Newborn screening for congenital adrenal hyperplasia: beyond 17-hydroxyprogesterone concentrations

Triagem neonatal para hiperplasia adrenal congênita: além das concentrações de 17-Hidroxiprogesterona

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The Congenital Adrenal Hyperplasias (CAHs) comprise a group of autosomal recessive disorders due to defects in adrenal steroidogenesis. The most common is 21-hydroxylase deficiency due to mutations in the 21-hydroxylase (CYP21A2) gene. The clinical spectrum ranges from life-threatening adrenal insufficiency to minimal symptoms depending on the specific CYP21A2 mutations. The clinical phenotype generally reflects the functional activity of the mildest mutation. The prevalence of the classical forms, salt-losing and simple virilizing, differs among populations ranging from approximately 1 in 6000 in India to 1 in 19,000 in Japan. The prevalence of non-classic or mild CAH is higher and was reported to be 1 in 2000 among Caucasians in the United States.

One goal of pediatrics is prevention of disease as exemplified by vaccine development. Another area for prevention is the development of programs to screen and detect newborn infants for whom early intervention is beneficial. For the pediatric endocrinologist, Newborn Screening (NBS) programs for congenital hypothyroidism provide a model in which early intervention prevents poor outcomes. For CAH, the high risk for morbidity and mortality in the classical forms led to the development of a microfilter paper 17-OHP assay in 1977. Subsequently, NBS protocols for CAH based on 17-OHP assays were established in over 40 countries.

Considerations prior to the establishment of a screening program include burden of the disorder, knowledge of disease prevalence, sensitivity and specificity of the screening test, and ethical considerations regarding privacy and informed consent. NBS programs are obligated to develop reliable methods to collect filter paper blood spot specimens from all newborn infants. The specimens need to be collected and processed within a specific timeframe. The intra-assay and inter-assay coefficients of variation for the laboratory test need to be low. And, finally, a system to locate and inform the parents and appropriate physician(s) is essential to an effective NBS program.

For CAH, the goal has been to identify infants with salt-losing and simple virilizing forms to prevent the morbidity and mortality due to acute adrenal insufficiency and to recognize affected females. NBS for CAH may also identify individuals with non-classic CAH. These individuals may have minimal symptoms and never need hormone replacement therapy. For the families, knowledge of a genetic diagnosis may generate years of anxiety due to the uncertainty regarding development of symptoms.

Hence, a challenge for CAH screening is to assure maximum sensitivity (proportion of positive tests among all individuals with the condition), maximum positive predictive...
value (proportion of true positive tests among all tests), and maximum specificity (proportion of negative tests among all unaffected individuals). Why is this such an obstacle for NBS for CAH? To achieve nearly 100% sensitivity, the cut-point for 17-hydroxyprogesterone (17-OHP) recall is set such that approximately 1% of all tests are reported as positive.\(^6\) At birth, 17-OHP concentrations are high and decline over the first 48–72 h of life. Early specimen collection can thus lead to a false positive result. False positive results (elevated 17-OHP values) can occur in preterm, heterozygous, and stressed infants.\(^7\) Some 17-OHP immunoassays cross-react with other steroids such as 17-hydroxypregnenolone sulfate and 15β-hydroxylated compounds leading to false positive results.\(^8\) Most 17-OHP assays are performed with commercial kits that may vary due to seasonal temperature fluctuations and unforeseen manufacturer kit changes.\(^9\) False positive results generate anxiety and costs inherent in follow-up evaluation to exclude the diagnosis of CAH.\(^10\) False negative results can occur, generally more commonly in girls than in boys.\(^11\) Maternal treatment with glucocorticoids can also lead to false negative results.

These challenges have led to development of strategies designed to improve the positive predictive value. In the cross-sectional study by Kopacek et al., their goals were to describe the results of a CAH newborn screening embedded in a public health program in Southern Brazil to assess the clinical features of infants identified through this program, and to ascertain the usefulness of CYP21A2 genetic testing in the NBS protocol.\(^13\) During the two years of this study, a total of 217,965 samples were obtained from 3rd to 40th postnatal days. A time-resolved immunofluorescence assay was used to measure 17-OHP concentrations in dried blood spots. To minimize the false positive results, Kopacek et al. chose to utilize cut-points for four birth-weight tiers stratified by both Brazilian and international experience.\(^14,15\) Repeat samples were obtained generally by 3 weeks of age for infants with elevated 17-OHP values or when mothers had used a corticosteroid late in pregnancy.

