 0 or A pathogenic variants on the other allele often led to more severe clinical manifestations.

CYP21A2 allele frequencies in our cohort are shown in Table 2. Only 2 pathogenic variants: c.293-13A/C>G and *CYP21A2* gene deletion/conversion occurred with a frequency greater than 20%. Together they accounted for more than half (55.5%) of the affected alleles in the cohort. The other 2 common pathogenic variants occurred at a frequency of 8%, namely p.Ile172Asn and p.Val282Leu. Country-specific differences were observed in the frequencies of occurrence of a particular pathogenic variant. The frequency of *CYP21A2* gene deletion was 24.5% in the whole cohort, 25.8% in Slovenian patients, and 22.5% in Slovak patients. The c.293-13A/C>G pathogenic variant with an allele frequency of 31% in the whole cohort was the most frequent pathogenic variant in both Slovak (37.5%) and Slovenian patients (26.7%).

In the study cohort, we found many alleles with multiple pathogenic variants: 6.5% of all alleles carried two pathogenic

TABLE 2 *CYP21A2* allele frequencies in individual populations of the cohort.

CYP21A2 allele	Slovakia	Slovenia	Together	
	n	n	n	%
del/conv.	18	31	49	24.5%
prom.conv.	1	1	2	1.0%
c.293-13C/A>G	30	32	62	31.0%
p.Pro31Leu	4	5	9	4.5%
del 8bp (ex3)	1	2	3	1.5%
p.Ile172Asn	6	10	16	8.0%
p.Val282Leu	5	11	16	8.0%
cluster ex6	0	1	1	0.5%
p.Arg409Cys	1	0	1	0.5%
p.Arg317Ter	1	0	1	0.5%
p.Arg357Trp	1	1	2	1.0%
p.Arg355Cys	1	0	1	0.5%
p.Arg357GIn	2	0	2	1.0%
p.Arg484Pro	0	1	1	0.5%
p.Gln319Ter	2	2	4	2.0%
p.Pro454Ser	0	2	2	1.0%
p.Leu307Phefs*5	5	0	5	2.5%
p.Asn493Ser	0	1	1	0.5%

(Continued)

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TABLE 2 Continued

CYP21A2 allele	Slovakia	Slovenia	Together	
	n	n	n	%
p.Asn493Ser + T-107C	0	1	1	0.5%
c.293-13A/C>G + p.Val282Leu	1	1	2	1.0%
c.293-13A/C>G + p.Gln319Ter	0	1	1	0.5%
del 8 bp + p.Pro454Ser	0	1	1	0.5%
c.293-13A/C>G + p.Pro454Ser	0	3	3	1.5%
p.Pro454Ser + cluster ex6 + p.Ile172Asn	0	1	1	0.5%
prom.conv + p.Pro31Leu	0	2	2	1.0%
prom.conv + c.293-13A/C>G	0	2	2	1.0%
prom.conv + del 8 bp	0	1	1	0.5%
prom.conv + c.293-13A/C>G + p.Pro31Leu	0	1	1	0.5%
prom.conv + p.Pro31Leu + Als15Thr	0	2	2	1.0%
prom.conv + p.Leu307Phefs*5 + p.Gln319Ter	0	2	2	1.0%
p.Leu307Phefs*5 + p.Gln319Ter	1	0	1	0.5%
c.293-13A/C>G + cluster ex6 + p.Val282Leu + p.Gln319Ter	0	2	2	1.0%
Alleles together	80	120	200	

del, CYP21 gene deletion; prom conv, gene conversion in promoter region; conv, large or small; del 8 bp, deletion in exon 3; cluster ex6, cluster mutation in exon6.

variants and 4% of alleles carried more than two pathogenic variants. The frequency of alleles with multiple pathogenic variants was higher in Slovenian patients (15.83% of all alleles). The most frequent pathogenic variant occurring on the same haplotype with another pathogenic variant was c.293-13A/C>G.

TARTs were identified in 7 of 24 male patients with available USG data, of whom all 7 (100%) patients had the SW form of CAH. (2 out of 7 Slovak patients and 5 out of 17 Slovenian patients with available USG data). The prevalence of TARTs was 29.2% in male patients with SW-CAH. All patients with TARTs had SW-CAH. Among patients with SV-CAH and NC-CAH, no TARTs were detected. The median age at diagnosis of CAH in patients with TARTs was 15 days. The median age at the diagnosis of TARTs was 13 years (range 8-24). Poor hormonal control due to non-compliance was present in all 7 patients. The average 17-OHP level at the diagnosis of TARTs was 217.88 nmol/l. Table 3 shows the characteristics of patients with TARTs including their genotypes. As 2 boys were brothers, they have the same mutations.

4 Discussion

In this study, we present a cohort of 100 individuals with CAH from Slovakia and Slovenia with genotype and phenotype information available. Since the population of the compared areas differs only by 400.000 inhabitants, we can say that we have compared two areas with similar demographic characteristics.

The distribution of the different forms of CAH was approximately the same for both countries. In both countries, SW-CAH was the predominant one in the cohort. However, the prevalence of SW-CAH was more frequent among Slovak patients than among Slovenian patients (72.5% versus 56.67%). NC-CAH was the second most frequent clinical form in both countries. The lower incidence of NC-CAH in the pediatric population may be explained by delayed diagnosis. When we compared the distribution of the different clinical forms of the disease in our cohort with the data from the other studies, we found differences in the distribution of SV-CAH and NC-CAH. In the compared sets, in 3 studies SV-CAH was the second most frequent and NC-CAH was the least frequent clinical form, while in the study by Marino et al. NC-CAH was more frequent than SV-CAH, similar to our data (3, 5–7).

The sex ratio for SW-CAH was balanced in the whole cohort. In NC-CAH the female sex was significantly predominant (85.71%), which can be explained by the fact that the manifestations of androgen excess are clinically clearer in women than in men, who can often be asymptomatic and diagnosed later in life (4).

In Slovak patients with SW-CAH (82.75% diagnosed after the introduction of NBS) the median age at diagnosis was 22.5 days lower than in the Slovenian patients. The difference was also large when comparing the median age at diagnosis of SW-CAH in boys from Slovakia and Slovenia (9 days versus 32.5 days). In Slovenian boys, the diagnosis of SW-CAH took four times longer than in females. This comparison confirms the importance of NBS, which

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TABLE 3 Characteristics of patients with TARTs.

Case/ phenotype	*CYP21A2* haplotype (allele 1/allele 2)	Age at dg. of CAH	Age at dg. of TARTs	Tanner stage at dg. of TARTs	Hormonal control 17-OHP (ug/l)	Treatment non-compliance
Patient 1/SW-CAH	c.293-13A/C>G/c.293-13A/C>G	7 days	9 years	3	69.7 (poor)	yes
Patient 2/SW-CAH	c.293-13A/C>G/p.Leu307Phefs*5	26 days	8 years	2	70 (poor)	yes
Patient 3/SW-CAH	c.293-13A/C>G/c.293-13A/C>G + p.Pro454Ser	30 days	22	5	241.6 (poor)	yes
Patient 4/SW-CAH	c.293-13A/C>G/c.293-13A/C>G + p.Pro454Ser	7 days	24	5	330 (poor)	yes
Patient 5/SW-CAH	p.Pro454Ser + cluster ex6 + p.Ile172Asn/c.293-13A/C>G	15 days	10	5	660.5 (poor)	yes
Patient 6/SW-CAH	c.293-13A/C>G + p.Val282Leu/del	30 days	13	4	85.7 (poor)	yes
Patient 7/SW-CAH	c.293-13A/C>G + cluster ex6 + p.Val282Leu + p.Gln319Ter/prom.conv + p.Leu307Phefs*5 + p.Gln319Ter	9 days	19	5	67.70 (poor)	yes

TART, testicular adrenal rest tumour; CAH, congenital adrenal hyperplasia; SW-CAH, salt-wasting form; SV-CAH, simple virilizing form; NC-CAH, non-classic form; dg, diagnosis; del, CYP21 gene deletion; prom conv, gene conversion in promoter region; cluster ex6, cluster mutation in exon6; 17-OHP, 17-hydroxyprogesterone.

has been in place in Slovakia since 2003, while it has not yet been introduced in Slovenia. During the analyses, we found that NBS in Slovakia most frequently detected patients with the SW form of the disease (92.59%), while the SV form was present in only 7.41% of the detected patients. In contrast, in patients not identified by NBS NC-CAH was predominant (46.15%).

In Slovak patients with SV-CAH, the median age at diagnosis was 0.91 years lower than in Slovenian group. This observation could be justified by earlier referral of patients from a general practitioner to a specialist or by parental notification of signs of gonadotropin-independent precocious puberty. Of course, a proportion of patients may also be detected by NBS. The age at diagnosis of NC-CAH was similar in Slovak and Slovenian patients.

The distribution of the most frequent pathogenic variants and the overall distribution of genotypes in the cohort was largely similar to the previously published European studies, whereas the differences were greater when compared to, for example, Mexican or Brazilian populations (8, 17). Deletion/large conversion and c.293-13A/C>G accounted for 55.5% of all pathogenic variants, followed by p.Ile172Asn and p.Val282Leu, both with a frequency of 8% (Table 2). The fifth most frequent pathogenic variant was p.Pro31Leu with a frequency of 4.5%. We found that c.293-13A/C>G was more frequent in Slovakia (37.5% versus 26.7%). The frequency of alleles with multiple pathogenic variants was higher in Slovenia (15.83% of all alleles), which may be the result of the founder effect. Clustering of point mutations on a single allele was previously reported in 1.9% of unrelated alleles in Dutch patients (6).

Direct sequencing of *CYP21A2* identified p.Asn493Ser substitution on both alleles and a concomitant T-307 pathogenic variant in the heterozygous form in a Slovenian patient with NC-CAH. The p.Asn493Ser substitution is also reported by some authors as a naturally occurring polymorphism, and by some as a disease-causing pathogenic variant, but its effect on the residual activity of the enzyme has never been analyzed *in vitro*. Ordonez-Sánchez et al. found a very high frequency of the p.Asn493Ser pathogenic variant in the Mexican population, and the proportion of homozygosity for the p.Asn493Ser substitution was higher in CAH patients than in the healthy population (17). Rodrigues et al. also reported a patient in whom the hemizygous Asn493Ser pathogenic variant was combined with the S268T pathogenic variant (18). It is possible that the Asn493Ser substitution may result in reduced enzymatic activity only when combined with the effect of another pathogenic variant.

A sporadic p.Arg357GIn pathogenic variant was found in a homozygous state in a Slovak patient with SV-CAH. This pathogenic variant with a residual enzymatic activity of 1.1% was first described in a Finnish study by Levo et al. (19) A Swedish study described 0.5% frequency of this pathogenic variant in the Swedish population studied, where it was associated with SW-CAH (20). In another Slovak patient, a rare pathogenic variant p.Arg409Cys was found in exon 10, previously found in two Brazilian patients, and *in vitro* testing resulted in an almost complete absence of enzymatic activity (21, 22). In a US study, the p.Arg409Cys pathogenic variant was associated with c.293-13A/C>G on the second allele and resulted in SV-CAH (22). In our cohort it led to SW-CAH with a *CYP21A2* deletion on the second allele. Other rare pathogenic variants included the p.Arg484Pro in exon 10, p.Arg317Ter in exon 8, which had not previously been described in the two populations studied. The relative frequency of the p.Arg484Pro pathogenic variant was very similar, 0.2% and 0.5% in the Chinese and US populations studied, respectively (23, 24). Based on a

Taiwanese study, it can be assumed that the p.Arg317Ter pathogenic variant has a higher frequency in Taiwan in the Minnan population (25).

Many studies have investigated genotype-phenotype relationships in large national and multiethnic cohorts. In general, severe genotypes leading to SW-CAH showed a strong correlation with clinical phenotype. In our cohort, the overall genotype-phenotype correlation was high for severe pathogenic variants (0: 94.74%; A: 97.3%) but extremely low for group B genotypes (50%). A higher concordance was observed for group C genotypes (70.83%) compared to 2 European studies. In comparison (Table 4), previous studies reported 97-100% concordance for null, 79-96% for A, 46-87% for B, and 58-100% for C genotypes (3–6, 9). One-third of the patients (33.33%) with genotype B were classified as SW-CAH. Among the patients with genotype C, 25% of them had the classic form of CAH (SW and SV).

The reasons for the observed differences are not fully elucidated. In some pathogenic variants, some residual enzyme activity may be present, for example in the case of c.293-13A/C>G, which led to SV-CAH (up to 20% of cases in our cohort), although it is classified as a severe pathogenic variant that usually leads to SW-CAH (3). Unusual chimeric genes are the cause of some genotype-phenotype discrepancies, and the sites of chimeric junctions resulting from genetic rearrangements may be of clinical significance. In addition to *CYP21A2* pathogenic variants, other genes that may affect the phenotype by modifying steroid action or salt balance include androgen receptor CAG repeat length; the highly polymorphic P450 oxidoreductase enzyme; splicing mutations in RNA splicing factors; and other genes encoding proteins other than the type II cytochrome P450 enzyme that have 21-OH activity (26). The role of *CYP2C19* as a potential modifier gene that contributes to extra-adrenal 21-hydroxylation of progesterone, which may alleviate mineralocorticoid deficiency in CAH, has been investigated. The results of a small Slovene study suggest that the *CYP2C19*1/*17* genotype could lead to a very subtle modification of the clinical phenotype of 21-OH deficiency (27).

In addition, the effects of other pathways that regulate the severity of enzymatic loss, such as the posterior androgen pathway, which causes more heterogeneous phenotypes, may play a critical role in individual patients.

In genotype-phenotype correlation analysis, we observed increased clinical severity in compound heterozygotes who had one severe and one non-severe pathogenic variant, which would be expected to result in a milder phenotype (Supplementary Table 2). According to the study, compound heterozygotes with one classic pathogenic variant versus homozygotes with two mild pathogenic variants had a greater biochemical androgen response to ACTH stimulation. Various studies have reported varying levels of residual *in vitro* activity, which can range from 2-5% for p.Ile172Asn and 10-60% for p.Pro31Leu. This indicates ambiguity from a functional point of view (28–31). The finding of a p.Pro31Leu pathogenic variant associated with gene conversion in the promoter region could explain the increased severity of the phenotypes in Slovenian patients.

TARTs are a common complication in CAH male patients with a reported prevalence of 18.3-48% in the pediatric population, while the prevalence is higher in adult patients (32–35). In our cohort, the prevalence of TARTs was 29.2% in male patients with SW-CAH, which represented the most common form of CAH in our sample (63%). There was a slight difference in the prevalence of TARTs between the two countries: 2 patients out of 7 with available USG data in Slovakia and 5 out of 17 patients with available USG data in Slovenia. The overall prevalence of TARTs in our study (29.2%) was similar to the other studies (32–34).

Poor hormonal control was reported in 58% of studies examining CAH and TART (34). In our study all patients with TARTs were found to have poor metabolic control due to treatment non-compliance. In addition, studies found TARTs in patients with good metabolic control, too (36, 37). In the present study, the median age at the diagnosis of TARTs was 13 years, which was like the study of Aycan et al. and Kocova et al, where the median at the diagnosis of TARTs was 15.5 and 13.2 years respectively (33, 34). These 2 studies were describing the prevalence of TARTs in paediatric populations aged 2-18 years. In the study of Claahsen et al. most of the TARTs were detected in children above 10 years old (32). It may be suspected that hormones whose levels are elevated during puberty, such as luteinizing hormone (LH), additionally stimulate tumour growth. In one of the patients after improved compliance the ultrasound of the testes was without signs of TARTs. Other studies also proved the possible remission of TARTs if identified early if compliance and treatment regimen are improved (33, 34).

The relationship between the CAH genotype and the development of TART is unknown (38). Most of the published studies indicate that TARTs are associated only with classical forms of the disease, e.g. the null group carrying null mutations with no enzyme activity, A group containing c.293-13A/C>G variant with negligible enzyme activity, or group B composed of patients with a homozygous p.I172N mutation causing the SV form (31, 32, 34, 35). Since all patients with TARTs in our cohort had SW-CAH, they

TABLE 4 Concordance of 0, A, B, C genotypes with assigned phenotypes compared to other studies.

Genotype/predicted phenotype	Our cohort (n=98)	USA (3) (n=1507)	Germany (2) (n=538)	Argentina (4) (n=454)	Netherlands (5) (n=198)	Poland (8) (n=44)
0/SW-CAH %	94.74	≤100	97	100	97	100
A/SW-CAH%	97.3	79	91	84	96	90.5
B/SV-CAH %	50	76	46	87	53	66.70%
C/NC-CAH %	70.83	>90	58	100	100	NA

CAH, congenital adrenal hyperplasia; SW-CAH, salt-wasting form; SV-CAH, simple virilizing form; NC-CAH, non-classic form.

belonged to group null (4 patients) and group A (3 patients). The most frequent variant in patients with TARTs was c.293-13C>G, similar to the study by Aycan et al. (38) Given that genotype-phenotype correlation in CAH is not found in all patients (1), genetic analysis of larger TART patient cohorts may reveal additional genotypes associated with TART (35, 38).

