REVIEW

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Antenatal Diagnosis and Treatment in Congenital Adrenal Hyperplasia Due to 21-hydroxylase Deficiency and Congenital Adrenal Hyperplasia Screening in Newborns

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Abstract

Signs of virilization, such as clitoromegaly, labio-scrotal fusion, and urogenital sinus may be observed in females with 21-hydroxylase deficiency (21-OHD) and other rare virilizing forms of congenital adrenal hyperplasia (CAH). This makes sex determination difficult, and multiple reconstructive surgeries in the postnatal period may be required. As 21-OHD is an autosomal recessive disease, the chance of any child being affected is one in four and so only one in eight will be an affected female. The primary objective of antenatal diagnosis is to identify only the affected fetus in the early gestational weeks before the onset of genital organogenesis and to treat that case. Therefore, studies aimed at antenatal diagnosis and preventing adrenal androgen exposure in the female fetus with CAH have long been of interest. Antenatal steroid treatment is considered experimental and controversial for safety reasons in recent clinical guidelines. If antenatal treatment is to be used, it is recommended that it should be performed in experienced centers that can collect data on a large number of cases which will help to define the benefits and harms of treatment better. In the postnatal period, a severe deficiency of the 21-hydroxylase enzyme leads to life-threatening adrenocortical insufficiency in both sexes and varying degrees of pathology of the external genitalia in females. This condition is also associated with high mortality in the first days of life and an increased risk of incorrect sex assignment. Neonatal screening for 21-OHD CAH effectively detects the severe forms and reduces mortality, and it is instrumental in the correct sex assignment of female cases.

Keywords: Congenital adrenal hyperplasia, 21-hydroxylase deficiency, antenatal diagnosis, antenatal dexamethasone, newborn screening

Introduction

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OHD) is an autosomal recessive disorder caused by pathogenic variants in the *CYP21A2* gene (MIM*613815). Many of the variants in the *CYP21A2* gene are caused by recombinations between the *CYP21A2* and its pseudogene *CYP21A1P* (aliases *CYP21A, CYP21P, P450C21A*). The complexity of this locus due to the chromosomal arrangement of the active gene and the pseudogene makes the genotyping of 21-OHD sophisticated. However, accurate molecular genetic diagnosis is crucial to provide families with appropriate genetic counseling concerning future pregnancies. Genetic testing should be performed by certified molecular laboratories with expertise in the analysis of the *CYP21A2* gene with sequence analysis and multiplex ligation-dependent probe amplification (MLPA) (1,2,3,4).

The adrenal cortex begins to function in the seventh gestational week (gw). Female fetuses with CAH are exposed to elevated levels of androgens between 8^{th}-12^{th} gw which is a critical period for sex differentiation. Exposure to excess androgens in the first trimester causes the formation of a

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urogenital sinus with a conjoined urethra and vagina, and continued exposure throughout pregnancy causes varying degrees of labial fusion and clitoral enlargement (5). Signs of virilization, like clitoromegaly, labio-scrotal fusion, and urogenital sinus may be observed in females with CAH due to 21-OHD and 11β-hydroxylase deficiency (11β-OHD) (6,7). This makes sex determination difficult and may lead to multiple reconstructive surgeries in the postnatal period (8). The routine practice of genital surgery in infancy has been questioned, and shared decision-making among parents, patients, surgeons, endocrinologists, and mental health providers is currently being promoted. Therefore, studies aimed at preventing adrenal androgen exposure in the female fetus with CAH have long been of interest (9).

This evidence-based review, which includes good practice points, was developed by the Adrenal Working Group of the Turkish Society for Pediatric Endocrinology and Diabetes. We have also developed this evidence-based review for "Antenatal Diagnosis and Treatment in Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency and Congenital Adrenal Hyperplasia Screening in newborns". The overall purpose of this evidence-based review is to provide good practice points, focusing on daily management recommendations.

Molecular Genetic Analyses in Antenatal Diagnosis

Antenatal diagnosis requires accurate and reliable *CYP21A2* sequence analysis and MLPA testing in the index case and confirmation of the variant through segregation analysis in the family (7).

Several approaches for antenatal diagnosis of affected fetuses with CAH have been used. The hormonal diagnostic test for 21-OHD is amniotic fluid 17-hydroxyprogesterone (17-OHP) but hormonal diagnosis is rarely used and considered only when molecular diagnosis is unavailable (10).

