Review Article

Pitfalls of prenatal and newborn screening in congenital adrenal hyperplasia: a narrative review※

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Background and Objective: Congenital adrenal hyperplasia (CAH) caused by 21-hydroxylase deficiency (21OHD) is now included in newborn screening (NBS) programs across the United States and in several other countries. This narrative review aims to update the reader on NBS methods, with a focus on their drawbacks and potential for improvement. In addition, the important role of preconception and prenatal genetic carrier screening in the parents, emerging fetal genetic testing, treatment, and preimplantation genetic testing has also been reviewed.

Methods: A literature search of publications from 1980 to 2020 for articles about NBS of infants with CAH in the United States was conducted using PubMed. References from selected articles were also reviewed and identified to add pertinent information. Articles that were not written in English were omitted.

Key Content and Findings: NBS methods vary widely across different states. Despite the varying methods of screening, the newborn screen continues to be an effective tool to screen for salt-wasting CAH, although milder forms of CAH (SV-CAH and NC-CAH) are more likely to be picked up on screens with two-tier screening methods. Newer methods of prenatal testing and carrier screening are shifting the landscape of the early diagnosis of CAH.

Conclusions: The newborn screen will remain an essential and cost-effective tool to screen for salt-wasting CAH across the United States and worldwide. It is important for the reader to be aware of newer methods of prenatal testing and parental carrier screening as these tests continue to become more readily available.

Keywords: Congenital adrenal hyperplasia (CAH); 21-hydroxylase deficiency (21OHD); newborn screening (NBS); adrenal steroid disorders

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Introduction

Congenital adrenal hyperplasia (CAH)

CAH is a group of inherited gene defects caused by deficiencies of various steroidogenic enzymes leading to inborn errors of cortisol biosynthesis. 21-hydroxylase deficiency (21OHD), which is caused by pathogenic variants in the *CYP21A2* gene, accounts for more than 90% of all cases of CAH. Deficiency of the 21-hydroxylase enzyme results in varying degrees of decreased production of cortisol and aldosterone and increased production of adrenal androgens.

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Depending on the specific gene variant, affected enzyme activity may vary, resulting in a wide phenotypic spectrum. As such, CAH is commonly classified into salt-wasting (SW-CAH), simple virilizing (SV-CAH), and non-classical CAH (NC-CAH). Salt-wasting CAH is the most severe form of CAH, resulting in a significant decrease in the production of cortisol and aldosterone. Salt-wasting CAH must be promptly recognized as untreated it can cause significant morbidity and mortality from hypotensive shock, hyponatremia, and hyperkalemia. Affected girls usually have atypical genitalia but boys' genitalia appear normal. The simple virilizing form typically causes prenatal virilization in affected females without evidence of salt-wasting. Treatment is warranted in males and females to prevent androgenization, rapid bone age advancement, and other sequelae. The most common form, non-classical CAH, is typically asymptomatic at birth and is diagnosed later in life where patients can present with early adrenarche, and clinical hyperandrogenism such as acne and hirsutism, menstrual irregularities, and impaired fertility. Patients with non-classical CAH can also remain asymptomatic.

The incidence of classic CAH due to 21OHD is 1:10,000 to 1:18,000 births, although the incidence may vary in certain populations like the Yupik people, in which the incidence of salt-wasting CAH is 1 in 282 (1). Other high-risk populations include the Ashkenazi Jewish and Caucasian populations (2).

Genetics of CAH

CAH due to 21OHD is an autosomal recessive disorder. The inactivation of the enzyme 21-hydroxylase may vary resulting in different disease severities and phenotypic expression. The salt-wasting phenotype results from large gene deletion causing the complete loss of 21-hydroxylase activity. The simple virilizing phenotype results from variants that cause a reduction in enzyme activity to approximately 2%, which preserves the mineralocorticoid production. In the non-classical phenotype, the enzyme activity is reduced to 10–75%, with the most common mutation being the *V281L*.