One of the limitations of our study is, despite clear diagnostic criteria and careful data editing, we cannot exclude arbitrary overestimation of the severity of clinical phenotypes. Especially the cut-off limit of 4 years to diagnose NC-CAH in boys may affect the SV/NC-CAH ratio concerning phenotyping, since there are dilemmas with precise definition (3, 7, 14). It's also important to mention that part of the patients was diagnosed more than 20 years ago, since then the definition of each form of CAH might have changed, too.

As our cohort includes a smaller set of especially Slovak patients, the frequency of less common mutations may not correspond to the full reality in the countries concerned. Regarding TARTs, we don't have available all data that would allow the comparison of the screened and unscreened population (TART size, full hormonal profile, etc.), which would be beneficial to see in the future. Ideally, all patients should be evaluated by a single endocrinologist, which was not possible with patients originating from two countries.

In this study, we investigated the phenotype and genotype in the largest group of 21-hydroxylase-deficient CAH patients from Slovakia and Slovenia to date. In previous European studies, the number of Slovak patients with complete phenotype and genotype data did not exceed 20 and Slovenian 38, while our cohort included 40 Slovak and 60 Slovenian patients. In addition, our study compared two European countries, where in one NBS has been introduced in 2003, while in the other NBS is not used yet. Our study clearly demonstrated the importance of NBS which was particularly evident in the speed of diagnosis of severe forms of the disease in the critical neonatal period. This work may therefore support the introduction of NBS in Slovenia as well. In the future, it would be ideal to create a map of the prevalence of pathogenic variants, especially the less frequent ones, in the different regions of the countries studied. In this work, we did not include patients from the Central and Eastern Slovak regions, but due to the relatively significant population fluctuation towards the west, it may be assumed that the studied sample may represent the entire population of Slovakia.

Genetic diagnosis remains important in confirming or excluding the diagnosis in the case of unclear biochemical parameters, in segregation analysis - identifying the origin of the pathogenic variant, in determining carriers in siblings, and in prenatal diagnosis. Due to the observed high prevalence of TARTs in boys with CAH, annual screening by USG of testes for early detection and treatment is strongly recommended since there is possible remission of TARTs if identified early.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by National Medical Ethics Committee of Slovenia (No.: 0120-290/2021/3). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

RS: first authorship, data acquisition and interpretation, statistical analysis, drafting and revision of the manuscript. ZP: senior authorship, data interpretation, revision of the manuscript. UG: senior authorship, study design, data interpretation, revision of the manuscript. Other authors: revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1134133/full#supplementary-material

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EDITED BY
Semra Çaglar Çetinkaya,
University of Health Sciences, Türkiye

REVIEWED BY
Avinaash Vickram Maharaj,
Queen Mary University of London,
United Kingdom
Mona Hafez,
Cairo University, Egypt

*CORRESPONDENCE
Ahmed Khattab
ak1684@rwjms.rutgers.edu

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Corticotropin releasing factor-1 receptor antagonism associated with favorable outcomes of male reproductive health biochemical parameters

Ahmed Khattab[1]* and R. Will Charlton[2]

[1]Division of Pediatric Endocrinology, Rutgers-Robert Wood Johnson Medical School, Child Health Institute of New Jersey, New Brunswick, NJ, United States, [2]Spruce Biosciences, Clinical Science, Inc., South San Francisco, CA, United States

Background: Disruption in androgen profiles and testicular adrenal rest tumors in males with congenital adrenal hyperplasia (CAH) can negatively affect sexual activity and fertility. Adrenal hyperandrogenism suppresses gonadotropin secretion and testicular adrenal rest tumors (TARTS), despite being noncancerous lesions, cause obstructive azoospermia and impaired testosterone (T) production. Circulating T in men with uncontrolled CAH is often predominantly adrenal in origin, which is reflected in high androstenedione/testosterone ratios (A4/T). Therefore, decreased luteinizing hormone (LH) levels and an increased A4/T are markers of impaired fertility in these individuals.

Methods: Oral tildacerfont 200 to 1000 mg once daily (QD) (n=10) or 100 to 200 mg twice daily (n=9 and 7) for 2 weeks (Study 201), and 400 mg QD (n=11) for 12 weeks (Study 202). Outcomes measured changes from baseline in A4, T, A4/T, and LH.

Results: Mean T levels increased in Study 201 from 375.5 ng/dL to 390.5 ng/dL at week 2 (n=9), 485.4 ng/dL at week 4 (n=4) and 420.7 ng/dL at week 6 (n=4). In Study 202, T levels fluctuated in the normal range from 448.4 ng/dL at baseline to 412.0 ng/dL at week 12. Mean LH levels increased in Study 201 from 0.68 IU/L to 1.59 IU/L at week 2 (n=10), 1.62 IU/L at week 4 (n=5) and 0.85 IU/L at week 6 (n=4). In Study 202, mean LH levels increased from 0.44 IU/L at baseline to 0.87 IU/L at week 12. Mean A4/T decreased across both studies. In Study 201, the mean A4/T changed from a baseline of 1.28 to 0.59 at week 2 (n=9), 0.87 at week 4 (n=4), and 1.03 at week 6 (n=4). In Study 202, the A4/T decreased from baseline of 2.44 to 0.68 at week 12. Four men were hypogonadal at baseline; all experienced improved A4/T and 3/4 (75%) reached levels <1.

Conclusion: Tildacerfont treatment demonstrated clinically meaningful reductions in A4 levels, and A4/T with concomitant increased LH levels indicating increased testicular T production. The data suggests improvement in hypothalamic-pituitary-gonadal axis function, but more data is required to confirm favorable male reproductive health outcomes.

KEYWORDS

congenital adrenal hyperplasia, tildacerfont, corticotropin releasing factor 1 receptor antagonists, CRF-1R, androstenedione, androstenedione/testosterone ratio

Introduction

Classic congenital adrenal hyperplasia (CAH) due to impairment of 21-hydroxylase enzyme activity causes dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis that results in cortisol deficiency and hyperandrogenemia. Defective cortisol synthesis impairs negative feedback on the hypothalamus and pituitary that normally modulates production of corticotropin releasing factor (CRF) and adrenocorticotropic hormone (ACTH). Consequently, both are increased, and excess ACTH leads to overstimulation and hyperplasia of the adrenal glands. 21-hydoxylase deficiency (21OHD) causes blockage of the glucocorticoid (GC) synthesis pathway and shunting of steroidogenesis towards the intact androgen pathway. Individuals with more severe 21OHD also have impaired mineralocorticoid synthesis and may manifest salt wasting in addition to hyperandrogenemia (1–3).

Cortisol and aldosterone deficiency can be replaced by GC and mineralocorticoid therapy at approximately physiologic doses. However, control of hyperandrogenemia often requires supraphysiological doses of GCs to adequately suppress ACTH. As a result, CAH management involves a delicate balance between risks associated with hyperandrogenemia and those associated with chronic GC overexposure.

In children, the clinical spectrum of CAH that is associated with adrenal androgen excess includes virilization of external genitalia in females, precocious adrenarche and pubarche, rapid somatic growth and accelerated skeletal maturation in early childhood which results in adult height compromise. In adults, elevated androgens are associated with insulin resistance/metabolic syndrome, acne, female hirsutism and androgenic alopecia, and reproductive concerns in men and women. Women may experience irregular menses and anovulation (1–4).

In men, disrupted androgen profiles and the relatively high prevalence of testicular adrenal rest tumors (TARTs) can affect sexual activity and fertility. Testosterone (T) levels may be normal in men with CAH, but it is often predominantly adrenal in origin, which is reflected in high androstenedione (A4) to T (A4/T) ratios. Testicular function can be impaired by suppression of the hypothalamic-pituitary-gonadal (HPG) axis due to adrenal hyperandrogenism or exogenous GC. Additionally, despite being noncancerous lesions, TARTs can impair T production and cause obstructive azoospermia (5). As a result, levels of luteinizing hormone (LH) and increased A4/T ratio are markers of impaired fertility in these individuals (6–9).

Supraphysiological GC administration, the current standard pharmacotherapy for CAH, aims to suppress the ACTH-driven adrenal hyperandrogenemia with biochemical therapeutic targets that are still higher than the normal reference ranges. In fact, normalizing 17-hydroxyprogesterone (17-OHP) levels in CAH management is likely indicative of overtreatment (2, 6, 10).

When treating patients with CAH, endocrinologists try to maintain the balance of achieving androgen control while minimizing GC overexposure. This balance is difficult to reach and most CAH patients are inevitably subjected to lifelong supraphysiological GC regimens that do not mimic typical cortisol profiles (6, 10). Supraphysiological GC levels and HPA axis dysfunction in CAH have been associated with growth failure, deranged carbohydrate and lipid metabolism, increase body mass index (BMI), decreased bone mineral density, increased cardiovascular risk, psychological morbidity and an overall impairment in quality of life (2, 10).

Due to the risks and challenges associated with the current standard of care CAH treatment, new therapies are needed. CRF1 antagonists originally became drug development targets after showing promise in animal models of anxiety, depression, and addiction. While similar studies in humans have not been successful, Schwandt and colleagues showed that CRF1 antagonism could dampen the HPA axis in humans by inhibiting CRF-mediated ACTH and cortisol release (11–14). Consequently, CRF1 antagonism became a potential target to address HPA axis dysfunction in CAH, a major unmet need. Two CRF1 receptor antagonists, tildacerfont and crinecerfont, are currently in late-stage development for CAH (10, 15, 16).

CRF1 antagonism has been shown to clinically reduce ACTH and adrenal androgen levels (17), potentially allowing the treatment goals of CAH – cortisol replacement and androgen control- to be addressed separately. Theoretically, controlling adrenal androgens by blocking CRF-mediated ACTH production should allow for cortisol replacement with physiologic doses, decreasing the compounding risk of lifelong overexposure to GCs.

This study examines changes in androgen profiles in men exposed to tildacerfont, a non-steroidal, once-daily oral CRF1

receptor antagonist, with the goal of gaining understanding of how CRF1 antagonism in men with CAH may impact gonadal Leydig cell function. The presented data highlights efficacy in suppression of adrenal hyperandrogenism and effect on TARTs (16). Novel preliminary data shows changes in gonadotropins and A4/T ratios, which may be related to positive male reproductive outcomes.

Methods

Ethics

Both studies were conducted in accordance with International Council for Harmonization Good Clinical Practice guidelines and the Declaration of Helsinki principals and applicable local and federal regulations. Institutional review boards at each study site approved the protocols and informed consent forms, and all participants provided written consent.

Study design

Study SPR001-201 was a Phase 2, open-label, first-in-CAH-patients, proof-of-concept, dose-ranging study that evaluated the safety, pharmacokinetics (PK), and efficacy of repeated doses of tildacerfont in adults with classic CAH at multiple sites in the US. The study population consisted of subjects with CAH who were not adequately controlled (based on elevated 17-OHP) despite a stable GC regimen. The study consisted of 3 cohorts. Subjects in Cohort A were enrolled into a 6-week dose-escalation treatment period to identify a range of safe and effective QD doses. Subjects in Cohort A (n=10 dosed) were each treated for 2 weeks at 200 mg QD, then 2 weeks at 600 mg QD, and then 2 weeks at 1000 mg QD, with no washout between dose escalations. Subjects in Cohort B (n=9 dosed) were treated for 2 weeks at 200 mg BID. Subjects in Cohort C (n=7 dosed) were treated for 2 weeks at 100 mg BID. Subjects were enrolled sequentially into each cohort, and each cohort was completed before the start of the next cohort.

Study SPR001-202 was a Phase 2, open-label study that evaluated the safety and efficacy of tildacerfont 400 mg QD over 12 weeks of dosing in adults with classic CAH. Subjects who previously participated in Study SPR001-201 were eligible to enroll in this study (after a washout period of at least 45 days), along with tildacerfont-naïve subjects. Nine of the 11 subjects enrolled were rollover subjects from Study SPR001-201.

During the studies, participants continued their previously prescribed regimen of GC ± mineralocorticoid replacement without dose adjustments.

Primary analyses have been published (16) but this *post-hoc* analysis only includes data from male participants, as it focuses on male reproductive health by evaluating changes in A4, T, LH, A4/T ratio, and ultrasound measurement of TARTs.

Study 201 included visits at baseline (Day 1) and week 2 for all cohorts, as well as weeks 4 and 6 for Cohort A dose escalation. Study 202 included visits at baseline (Day 1) and weeks 2, 4, 6, 8, 10, and 12. Biomarkers were drawn at 8am prior to administration of the morning GC dose. Study 202 participants had a follow-up visit 30 days after study drug discontinuation.

Statistical analyses

Sample size for Study 201 was estimated based on the assumption that 6-9 participants per cohort would provide adequate initial safety data and support proof of concept. For Study 202, estimates were based on the number of participants expected to enroll after completing Study 201. Power calculations were not performed, and all statistics are descriptive in nature.

The safety analysis population included all participants who received at least one dose of tildacerfont. The pharmacokinetic analysis population included all participants who had evaluable PK profiles. The efficacy analysis population included all participants who had both baseline and post-baseline 8am biomarker measurements. Due to the dynamic range of these biomarkers, and the nonnormality of the data, geometric means and geometric mean percentage changes were used to summarize the changes over time.

Results

10 men enrolled in Study 201: 5 in Cohort A, 1 in Cohort B, and 4 in Cohort C. 4 men enrolled in Study 202, 3 of whom had participated in Study 201. Due to the washout period between studies, results are evaluated by measurement instead of individual (i.e., measurements from 201 participants who enrolled in 202 were treated as new data). Also, while Study 202 results are presented as changes from baseline to post-treatment (week 12), results from Study 201 are presented at weeks 2, 4 and 6 due to dose increases.

Mean age of the men in both studies combined was 35.1 years (range 18-54): 46.3 years in Study 201 and 32.3 years in Study 202. All participants reported race as white except one who identified as white and Asian. Four reported ethnicity as Hispanic or Latino.

All the participants had elevated 17OHP at baseline, and all but one had elevated ACTH and A4 levels as well. One participant had modest elevation of baseline 17OHP with normal ACTH and A4 levels and normal A4/T ratio at 0.14. Aside from this individual and one non-responder, decreased ACTH levels resulted in altered androgen profiles across both studies.

At baseline, most of the men had normal T levels, but four 201 participants had T<300ng/dL. 6/11 (55%) had suppressed baseline LH (including the four with low T), and 11/14 (79%) had A4/T ratios > 1, indicating a predominantly adrenal origin of T.

During treatment with tildacerfont, mean T levels increased in Study 201 from 375.5 ng/dL to 390.5 ng/dL at week 2 (n=9), 485.4 ng/dL at week 4 (n=4) and 420.7 ng/dL at week 6 (n=4). In Study 202, mean T levels fluctuated within the normal range from 448.4 ng/dL at baseline to 412.0 ng/dL at week 12.

Mean LH levels increased in Study 201 from 0.68 IU/L to 1.59 IU/L at week 2 (n=10), 1.62 IU/L at week 4 (n=5) and 0.85 IU/L at week 6 (n=4). In Study 202, mean LH levels increased from 0.44 IU/L at baseline to 0.87 IU/L at week 12. Of the four men who had low T and suppressed LH (hypogonadotropic hypogonadism), all showed improvements in LH and 3/4 (75%) normalized LH levels.

Mean A4/T ratios decreased across both studies. In Study 201, the mean changed from a baseline of 1.28 to 0.59 at week 2 (n=9), 0.87 at week 4 (n=4), and 1.03 at week 6 (n=4). In Study 202, the A4/T ratio decreased from baseline of 2.44 to 0.68 at week 12. All the hypogonadal men experienced improved A4/T ratios, and 3/4 (75%) reached levels <1.

Three men had evaluable pre- and post-treatment scrotal ultrasounds to assess TARTs. In Study 201, one participant in Cohort A experienced a 23% reduction in TART volume after 6 weeks of treatment, correlated with an 87% reduction in ACTH. In Study 202, the non-responder showed no change in TART volume, correlated with no change in ACTH levels. Another 202 participant had 4 TARTs at baseline that were undetectable after treatment. However, assessment is confounded due to increased dexamethasone exposure during the study.

Discussion

Males with CAH may experience primary hypogonadism due to testicular damage caused by TARTs or secondary hypogonadism (hypogonadotropic hypogonadism) due to suppression of LH by adrenal T as well as adrenal androgens that have been aromatized to estrone or estradiol. Normal T levels with suppressed gonadotropins and high A4 levels suggest a higher contribution of adrenal T to circulating T levels.