For the antenatal diagnosis of CAH, invasive procedures may be used, such as chorionic villus sampling (CVS) at 10-12 gw and amniocentesis (AS) at 16-20 gw. CVS is preferred over AS for early diagnosis in the first trimester of pregnancy. However, even with CVS, diagnosis cannot be made before the ninth gw, leading to unnecessary treatment of male fetuses and unaffected female fetuses. CVS and AS are invasive procedures that may pose risks to both the mother and fetus. Therefore, it is important to identify cases of CAH, before the onset of genital organogenesis, so that only affected female fetuses receive treatment. As 21-OHD is an autosomal recessive disease, the risk of any one child being affected is one in four, and given the 50/50 chance of gender this trnaslates to a risk of one in eight being an affected female. This would equate to seven out of eight fetuses receiving antenatal steroid treatment unnecessarily if all at risk fetuses were treated before definitive diagnosis. The primary objective, therefore, is to identify only the affected fetus in the early weeks of gestation and to treat only those cases (6).

Advances in *CYP21A2* genotyping are expected to make molecular analysis of fetal DNA the ideal diagnostic tool for fetuses at risk for 21-OHD (11). Conditions such as undetectable mutations, allele drop-out (ADO), or maternal DNA contamination can complicate antenatal diagnosis (11,12,13). Analysis of CVS material may cause false positive or false negative results if there is a genetic mismatch between the fetus and the placenta however, this is not an issue in AS, because the fetal cells that are released into the amniotic fluid are analyzed (14).

Cell-free Fetal DNA

Non-invasive prenatal testing (NIPT), including cell-free fetal DNA (cffDNA), appears to be a promising technique for early diagnosis of 21-OHD (3). Lo et al. (14) first described the high concentration of cffDNA in maternal blood. Early in pregnancy, fetal DNA represents approximately 3.4% of maternal plasma DNA. In maternal blood, fetal DNA molecules are detected with maternal DNA sequences. The paternal allele is the fetal-specific sequence because the maternally inherited allele of the fetus is genetically identical to the maternal allele. The fetal genotype can be assessed by the detection of the specific molecular characteristics of the paternally inherited fetal allele using cffDNA. These specific characteristics include the Y chromosome or sex-determining region Y (*SRY*) and paternally inherited variants or polymorphisms. If the parents carry different variants (compound heterozygosity), CAH can be excluded if no variant is detected in the paternal allele (11).

Rijnders et al. (15) first reported successful sex determination from maternal blood in a fetus at risk for 21-OHD. The male fetus could be detected as early as 13th gw by demonstrating the presence of the *SRY* in maternal blood samples. Bartha et al. (16) were able to accurately identify the male fetus as early as the sixth gw. A proposed algorithm for antenatal diagnosis recommends testing for the *SRY* at the fifth gw. Further testing is recommended until there is evidence of a male fetus in two separate samples or until the 11th week of pregnancy. Procedures such as CVS are not required if these tests indicate that the fetus is 46,XY (17). This protocol eliminates the need for invasive diagnostic procedures and prevents unnecessary exposure of male fetuses to prenatal

steroid therapy (11). Some publications suggest that as early as four weeks and five days of gestation, *SRY* analysis can be used for antenatal sex determination (18). Although antenatal sex determination can be performed by cffDNA using NIPT, the results obtained by this protocol should be confirmed by CVS and AS (2).

Fetal sex determination is a relatively straightforward procedure based on the presence or absence of Y chromosome sequences (3,18). However, antenatal sex determination in CAH only prevents male fetuses from receiving treatment and does not preclude unaffected females from receiving steroid treatment. Since most variants in the *CYP21A2* gene are caused by gene conversions and are also present in the fetal (and maternal) DNA, the detection of variants in the antenatal period is technically challenging. Advances in next-generation sequencing techniques have been used to detect single nucleotide polymorphism (SNP) haplotypes in the *CYP21A2* region. This technique generates millions of small fragments (100 base pairs, bp) and assembles them into longer sequences. Determining the maternal allele is difficult because the maternal allele of the fetus is only 2.0% higher than the other allele. High sequencing depth is required for accurate quantification however, this is costly and technically challenging when using whole genome sequencing. Current approaches involve amplifying several hundred kilobases around the *CYP21A2* locus or capturing DNA from the target region prior to sequencing and all have the potential to reduce the amount of sequencing required (19,20). These techniques are still under development and are subject to improvement.