New *et al.* (3) studied a large cohort of 1507 families affected with different forms of CAH and found that there was a strong genotype-phenotype correlation among the salt-wasting and non-classical forms. The simple virilizing form was found to have a variable phenotypic presentation. The study found nine common allelic variants—*V281L* (23.9%), *IVS2-13 A/C>G* (22.9%), 30-kb deletion or genomic rearrangement/conversion fusing *CYP21* with *CYP21P* (20.0%), *I172N* (8.2%), *R356W* (3.6%), *Q318X* (3.5%), *P30L* (2.6%), and exon 6 cluster mutation (*I236N*, *V237E*, *M239K*) (2.1%). They noted other rare variants that accounted for the remainder of the alleles and often led to different allelic variations of known variants.

Fetal adrenal gland development and virilization

The formation of the fetal adrenal cortex occurs in the fourth week of gestation. By the seventh week post-fertilization, the fetal adrenal gland begins to produce steroids. Pituitary ACTH production regulates the synthesis of cortisol through feedback loops. In both salt-wasting and simple virilizing CAH, the lack of cortisol production leads to an increase in ACTH production. In turn, ACTH causes an increased production of adrenal androgens. This increased androgen production occurs in tandem with genital development in the fetus, leading to the virilization of female fetuses. Since genital development commences at the ninth week of gestation, androgenization of the genitalia can start at this time.

Maternal cortisol cannot suppress the ACTH-driven production of fetal androgens, because of cortisol inactivation by the 11beta hydroxysteroid dehydrogenase type 2 enzyme in the placenta.

Brief background on newborn screening (NBS) for CAH

Since CAH can be easily screened for in the blood with a 17-hydroxyprogesterone (17-OHP) level, it is well suited for NBS. The implementation of NBS for CAH expedites the diagnosis of CAH, thereby averting adrenal crisis and preventing serious morbidity and mortality. Although it is hard to determine the mortality rate in infants and neonates before the use of NBS, a case fatality rate of 4-11% has been reported in the literature (4). In addition, it is important to note that the cutoffs for the NBS are designed to pick up the salt-wasting form and not the milder forms of CAH, such as simple virilizing and non-classical CAH.

The earliest implementation of CAH to a state NBS program was in 1977 as a pilot program in Alaska, with gradual implementation by other states. By the time CAH was added to the United States Recommended Uniform Screening Panel (RUSP) in 2005, 38 NBS programs were already universally screening for the disorder. Currently, all 53 NBS programs universally screen for

Table 1 The search strategy summary

Items	Specification
Date of search	7/31/2021
Databases and other sources searched	PubMed
Search terms used	CAH, newborn screen
Timeframe	1980–2020
Inclusion	English articles on newborn screening of infants with CAH in the United States are included

CAH, congenital adrenal hyperplasia.

CAH, programs including the 50 states, the District of Columbia, Puerto Rico, and Guam (5,6). We present this article in accordance with the Narrative Review reporting checklist (available at https://pm.amegroups.com/article/view/10.21037/pm-21-104/rc).

Methods

A literature search of publications from 1980 to 2020 for articles pertaining to the NBS of infants with CAH in the United States was conducted using PubMed. References from selected articles were also reviewed and identified to add pertinent information (*Table 1*). Articles that were not written in English were omitted.

Methods of NBS across states

Cut-off points

Since 17-OHP is the main substrate for the enzyme 21-hydroxylase, levels of 17-OHP are significantly elevated in most newborns with CAH due to 21OHD. Hence 17-OHP levels are used to screen for CAH. It can be measured easily using dissociation-enhanced lanthanide fluoro-immunoassay (DELFIA) using the dried blood spots. Currently, all 53 NBS programs screen for CAH using fully integrated fluoro-immunoassay (FIA) as their first screen (5).

The cut-off points used to flag an abnormal 17-OHP level are not standard across the states. A data review conducted by Speiser *et al.* (7) using data from 17 states found that most states had their own cut-off points which varied from state to state. Most states used birthweight cut-off points with 17-OHP values ranging from 25 to 75 ng/mL (mean 41.2 ng/mL) for normal birthweights greater than 2,250 to 2,500 grams. Infants with lower birth weights had higher cut-off points. Only one state used cut-off points based on gestational age.