In a cross-sectional clinical outcome study involving 14 study centers in 6 European countries, Engels et al. evaluated a cohort of 121 men with CAH and extrapolated that approximately 20% of men with CAH have low T, and around half of those have suppressed gonadotropins. Our findings, albeit in a small cohort, included 29% (4/14) with low T. Interestingly, all four of these men had suppressed LH levels.

In a prospective longitudinal monocentric study of sexual well-being that included 20 male patients with CAH, serum A4/T ratio was used as a biomarker to differentiate testicular vs. adrenal T production. An A4/T of <0.2 indicated normal testicular T production, whereas an A4/T of >1 suggested T was predominately of adrenal origin. Auchus and Arlt believe that an A4/T ratio greater than 0.5 is an indicator of significant adrenal contribution to the total T level in males with CAH and if the A4/T ratio is greater than 1 with concomitant LH suppression then the majority of the circulating T is of adrenal origin (17, 18).

Our data demonstrates clinically meaningful decrease in A4 levels, increase in the testicular T proportion, and increase LH levels, collectively suggestive of recovery HPG axis function in males with CAH receiving tildacerfont. Table 1 shows the decrease in A4/T ratios with concomitant increase in LH levels in SPR001-201. Table 2 shows the decrease in A4 levels throughout the course of the 12 weeks of tildacerfont 400 mg daily administration and concomitant increase in LH levels in SPR001-202. Despite a mean decrease in T, levels were consistently normal and changes in the A4/T ratio suggest an increase in the testicular contribution. These results are consistent with clinical trial data reported from the CRF1 receptor inhibitor crinecerfont (9).

Table 2 also shows that 30 days after discontinuing tildacerfont (follow up column) levels of A4, T, LH, and A4/T ratio all reverted towards baseline, supporting the hypothesis that the changes observed during the study were due to tildacerfont exposure.

While conclusions cannot be drawn due to small numbers of participants, it is noteworthy that all four men with baseline hypogonadotropic hypogonadism showed improvement in LH

TABLE 1 Results from SPR001-201 showing changes in serum A4, Testosterone, LH, and A4/T ratio.

Laboratory Parameter		Baseline	Week 2	Week 4	Week 6
Androstenedione (ng/dL)	n	10	10	4	4
	Geometric Mean (CV%)	459.7 (143.8)	223.5 (304.6)	420.7 (428.5)	429.4 (411.1)
	Geometric Mean % change	n/a	-51.4%	-22.9%	-21.3%
Testosterone (ng/dL)	n	9	9	4	4
	Geometric Mean (CV%)	375.52 (63.8)	390.48 (40.7)	485.40 (60.8)	420.71 (72.0)
	Geometric Mean % change	n/a	+4.0%	+23.6%	+7.2%
Androstenedione: Testosterone	n	9	9	4	4
(Ratio)	Geometric Mean (CV%)	1.283 (193.9)	0.594 (594.9)	0.867 (960.8)	1.030 (1144.1)
	Geometric Mean % change	n/a	-53.7%	-37.3%	-25.5%
Luteinizing Hormone (IU/L)	n	10	10	5	4
	Geometric Mean (CV%)	0.680 (258.7)	1.585 (221.0)	1.619 (253.3)	0.851 (740.9)
	Geometric Mean % change	n/a	+133.1%	+232.1%	+30.0%

CI, confidence interval; CV, coefficient of variance.

TABLE 2 Results from SPR001-202 showing changes in serum A4, Testosterone, LH, and A4/T ratio.

Laboratory Parameter		Baseline	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12	Follow-up
			TILDACERFONT	400 mg	PO QD				
	n	4	4	4	4	4	4	4	4
Androstenedione (ng/dL)	Geometric Mean (CV%)	1096.5 (46.1)	437.8 (312.6)	427.3 (448.1)	338.6 (241.2)	419.5 (258.4)	256.4 (371.6)	281.2 (345.2)	591.6 (252.7)
	Geometric Mean % change	n/a	-60.1%	-61.0%	-69.1%	-61.7%	-76.6%	-74.3%	-46.0%
Testosterone (ng/dL)	Geometric Mean (CV%)	448.44 (26.7)	348.90 (109.2)	395.82 (118.6)	306.03 (116.1)	368.01 (115.7)	422.45 (127.9)	412.01 (127.5)	447.79 (34.9)
	Geometric Mean % change	n/a	-22.2%	-11.7%	-31.8%	-17.9%	-5.8%	-8.1%	-0.1%
Androstenedione: Testosterone (Ratio)	Geometric Mean (CV%)	2,444 (62.0)	1.253 (714.5)	1.078 (813.7)	1.094 (799.7)	1.139 (589.3)	0.602 (1014.9)	0.679 (1033.2)	1.310 (446.5)
	Geometric Mean % change	n/a	-48.7%	-55.9%	-55.3%	-53.4%	-75.4%	-72.2%	-46.4%
Luteinizing Hormone (IU/L	Geometric Mean (CV%)	0.436 (316.4)	0.545 (386.1)	0.653 (475.2)	0.812 (662.8)	0.667 (469.8)	0.874 (787.9)	0.866 (780.3)	0.600 (410.6)
	Geometric Mean % change	n/a	25.1%	50.0%	86.5%	53.2%	100.7%	98.8%	37.7%

CI, confidence interval; CV, coefficient of variance.

and A4/T ratios, and three of them normalized LH levels and reached A4/T ratios <1.

This *post-hoc* analysis supports prior studies showing that HPG axis disruption is common in men with CAH and suggests that modulation of adrenal androgen production through CRF1 antagonism may improve gonadal Leydig cell function. This analysis is primarily evaluating Leydig cell function using LH, T and A4/T rather than Sertoli cell function, as specific markers of Sertoli cell function such as inhibin levels or semen analysis, were not analyzed.

A potential pitfall is that the use of total testosterone rather than free testosterone may not accurately reflect hyperandrogenic states associated with low sex hormone binding globulin levels in individuals with a high BMI.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Both studies were conducted in accordance with International Council for Harmonization Good Clinical Practice guidelines and the Declaration of Helsinki principals and applicable local and federal regulations. Institutional review boards at each study site approved the protocols and informed consent forms, and all participants provided written consent. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication. Both authors contributed to data analysis and writing of the manuscript.

Conflict of interest

Author AK is an employee of Rutgers University, a principal investigator for clinical trials in congenital adrenal hyperplasia with Spruce Biosciences Inc, and a consultant for Antares Pharma. Author RC is an employee of Spruce Biosciences Inc.

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EDITED BY
Semra Çaglar Çetinkaya,
University of Health Sciences (Turkey), Türkiye

REVIEWED BY
Maria G. Vogiatzi,
University of Pennsylvania, United States
Arianne Dessens,
Erasmus Medical Center, Netherlands

*CORRESPONDENCE
Ibrahim Al Alwan
alwani@ksau-hs.edu.sa

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The clinical characteristics and quality of life of 248 pediatric and adult patients with Congenital Adrenal Hyperplasia

Edi A. Shafaay[1,2], Mohammed A. Aldriweesh[1,2], Ghadeer L. Aljahdali[1,2], Amir Babiker[1,2,3], Abdulrahman O. Alomar[1,2], Khulood M. Alharbi[4], Haneen Aldalaan[5], Ahmed Alenazi[6,7], Abdulaziz S. Alangari[2,8], Afaf Alsagheir[6,9], Bas P. H. Adriaansen[10], Hedi L. Claahsen – van der Grinten[10] and Ibrahim Al Alwan[1,2,3]*

[1]College of Medicine, King Saud Bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia, [2]King Abdullah International Medical Research Center, Riyadh, Saudi Arabia, [3]Department of Pediatrics, King Abdullah Specialized Children's Hospital, Ministry of National Guard Health Affairs, Riyadh, Saudi Arabia, [4]Department of Pediatrics, College of Medicine, Taibah University, Madinah, Saudi Arabia, [5]Department of Pediatrics, King Saud Medical City, Riyadh, Saudi Arabia, [6]Department of Pediatrics, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia, [7]Department of Pediatrics, Prince Mohammed Bin Abdulziz Medical City, Aljouf, Saudi Arabia, [8]Department of Epidemiology and Biostatistics, College of Public Health and Health Informatics, King Saud bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia, [9]College of Medicine, Alfaisal University, Riyadh, Saudi Arabia, [10]Department of Pediatrics, Radboud Amalia Children's Hospital, Radboud University Medical Center, Nijmegen, Netherlands

Background: Congenital Adrenal Hyperplasia (CAH) is a chronic disease that requires lifelong treatment. Patients may face stigmatization, which may affect their quality of life (QoL). Therefore, we assessed the clinical characteristics and QoL of patients with CAH in the Middle East.

Methods: This case-control study included patients with CAH aged >5 years from two tertiary centers (2020–2021). The patients were matched to a healthy control group and were then divided into pediatric and adult groups. Data were collected from their electronic medical records. Additionally, the EQ-5D-5L QoL questionnaire was completed by both the patients and control group to assess five domains (mobility, self-care, usual activities, pain/discomfort, and anxiety/depression).

Results: The study included 248 patients with CAH (females: 58.8%), with a family history of the condition (57.3%) and/or parental consanguinity (68.1%). The most frequently reported gene defect was CYP21A2, while the most commonly reported symptoms/signs were ambiguous genitalia and obesity. Almost all female patients had received corrective surgery. The questionnaire response rate was 86.3% (n=214/248). The CAH patient group's mean total QoL score was 85.2 compared with 99.8 in the control. Further, CAH patients had lower QoL scores in all domains compared to those in the control group ($p \leq 0.0001$–0.0023). The pain/discomfort and anxiety/depression domains were affected significantly more than the other domains were, with 47.7% and 44.4% participants, respectively, $p<0.0001$. Additionally, obesity was found to be a

predictor of reduced mobility following a logistic regression analysis ($p \leq 0.04$, OR (0.18-0.98)).

Conclusion: Patients with CAH reported lower QoL overall, particularly in the pain/discomfort and anxiety/depression domains. Based on this, we recommend the early involvement of psychologists in a multidisciplinary team approach, pre-marital screening, and the implementation of awareness programs for people diagnosed with CAH in communities with high consanguineous mating.

KEYWORDS

CAH, psychosocial, QOL, ambiguous genitalia, virilization

1 Introduction

Congenital Adrenal Hyperplasia (CAH) is an autosomal recessive disease caused by mutations or deletions among genes that encode enzymes producing sex steroids, mineralocorticoids, and glucocorticoids in the adrenal glands. Herein, 21-hydroxylase enzymes are the most commonly affected, resulting in impaired production of cortisol, mineralocorticoids, and elevated androgens and leading to virilization of varying degrees at birth in female patients (1). CAH is a chronic illness with a spectrum of clinical, biochemical, and genetic presentations that are likely to affect patients' quality of life (QoL) (2). Moreover, in some countries, patients with CAH suffer from a lack of access to affordable medical care and knowledge about their medical condition, societal stigmatization, and negative social beliefs related to intersex, cultural, and religious issues (2). For example, cultural factors, such as male predominance in certain societies, may influence peoples' decisions regarding gender assignment and reassignment, which may impact the QoL of patients with CAH throughout their life (2, 3). CAH patients' QoL can also be affected by their clinical features; for example, the recurrent presentation of severe vomiting, dehydration, and shock along with hypertension has been observed in patients with 11-hydroxylase deficiency (4). Excess androgens may also lead to excessive facial hair and early penile enlargement in males. Moreover, untreated female patients can experience excessive facial and body hair, a relatively deep voice, anovulation, and menstrual irregularities (4). In addition, both sexes can develop precocious puberty and a reduced final height due to rapid growth and early closure of the growth plate, in addition to puberty failure (1, 4–6). Herein, adjusting glucocorticoid doses is crucial to avoid overtreatment, which can lead to cushingoid habitus and undertreatment with the above features that further affect QoL in pediatric and adult patients (1, 5).

"QoL is defined as "individual's perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns" (7). Unfortunately, CAH patients often suffer from several physical and psychological complications despite early medical care. For instance, growth failure, obesity, infertility, adrenal masses, hypertension, cataract, and emotional and psychosocial sequelae may negatively affect their well-being and QoL. The extant literature indicates a discrepancy in QoL levels in these patients. However, there is lack of reports focusing on QoL in CAH patients in the Middle East (8–13). Additionally, most patients in the Middle East are affected by various social and religious factors, which require special consideration in the treatment of patients with CAH (4). A patient's cultural values and religious obligations can affect their decision-making in different aspects of CAH management. Furthermore, because of the social advantages associated with male assignments in Arab societies and other similar communities, early assignment with the male sex is likely to be valued more in cases of ambiguity.

Therefore, this study aimed to assess the QoL and clinical characteristics of pediatric and adult Saudi patients with CAH.

2 Materials and methods

2.1 Study design and setting

This retrospective case-control study was conducted at King Abdullah Specialized Children's Hospital (KASCH) and King Faisal Specialist Hospital and Research Centre (KFSH&RC) in Riyadh, Saudi Arabia.

2.2 Study participants

We included patients aged >5 years who were diagnosed with classic CAH from both KASCH and KFSH&RC from July 2020 to July 2021. The study sample comprised two groups: patients with CAH and matched healthy controls. For the CAH group, we included all pediatric and adult patients with a CAH diagnosis confirmed by clinical, genetic, and laboratory tests. In addition, the criteria for the control group consisted of 1) healthy siblings of patients with CAH, 2) with the nearest age, 3) and same-sex, as applicable. For CAH patients without siblings of the same sex, we enrolled siblings of the opposite sex. For patients without siblings, the sibling of another patient with CAH was enrolled considering the nearest age.

2.3 Questionnaire

A qualitative interview was conducted with all participants to deliver the questionnaire and discuss the impact of and concerns regarding CAH. Specifically, we used the EQ-5D-5L questionnaire to measure QoL in our study sample. This questionnaire consists of five domains (mobility, self-care, usual activities, pain/discomfort, and anxiety/depression) and uses a scale of 0–100 to represent a respondent's overall health status (14). A 5-point Likert scale is used for each domain. The total scores are positively proportional, meaning that higher scores indicate higher QoL. The EQ-5D-5L questionnaire was translated and validated in numerous languages by the EuroQol group. Further, permission was obtained from the EuroQol to use the Arabic version in this study. Additionally, the EQ-5D-5L questionnaire was validated in several studies for use among people with chronic diseases, such as asthma, diabetes mellitus, and cardiovascular diseases, and showed good validity and reliability (15–17).

2.4 Data collection

Owing to the COVID-19 pandemic, questionnaire responses were collected through telephonic interviews. Furthermore, informed consent was obtained from each participant. For those aged under 18 years, assent was taken from the child, with consent obtained from their parents. The patients completed the questionnaire independently, other than young children, who completed them with the assistance of their legal guardians.

All medical records were reviewed after obtaining consent via telephone. The collected data included patients' demographic details, including age, sex, sex of rearing, and clinical data (height, weight, body mass index (BMI) type of CAH, gene defect, date of diagnosis, presence of ambiguous genitalia, corrective surgery experienced, comorbidities, complications, parental consanguinity, family history of CAH, and medications used). In addition, these patients received medical follow-up from the day of diagnosis until their current age.

2.5 Statistical analysis

The descriptive variables are presented as percentages and frequencies based on the data distribution of the means (± SD) or medians (interquartile range). Inferential analyses were used to compare the pediatric and adult groups in terms of the questionnaire domains using a t-test for numerical data and a chi-square test for categorical data. Logistic regression analysis was performed to assess the predictors of reduced QoL among the various CAH complications. For the final inferences, results with p- values of less than 0.05 with two-sided testing were considered significant.

2.6 Ethical approval

The study was approved by the Institutional Review Board (IRB) of the King Abdullah International Medical Research Center (KAIMRC) and the KFSH&RC, Riyadh, Kingdom of Saudi Arabia.

3 Results

3.1 Description of the final study sample

The final sample comprised 248 eligible participants. The demographics and clinical characteristics of the pediatric group are shown in Table 1. The majority (58.8%, n=146/248) were in the pediatric group (mean age 11.5 ± 4.0 years for the males, 11.7 ± 3.9 years for females). Two pediatric males (46XY) were raised as females; both had a StAR gene deficiency. Additionally, CYP21 was the most frequently affected gene in the pediatric group (89%, n=130/146), followed by CYP11 (4.8%, n=7/146). The presence of ambiguous genitalia was the most common presentation (59.6%, n=87/146), followed by obesity (31.5%, n=46/146) and testicular adrenal rest tumors (TARTs) among 10 patients. Furthermore, parental consanguinity and a family history of CAH were reported in 65.8% and 56.8% of the patients, respectively.

TABLE 1 The demographic and clinical characteristics of the pediatric group aged 6–17 years with Congenital Adrenal Hyperplasia (CAH) (N = 146).