The use of targeted massively parallel sequencing (MPS) for the non-invasive diagnosis of CAH using cffDNA was first reported in 2014 (20). In this study, 14 families, each with an index diagnosis of classical 21-OHD and parents with at least one mutant allele in the *CYP21A2*, were analyzed by NIPT. The authors reported successful fetal sex and CAH genotype identification by NIPT. In one family, CAH was detected by the analysis of maternal plasma as early as five weeks and six days of gestation. In addition, it has been reported that this new NIPT avoids the risks associated with invasive procedures such as CVS and AS. The advantage of this method is that it ensures that only affected fetuses receive treatment. However, it is should be highlighted that cffDNA testing has not completely replaced invasive diagnostic procedures and that confirmation with invasive tests is still necessary (20,21).

CYP21A2 genotyping by MPS is more complex than simply detecting specific *CYP21A2* variants. This technique requires targeted MPS on the genomic DNA of the affected proband and parents. SNPs on both sides of the *CYP21A2* locus generate haplotype blocks, which are needed to detect paternal and maternal alleles. Dosage analysis quantifies the amount of DNA inherited from the parents, taking into account the mother's alleles as well as the alleles she passes on to the fetus. If the fetus carries a CAH-associated haplotype, more DNA will be present with a linked SNP than with no linked SNP (22,23). Distinguishing between maternal and fetal alleles in maternal blood poses a significant challenge. These technical limitations restrict the broad application of cffDNA in diagnosing fetal monogenic disorders (24). However, studies have reported successful results with the use of cffDNA to sequence the fetal genome as early as the first trimester of pregnancy (24,25). Advances in genome sequencing techniques will eliminate the controversial issues associated with the accurate detection of fetal alleles. Despite ethical and technical challenges in the interpretation of fetal genomic data, the use of cffDNA from maternal blood for the diagnosis of single-gene disorders such as CAH is an exciting development in fetal genetic diagnosis (24).

Preimplantation Genetic Test for Monogenic Gene Defects

Preimplantation genetic test for monogenic (PGT-M) is a valuable reproductive option for families who are at risk of having a child affected by CAH due to the ability to identify and prevent the transmission of monogenic diseases. PGT allows the identification of genetic abnormalities in preimplantation embryos before transfer, ensuring that only unaffected embryos are transferred (11). Preimplantation genotyping allows the identification of affected and unaffected embryos as well as the determination of the gender of the embryos. PGT-M can be used for any monogenic disease where the causative variant can be accurately identified. The objective of PGT-M is to select against embryos carrying a monogenic disease for which an individual or couple is at risk, to reduce the likelihood of a pregnancy resulting in the birth of an affected individual. As genetic technologies continue to improve and costs continue to fall, genetic testing is becoming more common in various settings, particularly in fertility clinics. This allows more patients to be eligible for PGT-M (26).

There are three PGT approaches: (1) polar body biopsy, which uses female gametes (oocytes); (2) blastomere biopsy, performed on day three of a 6- to 8-cell cleavage stage embryo; and (3) trophectoderm biopsy, performed on a 5- to 6-day blastocyst containing approximately 120 cells (27).

When performing PGT for single gene disorders, such as 21-OHD, it is recommended to use SNPs or short tandem repeats for linkage analysis. This is necessary to rule out ADO, which can lead to the loss of the mutant allele. ADO can be caused by dsDNA breaks or failure of the host DNA to bind with the targeted primer. If ADO occurs, it may lead to the erroneous conclusion that the allele detected is the only allele present in the embryo. If ADO carries the mutant recessive allele and is not detected, it may be falsely concluded that the embryo is homozygous wild-type. ADO is caused by preferential amplification of one allele over the other and can lead to misdiagnosis of the genetic status of the oocyte or embryo (28). PGT should include multiple linked polymorphic microsatellite markers around the disease-associated gene to mitigate these problems (29,30). Invasive diagnostic testing is recommended to confirm PGT results due to the technical limitations of non-invasive methods that may result in false negative results (14).

Although most of the literature is related to 21-OHD in the antenatal diagnosis of CAH, the same molecular approaches, and PGT procedures can be applied to 11β-OHD, 3β-hydroxysteroid dehydrogenase (HSD3B2) deficiency, and other rare CAH types.

