Outcome of Newborn Screening for Congenital Adrenal Hyperplasia at Two Time Points

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Abstract

Background/Aims: Screening newborns for congenital adrenal hyperplasia (CAH) is problematic owing to the dynamic changes in serum 17-hydroxyprogesterone (17-OHP) levels following birth. Our study objectives were to determine the accuracy of screening, severity of CAH, and biochemical and clinical outcomes of cases detected by our program which collects specimens at 2 time periods following birth.

Methods: We reviewed all CAH cases detected in the Northwest Regional Newborn Screening Program from 2003 through 2017. Comparison was made of screening and confirmatory serum 17-OHP, neonatal, maternal, and follow-up auxologic data, steroid treatment doses, and 21-hydroxylase genotype in cases detected on the first versus second test.

Results: Out of 164 cases of CAH, 25% were detected on the second screen. Infants detected on the second test had a lower screening 17-OHP (147 vs. 294 ng/mL), lower confirmatory serum 17-OHP (7,772 vs. 14,622 ng/dL), and were more likely to have simple virilizing CAH. There were no identifiable neonatal or maternal factors associated with detec-

tion on the second test. 21-Hydroxylase genotypes overlapped in first versus second screen cases. **Conclusion:** Early collection of specimens necessitated by early discharge resulted in milder CAH cases falling below the screening 17-OHP cutoff. In our program 25% of cases were detected on a routine second screen.

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Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disease, with 95% of cases caused by mutations in the *CP21A2* gene encoding the adrenal steroid 21-hydroxylase (21-OH) (P450c21) enzyme [1]. A 21-OH enzyme defect results in a deficiency of cortisol and, in the more severe salt-wasting form, aldosterone; the precursor steroids, including 17-hydroxyprogesterone (17-OHP) are shunted into the androgen biosynthetic pathway. Affected female infants present with virilization of the external genitalia, while babies with the severe salt-wasting form of CAH present with failure to thrive, hypovolemia, and shock in the first month of life [2]. CAH may be fatal if adrenal crisis is not detected and treated in the neonatal period.

Screening newborns for CAH by measurement of 17-OHP in the heel prick dried blood spot specimen is now performed in all 50 states in the USA and most developed countries in the world [3]. In the USA, the screening specimen is routinely obtained at 24–48 h of age, as guidelines recommend collection before discharge from the birthing unit. A number of factors influence the screening 17-OHP, including the age of specimen collection, birth weight, gestational age, associated comorbidities, and specificity of the assay, which may limit the accuracy of the initial screening test [4]. In healthy infants, serum levels of 17-OHP are normally high at birth and decrease rapidly in the first few postnatal days; in contrast, 17-OHP levels tend to rise in neonates with CAH. Most newborn screening (NBS) programs have developed birth weight and age-related 17-OHP cutoffs to reduce the number of false positive tests [5]. A few states have added “second-tier” screening (calculating ratios of precursor steroids to cortisol, or repeat measurement in babies with positive test results by liquid chromatograph-tandem mass spectrometry; LC-MS/MS) to reduce the number of false positive tests [6].

The Northwest Regional Newborn Screening Program (NWRSP) in the USA added CAH to its screening panel in 2003. The NWRSP collects 2 routine screening tests at 2 different time points. Annual summaries of CAH screening showed that a variable proportion of cases “passed” their first screening test and were then detected on the second routine test. While the explanation for why some babies passed their first screening test is unknown, we speculated that babies detected on the second routine test were more likely to have a milder form of CAH. The primary objective of our study was to determine the proportion of cases and severity of CAH detected on the first as compared to the second routine screening test. Secondary objectives included the natural history of CAH cases detected on the first versus second screening test, as assessed by growth, bone age, adrenal steroid hormone levels, and treatment doses of glucocorticoid and mineralocorticoid medications. In addition, we undertook 21-OH gene analysis in a subset of cases to determine if there might be a genotype-phenotype correlation in babies detected on the first versus the second screening test.