	Male (N = 57)	Female (N = 89)
Mean Age (years) (SD)	11.5 (4)	11.7 (3.9)
Opposite sex of rearing	0 (0)	2 (1.4)
Gene defect		
CYP21	50 (87.7)	80 (89.9)
CYP11	1(1.8)	6 (6.7)
StAR	2 (3.5)	2 (2.2)
HSD3	3 (5.3)	0 (0.0)
CYP17	1 (1.8)	0 (0.0)
StAR/ACY11A1	0	1 (1.1)
Medical and Surgical history		
Ambiguous Genitalia	4 (7.01)	83 (93.3)
Obesity	17 (29.8)	29 (32.6)
Precocious Puberty	15 (26.3)	19 (21.3)
Adrenal Crisis	5 (8.8)	9 (10.1)
Incorrect sex at birth	2 (3.5)	11 (12.4)
Testicular Adrenal rest tumor	10 (17.5)	0 (0.0)
Hyperglycemia	2 (3.5)	0 (0.0)
Primary Gonadal Failure	0 (0.0)	1 (1.1)
Corrective surgery	7 (12.3)	82 (92.1)
Two or more corrective surgeries	1 (1.8)	6 (6.7)
Any Surgery Complication	1 (1.8)	10 (11.2)
Parents Consanguinity	34 (59.6)	62 (69.7)
Family History of CAH	30 (52.6)	53 (59.6)

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The clinical characteristics of the adult patients with CAH are shown in Table 2. They constituted 41.1% (n=102) of the sample, with the majority (55.9%, n=57/102) being female (mean age for males was 25.6 ± 6.4 years and was 26 ± 6.6 years for females). A male sex was assigned to two adult females (46XX) with CYP21 and CYP11 gene mutations. The CYP21 gene defect was the most frequently observed (91.2%, n=93/102), followed by defects in CYP11 (4.9%, n=5/102) and HSD3 (3.9%, n=4/102). Among the reported CAH complications, a short stature was the most prominent (67.7%, n=69/102), with ambiguous genitalia being present in 52.9% (n=54/102), followed by obesity in 39.2% (n=40/102). TARTs were detected in 12 patients. Parental consanguinity and a family history of CAH were reported in 71.6% and 57.8% of patients, respectively.

TABLE 2 The demographic and clinical characteristics of the adult group aged 18–45 with Congenital Adrenal Hyperplasia (CAH) (N = 102).

	Male (N = 45)	Female (N = 57)
Mean age (years) (SD)	25.6 (6.4)	26.0 (6.6)
Opposite sex of rearing	2 (2)	0 (0.0)
Body measurement		
Mean Height (cm) (SD)	156.8 (11.1)	147.3 (14.4)
Mean Weight (kg) (SD)	69.3 (19.2)	61.7 (19.3)
Mean (BMI) (kg/m2) (SD)	28.6 (7.5)	28.2 (7.2)
Gene defect		
CYP21	40 (88.9)	53 (93.0)
CYP11	3 (6.7)	2 (3.5)
HSD3	2 (4.4)	2 (3.5)
Medical and Surgical history		
Ambiguous Genitalia	2 (4.4)	52 (91.2)
Obesity	14 (31.1)	26 (45.6)
Short Stature	27 (60.0)	42 (73.7)
Precocious Puberty	6 (13.3)	5 (8.8)
Adrenal Crisis	4 (8.9)	2 (3.5)
Incorrect sex at birth	2 (4.4)	2 (3.5)
Testicular Adrenal rest tumor	12 (26.7)	0 (0.0)
Oligomenorrhea	0 (0.0)	9 (15.8)
Primary Amenorrhea	0 (0.0)	12 (21.1)
Hyperglycemia	4 (8.9)	4 (7.0)
Primary Gonadal Failure	3 (6.7)	2 (3.5)
Corrective surgery	6 (13.3)	52 (91.2)
Two or more corrective surgeries	0 (0.0)	4 (7.0)
Any Surgery Complication	1 (2.2)	3(5.3)
Parents Consanguinity	33 (73.3)	40 (70.2)
Family History of CAH	24 (61.4)	35 (61.4)

3.2 QoL assessment

The response rate to the QoL questionnaire in CAH patients was 86.3% (n=214/248), and it was 100% (n=214/214) in the control group. The distribution of the questionnaire in adult CAH patients and the control group is shown in Table 3. Regarding the "mobility" domain, 12.3% (N=10/81) of adult CAH patients complained of various problems. For the "self-care" domain, only 6.2% (N=5/81) complained that they were affected, while the "usual activities" domain affected 16% (N=13/81) of the participants. The majority of adult patients with CAH (53.1%; N=43/81) were affected at variable degrees in the "pain/discomfort" domain, as well as in the "depression/anxiety" domain, which represents 46.9% (N=38/81) of the adult patients. The median total health score (0-100) was 90, with an IQR of 15. QoL was significantly different in adult patients with CAH in the five domains compared to the healthy control group. We encountered 21 adult CAH patients without siblings of the same sex. Thus, we enrolled controls of the opposite sex (20.6%; adult control group) (Table 3).

Similarly, 15.8% (N=21/133) of pediatric CAH patients reported problems in the "mobility" domain. For the "self-care" domain, only 8.3% (N=11/133) reported any negative experiences. Regarding the "usual activities" domain, 18.8% (N=25/133) reported experiencing various problems. For the "pain/discomfort" domain, 44.5% of patients (N=59/133), which includes the majority of pediatric CAH patients, indicated that they had experienced problems at varying degrees, similar to the "depression/anxiety" domain (42.9%; N=57/133). The median total health score (0–100) was 94, with an IQR of 20 in pediatric patients with CAH. The overall QoL in pediatric patients with CAH was found to be more significantly affected in the five domains compared to that in the healthy control group (Table 4). We encountered 63 pediatric CAH patients without siblings of the same sex. Hence, we enrolled controls of the opposite sex (56.3%; pediatric control group) (Table 4).

During the qualitative interviews, patients were asked about the factors that affect their QoL in terms of CAH. The answers mainly concerned the lifelong course of the disease, its medications, and the related long-term hospital visits. Some participants reported about CAH affecting their sexual life, either physically or psychologically, and expressed the fear of having affected children of their own. Other answers were those describing disease complications, such as short stature.

3.3 Predictors of QoL

Logistic regression analysis results in Table 5 show that obesity was significantly associated with impaired mobility in our patient groups (OR=0.4 (CI=0.2-1.0); p = 0.04), while precocious puberty was associated with pain/discomfort (OR=0.5 (CI=0.2-1.0); p = 0.04).

TABLE 3 Comparison between the adult CAH and control groups' responses to the EQ-5D questionnaire.

Variable		Adult CAH N=81	Control N=102	*P*
Mean age (years) (SD)		26 (6.6)	25.5 (5.6)	0.3
Gender, n (%)	Male	36 (44.4)	52 (51)	0.1
	Female	45 (55.5)	50 (49)	
Mobility, n (%)	No problem	71 (87.7)	102 (100)	***<.0001***
	Slight problem	6 (7.4)	0 (0)	
	Moderate problem	4 (4.9)	0 (0)	
	Severe problem	0 (0)	0 (0)	
	Unable/Extremely	0 (0)	0 (0)	
Self-care, n (%)	No problem	76 (93.8)	214 (100)	***<.015***
	Slight problem	2 (2.6)	0 (0)	
	Moderate problem	1 (1.2)	0 (0)	
	Severe problem	1 (1.2)	0 (0)	
	Unable/Extremely	1 (1.2)	0 (0)	
Usual Activities, n (%)	No problem	68 (84)	101 (99)	***<.0021***
	Slight problem	8 (9.9)	1 (1)	
	Moderate problem	4 (4.9)	0 (0)	
	Severe problem	0 (0)	0 (0)	
	Unable/Extremely	1 (1.2)	0 (0)	
Pain/discomfort, n (%)	No problem	38 (46.9)	102 (100)	***<.0001***
	Slight problem	19 (23.5)	0 (0)	
	Moderate problem	21 (25.9)	0 (0)	
	Severe problem	3 (3.7)	0 (0)	
	Unable/Extremely	0 (0)	0 (0)	
Depression/anxiety, n (%)	No problem	43 (53.1)	97 (95.1)	***<.0001***
	Slight problem	12 (14.8)	4 (3.9)	
	Moderate problem	17 (21)	0 (0)	
	Severe problem	6 (7.4)	0 (0)	
	Unable/Extremely	3 (3.7)	1 (1)	
Median total health scale 0-100 (IQR)		90 (15)	100 (0)	***<.0001***

The bold values denote statistically significant P values.

4 Discussion

In our study, the 21-hydroxylase deficiency was the most common gene defect found among CAH patients, followed by CYP11 and HSD3, which is consistent with previous literature (18). Most CAH cases are associated with parents' consanguinity and a family history of CAH. The most common complications observed in our patients were ambiguous genitalia in neonates, precocious puberty in childhood, and obesity and short stature in adulthood. Almost all female patients in our cohort had received corrective surgery. Further, patients with CAH reported lower QoL in all domains; however, our sample primarily experienced problems in the pain/discomfort and depression/anxiety domains. Moreover, patients reported a lower total health score than the healthy matched controls.

Globally, some studies have assessed QoL in pediatric and adult patients with CAH. Similar to our findings, these studies found CAH patients to have an impaired QoL in all domains compared to healthy participants (9, 19, 20). In the extant literature, QoL was either more significantly compromised in males or females or was equally compromised in both (13, 21–23). Some studies have found no effect of CAH on the QoL of adult patients compared to healthy participants, although males with CAH were found to be less sexually active (24, 25). In the Middle East, specifically, studies

TABLE 4 Comparison between the pediatric CAH and control groups' responses to the EQ-5D questionnaire.

Variable		Pediatric CAH N=133	Control N=112	*P*
Mean age (years) (SD)		11.7 (3.9)	12 (4)	0.5
Gender, n (%)	Male	47 (35.3)	68 (60.7)	***<.0001***
	Female	86 (64.6)	44 (39.3)	
Mobility, n (%)	No problem	112 (84.2)	112 (100)	***<.0001***
	Slight problem	10 (7.5)	0 (0)	
	Moderate problem	8 (6)	0 (0)	
	Severe problem	1 (0.8)	0 (0)	
	Unable/Extremely	2 (1.5)	0 (0)	
Self-care, n (%)	No problem	122 (91.7)	112 (100)	***<.007***
	Slight problem	7 (5.3)	0 (0)	
	Moderate problem	0 (0)	0 (0)	
	Severe problem	2 (1.5)	0 (0)	
	Unable/Extremely	2 (1.5)	0 (0)	
Usual Activities, n (%)	No problem	108 (81.2)	112 (100)	***<.0001***
	Slight problem	10 (7.5)	0 (0)	
	Moderate problem	13 (9.7)	0 (0)	
	Severe problem	1 (0.8)	0 (0)	
	Unable/Extremely	1 (0.8)	0 (0)	
Pain/discomfort, n (%)	No problem	74 (55.6)	112 (100)	***<.0001***
	Slight problem	28 (21.1)	0 (0)	
	Moderate problem	21 (15.8)	0 (0)	
	Severe problem	9 (6.8)	0 (0)	
	Unable/Extremely	1 (0.8)	0 (0)	
Depression/anxiety, n (%)	No problem	76 (57.1)	112 (100)	***<.0001***
	Slight problem	26 (19.6)	0 (0)	
	Moderate problem	23 (17.3)	0 (0)	
	Severe problem	6 (4.5)	0 (0)	
	Unable/Extremely	2 (1.5)	0 (0)	
Median total health scale 0-100 (IQR)		94 (20)	100 (0)	***<.0001***

The bold values denote statistically significant P values.

assessing QoL in patients with CAH are scant. One study in Egypt assessed QoL in children and adolescents with CAH without using a control group reported an overall reduction in QoL, similar to our study (10). Another Israeli study reported that CAH has no effect on health-related QoL. However, this could be explained by their cohort being clustered in non-classical CAH, which has a favorable disease course (26).

Regarding the QoL domains in our study, many patients had lower scores in the pain/discomfort domain. These complaints were mainly related to increased pain in the limbs, such as that caused by obesity, which significantly limits mobility. The association between precocious puberty and the pain/discomfort domain of QoL remains unexplored in the literature, and we could not find an explanation for this phenomenon.

For the psychological domain assessed in our study, many participants reported being affected by several factors, including the chronicity of the disease, need for lifelong medication, impacts on their sexual life, fear of having affected children, need for continuous hospital visits, and various disease complications, such as short stature. The literature also reports that the psychological domain is among the most concerning in this population (9, 10, 12, 13, 27). Other studies have found increased psychiatric symptoms and morbidity rates in children, adolescents, and adult patients with CAH (19, 27), as well as increased anxiety

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TABLE 5 Logistic regression with collapsed variables among adults and pediatrics.

Variables		Age groups (Adults vs. Pediatrics)	BMI (Obese vs. Non-obese)	Ambiguous Genitalia	Adrenal Crisis	Precocious Puberty	Short Stature
Mobility	**Unadjusted Odds Ratio**	1.4	0.4	0.9	0.7	1.1	0.6
	(95% CI)	(0.6 – 3.1)	***(0.18 – 0.98)***	(0.4 – 1.9)	(0.1 – 3.3)	(0.4 – 2.9)	(0.3 – 1.5)
	X^2	0.6	***4.1***	0.08	0.2	0.03	1.1
	P	0.4	***0.04***	0.8	0.7	0.9	0.3
Self-care	**Unadjusted Odds Ratio**	1.4	0.6	1.8	0.7	NA	0.7
	(95% CI)	(0.5 – 4.3)	(0.2 – 1.8)	(0.5 – 5.7)	(0.1 – 5.7)	NA	(0.2 – 2.2)
	X^2	0.4	0.9	0.9	0.1	NA	0.3
	P	0.5	0.3	0.3	0.7	NA	0.6
Usual Activities	**Unadjusted Odds Ratio**	1.1	0.7	1.5	1.4	0.5	0.8
	(95% CI)	(0.5 – 2.3)	(0.4 – 1.5)	(0.7 – 3.2)	(0.4 – 4.4)	(0.2 – 1.4)	(0.4 – 1.8)
	X^2	0.1	0.6	1.1	0.3	1.8	0.2
	P	0.8	0.4	0.3	0.6	0.2	0.7
Pain/ Discomfort	**Unadjusted Odds Ratio**	0.6	0.7	1.01	1.8	0.5	1.4
	(95% CI)	(0.4 – 1.1)	(0.4 – 1.2)	(0.6 – 1.8)	(0.7 – 4.9)	(0.2 – 1.0)	(0.8 – 2.4)
	X^2	2.3	1.5	0.003	1.4	3.9	1.2
	P	0.1	0.2	0.9	0.2	0.04	0.3
Depression/ Anxiety	**Unadjusted Odds Ratio**	0.9	0.8	0.7	1.6	0.9	0.9
	(95% CI)	(0.5 – 1.6)	(0.5 – 1.4)	(0.4 – 1.2)	(0.6 – 4.3)	(0.5 – 1.9)	(0.5 – 1.7)
	X^2	0.1	0.4	1.6	0.9	0.01	0.02
	P	0.7	0.5	0.2	0.3	0.9	0.9

The bold values denote statistically significant P values.

and suicidal thoughts (12). This is supported by a Swedish study that reported fewer chances of marriage, a lack of sexual activity, and delays in psychosexual development in this population (28). In particular, males with CAH have fewer chances of having a lifetime partner and often experience erectile dysfunction, decreased sexual activity, and reduced fertility, which leads to substantial psychological issues (25, 27). A study on sexual orientation found that females with CAH had increased rates of homosexuality and bisexuality than the control group, which might be explained by excess prenatal androgen exposure (29).

A systematic review concluded that adult CAH males experienced more psychological issues than their female counterparts (11). In contrast, other studies reported that CAH did not significantly affect adult QoL compared to the healthy population, except that CAH patients were less sexually active (24, 25). Another study reported that males with CAH were satisfied with their sexual life with reasonable hormonal control, with the results being comparable to those of a healthy control population (30). Moreover, the frequency of sexual intercourse in patients with CAH did not differ from that in healthy control groups in another study (24). In our study, patients experienced psychosocial challenges similar to those mentioned in previous research. With some limitations to open discussions about psychosexual issues in our communities, most of these challenges should preferably be explored regularly in the routine follow-up of patients with CAH in the Middle East.

Our study has some limitations. First, due to the COVID-19 pandemic, we had to conduct phone interviews, which may have affected our response rate. Second, this was a retrospective study, meaning that we may potentially have missed some documentation in the clinical characteristics section. Third, the questionnaire was a generic tool rather than a disease-specific measure, which could have missed various patients' concerns and unique issues, such as those concerning their sexuality and the effects of genital surgeries.