Couples who have a child with CAH and who are at risk for this condition are candidates for genetic counseling at the time of pregnancy planning. The accuracy of the molecular genetic diagnosis of the proband is important for genetic counseling. During counseling, parents should be informed about the possibility of the potential fetus being affected and the consequences of this condition. Another goal of antenatal diagnosis of CAH is to ensure that treatment is initiated in the early weeks of pregnancy to prevent virilization of the affected female fetus and so the need for genital surgery and gender confusion may be minimized, or even avoided (9). Several approaches have been used for the identification of fetuses with CAH during the antenatal period. Due to the rapid advances in molecular genetic methods, targeted approaches will become progressively more achievable.

Good practice points:

1. The protocol should include testing of maternal blood for Y chromosome material to distinguish male fetuses from the potential antenatal treatment group in research protocols (ungraded good practice statement).

2. Genetic counseling should be provided to families of a proband with CAH, adolescents and young adults transitioning to adult clinics, those diagnosed with non-classical CAH in adulthood, and their spouses when individuals diagnosed with CAH plan to have children.

3. Counseling must be given by clinicians experienced with CAH and its genetic characteristics (1⊕⊕⊕O).

Antenatal Treatment

Antenatal treatment of CAH, particularly 21-OHD, has been the focus of interest for many years. Nevertheless, this treatment is still considered controversial and experimental. In 1984, Forest et al. (30) first reported maternal treatment during pregnancy to reduce the virilization of female fetuses with CAH. Dexamethasone (Dex) is the glucocorticoid of choice for antenatal treatment due to its long half-life, its ability to cross the placenta as Dex is not metabolized by placental 11β-HSD2, and its ability to reduce androgen levels by suppressing fetal ACTH secretion (2).

The experimental approach for antenatal treatment of 21-OHD is the use of Dex in early pregnancy (before 6th gw) with the consent of parents who are heterozygous for a *CYP21A2* variant. Treatment may be discontinued in 46,XY fetuses following AS or CVS. The primary objective is to identify the affected fetus early in the gestation period and provide antenatal treatment only if necessary (2).

The antenatal treatment protocol includes the use of Dex at a dose of 20 μg/kg (maximum 1.5 mg/day), calculated based on the mother's pre-pregnancy weight. For a mother weighing 60 kg pre-pregnancy, this dose is 1.2 mg/day, which is approximately six times the physiological dose (31,32). Lower doses of Dex for antenatal treatment have not been reported, and the reason for the recommendation of such a high dose is not clear (18). Fetal cortisol concentration is low in early pregnancy but rises during external genital differentiation between 8-12 gw. It is only 10% of maternal levels in mid-pregnancy and then increases in the last trimester of pregnancy (31,32,33). This high therapeutic dose can result in fetal levels that are 30-60 times higher than normal, which can cause elevations in glucocorticoid concentrations that exceed physiologic levels in the second trimester (34,35).

In pregnancies in which the fetus is treated to term, treatment efficacy is monitored by maternal serum dehydroepiandrosterone sulfate DHEA-S (beginning at the 7th gw) and estriol measurements. Low DHEA-S levels indicate sufficient fetal adrenal suppression, while low estriol levels indicate maternal adrenal suppression and poor compliance (36). Many studies have reported that infants with CAH treated antenatally, are less virilized than their affected sisters who did not receive treatment, provided that treatment is initiated on time and the mother is compliant with the

treatment (37). To prevent genital virilization in females with 21-OHD or 11β-OHD, treatment should be initiated before an increase in genital sensitivity to androgens occurs, at the latest in the 7th-9th week of amenorrhea (37,38). This protocol may result in unnecessary steroid treatment for the majority of potential fetuses, as fetal genotyping is typically not feasible before 10-12 weeks of gestational age (9,38). The timing of Dex initiation is crucial for the genital morphology of females with CAH. To ensure the effectiveness of Dex treatment, it is recommended to continue until delivery in female fetuses with CAH (39).

Antenatal Dex treatment has the potential to prevent or reduce virilization of the external genitalia and brain, as well as reduce the need for multiple corrective genital surgeries in the postnatal period. This treatment option should only be offered to parents with an affected child with CAH whose heterozygous carrier status is established by molecular analysis. Antenatal diagnosis typically begins at 6 to 8 gw, even when fetal sex can be determined from maternal plasma by cffDNA analysis. This diagnosis may lead to the avoidance or early discontinuation of Dex in male fetuses. In female fetuses, the diagnosis of CAH can be made by CVS at 12-13 gw (2).