One screen vs. two screen models

There are two models for NBS used across the United States: a one-screen model and a two-screen model. Based on current data from Health Resources & Services Administration (HRSA), most states use the one-screen model, in which a sample is obtained only once between 24 to 48 hours of life. Even in one screen states improper specimen collection and the collection of a specimen very early will prompt an additional screen even in the one-screen states. If the result is negative, no further screening is obtained.

Currently, 13 states across the United States use a two-screen model. In this model, the first sample is obtained between 24 to 48 hours of life, followed by a routine second sample obtained at one to two weeks of life as part of a well-child visit.

In an extensive review of NBS for CAH in the United States, Edelman *et al.* (5) showed that of the second-tier screen programs, six programs used FIA as a second-tier screen while five used extracted 17-OHP. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used by four screening programs with the addition of other androgens in addition to the 17-OHP, with two programs using androstenedione, two programs using cortisol, two using a combination of androstenedione/cortisol, and one program using 11-deoxycorticosterone.

A retrospective chart review by Held *et al.* (8) comparing the results between one and two-screen states found that there was no statistically significant difference between the detection rates of classical CAH. Most two-screen states had higher detection rates for SV-CAH and NC-CAH, with most of these non-salt wasting forms being caught on the second screen. Some argue that since the main purpose of the NBS is to detect newborns with the severe salt-wasting form, the benefit of the second screen is difficult to ascertain.

False positives

Several factors cause false-positive results on the NBS, thereby limiting its accuracy. The most common false positive occurs due to the collection of a specimen prior

to 24 hours of life. 17-OHP levels are high in the cord blood and fall in the first one to two days of life, even in unaffected neonates. Hence, the premature collection of the specimen before 24 hours can cause a false positive value in babies without 21OHD, prompting a second screen.

In addition, the 17-OHP levels can be elevated in premature infants due to the increased production of 17-OHP from the fetal adrenal glands. Currently, most newborn screens use birth weight to determine cut-off points for 17-OHP levels instead of gestational age-related cut-off points. Lastly, sick infants can have higher levels of 17-OHP due to stress from illness, also causing a false positive on the NBS. Other rarer forms of CAH, including 11-hydroxylase deficiency, 3β-hydroxysteroid dehydrogenase deficiency, and P450 oxidoreductase deficiency may also result in a false positive due to elevated 17OHP values detected on NBS, but it is important to note that the test was not designed to screen for these conditions.

False-negative results

A false negative NBS results from a falsely low level of 17-OHP in a baby with true 21-OHD, hence escaping detection on the NBS. It is challenging to determine the number of false-negative results since not all the children ultimately diagnosed with CAH with an initial negative screen are reported back to the NBS programs. Speiser *et al.* (7) suggested the use of a centralized CAH registry to document cases diagnosed after the newborn period.

Maternal and infant steroid use suppressing 17-OHP levels are a cause of false-negative results. In the case of maternal steroid use, a second screen could eliminate the false-negative result.

However, it is unclear if the use of a second screen eliminates false-negative results. Some states have published data about their false-negative rates. New York state reported only three false-negative cases from their single screen over 7 years but all three false-negative cases were female infants with simple virilizing CAH (2007 to 2014) (9). Some states could determine a false negative rate once a second screen was implemented. In Colorado, the research found that there was a 28% false-negative rate on the first screen (10). A total of 28 infants with classical CAH and 1 with NC-CAH were identified on the first screen, and an additional 11 patients with classical CAH and 6 patients with NC-CAH were identified on the second screen. Researchers from Minnesota (11) found a 32% false-negative rate over 11 years from 1999 to 2010, even in the context of two screens, with the second screen implemented in the year 2004. Of the 15 false negatives noted, 11 had SV-CAH, and 4 had SW-CAH. Eshragh *et al.* (12) reviewed CAH cases detected from 2003 to 2017 by the Northwest Regional Newborn Screening Program. They found that 25% of the 164 diagnosed cases were caught on the second screen, with one-third of the cases being salt wasters.