5 Conclusion

In this study, participants with CAH reported a significantly reduced QoL in all domains, particularly in the pain/discomfort and

anxiety/depression domains, as well as lower total health scores compared to healthy controls. As such, we recommend the early involvement of a psychologist in a multidisciplinary team approach for treating CAH patients to improve their mental health outcomes. Further, in areas with a high prevalence of parental consanguinity, testing for CAH during pre-marital screening and awareness programs about genetic diseases such as CAH should be implemented nationwide.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

Author contributions

ES, MA, GA, IA, AB, AOA, and AfA contributed to the design and implementation of the study and writing of the manuscript. MA, ES, and ASA analyzed the data and wrote the methodology. KA, HA, AhA, AOA, ES, GA, and MA collected data and co-wrote the manuscript. IA, ES, MA, AB, AfA, HC-G, and BA reviewed and edited the final manuscript. IA, AB, and AfA supervised the project. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Semra Çaglar Çetinkaya,
University of Health Sciences, Türkiye

REVIEWED BY
Maria G. Vogiatzi,
University of Pennsylvania, United States
Elizabeth Reynolds,
RTI International, United States

*CORRESPONDENCE
Kyriakie Sarafoglou
saraf010@umn.edu

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Depressive and anxiety disorders and antidepressant prescriptions among insured children and young adults with congenital adrenal hyperplasia in the United States

Lauren A. Harasymiw[1], Scott D. Grosse[2], Kathryn R. Cullen[3], Rebecca H. Bitsko[2], Ruth Perou[2] and Kyriakie Sarafoglou[4,5]*

[1]Department of Pediatrics, University of Minnesota Medical School, Minneapolis, MN, United States, [2]National Center on Birth Defects and Developmental Disabilities, Centers for Disease Control and Prevention, Atlanta, GA, United States, [3]Department of Psychiatry and Behavioral Science, University of Minnesota Medical School, Minneapolis, MN, United States, [4]Department of Pediatrics, Division of Pediatric Endocrinology, University of Minnesota Medical School, Minneapolis, MN, United States, [5]Department of Experimental and Clinical Pharmacology, University of Minnesota College of Pharmacy, Minneapolis, MN, United States

Background: Dysfunction in the hypothalamic-pituitary-adrenal axis has been associated with depressive and anxiety disorders. Little is known about the risk for these disorders among individuals with congenital adrenal hyperplasia (CAH), a form of primary adrenal insufficiency.

Objective: We investigated the prevalence of depressive and anxiety disorders and antidepressant prescriptions in two large healthcare databases of insured children, adolescents, and young adults with CAH in the United States.

Methods: We conducted a retrospective cohort study using administrative data from October 2015 through December 2019 for individuals aged 4–25 years enrolled in employer-sponsored or Medicaid health plans.

Results: Adjusting for age, the prevalence of depressive disorders [adjusted prevalence ratio (aPR) = 1.7, 95% confidence interval (CI): 1.4-2.0, p<0.001], anxiety disorders [aPR = 1.7, 95% CI: 1.4-1.9, p<0.001], and filled antidepressant prescriptions [aPR = 1.7, 95% CI: 1.4-2.0, p<0.001] was higher among privately insured youth with CAH as compared to their non-CAH peers. Prevalence estimates were also higher among publicly insured youth with CAH for depressive disorders [aPR = 2.3, 95% CI: 1.9-2.9, p<0.001], anxiety disorders [aPR = 2.0, 95% CI: 1.6-2.5, p<0.001], and filled antidepressant prescriptions [aPR = 2.5, 95% CI: 1.9-3.1, p<0.001] as compared to their non-CAH peers.

Conclusions: The elevated prevalence of depressive and anxiety disorders and antidepressant prescriptions among youth with CAH suggests that screening for symptoms of depression and anxiety among this population might be warranted.

KEYWORDS

congenital adrenal hyperplasia, depression, anxiety, antidepressants, glucocorticoids

Introduction

Congenital adrenal hyperplasia (CAH) is an inherited form of primary adrenal insufficiency characterized by impaired cortisol synthesis and increased adrenal androgen production (1). Classic CAH requires life-long glucocorticoid replacement (2). Individuals with the salt-wasting form of classic CAH may also require mineralocorticoid replacement to counteract aldosterone deficiency (2). Non-classic CAH is a milder disorder with normal cortisol production in most cases; individuals with non-classic CAH typically require glucocorticoid replacement only when symptomatic (3). In the United States, the frequency of CAH detected by newborn screening programs ranges from approximately 1 in 16,000 to 1 in 18,000 (4, 5). Non-classic CAH is more common, with an estimated prevalence of approximately 1 in 200 US adults of European ancestry (6).

During childhood, hydrocortisone, a short-acting glucocorticoid, is used for replacement therapy to minimize the adverse impact on growth from long-acting glucocorticoids. Due to its short half-life, treatment with hydrocortisone can lead to alternating states of hypercortisolemia and hypocortisolemia with resultant hyperandrogenemia (7, 8). Chronic hypercortisolemia can lead to growth failure, iatrogenic Cushing syndrome, hypertension, increased weight gain, infertility, metabolic syndrome, hypertension, and osteoporosis in adulthood (9–13). Chronic hypocortisolemia and exposure to excess androgen can lead to virilization, peripheral precocious puberty, advanced bone age, growth acceleration and early epiphyseal closure leading to short stature (1, 10, 14).

The potential effects of CAH on mental health, either due to coping with a life-long disease or from the inherent hypothalamic-pituitary-adrenal axis dysfunction and associated therapeutic limitations, are less well understood. Some evidence suggests that individuals with CAH are more likely to experience symptoms of depression or anxiety compared to the general population (15–17). In a recent matched-cohort study from the United Kingdom using a primary-care based administrative database (UK Clinical Practice Research Datalink), Jenkins-Jones and colleagues (17) reported a higher prevalence of depression among 255 CAH patients younger than 18 years old, but no difference in the lifetime prevalence of depression diagnoses or antidepressant use among individuals aged 18–40 years. Two other recent studies utilized a national patient registry in Sweden to evaluate mental health diagnoses in large samples of patients with CAH (18, 19). Engberg and colleagues (18) reported that the odds ratio of a mood or anxiety disorder was 1.7 for females over the age of 18 years with CAH in Sweden compared to females the same age without CAH. However, there was no increase in risk of those diagnoses among females 12–18 years old with CAH, although substance misuse was significantly elevated among both adolescents and adults with CAH. Falhammar and colleagues (19) reported statistically insignificant odds ratios of 1.6 and 1.2 for mood and anxiety disorders for 239 males with CAH (median age 23.2 years) in Sweden compared to age-matched controls from the general population. Because they did not report age-stratified analyses, it is difficult to know how these findings apply to the pediatric population.

In particular, there is a paucity of data describing the prevalence of diagnosed depressive and anxiety disorders among large cohorts of pediatric and young adult patients with CAH. Most prior pediatric studies have reported information on relatively small samples of children and adolescents with CAH and utilized behavioral scales to measure parent-reported symptoms (20–23). For instance, Messina and colleagues conducted an observational study involving Swedish and Italian children aged 7-17 years with CAH (n = 57). They found that parents of CAH patients rated their children as having more social problems, as measured by the Child Behavior Checklist (CBCL), compared to the control group. No differences in internalizing problems or anxiety/depression symptoms were reported (20–23). In contrast, Idris and colleagues reported that parents disclosed a higher rate of both internalizing and externalizing problems among children aged 6-18 years with CAH in Malaysia (n = 49) as compared to a control group made up of non-affected relatives. However, there was no difference in the proportion of children with CAH with clinically significant scores on the anxious/depressed or withdrawn/depression syndrome subscales of the CBCL (20–23). In a study of 81 children ages 4-11 years with CAH in the United Kingdom, Kung and colleagues using found increased scores for conduct problems and hyperactivity/inattention and lower scores for pro-social behaviors via parent-report on the Strengths and Difficulties Questionnaire for girls with CAH as compared to unaffected relatives. No differences in emotional symptoms were noted for boys or girls, either as compared to unaffected relatives or the general population (20–23). Finally, in a study of 114 children and young adults with CAH (ages 3-31 years) in the United States, Berenbaum and colleagues did not find any differences in parent-report of internalizing or behavioral problems as measured by the CBCL among boys or girls CAH as compared to unaffected

relatives. However, a more negative affect was noted for adolescent and adults males with CAH as measured by the Self-Image Questionnaire for Young Adolescents (20–23).

The current study aimed to investigate the prevalence of depressive and anxiety disorders and antidepressant prescriptions among insured children, adolescents, and young adults in the United States with and without CAH who were enrolled in health plans contributing records to one of two large administrative healthcare databases. Prior research has consistently demonstrated that starting in adolescence, depressive and anxiety disorders have higher prevalence rates among females (24). We therefore also evaluated for sex-specific differences in the prevalence of depressive disorders, anxiety disorders and antidepressant prescriptions among adolescent and young adults with CAH.

Materials and methods

Data

We utilized the Merative™ MarketScan® Commercial and Multi-State Medicaid Research Databases to identify eligible patients. Administrative databases contain records generated as byproducts of reporting or paying for services and do not contain patient-reported or clinical information. We reviewed health insurance encounter records from October 1, 2015 through December 31, 2019, including data on outpatient and inpatient services and filled outpatient pharmacy prescriptions. Data from both databases were accessed and tabulated at the Centers for Disease Control and Prevention (CDC) using Merative MarketScan Treatment Pathways, an online analytic platform that is licensed to CDC and restricted to health plans that report outpatient pharmacy records for their enrollees, i.e., no pharmacy carve-outs. Data from the Commercial database relate to employees and their dependents enrolled in participating employer-sponsored health insurance plans throughout the United States. The Medicaid database includes data for children, adolescents and young adults enrolled in Medicaid or Children's Health Insurance programs from participating states, varying in number from 6 to 13 states.

We restricted both the Commercial and Medicaid samples to plans that report mental health encounters for their enrollees, i.e., no mental health carve-outs. We included records from all health plans, both capitated plans, which report records of services provided during encounters despite not filing claims for reimbursement, and non-capitated or fee-for-service plans with billing claims; by convention, we refer to both types of encounter records as claims.

Patients in eligible plans were included if they were enrolled at any point during October 1, 2015 through December 31, 2019, with no minimum length of enrollment specified, and had one or more claims during that period. For eligible patients, recorded age in years and sex were abstracted from the database at the start of the study period. We initially evaluated four age groups: preschool-aged children (3-5 years), school-aged children (6-11 years), adolescents (12-17 years), and young adults (18-25 years). Due to the small number of eligible patients aged 3 to 5 years, we created a single group of eligible children 4-11 years old, after excluding those with an age of 3 years.

Individuals who met the case definitions for mental health diagnoses or prescriptions were included in the age-specific prevalence calculations if they remained within the same age group at the time of the first mental health diagnostic or prescription claim. For example, a child who was initially aged 10 and became diagnosed with depression at age 12 is not included in the calculation of the prevalence of depression for the 4-11 age group. The denominators for those calculations were the numbers of people in the age group at the start of the period. Sex was defined as "Male" or "Female" but is not specified in either database as referring specifically to biological sex versus self-identified gender. No demographic variables other than age and sex were available in both databases.

Congenital adrenal hyperplasia

We defined Individuals with CAH using a previously-defined algorithm for pediatric CAH cases based on diagnostic codes from the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision, Clinical Modification (ICD-10-CM) as well as prescription drug data (25). Individuals were classified as having CAH if they had at least one claim with the ICD-10-CM code E25.0 ("Congenital adrenogenital disorders associated with enzyme deficiency") in any setting, no ICD-10 claims with a diagnosis code for a pituitary disorder (ICD-10: E228.x, E229.x, E236.x, E237.x), and at least two filled prescriptions for a glucocorticoid, with the second fill at least 28 days after and within 365 days of the first fill. The prevalence of CAH was defined as the percentage of children, adolescents, and young adults meeting these criteria.

Depressive and anxiety disorders

We classified individuals as having a "depressive disorder" if they had two or more outpatient claims or encounters at least 1 week apart or at least one claim from an inpatient setting with an ICD-10 diagnosis code for a depressive disorder (F32.0-32.4, F32.8, F32.9, F33.0-33.41, F33.8, F33.9, F34.1, F34.9, F43.21, F43.23, O90.6). We classified individuals as having an "anxiety disorder" if they had two or more outpatient claims a week apart or at least one claim from an inpatient setting with an ICD-10 diagnosis code for an anxiety disorder (F40.0, F40.1, F40.2, F40.8, F40.9, F41.0-41.3, F41.8, F41.9, F43.22, F43.23, F93.0). Specific ICD-10 codes were identified through review of the published literature (17, 26–42) and a methodology for syndrome surveillance established by the Centers for Disease Control and Prevention (43). This comprehensive list of ICD-10 codes was refined through expert review for application to the pediatric population.

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Antidepressant prescriptions

Individuals met the criteria for a filled antidepressant prescription if they had at least one diagnosis code for either a depressive or anxiety disorder, as defined above, and two or more outpatient pharmacy filled prescriptions for an antidepressant medication separated by at least 14 days with no maximum (see Supplemental Table 1 for the list of included medications).

Statistical analysis

To compare the prevalence of depressive disorders, anxiety disorders, and filled antidepressant prescriptions between the non-CAH and CAH groups, we calculated prevalence differences and prevalence ratios (PRs). We calculated 95% confidence intervals (CIs) for PRs using a Taylor series linearization of estimated variance and p-values for PRs using a two-sided Mantel-Haenszel chi-square test in MATLAB (Mathworks, Natick, MA). Because a few comparisons had expected prevalence numbers below the cutoff of expected numbers for an asymptotic chi-square test we also calculated p-values using an exact test. Almost all p-values were similar in terms of statistical significance above or below a p value of 0.05; exceptions are noted below (results available on request).

Analyses were calculated separately by payer type (Commercial sample and Medicaid sample) with stratification by age group. Owing to the appearance of sex-based differences beyond age 11, we also reported sex-specific estimates for the adolescent (12-17 years) and young adult (18-25 years) groups. For the main effect of CAH in each subsample, Mantel-Haenszel adjusted prevalence ratios (aPRs) are presented after stratification by age. The PRs presented for each age strata are unadjusted. Prevalence data for groups with fewer than five individuals meeting the case definition are not presented.

Results

Using our claims-based algorithm, we identified a total of 1056 individuals with CAH in the Commercial sample (N=12,313,882) and 570 individuals in the Medicaid sample (N=9,316,824). Descriptive data are presented for each sample in Tables 1, 2, respectively. In the general pediatric and young adult population, mental disorders and antidepressant prescriptions were higher within the Medicaid sample compared with the Commercial sample (Supplemental Figure 1). In the general pediatric population not treated for CAH, there was little difference between males and females prior to adolescence, i.e., aged 4-11, in the prevalence of depressive disorders and anxiety disorders (Supplemental Figure 2).

Within the Commercial sample, depressive disorders (Figure 1A) were significantly more likely among children (PR=1.9, 95% CI:1.1-3.4, p=0.026), adolescents (PR=1.4, 95% CI:1.0-1.9, p=0.044), and young adults (PR=1.9, 95% CI:1.5-2.4, p<0.001) with CAH when compared with their non-CAH peers in the same age group using the Mantel-Haenszel chi square test. However, the differences for the 4-11 and 12-17 years age groups were not significant when calculated using a Fisher exact test. Anxiety disorders (Figure 1B) were also more likely among children (PR=1.8, 95% CI:1.2-2.5, p=0.002), adolescents (PR=1.5, 95% CI:1.2-2.0, p=0.003), and young adults (PR=1.7, 95% CI:1.4-2.1, p<0.001) with CAH when compared with their non-CAH peers. Filled antidepressant prescriptions were significantly elevated only among young adults with CAH (PR=1.9, 95% CI:1.5-2.4, p<0.001) (Figure 1C).

Within the Medicaid sample, depressive disorders (Figure 1A) were more likely among children (PR=3.5, 95% CI:2.5-5.1, p<0.001), adolescents (PR=1.9, 95% CI:1.4-2.7, p<0.001), and young adults (PR=1.9, 95% CI:1.2-3.0, p=0.005) with CAH as compared with their non-CAH peers. Anxiety disorders

TABLE 1 Commercial insurance sample demographics based on congenital adrenal hyperplasia (CAH) diagnosis and the presence of a mental disorder diagnosis or a filled antidepressant prescription.