While animal studies have shown that early *in utero* exposure to Dex can cause adverse neurodevelopmental effects, human evidence is inconclusive. Several studies have reported a potential link between antenatal Dex use and neuropsychological development, but a consensus has not yet been reached (40).

Exposure to Dex between 7-12 gw coincides with neurogenesis and neuronal migration (38,41). Epigenetic processes may also have long-term programming effects on brain development and so questions remain about the behavioral and developmental effects of Dex. Although a Swedish study reported good school performance and psychological well-being, it did not identify concerns about memory and gender behavior (42). Impaired verbal memory and social anxiety were reported in cases that were not affected by CAH but received antenatal treatment (40). However, a larger follow-up study examining cognitive outcomes in Dex-exposed fetuses detected no impairment in memory. Instead, the study reported slower mental processing in antenatally treated females with CAH compared to controls (43).

The effects of steroids on neurodevelopment during the second and third trimesters have been the primary focus of research to date. Synthetic glucocorticoids are commonly administered to induce fetal lung maturation in fetuses at risk of preterm birth during the third trimester, but their use has been associated with a decrease in rostral anterior cingulate cortex thickness (44). In addition, high antenatal maternal cortisol concentrations have been linked to reduced fetal brain growth and altered functional and structural connectivity during childhood (44,45). The amygdala develops early in fetal life and is particularly sensitive to early abnormalities in cortisol concentrations. Increased amygdala volume has been associated with depression risk in girls (38,45). The effect of Dex treatment in the first trimester on changes in brain structure in adulthood was also investigated in a recent study (38). Magnetic resonance imaging scans of male and unaffected females at risk of CAH, as well as antenatally treated subjects, were compared with controls. It was reported that Dex exposure during the first trimester was associated with structural changes in the brain during adulthood. Moreover, methylation changes have been reported. In a previous study, changes in gene methylation associated with brain development were detected in individuals treated with Dex but not diagnosed with CAH. The candidate genes were brain-derived neurotrophic factor (*BDNF*), glucocorticoid receptor (GR) (*NR3C1*), GR co-chaperone *FKBP5*, and mineralocorticoid receptor gene (*NR3C2*) (46). It has been suggested that altered methylation of some of these genes may be associated with depression (46,47). Changes in brain structure may occur as a result of the effects of synthetic steroids on antenatal programming. Antenatal Dex treatment may have detrimental effects on cognitive function, as cognitive function and mood regulation depend on networks with high GR density. These findings raise concerns about the safety of antenatal steroid treatment in CAH (2).

Recent clinical guidelines consider antenatal steroid treatment to be an experimental treatment that is controversial for safety reasons. Therefore, Endocrine Society Guidelines also do not recommend specific antenatal treatment protocols (2).

Good practice points:

1. Clinicians should continue to regard antenatal therapy as experimental. Thus, we do not recommend specific antenatal treatment protocols (ungraded good practice statement).

2. In pregnant women at risk for having a fetus with CAH and who are considering treatment, we recommend therapy only through protocols approved by Institutional Review Boards at centers capable of reporting the outcomes in a large number of patients, so that risks and benefits can be defined more precisely (2⊕⊕OO).

Neonatal Congenital Adrenal Hyperplasia Screening

Newborn CAH screening is implemented in over 50 countries (48). According to these screening data, the incidence of classical CAH has been found to be approximately 1:14,000 to 1:18,000 in most populations (2). In the screening program conducted in our Turkey, this ratio has been similarly determined to be 1:15,067 (49).

The CAH screening program significantly reduces the time to diagnosis for infants with CAH, thereby decreasing morbidity and mortality (50,51,52,53). In a study involving 242 cases diagnosed with sudden infant death syndrome, dry blood samples obtained during the neonatal period were retrospectively analyzed, and classical CAH diagnosis was genetically confirmed in 3 (1.2%) cases (54). In contrast, another study found no cases of CAH in dry blood samples collected from 1,198 infants who died between 5 days and 6 months of age (55). In males with salt-wasting CAH, where there is no genital ambiguity, diagnostic delay, and misdiagnosis are more likely compared to female CAH infants. Therefore, the relative rarity of males with salt-wasting CAH in the patient population may serve as indirect evidence of unreported deaths (2).