Methods

Description of the NBS Program to Detect CAH

The NWRSP began screening for CAH in 2003. Five states within our regional program undertook collection of 2 routine specimens (heel prick blood spotted on filter paper): Oregon, Idaho,

Table 1. Cutoffs for DBS 17-OH progesterone based on birth weight and age of specimen collection

	Normal range	Request for repeat DBS specimen	Request for confirmatory serum testing
<i>Birth weight >2,500 g</i>			
<i>Age of collection</i>			
<24 h	0–90	90–140	>140
1–10 days	0–60	60–90	>90
>10 days	0–25	25–50	>50
<i>Birth weight <2,500 g</i>			
<i>Age of collection</i>			
<24 h	0–120	120–180	>180
1–10 days	0–120	120–180	>180
10 days	0–40	40–70	>70

Data are expressed in serum units, ng/mL. DBS, dried blood spot.

Alaska, Nevada, and New Mexico, and some military bases in Washington and the South Pacific (Nevada withdrew from the NWRSP in 2014). The first specimen was obtained at 24–48 h of life, often just before discharge home, and the second typically at 10–14 days of age (the timing of collection of the second specimen was at the discretion of the baby’s primary care provider). All filter paper specimens were mailed to the Oregon State Public Health Laboratory where the screening test was performed. Detection of CAH was based on measurement of serum 17-OHP in the dried blood spot specimen. Throughout the study period, 17-OHP was measured by an automated time-resolved dissociation-enhanced lanthanide fluoroimmunoassay, with the exception of the first few months in 2003 when 17-OHP was measured by radioimmunoassay. The cutoffs for 17-OHP were stratified by birth weight and the age of specimen collection. 17-OHP cutoffs have undergone periodic minor revisions based on program evaluation; current cutoffs are presented in Table 1 (expressed in serum units, ng/mL). Using these cutoff levels, the positive predictive value was 7.35% on the first screening test and 8.6% on the second screening test. The false positive rate was 0.045% on the first screening test and 0.037% on the second test. The false negative rate was 0.43 on the first screening test; no false negative cases were known on the second screening test. When an abnormal result was identified, it was rapidly communicated to the infant’s primary care provider with a follow-up telephone call by an Oregon Health and Science University (OHSU) Pediatric Endocrine consultant, requesting confirmatory serum 17-OHP, androstenedione, serum electrolytes, and plasma renin activity. The diagnosis of CAH was based on a confirmatory serum 17-OHP >3,000 ng/dL. Recommendations were provided at this time regarding therapy initiation and follow-up care with a pediatric endocrinologist. Classification of CAH was made by the treating pediatric endocrinologist, based on biochemical results and clinical findings at diagnosis and follow-up. Cases were categorized as salt-wasting CAH if they manifested hyponatremia with an elevated plasma renin activity, either in the neonatal period or later, requiring treatment with both hydrocortisone and fludrocortisone. Cases

Fig. 1. a CAH cases from NWRSP from 2003 to 2017. Cases detected on first and second NBS test, total number, and number of cases included. **b** CAH cases included for follow-up data analysis.

without hyponatremia at diagnosis were categorized as simple virilizing CAH; these patients typically manifested signs of premature adrenarche, accelerated growth, advanced bone age, and occasional elevation of serum 17-OHP >3,000 ng/dL during follow-up, features compatible with their diagnosis of simple virilizing CAH.

Study Population

For the initial NBS and confirmatory serum test results, our study population consisted of all cases of confirmed CAH detected in the 5-state Regional Program born between 2003 and 2018 ($n = 164$; Fig. 1). Initial NBS results and confirmatory testing results were available from an existing database ($n = 164$; Fig. 1a). Using this existing database, we received permission to obtain follow-up data on CAH cases from 3 states (Oregon, Idaho, and Nevada, $n = 105$; Fig. 1b).

Collection of Follow-Up Data

We contacted individual patient primary care physicians or treating pediatric endocrinologists to obtain follow-up data. Birth history included the delivery method (vaginal or cesarean), gestational age, birthweight, and history of prenatal steroids. The anthropometric data collected included current height and weight.