New directions

Given all the benefits of the early diagnosis and intervention for CAH in preventing morbidity and mortality, the CAH guidelines state that all NBS programs incorporate screening for CAH due to 21OHD (13). However, due to the high false-positive rate, there are often many neonates not affected by 21 OHD that are subjected to confirmatory testing, leading to undue parental anxiety and healthcare burdens.

Several suggestions have been made to alleviate the false-positive rates by use of gestational age-based cut-offs or cut-offs using a combination of birthweight and gestational age-related points (14). The implementation of a second screen for all states has been suggested, although based on a study by Held *et al.* (8), this only improves the positive predictive value by diagnosing more babies with simple virilizing or non-classical CAH. LC-MS/MS is a more accurate test to detect 17-OHP levels. The use of LC-MS/MS led to an increase in the positive predictive value on CAH screens in New Zealand from 1.71% to 11.1% in a study done by de Hora *et al.* (15). Presently, LC-MS/MS is not suitable for high-volume screening as first-tier testing due to time constraints.

Miller also suggested the benefits of using 21-deoxycortisol instead of 17 OHP as the analyte on the NBS (16). The use of 21-deoxycortisol would prevent false positives since 21-deoxycortisol is not elevated in premature infants or other forms of CAH, and therefore this may be a more specific test for 21OHD. Presently, the use of 21-deoxycortisol is not validated for NBS, and adding it as the primary analyte will require revision of current procedures.

The extraction of DNA from the same dried blood spot that is used for hormonal screening as a second-tier test has also been suggested. Since a 17-OHP level does not always correlate with the phenotype, the 17-OHP level alone may not always help differentiate between salt-wasting and simple virilizing CAH. The use of genetic testing as a

second-tier screen may help determine the exact phenotype at the time of diagnosis. In CAH 90% to 95% of affected alleles carry a known variant (3). Samples that do not carry these variants may be presumed with more than 99% confidence to be unaffected. Genetic samples noted to have only a single variant would still need confirmatory genetic testing given that the other allele could be affected by a rare mutation, which is seen in less than 2% of individuals who are carriers of classic 21-OHD alleles (1). Two groups have studied the utility of adding genotyping to current screening programs (17,18). Fitness *et al.* determined that the cost per genetic test on a sample would be close to 5 dollars (17). Presently, this is not a feasible option but may become available in the future as more laboratories are able to perform rapid, accurate, and large-scale CYP21 genotyping.

Prenatal genetic testing

With advances in medical science, parents of high-risk infants can now determine their CAH status as well as that of the fetus. This provides the added benefit of knowing the phenotype at birth, allowing for quicker and more specific treatment. It also allows for more targeted family counselling and parental preparation, given that in many cases there is a good genotype and phenotype correlation in CAH. In addition, parents may use the prenatal diagnostic data to make an informed decision on the use of prenatal dexamethasone treatment to avoid genital atypia in affected female fetuses.

Fetal genetic testing

The gold standard tests for fetal genetic testing remain chorionic villus sampling (CVS) and amniocentesis. Genetic testing on fetal DNA is typically obtained via CVS at approximately 10–14 weeks of gestation or amniocentesis at approximately 15–20 weeks of gestation. In addition to the primary drawback of amniocentesis and CVS being invasive techniques, they can only be completed later in the gestational period, after genital virilization has already begun. In 1997, Lo *et al.* discovered circulating cell-free fetal DNA (cffDNA) in maternal plasma which shifted the direction of prenatal testing (19). Obtaining cffDNA from maternal plasma is non-invasive and is done via a blood draw. Monogenic disorders are identified by detecting a maternal and paternal variant in the mother's blood, suggesting that the fetus is affected. In addition, fetal sex determination can also be performed by the detection of a Y sequence in maternal plasma that complements traditional polymerase chain reaction using an SRY probe (20).