Regarding morbidity, infants diagnosed through neonatal screening tend to experience milder hyponatremia and shorter hospital stays (51,53,55,56). Although males with salt-wasting CAH may seem to benefit more from screening, it also enables early determination of the correct gender in severely virilized girls identified through screening (53,57). Furthermore, the CAH screening program prevents diagnostic delays in male virilized CAH cases. In such cases, delayed diagnosis will result in rapid growth and accelerated bone maturation, leading to loss of final height in adulthood.

Screening is performed using methods that allow rapid results to be obtained from samples absorbed onto Guthrie cards and dried. A commonly used measurement method is time-resolved fluorescence immunoassay (DELFIA), which is an immunoassay technique (2). Several factors limit the accuracy of this test. Firstly, the level of 17-OHP is elevated at birth in healthy infants and decreases gradually in the following days. In contrast, in infants with CAH, 17-OHP increases progressively (58). Therefore, samples taken within the first two days have poor diagnostic accuracy, and follow-up samples are required for an accurate diagnosis. According to some reports, female infants have lower 17-OHP levels than males (58). Since nearly all of these infants exhibit virilization and salt loss, medical intervention is promptly sought, mitigating a significant issue. Thirdly, in premature, sick or stressed infants, the level of 17-OHP tends to be higher, which increases the likelihood of false positives.

For example, in a 26-year screening program in Sweden (evaluated using immunoassay for 17-OHP levels), the positive predictive value for term infants was found to be 25%. In contrast, for preterm infants, it was only 1.4%, and the predictive value of the test was reported to be strongly correlated with gestational age (59). Lastly, immunoassays may lack specificity. No universally standardized criteria are categorized according to infants, but most laboratories use cutoff limits based on birth weight.

Repeating the screening a few days after birth improves sensitivity and positive predictive value (60,61). It is indicated that repeated sampling should be done in the 2nd and 4th weeks for preterm infants and hospitalized babies (61). In a study, a positive predictive value of 5.6% was obtained between 48-72 hours, while samples taken after 72 hours yielded a positive predictive value of 14.1%, using the same cutoff value for CAH newborn screening (62). In a study conducted in the United States, the incidence of CAH was found to be 1:9,500 in the states where only a single sample was taken, whereas, in states where a second sample was taken, the incidence of CAH was 1:17,500 (63).

It has been found that classifying 17-OHP levels used in screening according to gestational age increases the specificity of newborn screening compared to birth weight (64). In a study where classification was made according to gestational age, screening increased the positive predictive value from 4.5% to 16% (56). In early gw, in addition to cross-reactions in immunologic tests, elevated levels of 17-OHP are observed due to functional deficiencies in several adrenal steroidogenic enzymes (adrenal steroidogenic enzymes are at their lowest point at the 29th week of gestation) (65). For example, due to cross-reaction with 17OH-pregnenolone sulfate, immunoassay may yield elevated levels of 17-OHP (66). Using organic extractions to remove steroid sulfates will increase the specificity of immunoassays (67). The corticosteroids used by the mother during the antenatal period may reduce 17-OHP levels in the baby and increase the likelihood of false negatives. Taking a second sample will minimize this issue.

Limitations of immunoassays for 17-OHP include proper elevation of levels in premature infants or those who are sick or stressed and lack of antibody specificity. In the second tier, direct measurement of steroids using the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method is more effective than immunoassays in addressing these issues (68). However, since each sample takes 6-12 minutes to analyze, it is not practical for evaluating a large

number of samples. This type of analysis is only suitable for smaller numbers of samples (69). Approximately 40% of the positive tests in the first sample have normal levels of 17-OHP. This supports the suboptimal antibody specificity in the first tests.

Measuring steroid ratios further enhances the specificity of LC-MS/MS used in screening. Measuring additional analytes or analyte ratios can also improve screening results. For example, 21-deoxycortisol (produced by 11β-hydroxylation of 17-OHP) is not normally secreted in large amounts (even in premature infants), so elevated levels are highly specific for 21-OHD. In a study using a modified LC-MS/MS protocol, where the ratio obtained by dividing the sum of 17-OHP and 21-deoxycortisol values by cortisol was used, a 100% positive predictive value was observed, and no false positives were reported (62).

Good practice points:

1. The most common cause of CAH is 21-OHD. Due to the significant reduction in mortality and morbidity with early detection and treatment of the disease, it is recommended to be included in the neonatal screening program (1⊕⊕⊕O).