	All		Depressive Disorder[1]			Anxiety Disorder[2]			Antidepressant Prescription[3]		
	Non-CAH n	CAH n	Non-CAH Cases (%)	CAH Cases (%)	PD	Non-CAH Cases (%)	CAH Cases (%)	PD	Non-CAH Cases (%)	CAH Cases (%)	PD
4-11 y	3,935,932	370	60,867 (1.5)	11 (3.0)	1.4	167,453 (4.3)	28 (7.6)	3.3	49,250 (1.3)	7 (1.9)	0.6
12-17 y	3,262,212	313	254,529 (7.8)	34 (10.9)	3.1	308,607 (9.5)	45 (14.4)	4.9	227,867 (7.0)	29 (9.3)	2.3
18-25 y	5,114,682	373	396,310 (7.7)	54 (14.5)	6.7	532,236 (10.4)	66 (17.7)	7.3	487,041 (9.5)	68 (18.2)	8.7
Males											
12-17 y	1,628,564	129	91,603 (5.6)	16 (12.4)	6.8,	168,895 (10.4)	17 (13.2)	2.8	80,125 (4.9)	13 (10.1)	5.2
18-25 y	2,266,275	123	141,147 (6.2)	15 (12.2)	6.0	281,249 (12.4)	16 (13.0)	0.6	156,173 (6.9)	19 (15.4)	8.6
Females											
12-17 y	1,633,648	184	162,926 (10.0)	18 (9.8)	-0.2	139,712 (8.6)	28 (15.5)	6.7	147,742 (9.0)	16 (8.7)	-0.3
18-25 y	2,848,407	250	255,163 (9.0)	39 (15.6)	6.6	250,987 (8.9)	50 (20.0)	11.2	330,868 (11.6)	49 (19.6)	8.0

Years (y). prevalence difference (PD), expressed as percentage points. [1]Defined as 2 or more outpatient claims or 1 or more inpatient claims for a depressive disorder. [2]Defined as 2 or more outpatient claims or 1 or more inpatient claims for an anxiety disorder. [3]Defined as at least 2 filled antidepressant prescriptions and at least 1 claim for either a depressive or anxiety disorder.

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TABLE 2 Medicaid sample demographics based on congenital adrenal hyperplasia (CAH) diagnosis and the presence of a mental disorder diagnosis or a filled antidepressant prescription.

	All		Depressive Disorder[1]			Anxiety Disorder[2]			Antidepressant Prescription[3]		
	Non-CAH n	CAH n	Non-CAH Cases (%)	CAH Cases (%)	PD	Non-CAH Cases (%)	CAH Cases (%)	PD	Non-CAH Cases (%)	CAH Cases (%)	PD
4-11 y	2,956,016	208	104,316 (3.5)	26 (12.5)	9.0	166,825 (5.6)	23 (11.1)	5.4	64,708 (2.2)	13 (6.3)	4.1
12-17 y	1,836,169	115	224,997 (12.3)	27 (23.5)	11.2	189,213 (10.3)	24 (20.9)	10.6	168,435 (9.2)	25 (21.7)	12.6
18-25 y	1,343,950	66	156,853 (11.7)	15 (22.7)	11.1	160,306 (11.9)	15 (22.7)	10.8	145,926 (10.9)	17 (25.8)	14.9
Males											
12-17 y	914,446	44	79,866 (8.7)	9 (20.5)	11.7	66,702 (7.3)	8 (18.2)	10.9	60,335 (6.6)	9 (20.5)	12.0
18-25 y	364,496	21	36,909 (10.1)	–	–	36,707 (10.1)	–	–	33,023 (9.1)	–	–
Females											
12-17 y	921,723	71	145,131 (15.7)	18 (25.4)	9.6	122,511 (13.3)	16 (22.5)	9.2	108,100 (11.7)	16 (22.5)	10.8
18-25 y	979,454	45	119,944 (12.2)	15 (33.3)	21.1	123,599 (12.6)	14 (31.5)	18.5	112,903 (11.5)	15 (33.3)	21.8

Years (y). prevalence difference (PD), expressed as percentage points. Data not presented, <5 cases in subgroup (–). [1]Defined as 2 or more outpatient claims or 1 inpatient claim for a depressive disorder. [2]Defined 2 or more outpatient claims or 1 inpatient claim for an anxiety disorder. [3]Defined as at least 2 filled antidepressant prescriptions and at least 1 claim for either a depressive or anxiety disorder. Males with CAH in the Medicaid sample aged 18-25 years were excluded from this analysis as fewer than five subjects met the case definitions for depressive disorders, anxiety disorders, or antidepressant prescriptions.

(Figure 1B) were also more likely among children (PR=2.0, 95% CI:1.3-2.9, p=0.001), adolescents (PR=2.0, 95% CI:1.4-2.9, p<0.001), and young adults (PR=1.9, 95% CI:1.2-3.0, p=0.007) with CAH when compared with their non-CAH peers. The prevalence of filled antidepressant prescriptions (Figure 1C) was also elevated among individuals with CAH for all age groups (children: PR=2.9, 95% CI:1.7-4.8, p<0.001; adolescents: PR=2.4, 95% CI:1.7-3.4, p<0.001; young adults: PR=2.4, 95% CI:1.6-3.6, p<0.001) as compared to their peers without CAH.

We next compared prevalence ratios for depressive disorders, anxiety disorders, and filled antidepressant prescriptions among adolescents and young adults after stratifying by sex. Within the Commercial sample, adolescent females with CAH were more likely to have an anxiety disorder than adolescent females without CAH (PR=1.8, 95% CI:1.3-2.5, p=0.001), but not a depressive disorder or filled antidepressant prescription (Figure 2). Young adult females with CAH were more likely to have a depressive disorder (PR=1.7, 95% CI:1.3-2.3, p<0.001) or an anxiety disorder (PR=2.3, 95% CI:1.8-2.9, p<0.001), and to fill a prescription for an antidepressant (PR=1.7, 95% CI:1.3-2.2, p<0.001) compared to young adult females without CAH.

Adolescent and young adult males in the Commercial sample were approximately twice as likely to have a depressive disorder (adolescents: PR=2.2, 95% CI:1.4-3.5, p=0.001; young adults: PR=2.0, 95% CI:1.2-3.1, p=0.006) and to fill a prescription for an antidepressant (adolescents: PR=2.0, 95% CI:1.2-3.4, p=0.007; young adults: PR=2.2, 95% CI:1.5-3.4, p<0.001) compared to adolescent and young adult males without CAH. There were no significant differences for anxiety disorders among adolescent or young adult males.

Adolescent females with CAH in the Medicaid sample were 1.6-1.9 times as likely to have a depressive disorder (PR=1.6, 95% CI:1.1-2.4, p=0.026) or an anxiety disorder (PR=1.7, 95% CI:1.1-2.6, p=0.022), and fill a prescription for an antidepressant (PR=1.9, 95% CI:1.2-3.0, p=0.005) as compared to adolescent females without CAH (Figure 2). Young adult females with CAH were 2.5-2.9 times as likely to have a depressive disorder (PR=2.7, 95% CI:1.8-4.1, p<0.001) or an anxiety disorder (PR=2.5, 95% CI:1.6-3.8, p<0.001), and to fill a prescription for an antidepressant (PR=2.9, 95% CI:1.9-4.4, p<0.001) as compared to young adult females without CAH.

The prevalence ratio was 2.3-2.5 times higher for adolescent males with CAH in the Medicaid sample for a depressive disorder (PR=2.3, 95% CI:1.3-4.2, p=0.006) or an anxiety disorder (PR=2.5, 95% CI: 1.3-4.7, p=0.005), and a filled antidepressant prescription (PR=2.4, 95% CI:1.4-4.3, p=0.004) as compared to adolescent males without CAH. Because fewer than five young adult males in the Medicaid sample with CAH met the case definitions for depressive disorders, anxiety disorders, or antidepressant prescriptions, results for this group were not reported (Table 2).

Discussion

In this retrospective cohort study, we examined the administrative prevalence of diagnosed depressive disorders, anxiety disorders, and filled antidepressant prescriptions among children, adolescents, and young adults with and without CAH using two large administrative healthcare databases. Depression and anxiety are common mental disorders that often emerge in late childhood or adolescence and can result in significant disability (44, 45). If untreated, symptoms that begin during childhood and adolescence may recur later in life (46, 47) and can lead to long-term functional impairment in adulthood (48). Thus, determining whether the risks for these conditions are elevated among the pediatric and young adult CAH population is important to improve clinical care for these individuals.

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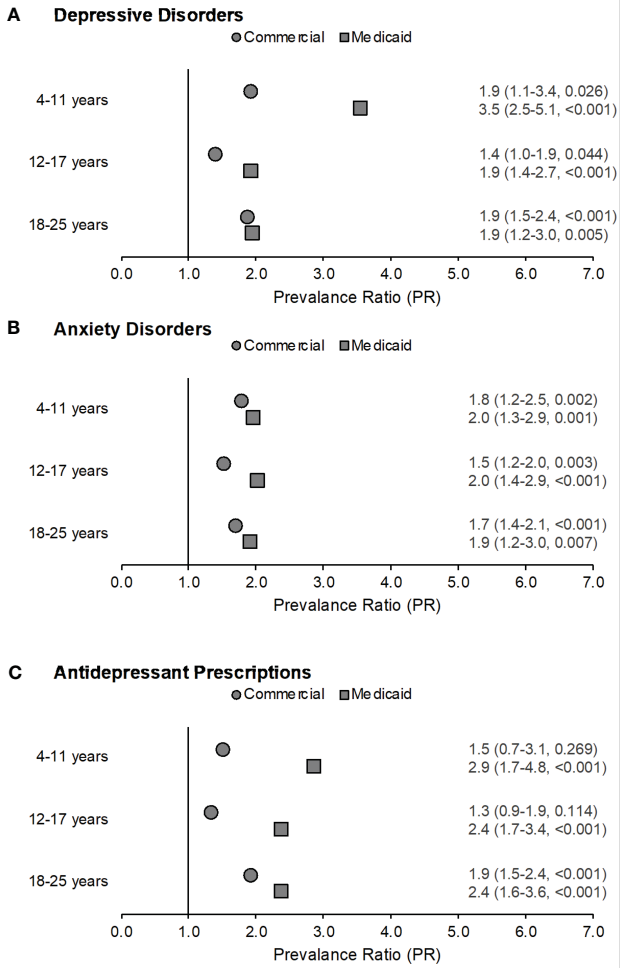

FIGURE 1
Prevalence ratios by age across both samples for depressive disorders (part **A**), anxiety disorders (part **B**), and filled antidepressant prescriptions (part **C**) among those with congenital adrenal hyperplasia (CAH) as compared to non-CAH peers in the same age group. Data labels reflect prevalence ratio (95% confidence interval, p-value). Males with CAH in the Medicaid sample ages 18-25 years were excluded from this analysis as fewer than five subjects met the case definitions for depressive disorders, anxiety disorders, and antidepressant prescriptions.

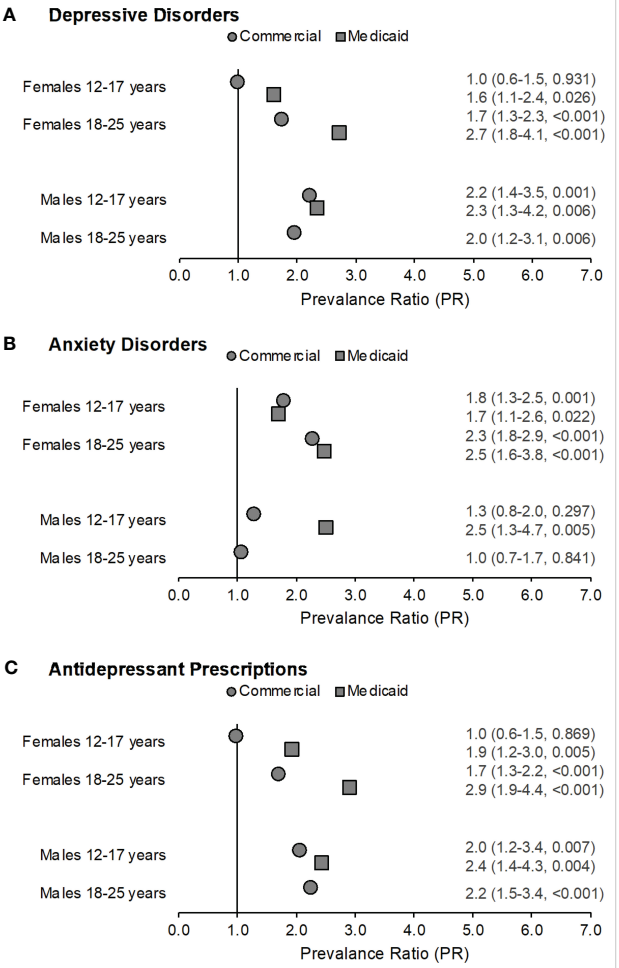

FIGURE 2
Prevalence ratios by gender across both samples for depressive disorders (part **A**), anxiety disorders (part **B**), and filled antidepressant prescriptions (part **C**) among those with congenital adrenal hyperplasia (CAH) as compared to their non-CAH peers. Data labels reflect prevalence ratio (95% confidence interval, p-value). Males with CAH in the Medicaid sample aged 18-25 years were excluded from this analysis as fewer than five subjects met the case definitions for depressive disorders, anxiety disorders, or antidepressant prescriptions.

School-aged children, adolescents, and young adults with CAH were relatively more likely to have records of a depressive disorder or an anxiety disorder compared to their peers without a CAH diagnosis in both the commercially and publicly insured samples (Figure 1). The finding of a lower administrative prevalence of the studied disorders prior to age 11 with no variation by sex (Supplementary Figure 2) is consistent with published evidence that male-female differences in depressive and anxiety disorders emerge in early adolescence (with consistently higher prevalence rates of depression and anxiety in female adolescents) (24). Also consistent with prior research in the general pediatric and young adult population (49), we found that the absolute administrative prevalence estimates for depressive disorders and anxiety disorders among individuals with and without CAH increased from childhood to adolescence (Tables 1, 2).

Our findings extend those of other large studies of CAH in children, adolescents, and young adults. In particular, In particular, Sewell and colleagues (50) reported no increased risk for depressive disorders among a cohort of 1,647 patients under the age of 18 years with a diagnosis of CAH recorded in electronic health records at one of six U.S. children's hospitals who had at least one outpatient visit during 2009–2019. In the same pediatric population, the investigators also found no increased risk for anxiety among males under the age of 18 with CAH, and lower odds of anxiety for females under the age of 18 with CAH compared with females without CAH (odds ratio = 0.7). The differences between our findings and Sewell's finding may reflect differences in classification of CAH case status. Unlike our study, Sewell et al. did not restrict their analysis to individuals with treated CAH. That might have biased associations in their study towards the null if

untreated individuals with diagnostic codes for "congenital adrenogenital disorders associated with enzyme deficiencies" did not actually have CAH. In our sample, fewer than half of individuals with a diagnosis code for CAH had a minimum of two filled prescriptions for glucocorticoids.

In our study, sex differences in the relative prevalence of diagnosed depressive and anxiety disorders and filled antidepressant prescriptions among adolescents with CAH were attenuated compared to the general population. Adolescent males with CAH had much higher rates of the mental health outcomes than their non-CAH male agemates. Prevalence ratios for depressive disorder and antidepressants were slightly higher for young adult males than for females in the Commercial sample; results were not reported for the Medicaid sample as noted in the Methods section. Anxiety disorders were more common for females in both age groups in both samples as well as for Medicaid-enrolled male adolescents (Figure 2). Commercially insured young adults with CAH as well as publicly insured children, adolescents, and young women with CAH were also approximately twice as likely to fill prescriptions for an antidepressant (Figures 1, 2). The findings of increased prevalence of filled antidepressant prescriptions among CAH patients relative to the general population are consistent with data from the UK Clinical Practice Research Datalink (17). This speaks to the impact and burden of depressive disorders in this population.

The higher absolute administrative prevalence estimates for depressive and anxiety disorders observed in the Medicaid sample may be in part explained by the Medicaid qualification process. Mood and anxiety disorders can be qualifying conditions for disability benefits under the Supplemental Security Income program, which in turn makes individuals eligible for Medicaid (51). Many children also qualify for Medicaid coverage based on household income, and the prevalence of mental, behavioral and development disorders among children living in lower-income households in the United States is higher than among those in higher-income households (52). Children and young adults with public insurance may also be at greater risk for adverse childhood experiences, such as witnessing violence and trauma, relative to those with private insurance. That would in turn raise their risk for negative health outcomes, including mental disorders (i.e., the toxic stress hypothesis) (53). The higher prevalence ratios for depressive disorders, anxiety disorders, and filled antidepressant prescriptions seen in some CAH groups in the Medicaid sample as compared to the Commercial sample could suggest that the presence of CAH may interact with or enhance these risks.

The pattern of sex-based differences in the prevalence of depressive disorders, anxiety disorders, and filled antidepressant prescriptions among adolescents and young adults with CAH was somewhat unexpected. While depression and anxiety are more commonly diagnosed among females than males beginning in adolescence (24, 54–56), the findings indicate that the likelihood of meeting criteria for a depressive or anxiety disorder or filling an antidepressant prescription among males with CAH was comparable to or higher than among females with CAH in the same age groups, except for anxiety among publicly insured males with CAH. These findings suggest a narrowing of the gender gap in depressive disorders and anxiety disorders among youth with CAH. This is consistent with data from the UK Clinical Practice Research Datalink (17), which showed a substantially narrower gender gap in depression diagnoses or antidepressant prescriptions in children or adolescents with CAH than among the general population, with a higher prevalence ratio relative to population controls for males with CAH (PR=2.2) than females (PR=1.2).