In 2014, Lo and New *et al.* (21) used massively parallel sequencing (MPS) of cell-free DNA of the maternal plasma to determine the presence of a mutation in the CYP21A2. This technique used targeted MPS of genomic DNA from the trio of both parents and the affected proband. Single-nucleotide polymorphisms (SNPs) on both sides of the *CYP21A2* locus allowed one to assemble haplotype blocks that are needed to determine paternal and maternal allelic inheritance. In the study, 14 families were studied, and the fetal CAH status was detected as early as 5 weeks and 6 days.

Treatment with dexamethasone

The goal of prenatal treatment with dexamethasone is to prevent virilization in an affected female fetus prior to the onset of genital development. As mentioned above, in an affected fetus, the adrenal gland begins to make excess androgens by the seventh week of gestation. This leads to virilization in affected female fetuses which starts at nine weeks gestation. Maternal cortisol is inactivated by placental 11 beta-hydroxysteroid dehydrogenase type 2 and therefore cannot suppress fetal androgen production. The rationale for the use of dexamethasone is that it can cross the placenta in its active form and can suppress fetal ACTH and, in turn, fetal androgen production thus preventing virilization. To be most effective, treatment should be initiated early in the pregnancy or after confirmation of pregnancy and before the onset of genital development.

The use of prenatal low-dose dexamethasone treatment for CAH was presented in 1989 by Forest *et al.* in France (22), and in 1986 in the United States by Speiser and New *et al.* (23). The dose that is used is 20 microgram/kg per day based on pre-pregnancy weight to a maximum of 1.5 mg/day divided into two or three doses. The administration of dexamethasone at appropriate doses, at or prior to the ninth week of gestation used throughout pregnancy has been shown to lead to the significant reduction of genital virilization in affected females (22-24).

Presently the data on the effect of dexamethasone on long-term cognitive and behavioral outcomes remain inconclusive (25). In a 2004 study of 174 children (including 48 children with CAH) with prenatal exposure to dexamethasone, Meyer-Bahlburg *et al.* found no difference in cognitive and motor outcomes when compared to 313 children (including 195 children with CAH) without

prenatal exposure to dexamethasone (26). A 2007 study by Hirvikoski *et al.*, comparing 40 children who received prenatal treatment with dexamethasone with 35 healthy age-matched controls, found that the children who received treatment with dexamethasone performed poorer than the controls on a test assessing verbal working memory, but found no differences in intelligence, learning, or long-term memory (27). A 2012 study found that there was no effect on working memory in short-term exposed unaffected children or short-term exposed boys with CAH, but girls with CAH treated throughout pregnancy had slower mental processing than did controls by several assessments (28). Another study found that CAH unaffected girls that had received prenatal dexamethasone scored lower than control girls on measures of verbal and nonverbal intelligence and verbal working memory tasks, but without effects on long-term memory, handedness, speed of processing, nor self-perceived or parentally reported scholastic performance (29).

Due to potential safety concerns of dexamethasone with regards to brain development, pre-emptive treatment with low-dose dexamethasone is considered an experimental therapy. The current CAH guidelines advise that treatment be conducted through IRB-approved protocols so that the risks and benefits of treatment can be better identified and defined. The guidelines also recommend testing for Y-chromosomal DNA in maternal blood to eliminate male fetuses from potential treatment groups.

As highlighted above, with invasive methods such as CVS and amniocentesis, the prenatal diagnosis cannot be made before the onset of genital development, which typically begins at the 9th week of gestation (30). Therefore, this either leads to a diagnosis of CAH after virilization has begun or can lead to the unnecessary prolonged treatment of unaffected females and all male fetuses. With the advent of maternal cell-free DNA, the diagnosis of CAH can potentially be made earlier closer to six weeks. Simultaneously, a fetal karyotype and genetic analysis can help determine if the fetus is male or an unaffected female. Treatment can then be discontinued for fetuses who are male or unaffected females. If the fetus is identified as an affected female, prenatal dexamethasone treatment can be continued for the full duration of the pregnancy. Given that CAH is an autosomal recessive disease, the chances of having an affected female fetus in parents who are both carriers of the mutation is 1 in 8. Therefore, early diagnosis can aid in targeting treatment for an affected female fetus and eliminate unnecessary treatment in seven out of eight fetuses.