Parental heights were obtained and mid-parental target height was calculated. Current glucocorticoid and mineralocorticoid doses were obtained. Laboratory evaluation obtained included the most recent monitoring test results, including serum 17-OHP, androstenedione, and renin, and the most recent bone age, if available.

Genetic analysis of the 21-OH gene was performed as a study-related procedure, at no cost to the family. The number of patients undergoing gene testing ($n = 17$) was limited by the available grant funding; all were followed at OHSU. 21-OH gene analysis was performed by Baby Genes Inc.; the entire *CYP21A2* gene was sequenced.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel 2013, version 15.0.5031.1000. Laboratory data are presented as the median and interquartile range, while clinical data are presented as the mean $\pm$ SD. Comparison of mean 17-OHP levels on screening tests and confirmatory testing in cases detected on the first versus the second newborn screen was analyzed using an unpaired 2-sided t test. Comparison of demographic and follow-up data between patients detected on the first versus second newborn screen was performed with an unpaired 2-sided t test or χ^2 test, where applicable. The frequency of fludrocortisone

Table 2. Demographic data for confirmed cases of CAH detected on the first versus second NBS

	1st NBS	2nd NBS	<i>p</i> value
<i>n</i>	123	41	
Female, %	45	53	0.359
Spontaneous vaginal delivery, %	72	79	0.203
Cesarean, %	28	21	0.729
Term (>36 weeks), %	91	87	0.631
Pre-term (<36 weeks), %	9	13	0.625
Birth weight (mean ± SD), g	3,353±758	3,220±681	0.700

p < 0.05 was considered significant. CAH, congenital adrenal hyperplasia; NBS, newborn screening.

use on follow-up of cases detected on the first versus second test was analyzed by the Fischer test. *p* values <0.05 were considered statistically significant.

Informed Consent and Institutional Review Board Approval

Data were collected from the OHSU electronic medical record and recorded in a de-identified manner for cases under the care of pediatric endocrinologists at OHSU. Those cases that were not current pediatric endocrinology patients at OHSU were contacted to obtain informed consent to access pertinent medical records. Once written consent was obtained, follow-up data were collected from treating pediatric endocrinologists by letter. Written consent was obtained from a parent/guardian and child assent was obtained for children between 7 and 18 years of age for the 21-OH gene testing. This study was reviewed and approved by the OHSU Institutional Review Board.

Results

Over the 15-year period (2003–2017) once CAH was added to NWRSP, a total of 164 confirmed cases of CAH were detected by screening 2,212,550 newborns, an incidence of 1:13,491. Of these 164 cases, 123 were detected on the first newborn screen (75%) and 41 cases were detected on the second newborn screen (25%; Fig. 1a). Of infants with CAH detected on the first newborn screen, 61.4% had salt-wasting CAH and 38.6% were categorized as simple virilizing CAH. Of infants detected on the second newborn screen, 39% had salt-wasting CAH and 61% were categorized as simple virilizing CAH.

Demographic characteristics compared between cases detected on the first versus the second newborn screen are presented in Table 2. There was no statistically significant difference in sex, mode of delivery, or whether the babies were born at term or pre-term. The mean gestational age

for infants detected on the first newborn screen was 38 weeks (SD 2.4) and those detected on the second newborn screen was 38.5 weeks (SD 2.0). There were 2 cases detected on the first newborn screen that had a birth weight <2,500 g, and there were 3 cases detected on the second newborn screen with a birth weight <2,500 g. There was no history of maternal steroid administration in any of the cases detected on the second screening test.

Cases detected on the first newborn screen had a significantly higher level of 17-OHP on screening compared to those detected on the second newborn screen (*p* < 0.001; Table 3). Confirmatory serum 17-OHP levels showed a similar statistically significant relationship (*p* = 0.005; Table 3).