Pediatric and young adult patients with CAH may be at greater risk for mood and anxiety disorders due to multiple mechanisms. First, the burdens of living with a chronic disease, such as CAH, including recommended daily medication adherence and frequent healthcare contacts, may increase children's risk for anxiety and depression. Multiple studies have shown an increased risk for anxiety and depression among children with a wide range of chronic physical illnesses (57, 58) and life-limiting conditions (59). Second, children and young adults with CAH may be at increased risk for depression and anxiety due to the specific disease pathology and side effects from treatment. Dysfunction in the hypothalamic-pituitary-adrenal (HPA) axis and hypothalamic-pituitary-gonadal axis is known to impact mental health (60–62), and in particular, may alter stress reactivity and lead to downstream effects on mood and anxiety. In CAH patients, hypocortisolemia disrupts the endogenous negative feedback loop of the HPA axis and leads to overproduction of adrenal androgens. Further, cortisol replacement with glucocorticoids is unable to fully replicate the circadian and ultradian cortisol secretion rhythms associated with normal adrenal function (63). In a recent clinical trial utilizing a block and replace design among healthy young adult volunteers to test the significance of glucocorticoid replacement rhythmicity in mood regulation and neural dynamics, oral glucocorticoid replacement three times a day (which is the standard of care for CAH patients) showed a decrease in positive mood and an increase in negative mood throughout the day (64). This contrasted with individuals undergoing pulsatile glucocorticoid replacement (which more closely approximated physiological ultradian cortisol rhythms), in which mood variation more closely approximates what is thought to be normal variation in daily mood. Thus, excessive adrenal androgens, fluctuating cortisol levels, or over-suppression of the HPA axis could contribute to the development of depressive and anxiety disorders among CAH patients.

To elucidate potential causal pathways from CAH to prevalence of depression and anxiety, future work might examine the extent to which these associations vary relative to patients' specific enzyme defects, disease severity and phenotype, glucocorticoid form and dosage, and medication adherence. For example, the two Swedish registry-based studies assessed disease severity and phenotype. One study reported that the odds ratio for mood disorders was 2.0 among males with salt-wasting phenotypes vs 1.1 among those with the simple virilizing form, while the other study found no difference among female patients (18, 19). Jenkins-Jones and colleagues reported lower medication adherence among adult patients than pediatric patients but did not assess how adherence was related to

anxiety and depression (17). The present study is limited in that it did not assess medication possession ratios.

There are several additional limitations to our study. Despite the overall large number of individuals with CAH identified in our study, we could not include young adult males in the Medicaid sample Diagnosis codes for medical and mental health conditions in claims and encounters data are subject to miscoding or incomplete coding, which may have resulted in individual misclassification. We sought to minimize the impact of miscoding, which is more common in outpatient records, by using algorithms that require diagnosis codes be present on at least two outpatient records on different dates. Further, while the current results build upon our prior study detailing the development of our CAH case algorithm, the sensitivity and specificity of our CAH algorithm has yet to be validated through comparison with external data sources, such as medical records. Additionally, because Treatment Pathways reports age in years, we used broad age groups. Because we excluded mental health diagnoses for individuals who moved between age groups during the 3-year study period, we did not include all cases of mental health diagnoses in our age-specific prevalence estimates. Cumulative age-specific prevalence estimates without exclusion of those who aged out were higher for both the CAH and non-CAH populations, especially for the youngest age group, but prevalence ratios were almost all within 10% of those reported (results not reported).

The MarketScan datasets are convenience samples and hence the findings may not be generalizable to the populations of people with employer-sponsored or Medicaid insurance. Finally, we were unable to control for risk factors and cofounders not included in both databases, such as race and ethnicity (included in Medicaid only), geographical location (included in Commercial only), stated gender identity versus biological sex, parent education level, and socioeconomic status (neither database). Additional research using other data sources could potentially elucidate the impact of these factors.

In conclusion, in our retrospective cohort study we found that children, adolescents, and young adults with CAH in the United States were more likely to be diagnosed with a depressive or anxiety disorder and to be prescribed antidepressants as compared to their age and sex matched peers. The likelihood of these conditions increased with age and did not follow the same gender distribution commonly observed in the pediatric and young adult population, with a concentration of cases among males with CAH. If these associations are confirmed in further research, enhanced screening for symptoms of depression and anxiety among the pediatric and young adult population with CAH may be warranted.

Data availability statement

The datasets analyzed for this study are the property of Merative (Ann Arbor, Michigan, USA), formerly IBM Watson Health, and may be accessed by researchers through a contract and data use agreement with the company.

Author contributions

LH, SD, and KS contributed to the study conception and design. Data analysis was performed by LH and SD. The first draft of the manuscript was written by LH.SD and KS wrote sections of the manuscript. KC, RB and RB contributed to manuscript revision and additional data interpretation. All authors read, and approved the submitted version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1129584/full#supplementary-material

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EDITED BY
Semra Çaglar Çetinkaya,
University of Health Sciences, Türkiye

REVIEWED BY
Dulanjalee Kariyawasam,
Hópital Necker-Enfants Malades, France
Agustini Utari,
Diponegoro University, Indonesia

*CORRESPONDENCE
Mark de Hora
mdehora@adhb.govt.nz

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The use of liquid chromatography-tandem mass spectrometry in newborn screening for congenital adrenal hyperplasia: improvements and future perspectives

Mark de Hora[1,2]*, Natasha Heather[1,2], Dianne Webster[1,2], Benjamin Albert[2] and Paul Hofman[3]

[1]Newborn Screening, Specialist Chemical Pathology, LabPlus, Auckland City Hospital, Auckland, New Zealand, [2]Liggins Institute, University of Auckland, Auckland, New Zealand, [3]Clinical Research Unit, Liggins Institute, University of Auckland, Auckland, New Zealand

Newborn screening for congenital adrenal hyperplasia using 17-hydroxyprogesterone by immunoassay remains controversial despite screening been available for almost 40 years. Screening is confounded by poor immunoassay specificity, fetal adrenal physiology, stress, and illness which can result in a large number of false positive screening tests. Screening programmes apply higher screening thresholds based on co-variates such as birthweight or gestational age but the false positive rate using immunoassay remains high. Mass spectrometry was first applied to newborn screening for congenital adrenal hyperplasia over 15 years ago. Elevated 17-hydroxprogesterone by immunoassay can be retested with a specific liquid chromatography tandem mass spectrometry assay that may include additional steroid markers. Laboratories register with quality assurance programme providers to ensure accurate steroid measurements. This has led to improvements in screening but there are additional costs and added laboratory workload. The search for novel steroid markers may inform further improvements to screening. Studies have shown that 11-oxygenated androgens are elevated in untreated patients and that the adrenal steroidogenesis backdoor pathway is more active in babies with congenital adrenal hyperplasia. There is continual interest in 21-deoxycortisol, a specific marker of 21-hydroxylase deficiency. The measurement of androgenic steroids and their precursors by liquid chromatography tandem mass spectrometry in bloodspots may inform improvements for screening, diagnosis, and treatment monitoring. In this review, we describe how liquid chromatography tandem mass spectrometry has improved newborn screening for congenital adrenal hyperplasia and explore how future developments may inform further improvements to screening and diagnosis.

KEYWORDS

congenital adrenal hyperplasia, newborn screening, steroid profiling, bloodspots, LCMSMS congenital adrenal hyperplasia, LCMSMS

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1 Introduction

Congenital adrenal hyperplasia (CAH) caused by mutations in CYP21A2 results in reduced activity of 21-hydroxylase, an enzyme essential to the synthesis of aldosterone and cortisol. If not detected, the severest form of CAH, leads to life-threatening salt-wasting (SW-CAH) and hypoglycaemia in the early neonatal period. Patients with the simple virilising form of CAH (SV-CAH) have sufficient enzyme activity to maintain electrolyte balance under all but the most extreme conditions (1). The metabolic block in CAH diverts adrenal steroidogenesis towards excessive androgen production, stimulated by a lack of negative feedback on the hypothalamus and pituitary glands by cortisol, causing pre-natal virilisation. Without careful clinical management, increased adrenal androgens (and the aromatization to oestrogens) causes rapid post-natal growth, epiphyseal maturation, premature puberty and subfertility in both sexes. Undetected cases also carry a risk, particularly during childhood, of an acute adrenal crisis during periods of fever or infection. A milder non-classic form of CAH results from a partial enzyme deficiency (NC-CAH) and is considered one of the most common recessive inherited disorders (2), with variable degrees of androgen excess which may lead to rapid growth and premature puberty in childhood and subfertility in adulthood (1).

2 Newborn screening for CAH

Newborn screening (NBS) for CAH, available in most developed countries, is successful in preventing salt-wasting adrenal crises with the additional benefits of earlier treatment and reversal of incorrect sex assignment. Measurement of bloodspot 17-hydroxyprogesterone (17OHP), the main accumulating adrenal steroids, by high throughput automated immunoassay in the newborn period is usually used as a screening test. Mutation analysis or characteristic steroid profiles in plasma, with or without an adrenal stimulation test, can confirm the diagnosis (1).

The accuracy of screening has been limited by two main confounding factors. Firstly, dynamic changes in the fetal hypothalamic-pituitary-adrenal (HPA) axis in the early third trimester can lead to the accumulation of large quantities of adrenal steroids and their sulfated conjugates in blood that interfere with immunoassays resulting in falsely elevated measurements of bloodspot 17OHP (3). Secondly, the late expression of some adrenal enzymes in babies born before term, and HPA stimulation of steroidogenesis in stressed or ill neonates, increases the blood concentration of 17OHP (4, 5).

Many NBS laboratories use birthweight (BW) or gestational age (GA) adjusted laboratory thresholds for 17OHP immunoassays as there is a negative correlation between GA and BW with 17OHP measurements (6, 7). Additional improvements may be possible with the combined use of BW and GA (8) or with the collection of additional screening samples (9). Another approach is to use a second-tier immunoassay after the removal of interfering metabolites with a non-polar volatile solvent. While reducing the number of falsely elevated results, the positive predictive value (PPV) of CAH screening remains one of the lowest of disorders included in some NBS programmes (10). Indeed, a review of screening in France led to the recommendation for the discontinuation of screening in premature babies due to the unacceptably low PPV (0.4%) of screening and the close clinical monitoring that is available in hospitalised babies (11), while screening has not yet been recommended in the United Kingdom (12) in part due to the limitations of immunoassay.

Newborn screening using 17OHP immunoassays has high sensitivity in identifying SW-CAH, but some cases of SV-CAH and most NC-CAH will not be detected. In a retrospective analysis of 143 cases of CAH identified by newborn screening over 26 years in Sweden, the sensitivity of screening for SW-CAH, SV-CAH and NC-CAH were 100%, 79.7% and 32.4% respectively when molecular analysis was used to define disease classification (13). Higher 17OHP thresholds for babies born before term will improve the PPV of screening but may reduce the screening sensitivity for SV-CAH, although cases will be missed even with low screening thresholds for 17OHP (14, 15). In general, variations in screening accuracy can also be associated with differences in the timing of sample collection (16), the number of repeat samples collected (9, 16), whether second tier testing is used as part of the screening pathway (17) and how different screening programmes define a positive screening test. The most frequent recommended age for screening sample collection is between 24-72 hours, as later collections increase the likelihood of progressive salt wasting prior to screening notification. However, collection of additional later samples, such as occurs routinely in two-screen states, increases the sensitivity of screening for SV-CAH and NC-CAH (9). In recent years, many screening protocols have incorporated second-tier liquid chromatography-tandem mass spectrometry testing, which accurately measures multiple informative steroids simultaneously and can further improve the efficiency of screening (15, 18, 19).

3 Blood spot steroids by liquid chromatography –tandem mass spectrometry

Tandem mass spectrometry (LCMSMS) is a core analytical technology in many clinical and public health laboratories. The technique facilitates the simultaneous quantitation of low molecular weight metabolites in biological specimens. In NBS, target metabolites are extracted from punched bloodspot disks with a suitable volatile polar solvent, then nebulized and ionised through a heated high voltage probe. The resulting molecular ions enter the mass spectrometer in a gaseous state for mass filtering and detection. Mass detection is usually in the multiple reaction monitoring (MRM) mode due to the high signal to noise ratios that can be achieved. Molecular ions are focused through the first mass filter, then fragmented, after which specific mass fragments associated with the molecular ion can be detected and measured. Rapid switching of both mass filters allows specific molecular to fragment ion transitions to be collected for each target metabolite.

For steroid analysis, additional sample clean up using liquid chromatography removes ion suppressing compounds that are present in bloodspots, enabling the sensitive measurement of nanomolar concentrations of steroids. The use of matched isotopic labelled steroids improves the accuracy of measurement as they can be used to correct for any loss of target steroids during the sample preparation procedure.

Improvements in NBS for CAH have been possible with the introduction of LCMSMS to measure steroids in bloodspots (18–20). Commonly measured steroid metabolites such as 17OHP, androstenedione (A4), 11-deoxycortisol (11DF), 21-deoxycortisol (21DF) and cortisol (F) are particularly suited to LCMSMS analysis. These and other steroids with a Δ4 ring structure (4-pregnene or 4-androstene) are proton acceptors and are readily ionised using the electrospray technique universally used by newborn screening laboratories. Other informative adrenal steroids in the Δ5 pathway such as dehydroepiandrosterone (DHEA) are more difficult to ionise while testosterone (T) and dihydrotestosterone (DHT) are not adrenal specific and are normally not used in screening protocols.

Early LCMSMS methods described the measurement of 17OHP, A4 and F in bloodspots as a second-tier test on the same bloodspot specimen when the initial 17OHP immunoassay measurements were elevated (19). Steroids are extracted from bloodspots using a solvent such as acetonitrile, methanol or diethylether after which eluants are dried and reconstituted in a suitable solvent for LCMSMS or undergo further purification using an additional solid phase extraction step (21, 22) before analysis. Additional informative steroids such as 11DF and 21DF were incorporated and methods now have established screening protocols using 17OHP, 21DF and a combination of steroid ratios (18, 20). The metabolic block in CAH leads to accumulation of blood 17OHP, A4 and 21DF while the distal metabolites 11DF and F will be reduced. Several methods have incorporated an expanded profile that includes other Δ4 steroids such as progesterone, corticosterone, 11-deoxycorticosterone, testosterone and cortisone but there is no indication that these have been incorporated into NBS protocols (23, 24). Most detailed published methods (Supplementary Table) use standard C18 reverse phase chromatography columns which are particularly suited to separate informative steroids in less than 10 minutes (18–21, 23–30).

Accurate measurement is made possible with the incorporation of bloodspot steroid calibrators. Whole blood is washed with saline to remove endogenous steroids and then enriched with known quantities of target steroid metabolites in a serum substitute to manufacture bloodspot calibrators (19, 20, 24). In 2006, a pilot proficiency scheme was made available by the Newborn Quality Assurance Programme provided by the Center for Disease Control and Prevention (CDC, Atlanta, USA). The scheme distributed bloodspots enriched with known quantities of 17OHP, A4 and F to each participating laboratory. Participation in the scheme resulted in improved analyte recoveries and enhanced sample preparation and continuous improvements to second tier testing for CAH (31). The scheme has now expanded to include 11DF and 21DF while participation in the United States has grown from 11 in 2009 to 17 in 2019. Newborn screening for CAH is now available in Australia and the Royal College of Pathologists of Australasia has introduced a pilot scheme for bloodspots steroids to assist six regional laboratories in assessing their performance. Use of short analytical chromatography columns (≤50mm) can speed up analysis times (26) that may be sufficiently rapid for urgent analysis. Several studies have performed prospective and retrospective analyses of bloodspots to determine newborn reference intervals for steroids included in methods (20, 23, 24) but ranges are highly variable due to age, BW and GA ranges that are applied. There remains an ongoing need for the development of local population reference ranges after assay validation.

4 Use of LCMSMS to improve screening accuracy

The primary goal of bloodspot steroid analysis in NBS for CAH is to reduce the number of false positive tests encountered using immunoassay while maintaining screening sensitivity. False positive tests result in increased healthcare costs and can lead to lasting anxiety in families (32). LCMSMS is not suitable as a primary screening test as samples are processed sequentially and the cost of dedicated instrumentation is high. The method is almost universally used as a second-tier test when first tier immunoassay 17OHP measurements are elevated.

Analysis of 17OHP by LCMSMS in residual bloodspots revealed that measurements were lower in NBS specimens when compared to immunoassay and that correlation between the methods of measurement was poor (19, 20) due to the improved analytical specificity of LCMSMS. In a retrospective study from Minnesota, measurement of 17OHP by LCMSMS reduced the false positive rate by 55% due to the more specific nature of the second-tier analysis (18). However, prospective data over a 3-year period from the same screening programme, revealed that 41% of specimens reflexed to second tier LCMSMS had 17OHP measurements above the laboratory threshold for a positive screening test (33), indicating a need for alternative approaches to improve the positive predictive value.