Preconception carrier testing

With decreasing costs of DNA analysis and sequencing, carrier screening is now readily available. In the past, most screens were ethnicity-based tests that were directed toward specific populations known to be at a higher risk of particular disorders. It has now become possible to screen for multiple conditions simultaneously, with the means of expanded carrier screens (31). Most expanded carrier screens require the conditions tested for to have a carrier frequency of 1 in 100 or greater, a well-defined phenotype, a detrimental effect on the quality of life, cause cognitive or physical impairment, require surgical or medical intervention, or have an onset early in life (32). Many prenatal carrier screens routinely screen parents for variants in the *CYP21A2* gene. If the parents are both carriers of a variant, they can undergo genetic counseling before pregnancy. However, there are a few carrier screening panels that do not routinely test for mutations in the *CYP21A2* gene, and so obstetricians should be wary of this, especially while screening patients in high-risk populations.

The American College of Obstetricians and Gynecologists (ACOG) (33) currently recommends that the option of genetic carrier screening should be provided to every pregnant woman. To provide informed genetic counselling, and counseling about reproductive outcomes, carrier screening should be recommended prior to the pregnancy. If the individual is found to be a carrier, then the partner should also be tested. If CAH carrier screening is done post-conception, parents can be referred for genetic counseling and may be offered fetal genetic testing and the option of treatment with dexamethasone.

Pre-implantation testing

Pre-implantation genetic testing may be offered to high-risk mothers to implant only unaffected embryos via in vitro fertilization (IVF). The chromosomal sex and CAH status of the embryo can be determined by molecular techniques. Preimplantation genetic testing can be carried out for any single gene disorder whose chromosomal location is known (34). This is possible even if the causative nucleotide mutation is not known and is readily available for CAH. While IVF is accompanied by certain challenges, pre-implantation genetic testing has been used successfully for several years and is a promising method that allows one to

Table 2 Timing, advantages and disadvantages of the various tests used for the diagnosis of CAH in the prenatal and newborn period

Test	Timing	Advantages	Disadvantages
Newborn screening	24–48 hours after birth; 1–2 weeks of life (2nd screen for 2 screen states)	Cost effective; non-invasive; can be used for mass screening	Designed to pick up only SW-CAH; does not identify specific variants or differentiate between different types of CAH
Preconception carrier testing	Anytime (preferably prior to conception)	Non-invasive; useful for future pregnancies; can screen for multiple conditions simultaneously	All panels may not screen for (CYP21A2)
Post-natal genetic testing	Anytime postnatally	Non-invasive; can identify specific variants	Testing is labor-intensive and cannot be solely relied on for diagnosis in a newborn; not cost effective; difficult to use for mass screening (not all laboratories are able to perform rapid, accurate, and large-scale CYP21 genotyping)
Fetal genetic testing via CVS/ Amniocentesis	10–14 weeks (CVS); 15–20 weeks (Amnio)	Gold standard	Invasive; can only be completed later in the gestational period, after the onset of genital androgenization
Maternal cell fee DNA	6 weeks	Non-invasive; prenatal results available sooner than with any other testing methods; useful for high-risk populations	Not cost-effective; not commercially or widely available

CAH, congenital adrenal hyperplasia; CVS, chorionic villus sampling; SW-CAH, salt-wasting congenital adrenal hyperplasia.

circumvent treatment with dexamethasone.

A summary of the various modalities of testing is listed in *Table 2*.

Conclusions

The newborn screen will remain an essential and cost-effective tool to screen for salt-wasting CAH across the United States and worldwide. In recent years, there have been many changes in the landscape of the diagnosis of CAH which have moved beyond the NBS. It is important for the reader to be aware of these changes, as these tests continue to become more readily available.

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Footnote

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