We pursued follow-up data in CAH cases from Oregon, Idaho, and Nevada (Fig. 1b). A total of 105 cases over the 15-year period were diagnosed in Oregon, Idaho, and Nevada, with 33 cases being detected on the second newborn screen (31.4%). We were able to obtain follow-up data on 55/105 cases (52%; Fig. 1b). Mean age at follow-up was 8.58 years (SD 3.67) for cases detected on the first newborn screen and 4.08 years (SD 4.17) for those detected on second newborn screen (Table 4). Serum 17-OHP levels at the most recent evaluation on follow-up were significantly higher in the group detected on the first screen (*p* = 0.02). Mean androstenedione on follow-up in cases detected on the first screen was 115 ng/dL (SD 222) as compared to 37 ng/dL (SD 55) in cases detected on the second screen; this difference was not significant. We also examined height SDS on follow up and compared it to mid-parental target height SDS (Table 4); neither group was significantly different when comparing patient height on follow-up to mid-parental target height. Also, there was no significant difference in bone age advancement between the 2 groups.

CYP21A gene testing was performed on a subset of CAH patients (total *n* = 17; Fig. 2; Table 5). Each patient was classified into a gene severity group based on previous reports correlating the gene mutation with 21-OH enzyme activity [7–13]. Of the 8 CAH cases detected on the first screening test, 5 were classified as gene severity group A (0–2% enzyme activity), 2 as group B (3–7% enzyme activity), and 1 as group C (case 3). Case 3 had hyponatremia at diagnosis (serum Na = 125 mmol/L, K = 6.7 mmol/L) with a confirmatory serum 17-OHP of 9,120 ng/dL. We suspect that a second mutation on the allele with the V282L was missed. Of the 9 CAH cases detected on the second screening test, 7 were classified as gene severity group B (enzyme activity 3–7%). Although group B is associated with the simple virilizing phenotype, 4 of these 7 had biochemical evidence of salt wasting, often typically

Table 3. NBS and confirmatory serum 17-OHP for confirmed CAH cases detected on the first versus second NBS test

Detection	1st NBS	2nd NBS	<i>p</i> value
<i>n</i>	123	41	
1st NBS 17-OHP, ng/mL	252.7 (174.7–383.6)	31.1 (14.1–48.89)	–
2nd NBS 17-OHP, ng/mL	N/A	80.2 (58.03–216.9)	<0.001*
Confirmatory serum 17-OHP, ng/dL	11,000 (3,966–22,000)	4,128 (2,480–9,891)	0.005

Data are the mean and interquartile range (Q1, Q3). * *p* value for comparison of the abnormal NBS 17-OHP values: 1st NBS 17-OHP result for babies detected on the first screening test versus 2nd NBS 17-OHP results for babies detected on the second screening test. N/A, not applicable as the CAH cases detected by the 1st NBS were now on treatment; CAH, congenital adrenal hyperplasia; NBS, newborn screening.

Table 4. Follow-up data from the most recent evaluation while on treatment for CAH detected on the first versus second NBS

	1st NBS	2nd NBS	<i>p</i> value
<i>n</i>	33	20	
Age, years	8.58±3.67	4.08±4.17	0.002
Hydrocortisone dose, mg/m ² /day	14.9±4.04	14.0±6.13	0.347
Fludrocortisone treatment, %	100	50	<0.001
Serum 17-OHP, ng/dL	788 (311–3,815)	188.5 (48.9–458)	0.020
Serum androstenedione, ng/dL	30.9 (10–100)	11 (5.6–25)	0.211
Plasma renin activity, ng/mL/h	2.1 (0.5–6.08)	3.9 (0.6–6.3)	0.981
Height SDS	0.128±1.13	–0.379±1.54	0.226
Target height SDS	–0.386±0.92	–0.357±1.01	0.923
Height corrected for target height, SDS	0.514	–0.022	0.061
Bone age advancement, months	22.7 ± 28.1 (+2.27 SD)	15.4 ± 13.6 (+2.17 SD)	0.262

Data are the mean ± SD, median (interquartile range), or as indicated. The hydrocortisone dose was the dose at last visit. CAH, congenital adrenal hyperplasia; NBS, newborn screening; SDS, standard deviation score.

manifested during acute illness. In 2 patients detected on the second NBS test (siblings), only a single 21-OH mutation was identified. Both parents underwent 21-OH gene testing; the single mutation in the patients was present in the father, whereas no mutation was identified in the mother. Their mutation appears to be novel and likely falls into gene severity group C (>20% enzyme activity).