One suggested approach is to use the steroid ratio (17OHP +A4)/F to further distinguish between babies with CAH and unaffected newborns. When used in combination, 17OHP and (17OHP+A4)/F, was modelled to reduce the FP rate by 93% in Minnesota (USA) when compared to an immunoassay only approach (18, 19). Similar improvements in the Utah (USA) screening programme found that the false positive rate was reduced from 2.6% to 0.09% (94% improvement) when using 17OHP with (17OH+A4)/F as second tier markers (34). In an Australian prospective study using 2 years of screening data, the positive predictive value of screening was 71.4% with the combined use of 17OHP and (17OHP+A4)/F (28). The use of BW or GA adjusted screening cut-offs for both 17OHP and (17OHP+A4)/F remain necessary as both parameters show a negative correlation with both GA and BW (8, 29). Incorporation of 11DF and 21DF measurements facilitated the calculation of additional informative steroid ratios such as 17OHP/11DF and (17OHP+21DF)/F (20, 21).

Most studies showed in improvements in specificity without any loss of screening sensitivity, however retrospective analysis of screening data from Minnesota revealed a reduction in screening sensitivity when second tier LCMSMS was introduced, partly due to the selection of screening thresholds for LCMSMS (14). It should be noted that 11DF and F are not stable when stored at room temperature for extended periods. The use of LCMSMS analysis of stored NBS specimens to set steroid ratio parameter screening threshold such as (17OHP+A4)/F or 17OHP/11DF may lead to inappropriately high thresholds that impact screening accuracy (35).

Incorporating 21DF as an additional bloodspot marker offers further improvements to screening for CAH as 21DF has long been recognised as a sensitive and specific marker of 21-hydroxylase deficiency (36, 37). In CAH, accumulating 17OHP is predominantly converted to 21DF by adrenal specific cytochrome P450 11β-hydroxylase (CYP11B1). In a retrospective and prospective study from Germany, 21DF showed a clear distinction between CAH affected and unaffected newborns. Additionally, the use of (17OHP +21DF)/F led to further improvements in sensitivity (20). A further benefit of using 21DF is that levels do not appear to correlate with BW or GA (37).

Two screening programmes have evaluated the use of 21DF alone as a second-tier screening marker. In a study from Wisconsin, 906 newborn screening specimens were subjected to 21DF analysis (851 unaffected, 55 affected with CAH) that yielded a test PPV of 91.7% when the laboratory threshold for 21DF was optimised for 100% sensitivity (37). A similar outcome was found in a screening pilot from the Netherlands which found the 21DF eliminated false positive results if used following 17OHP immunoassay (38). In both studies there were mild elevations in 21DF in some specimens from babies that were presumed not to have CAH which would result in a few additional sample recollections.

To date, prospective studies from newborn screening programmes, summarised in Table 1, have shown that the use of LCMSMS as a second tier test incorporating 17OHP, steroid ratios and 21DF can improve the PPV of NBS for CAH. Some milder cases of CAH may be missed by this two-tier approach.

5 Future directions – bloodspot androgen markers in CAH

5.1 Pathways to androgen synthesis in CAH

The enzyme deficiency in CAH diverts adrenal steroidogenesis towards excessive adrenal androgen production. There has been significant progress in our understanding of the pathways to androgen synthesis in CAH that raises the possibility of using less recognised androgen metabolites as biomarkers of CAH (42). In the classic adrenal steroidogenesis pathway, A4 and T are synthesised via DHEA as 17OHP is poorly converted directly to A4 by 17α-hydroxylase/17,20 lyase (CYP17A1). In CAH, accumulating 17OHP may overcome the low 17,20 lyase activity of CYP17A1

TABLE 1 Prospective studies on newborn screening for CAH using second tier LCMSMS analysis.

Author	No Screens	No. 2nd Tier Tests	CAH cases	2nd tier parameters	PPV before LCMSMS	PPV After LCMSMS
Janzen et al 2007 (20)	242,500	1609	16	17OHP (17OHP+21DF)/F	1.0%	100%
Matern et al., 2008 (33)	204,281	1298	9	17OHP (17OHP+A4)/F	0.8%	7.3%
Schwarz et al., 2009 (34)	64,115	1709	6	17OHP (17OHP+A4)/F	<1.0%	9.4%
Dhillon et al., 2011 (25)	2,702,000	10,932	143	17OHP (17OHP+A4)/F	1.3%	7.0%
Seo et al 2014 (39)	5852	104	2	17OHP (17OHP+A4)/F	1.9%	100%
Bialk et al 2019 (29)	63,725	472	5	17OHP (17OHP+A4)/F	1%	17%
Lai et al 2020 (28)	202,960	4218	12	17OHP (17OHP+A4)/F	–	71.4%
Stroek et al 2021 (38)	–	350	37	21DF	24.7%	53%
Cavarzere et al., 2022 (40)	99518	–	3	17OHP, 21DF,11DF (17OHP+A4)/F	0.24%	2.54%
de Hora et al., 2022 (15)	236,835	1915	11	17OHP, 21DF (17OHP+A4)/F	1.7%	45.8%
Lind Holst et al., 2022 (41)	593,435	15121	29	17OHP (17OHP+A4)/F	–	55.8%

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and drive the direct conversion of 17OHP to A4. In target tissues T is converted by 5α reductase (types 1 or 2) to the potent androgen dihydrotestosterone (DHT). Additionally, A4 may be 5α-reduced to 5α-androstandione (5αdione) before 17β-reduction by to DHT (43).

In 2004, a "backdoor" pathway was described with a metabolic route from 17OHP to DHT that does not involve A4 or T. In CAH, accumulating 17OHP is 5α- and 3α- reduced before being converted to androsterone by CYP17A1 with subsequent reduction and oxidation steps yielding DHT (44). Urinary steroid profiles in babies with CAH revealed that this pathway is active in CAH in the newborn period (45). The backdoor pathway may also make further contributions to the total androgen pool in CAH in the newborn period. *In vitro* studies have demonstrated that 11-hydroxylated corticosteroids such as 21DF, 21-deoxycortisone (21DE) and 11β-hydroxyprogesterone (11βOHP) can be converted by backdoor pathway enzymes to yield 11-ketodihydrotestosterone (11KDHT) (46), an androgen with a similar potency to DHT (Figure 1). Almost 60 years ago, Jailer and colleagues demonstrated that 21DF and not 17OHP dosing resulted in increased 11-hydroxyandrosterone (11OHAST) excretion, an indication that 21DF is an androgen precursor (47). Whether the route is via the backdoor pathway or by the direct conversion of 21DF to 11OHA4 via CYP17A1, 21DF may be an important contributor to the androgen pool in CAH.

More recently, there has been growing interest in the role of 11-oxygenated androgens in CAH. The adrenal glands produce a series of C19 steroids via the adrenal specific enzyme, CYP11B1. The most abundant of these 11-oxygenated androgens is 11-hydroxyandrostenedione (11OHA4), which normally circulates at higher concentrations than A4 (48). In CAH, accumulating A4 is readily hydroxylated by CYP11B1 leading to higher circulating concentrations of 11OHA4 and then oxidised in the periphery to 11-ketoandrostenedione (11KA4). Additionally, accumulating T is converted by adrenal CYP11B1 to 11OH-testosterone and then converted to 11-ketotestosterone (KT). Both 11KT and T have similar androgenic potency. Accumulating 11KA4 can be converted to 11KT by 17β-reduction in peripheral tissues (Figure 1). At specific target tissues such as adipose, prostate and skin, T, 11OHT and 11KT can be converted to the most potent androgens, DHT and KDHT. In treated classical CAH patients, plasma studies have shown that 11-oxygenated C19 steroids are the dominant circulating adrenal specific androgen precursors (48). Additionally, Turcu and colleagues revealed that adrenal 11-oxygenated androgen are disproportionally elevated compared to T and A4 in non-classical CAH in unstimulated blood tests (49).

5.2 Bloodspot androgen measurements for NBS for CAH

The challenge for NBS laboratories is the development of methods that can measure androgens and androgen precursor steroids in bloodspot specimens in a reliable way and to characterize the typical profiles in bloodspots from babies with classical CAH and unaffected newborns. While studies have

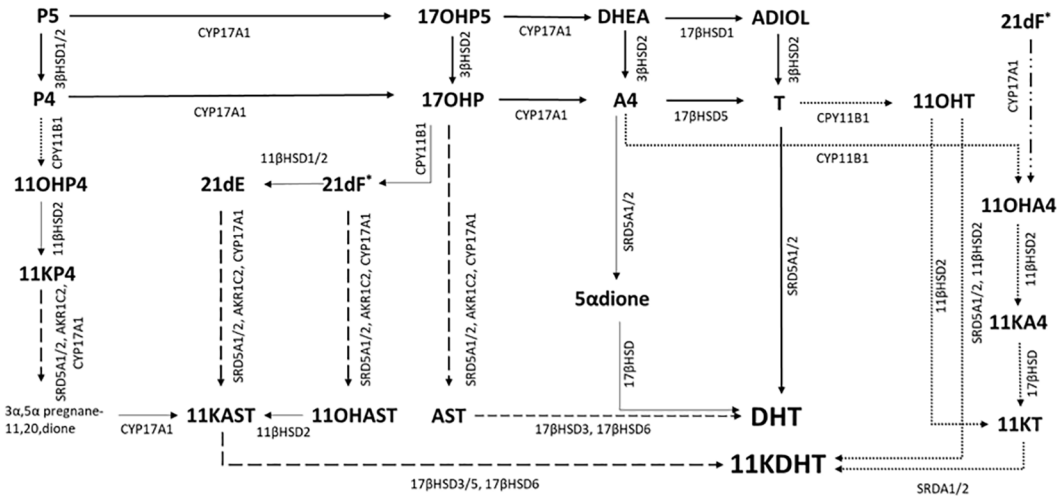

FIGURE 1
Possible pathways to androgen synthesis in CAH. Steroidogenesis is diverted by the metabolic block in CAH in classic pathway via A4 and DHEA. Accumulating 17OHP can overcome the relatively low A4 substrate affinity for 17α-hydroxylase/17,20 lyase CYP17A1 that results in A4 been directly converted to T. Subsequent 5α reduction (SRD5A) of T to DHT, one of the 2 most potent androgens, occurs in target tissues (Solid arrows). Accumulating 17OHP can also be metabolised to DHT via the steroidogenesis backdoor pathway, after 5α and 3α reduction the 17,20-lyase activity of CYP17A1 forms the C19 steroid androsterone (dashed arrows) which is then converted to DHT by subsequent reductive (17βHSD6) and oxidative reactions (17βHSD3). *In vitro* studies have suggested that additional accumulating C21 steroids, such as 21-deoxycortisol (21dF), 21-deoxycortisone (21dE) and 11-hydroxyprogesterone (11OHP4) can be metabolised by the backdoor pathway to 11-ketodehydrotestosterone (11KDHT), a C19 steroid with similar androgenic potency as DHT (dashed arrows). An additional route for androgen synthesis is through the C19 oxygenated steroid pathway (dotted arrows). Accumulating A4 and T are converted to 11OHA4 and 11OHT via the adrenal specific enzyme CPY11B1. 21DF may also be converted directly to 11OHA4 BY CYP17A1. 11OHA4 is the most abundant 11-oxygenated steroid. KT is synthesised via 11KA4 (11βHSD2, 17βHSD) and subsequently 5^{α}-reduced to 11KDHT. Enzymes are denoted by their coding gene. 17βHSD, 17β-hydroxysteroid dehydrogenase; CYP11B1, 11β-hydroxylase; SRDA1/2, 5α-reductase; AKR1C2, aldo-keto reductase.

revealed the 21DF is the most specific single corticosteroid marker for CAH, it is not 100% sensitive or specific in newborn screening (37, 38). The development of methods to include the 11-oxygenated C19 steroids along with DHT and KDHT in newborn screening for CAH may offer further improvements to screening accuracy. The C19 oxygenated steroids will undergo sufficient ionization in LCMSMS due to their 4-androstene structure while the saturated androgens (11KDHT, DHT) and the 5-androstenes (DHEA) have much lower ionization efficiencies that require more sensitive and expensive instrumentation for reliable quantitation.

Alternatively, chemical derivatisation can be used to improve the ionisation of steroids by electrospray ionisation. Many screening laboratories butylate endogenous amino acids and acylcarnitines to increase the ionisation efficiency of target compounds to screen for amino and fatty acid breakdown disorders. Use of derivatisation to enhance the sensitivity of steroid measurements has been reviewed (50, 51) but the most common method for enhancing the sensitivity of Δ5 steroids and C19 androgens is an oximation reaction. This is usually done as a last step in sample preparation and does not require any additional sample clean up after the derivatisation reaction is complete. In a method described by Caron and colleagues, hydroxylamine derivatised C19-oxygenated steroids were measured in plasma at a lower limit of quantification than other non-derivatised methods for 11A4OH, 11KA4, 11OHT, 11KT, 11KDHT, 11OHAST and 11KAST (52). The derivatisation method has also been applied to measure a broader range of steroids including DHT, corticosteroids and the steroids of the Δ5 pathway (53). while methoxylamine derivatisation was used to measure 17 ketosteroids in plasma (54). In a further study, oximation of ketosteroids before LCMSMS improved the lower limit of quantification for DHT by 25-fold when compared to an underivatized approach and greater improvements were achieved for DHEA and 21DF (55).

One of the limitations of derivatising steroids before LCMSMS analysis is that there is no universal derivatising chemical available for all steroid classes. Oximation only targets steroids with a carbonyl group, however almost all informative steroids in CAH have carbonyl functional groups. A second limiting factor of using hydroxylamine as a derivatising reagent is that several isoforms of target steroids can occur as hydroxylamine groups can form in an α or β configuration and steroids with 2 or more carbonyl group generally result in 2 chromatographic peaks. Lastly, isobaric androgen derivatives may also co-elute and chromatographic conditions should be sought to ensure they are appropriately separated on the chosen chromatography column. In a technical report, Hakkinen and colleagues assessed 3 types of reversed phase columns and found that a biphenyl column had enough selectivity to separate most of the ketosteroids that may be informative for NBS for CAH (51).

In summary, the accuracy of newborn screening for CAH due to 21-hydroxylase deficiency is improved with the use of second tier LCMSMS analysis. Screening programmes that use this approach have a lower false positive rate than programmes that use immunoassay alone. The use of additional steroid parameters such as (17OHP+A4)/F and 21DF offer even better sensitivity and specificity while incorporating oxygenated C19 steroids and the potent androgens DHT and KDHT may, in the future, offer further improvements to screening performance. To date, the markedly improved PPV due to the use of LCMSMS has strengthened the case for CAH newborn screening, that may be further strengthened as new markers are incorporated in NBS protocols.

Author contributions

Conceptualization; MD, DW, NH, BA, PH. Methodology; MD. Formal analysis; MD. Investigation; MD; Resources, MD; Data curation, Original draft preparation; MD. Writing, review and editing; NH, DW, BA, PH. Visualization; MD. Supervision; PH, NH, DW, BA. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1226284/full#supplementary-material

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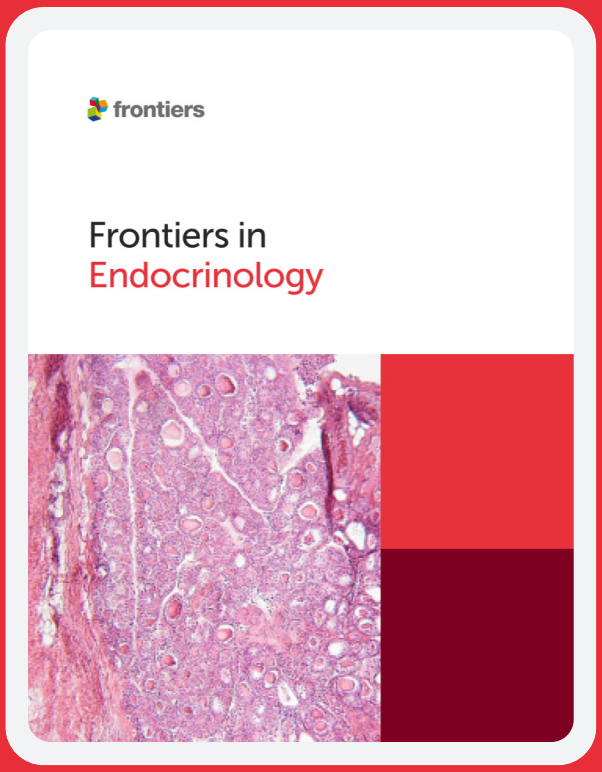

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