To assess the value of a second-tier molecular test, the authors performed 17-OHP determinations and CYP21A2 genetic testing on the second sample. They utilized a SNPshot assay to detect 12 common CYP21A2 mutations and Multiplex Ligation-dependent Probe Amplification (MLPA) assay to detect rearrangements.\(^16\) Elevated 17-OHP values for birth weight cut points were detected in 147 infants. Of these 147 infants, 15 cases were confirmed to have classical CAH. As would be anticipated, infants found to have classical CAH had the highest 17-OHP concentrations at both timepoints. Of the 15 patients found to have classical CAH, all were identified by NBS prior to clinical diagnosis, thus confirming the benefits of the NBS program. Of the remaining 132 positive 17-OHP screening results, the second-tier genetic testing revealed that 7 infants had non-classical CAH, 14 were heterozygotic carriers, and 96 had false positive results. In this situation, knowledge of the specific diagnosis was able to direct appropriate management.

Kösel et al. also tested a two-tiered approach using CYP21A2 molecular testing for samples with elevated 17-OHP values and concluded that despite the slightly higher cost, this testing paradigm could reduce the need for second samples and prevent parental anxiety.\(^17\) However, molecular genetic analysis can be complicated because CYP21A2 is located in a complex genetic locus in close physical proximity to a highly homologous nonfunctional pseudogene (CYP21A1P). Most CYP21A2 mutations associated with CAH comprise normal CYP21A1P sequence and represent recombination (gene conversion) events between CYP21A2 and CYP21A1P. Three neighboring genes, serine/threonine kinase (RP), complement C4, and Tenascin (TNX), map to this locus. These four genes form a unit known as the RCCX module, which can be deleted or duplicated. In addition to this copy number variation, the high sequence homology between the functional CYP21A2 gene and its nonfunctional pseudogene CYP21A1P complicates the genetic testing.\(^18\) Discriminating deleterious mutations from Variants of Unknown Significance (VUS) discovered by whole genome and exome sequencing may be problematic.\(^19\) In some instances, multiple laboratory methods and parental genotyping are necessary to accurately determine a child’s genotype.\(^20\)

Collection of a second blood sample is being used to improve NBS performance parameters. Currently, approximately 22% of American infants are routinely screened a second time; this has improved detection of infants with simple virilizing and NCAH.\(^21\) Other paradigms involve use of cut-points based on gestational age, collecting a second sample for 17-OHP immunoassay, liquid chromatography followed by tandem mass spectrometry (LC-MS/MS), determination of 21-deoxycortisol concentrations, or hormone ratios such as 17-OHP/11-deoxycortisol ratio.\(^22,23\)

Reflection on the details of this study can provide insight and additional perspectives. Two aspects of this study warrant additional comments: (1) maternal use of glucocorticoids and (2) characterization of the IVS2-13A/C>T variant. Some mothers were taking glucocorticoids (GC) prior to delivery. The indications for GC use and type of GC were not described. Did mothers take GC for prenatal treatment to prevent genital virilization for presumably affected infants, for threatened preterm labor, or for another reason?

The authors reported a novel variant, IVS2-13A/C>T, in two individuals. These patients were labeled as being heterozygous carriers. The CYP21A2 mutation, IVS2-13A/C>G, involves the same nucleotide and was the most commonly identified mutation in this cohort. The A/C>G mutation creates a novel upstream splice acceptor site resulting in aberrant splicing of intron 2, retention of 19 intronic nucleotides, and a downstream premature termination codon.\(^24\) However, the possibility that the A/C>T variant represents a benign variant or VUS has not been addressed. Functional characterization using in vitro expression studies or in silico analysis utilizing bioinformatics prediction software could be used to characterize the functional significance of this intronic variant.

Early detection, confirmation of diagnosis, and treatment are beneficial for individuals with salt-losing and simple virilizing forms of CAH. Pediatricians, family physicians, pediatric endocrinologists, obstetricians, and neonatologists need to be knowledgeable regarding local NBS programs and understand the current limitations of screening for CAH. The future holds promise as the methodology to achieve improved positive predictive values for NBS for CAH evolves.
Conflicts of interest

The author declares no conflicts of interest.

References