Discussion

Over the 15-year period since the inception of screening for CAH in the NWRSP, the incidence of 1:13,491 was similar to other reports of 1:14,000 to 1:18,000 [2, 3]. Experience with collection of 2 routine specimens at 24–48 h and 10–14 days of age in the NWRSP has shown that

a quarter of CAH cases fall below the screening cutoff on the first test and are detected on the second test. Collection of 2 routine specimens is not unique to screening for CAH, as it has been in place in the NWRSP since the 1970s. The primary objective of screening for this disorder is detection of newborns with severe or salt-wasting CAH [2]. In the NWRSP, while the majority of salt-wasting cases were detected by the first test, approximately 15% of salt-wasting cases were detected on the second test. The majority of CAH cases detected on the second test were categorized as simple virilizing CAH. While detection of this form of CAH is a secondary objective, simple virilizing cases benefit as early diagnosis and treatment can avert the development of premature adrenarche with its associated adverse effects. Given that CAH phenotypes represent a continuum of clinical disease, it is possible that

Fig. 2. Frequency of allelic mutation in cases detected on the first versus second NBS test.

some of our cases classified as simple virilizing CAH actually had the milder, non-classic form of CAH.

Screening newborns for CAH is problematic, primarily owing to the dynamic changes in adrenal steroid levels after birth, but also by the impact of gestational age, birth weight, perinatal stress, and unrelated acute illnesses on serum 17-OHP levels. NBS programs set 17-OHP cutoffs with the goal of detection of all babies with salt-wasting and most with simple virilizing CAH, while reducing the number of false positive cases. The 17-OHP cutoffs employed in the NWRSP vary with birth weight and age of collection, with lower cutoffs on the second test (Table 1). The positive predictive value of the first and second test is similar to that reported by other NBS programs [2]. At the set cutoffs, approximately 12 false positive cases were detected for every true positive CAH case on the first test and 10 false positive cases for every true positive on the second test. In CAH cases detected on the second test, the screening 17-OHP level rose from 34 ng/mL on the first test to 147 ng/mL on the second test. Detection of these cases on the second test reflects the finding that serum 17-OHP levels increase over time in babies with CAH, whereas they fall in healthy babies [2].

Other NBS programs have reported their experience detecting CAH on a second screen. Chan et al. [7] from Colorado reported that 28.2% of classic CAH cases were detected on the second newborn screen. Therrell et al. [8] from

Texas, also a two-screen state, reported that 14% of infants with CAH were detected on second newborn screen over a 6-year period. All salt-wasting CAH cases were detected on the first screen in this report. A study of screening results from 7 states (including Oregon) reported that 15% of CAH cases were detected on a second screening test [9]. Of those detected on the second test, 72% were categorized as simple virilizing cases, while of those detected on the first test, 75% were categorized as salt-wasting CAH. Sarafoglou et al. [10] from Minnesota, a single-screen state, reported that 15 CAH cases (22.5%) were missed by NBS over a 12-year period. Of the 15 CAH cases, 5 had salt-wasting while 10 had simple virilizing CAH. Minnesota introduced two-tier screening in 2004 and compared this methodology to one-tier screening. Cases with a positive first-tier 17-OHP result on fluoroimmunoassay then underwent second-tier steroid profiling by LC-MS/MS on the initial blood spot; cases with a positive second-tier test were then referred to a pediatric endocrinologist to confirm the diagnosis of CAH. They described 11 false negative cases under two-tier screening; 4 were correctly identified on first-tier screening but had a negative second-tier screen which supersedes first-tier results [11]. The remaining cases had negative first-tier screens. Thus, it appears that despite additional methods for screening, such as two-tier testing employing LC-MS/MS in addition to routine NBS, there may be cases of CAH that will not be detected.