2. The first-line test for screening should include measurement of 17-OHP levels. Screening using standardized technological methods and evaluating the results according to gestational age is recommended (1⊕⊕OO).

3. Individuals with high results in the first-tier test should be recommended for a second-tier test (1⊕⊕⊕O). To improve the positive predictive value of CAH screening, the use of the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method is recommended over all other methods (e.g., genotyping) to improve the positive predictive value of CAH screening (1⊕⊕OO).

Molecular Genetic Screening

CYP21A2 mutations can be detected from the DNA extracted from the same dried blood spots used for hormonal screening. Genotyping is a valuable diagnostic tool and a good complement to neonatal screening, especially in confirming or discarding the diagnosis in cases with slightly elevated 17-OHP. Because over 90% of mutant alleles carry one or more of a discrete number of mutations, patients carrying none of these mutations are unlikely to be affected. If at least one mutation is detected, the patient undergoes further evaluation (2). Genotyping of samples from screening programs has been suggested as a potentially helpful adjunct to hormonal measurements, but no large-scale study of efficacy has been reported as a second-tier screen in actual use (2). Also, genotyping, which has a lower positive predictive value, is time-consuming and more costly than LC-MS/MS on a per-sample basis, so it is not recommended as a second-tier screening test (2).

Newborn Congenital Adrenal Hyperplasia Screening in Turkey

The first CAH screening in Turkey was conducted in 2017 in four pilot provinces (Konya, Kayseri, Samsun, Adana) by the Ministry of Health. In this study, 38,935 infants were evaluated to determine the incidence of CAH and investigate the effectiveness of screening. Heel blood spot samples taken from infants at 3 to 5 days or after 48 hours were measured for 17-OHP values using fluoroimmunoassay, and those with high values underwent second-tier evaluation with LC-MS/MS. In this study, cutoff values were determined based on the infants' gestational age and birth weight. For infants with gestational age ≥37 weeks and/or birth weight ≥3500 grams, 10 ng/mL was accepted, while for infants with gestational age between 32-37 weeks and/or birth weight between 2500-3500 grams, 15 ng/mL was considered. In the second tier, 17-OHP, 21-deoxycortisol, cortisol, 11-deoxycortisol, and androstenedione were measured using the LC-MS/MS method, and infants with a (21-deoxycortisol + 17-OHP)/cortisol ratio ≥0.5 were referred to pediatric endocrinology clinics for further evaluation. It was found that second-tier steroid profiling increased the effectiveness of screening and reduced the number of false positives. In this pilot study, the frequency of 21-OHD was found to be 1:7,787 in the screened population (70).

Subsequently, in 2018, this pilot study was expanded and conducted in 241,083 infants in 14 provinces. According to the data from the initial study, the cutoff value used in the second-tier test was changed to (21-deoxycortisol + 17-OHP)/cortisol ratio ≥1 in this screening. No salt-wasting CAH cases were missed by newborn screening (sensitivity 100%). According to the results of this larger study, the frequency of classical 21-OHD in the screened population was 1:15,067, in line with other published frequencies, and 11β-OHD was 1:60,270 (49). The current screening does not detect non-classical CAH cases. If a CAH diagnosis is present as a result of the screening program, cortisol therapy initiation is appropriate. According to the screening results conducted in our country (49,70), if the first-tier screening using the fluorescent immunoassay (FIA) shows 17-OHP >90 ng/mL, treatment should be initiated immediately. In retrospectively analyzed cases diagnosed with CAH (49,70), the first-tier screening 17-OHP levels using the FIA method

were determined as >15 ng/mL in term and normal birth weight infants and >50 ng/mL in preterm and low birth weight infants. For these values, further investigations should be performed directly without waiting for second-tier test results, if possible. In addition, if the 11-deoxycortisol level is >10 ng/mL, further investigation for 11β-OHD should be conducted (Figure 1).

Methods are described at Part 1 (Clinical, Biochemical and Molecular Characteristics of Congenital Adrenal Hyperplasia Due to 21-hydroxylase Deficiency) of this supplement (71).

Good practice point:

1. In the first tier, if 17-OHP >90 ng/mL using the immunoassay method (FIA), treatment should be initiated immediately. In the first tier, if FIA 17-OHP >15 ng/mL (term, normal birth weight), >50 ng/mL (preterm, low birth weight), further investigations should be conducted. In the second tier, if (21-S + 17-OHP)/Cortisol ≥1 and 21-deoxycortisol >0.4 ng/mL and/or 17-OHP >2 ng/mL, further investigations should be conducted (1⊕⊕OO).