Table 5. Allelic mutations and CAH type and gene severity group for cases detected on the first and second NBS test

Case No.	Detection on 1st or 2nd NBS test	1st mutation	2nd mutation	Type CAH	Gene severity group
1	1st	Intron 2 g.655A/C>G	c.518T>A(p.Ile173Asn)	SV	B
2	1st	30 kb del	c.518T>A(p.Ile173Asn)	SV	B
3	1st	c.290C>G intron var	c.844G>T (p.Val282Leu)	SW	C
4	1st	c.290C>G intron var	c.290C>G intron var	SW	A
5	1st	30 kb del	c.290C>G intron var	SW	A
6	1st	c.293C>G	c.293C>G	SW	A
7	1st	c.332_339del (p.Gly111Valfs)	c.332_339del (p.Gly111Valfs)	SW	A
8	1st	30 kb del	c.518T>A(p.Ile173Asn)	SV	B
1	2nd	30 kb del	c.518T>A(p.Ile173Asn)	SV	B
2 ^a	2nd	c.1024C>T(p.Arg342Trp)	None detected	SV	B/C?
3 ^a	2nd	c.1024C>T(p.Arg342Trp)	None detected	SV	B/C?
4	2nd	Intron 2 A/C>G	c.518T>A(p.Ile173Asn)	SW	B
5	2nd	c.185A>T (p.His62Leu) c.290C>G intron var	c.518T>A(p.Ile173Asn)	SW	B
6	2nd	30 kb del	c.518T>A(p.Ile173Asn)	SW	B
7	2nd	Intron 2 g.655A/C>G	Intron 2 g.655A/C>G	SW	B/C
8	2nd	c.327-336del (p.Gly111_Try113fs)	c.518T>A(p.Ile173Asn)	SW	B
9	2nd	c.92C>T (p.Pro31Leu) c.293-13C>G c.332-339fs	c.518T>A(p.Ile173Asn) 710-719 10 bp del/Inser c.844G>T (p.Val282Leu) c.923-924 1bp dupT→fs	SW	B/C

Gene severity group: A = 0–2%, B = 3–7%, and C = 10–20% 21-OH enzyme activity [13]. CAH, congenital adrenal hyperplasia; NBS, newborn screening.

^a Cases 2 and 3 are siblings; gene severity “B/C” was based on “simple virilizing” clinical status, but described as “B/C?” as only one mutation was found.

Our study found similar modes of delivery, gestational age, and birth weight between cases of CAH detected on the first versus the second newborn screen. The majority of infants detected on both the first and second screen were born at full term. Our findings are in line with previous studies; Chan et al. [7] reported no difference in gestational age and birthweight in cases detected on the first versus second newborn screen.

On follow-up, CAH cases detected on the first and second newborn screen were on similar treatment doses of hydrocortisone, whereas all cases detected on the first newborn screen were treated with fludrocortisone as compared to half of cases detected on the second screening test. It is generally accepted that a degree and spectrum of aldosterone deficiency exists in all forms of CAH [2]. Thus, even though approximately a third of cases detected on the first screening test and the majority of those detected on the second test were classified as simple virilizing CAH in the neonatal period, some of these simple vir-

ilizing patients were treated with fludrocortisone on follow-up. Serum 17-OHP levels on follow-up were higher in those cases detected on the first NBS compared to those detected on the second, likely reflecting the greater severity of their CAH. This same pattern was seen with androstenedione levels on follow-up between the 2 groups; however, this finding was not statistically significant.

On follow-up, children detected on the first and second screening test were similar in height; however, when corrected for target height, those detected on the first test tended to be taller than those detected on the second test ($p = 0.06$). One could speculate that a greater bone age advancement might be seen in the more severely affected CAH cases detected on the first test to explain the taller height. Cases detected on the first newborn screen had a mean bone age advancement of 22.7 months compared to 15.4 months in those detected on the second newborn screen; however, this finding was not statistically significant.

Several studies have demonstrated a genotype-phenotype correlation in 21-OH-deficient CAH [12–19]. We performed *CYP21A* gene testing on a subset of CAH patients in order to investigate the frequency of allelic mutations in cases detected on the first compared to the second NBS test (Table 5). Most patients were compound heterozygotes, with disease severity correlating with the milder of the 2 mutations. The majority of CAH cases detected on the first screening test were categorized as group A (0–2% enzyme activity), while the majority of cases detected on the second screening test were categorized as group B (3–7% enzyme activity). It is likely that the discrepancy between genotype and phenotype manifested in some of our cases represents problems with genotyping owing to the complexity of gene duplications, deletions, and rearrangements within the *CYP21A2* locus [2].

In conclusion, over a 15-year period in the NWRSP, we observed that 25% of CAH cases were detected on the second NBS test. Although infants with CAH detected on the second newborn screen had a milder form of the disease, as evidenced by significantly lower 17-OHP levels on screening, confirmatory testing, and follow-up, approximately one-third were classified as salt wasters. These cases had 17-OHP levels that fell below the threshold on the first screening test, most likely related to the necessity of having relatively high cutoffs using the currently employed fluoroimmunoassay to reduce the number of false positive tests. Second-tier testing using LC-MS/MS has the potential to improve the accuracy of NBS, but its use has not eliminated all false negatives [11]. A recent proposal recommended replacing 17-OHP with measurement of 21-deoxycortisol, a more specific marker for 21-OH deficiency [20]. Based on our experience, we believe modifications of the screening test for CAH along these lines are needed to reduce false negative cases.

References

- White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Endocr Rev*. 2000 Jun;21(3):245–91.
- Speiser PW, Arlt W, Auchus RJ, Baskin LS, Conway GS, Merke DP, et al. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*. 2018 Nov;103(11):4043–88.
- Gidlöf S, Wedell A, Guthenberg C, von Döbeln U, Nordenström A. Nationwide neonatal screening for congenital adrenal hyperplasia in Sweden: a 26-year longitudinal prospective population-based study. *JAMA Pediatr*. 2014 Jun;168(6):567–74.
- Anandi VS, Shaila B. Evaluation of factors associated with elevated newborn 17-hydroxyprogesterone levels. *J Pediatr Endocrinol Metab*. 2017 May;30(6):677–81.
- Pode-Shakked N, Blau A, Pode-Shakked B, Tiosano D, Weintrob N, Eyal O, et al. Combined gestational age- and birth weight-adjusted cutoffs for newborn screening of congenital adrenal hyperplasia. *J Clin Endocrinol Metab*. 2019 Aug;104(8):3172–80.
- Schwarz E, Liu A, Randall H, Haslip C, Keune F, Murray M, et al. Use of steroid profiling by UPLC-MS/MS as a second tier test in newborn screening for congenital adrenal hyperplasia: the Utah experience. *Pediatr Res*. 2009 Aug;66(2):230–5.
- Chan CL, McFann K, Taylor L, Wright D, Zeitler PS, Barker JM. Congenital adrenal hyperplasia and the second newborn screen. *J Pediatr*. 2013 Jul;163(1):109–13.e1.
- Therrell BL Jr, Berenbaum SA, Manter-Kaplanke V, Simmank J, Korman K, Prentice L, et al. Results of screening 1.9 million Texas newborns for 21-hydroxylase-deficient congenital adrenal hyperplasia. *Pediatrics*. 1998 Apr;101(4 Pt 1):583–90.
- Held PK, Shapira SK, Hinton CF, Jones E, Hannon WH, Ojodu J. Congenital adrenal hyperplasia cases identified by newborn screening in one- and two-screen states. *Mol Genet Metab*. 2015 Nov;116(3):133–8.