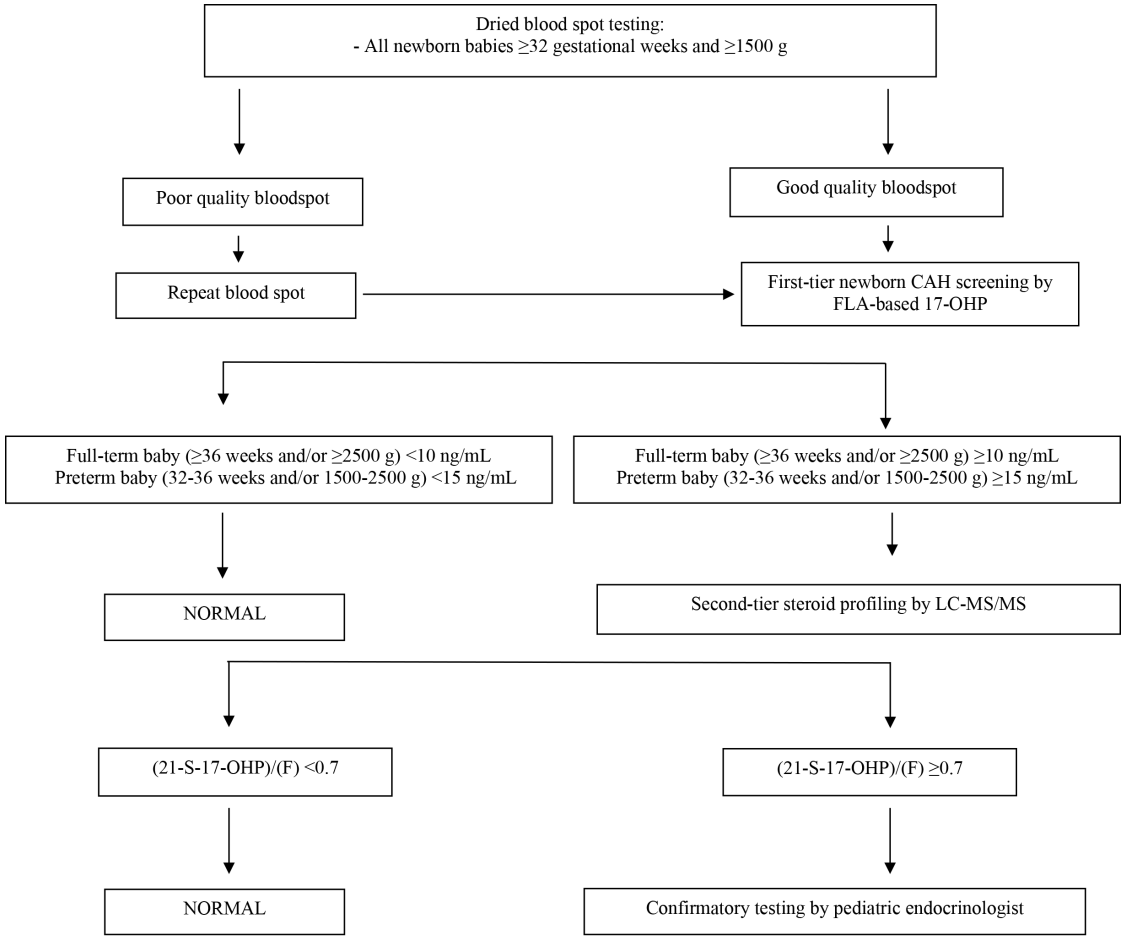

Figure 1. Flowchart for extended neonatal congenital adrenal hyperplasia screening initiated by the Turkish Directorate of Public Health (17-OHP conversion factor from ng/mL to nmol/L: multiply by 3.02)

CAH: congenital adrenal hyperplasia, FIA: fluoroimmunoassay, LC-MS/MS: liquid chromatography-tandem mass spectrometry, 17-OHP: 17-hydroxyprogesterone, 21-S: 21-deoxycortisol, F: cortisol

Footnotes

Authorship Contributions

Concept - Design - Data Collection and Processing - Analysis or Interpretation - Literature Search - Writing: Zehra Yavaş Abalı, Erdal Kurnaz, Tülay Güran.

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