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Statement of Ethics

The parents and/or guardians of the subjects gave their written informed consent and the study protocol was approved by the Oregon Health and Science University's committee on human research.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

N.E. conceived and designed the study, carried out the investigation, collected the data, performed the analysis, contributed to the interpretation of the results and took the lead in writing the manuscript, and provided critical feedback and helped shape the research, analysis, and manuscript. L.D., K.C., and S.L. contributed data, analytic calculations, analysis and interpretation of the results, provided critical feedback and helped shape the research, analysis, and manuscript. S.D. provided annual newborn screening test results and contact information for research subjects, provided critical feedback and helped shape the research, analysis, and manuscript. S.W. contributed to data by analysis of false negative rates, provided critical feedback and helped shape the research, analysis, and manuscript.

- 10 Sarafoglou K, Banks K, Kylo J, Pittock S, Thomas W. Cases of congenital adrenal hyperplasia missed by newborn screening in Minnesota. *JAMA*. 2012 Jun;307(22):2371–4.
- 11 Sarafoglou K, Banks K, Gaviglio A, Hietala A, McCann M, Thomas W. Comparison of one-tier and two-tier newborn screening metrics for congenital adrenal hyperplasia. *Pediatrics*. 2012 Nov;130(5):e1261–8.
- 12 Speiser PW, Dupont J, Zhu D, Serrat J, Buegeleisen M, Tusie-Luna MT, et al. Disease expression and molecular genotype in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J Clin Invest*. 1992 Aug;90(2):584–95.
- 13 Wilson RC, Mercado AB, Cheng KC, New MI. Steroid 21-hydroxylase deficiency: genotype may not predict phenotype. *J Clin Endocrinol Metab*. 1995 Aug;80(8):2322–9.
- 14 Bachega TA, Billerbeck AE, Madureira G, Marcondes JA, Longui CA, Leite MV, et al. Molecular genotyping in Brazilian patients with the classical and nonclassical forms of 21-hydroxylase deficiency. *J Clin Endocrinol Metab*. 1998 Dec;83(12):4416–9.
- 15 Krone N, Braun A, Roscher AA, Knorr D, Schwarz HP. Predicting phenotype in steroid 21-hydroxylase deficiency? Comprehensive genotyping in 155 unrelated, well defined patients from southern Germany. *J Clin Endocrinol Metab*. 2000 Mar;85(3):1059–65.
- 16 Silveira EL, Elnecave RH, dos Santos EP, Moura V, Pinto EM, van der Linden Nader I, et al. Molecular analysis of CYP21A2 can optimize the follow-up of positive results in newborn screening for congenital adrenal hyperplasia. *Clin Genet*. 2009 Dec;76(6):503–10.
- 17 New MI, Abraham M, Gonzalez B, Dumic M, Razzaghy-Azar M, Chitayat D, et al. Genotype-phenotype correlation in 1,507 families with congenital adrenal hyperplasia owing to 21-hydroxylase deficiency. *Proc Natl Acad Sci USA*. 2013 Feb;110(7):2611–6.
- 18 Yoo Y, Chang MS, Lee J, Cho SY, Park SW, Jin DK, et al. Genotype-phenotype correlation in 27 pediatric patients in congenital adrenal hyperplasia due to 21-hydroxylase deficiency in a single center. *Ann Pediatr Endocrinol Metab*. 2013 Sep;18(3):128–34.
- 19 Santos-Silva R, Cardoso R, Lopes L, Fonseca M, Espada F, Sampaio L, et al.; on behalf of the Portuguese Society of Pediatric Endocrinology and Diabetology. CYP21A2 gene pathogenic variants: a multicenter study on genotype-phenotype correlation from a Portuguese pediatric cohort. *Horm Res Paediatr*. 2019;91(1):33–45.
- 20 Miller WL. Congenital adrenal hyperplasia: time to replace 17OHP with 21-deoxycortisol. *Horm Res Paediatr*. 2019;91(6):416–20.