

Weight-adjusted neonatal 17OH-progesterone cutoff levels improve the efficiency of newborn screening for congenital adrenal hyperplasia

Determinação de pontos de corte para 17OH-progesterona de acordo com o peso ao nascimento melhora a eficiência da triagem neonatal da hiperplasia adrenal congênita

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ABSTRACT

Objective: To evaluate weight-adjusted strategy for levels of neonatal-17OHP in order to improve newborn screening (NBS) efficiency. **Subjects and methods:** Blood samples collected between 2-7 days of age from 67,640 newborns were evaluated. When N17OHP levels were ≥ 20 ng/mL, and a second sample was requested. We retrospectively analyzed neonatal-17OHP levels measured by Auto DELFIA- B024-112 assay, grouped according to birth-weight: G1: $< 1,500$ g, G2: $1,501-2,000$ g, G3: $2,000-2,500$ g and G4: $> 2,500$ g. 17OHP cutoff values were determined for each group using the 97.5th, 99th, 99.5th and 99.8th percentiles. **Results:** 0.5% of newborns presented false-positive results using the cutoff level ≥ 20 ng/mL for all groups. Neonates of low birthweight made up 69% of this group. Seven full-term newborns presented congenital adrenal hyperplasia (CAH) and, except for one of them, 17OHP levels were > 120 ng/mL. Only the 99.8th percentile presented higher predictive positive value (2%), and lower rate of false-positives in all groups. **Conclusions:** We suggest the use of 99.8th percentile obtained by weight-adjusted N17OHP values of healthy newborns to reduce the rate of false-positive results in NBS. *Arq Bras Endocrinol Metab.* 2011;55(8):632-7

Keywords

21-hydroxylase deficiency; congenital adrenal hyperplasia; newborn screening; neonatal 17OH-progesterone levels; preterm and full-term newborns

RESUMO

Objetivo: Avaliamos retrospectivamente os valores da 17OHP ajustados para o peso ao nascimento para melhorar a eficiência da triagem neonatal. **Sujeitos e métodos:** 67.640 recém-nascidos com amostras coletadas entre 2-7 dias de vida. Uma segunda amostra foi solicitada na presença de testes com valores da 17OHP ≥ 20 ng/mL. 17OHP dosada pelo método DELFIA- B024-112 e correlacionada com o peso ao nascimento: G1 < 1.500 g, G2 $1.501-2.000$ g, G3 $2.000-2.500$ g e G4 > 2.500 g. Pontos de corte da 17OHP foram determinados para cada grupo usando os percentis 97,5th, 99th, 99,5th e 99,8th. **Resultados:** Falso-positivos ocorreram em 5% dos resultados com o ponto de corte ≥ 20 ng/mL, dos quais 69% eram prematuros. Sete recém-nascidos apresentaram deficiência da 21-hidroxilase e, exceto em um, os valores da 17OHP foram > 120 ng/mL. Somente o valor da 17OHP do 99,8th apresentou maior valor preditivo positivo (2%) e menor índice de falso-positivos. **Conclusões:** Sugerimos o valor da 17OHP do 99,8th ajustado para o peso ao nascimento para se reduzir a taxa de resultados falso-positivos da triagem neonatal. *Arq Bras Endocrinol Metab.* 2011;55(8):632-7

Descritores

Deficiência da 21-hidroxilase; hiperplasia adrenal congênita; triagem neonatal; 17OH-progesterona; recém-nascidos prematuros e de termo

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Received on 5/Oct/2011
Accepted on 16/Oct/2011

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder caused by a deficiency in one of the five enzymes necessary for adrenal biosynthesis of cortisol from cholesterol. The most frequent form of CAH, accounting for 90%-95% of all cases, is caused by 21-hydroxylase deficiency (1).

CAH is caused by mutation of *CYP21A2* gene, which results in a spectrum of enzymatic deficiencies with a wide range of clinical manifestations, varying from prenatal external genitalia virilization in females, precocious pseudopuberty in males (simple virilizing form) to late onset hyperandrogenic symptoms (non-classical form) (2-4). Nearly 75% of newborns also present mineralocorticoid deficiency, characterized by extreme dehydration with hyponatremia and shock in the first weeks of life (salt-wasting form). The incidence of severe forms varies from approximately 1:16,000 to 1:20,000 depending on the population (1). In Brazil, a recent report in the state of Goiás, which performed CAH newborn screening (NBS) by means of a government-funded mandatory program, identified an incidence of 1:10,300 live births (5).

Despite an apparently high potential for symptomatic diagnosis, clinical diagnosis of CAH is poor in the newborn period. Males with the most severe salt-wasting form have a high rate of neonatal mortality due to lack of recognition of adrenal insufficiency signs. Furthermore, external genitalia virilization in females can be severe enough to result in incorrect gender assignment at birth. High morbidity and mortality of the classical forms, as well as the existence of accessible treatment, and the possibility of screening using samples routinely obtained for other diseases, justify the inclusion of CAH in newborn screening programs (NBS) (6,7).

CAH-NBS was developed in the late 1970s using a radioimmunoassay for 17-hydroxyprogesterone (17OHP) in dried blood spots collected from infants soon after birth on filter paper cards (8). Nowadays, CAH-NBS has been adopted in all states of the USA and in at least 16 other countries, including New Zealand, Japan, Israel, Sweden, Switzerland, France, Austria and the Netherlands (1). The main objective of CAH-NBS is to identify newborns at high risk of being affected by the salt-wasting form of the disorder, in order to prevent the adrenal crisis; moreover, NBS also enables earlier correction of gender assignment in affected females (9,10).

One of the major shortcomings of CAH-NBS is the occurrence of false-positive results in preterm or low birthweight newborns, or in association with unrelated neonatal diseases (7,10). These infants should be followed up until there is a definite diagnosis or normalization of serum 17OHP levels, as these situations add not only to the cost of screening programs, but can also be the cause of great anxiety to parents. In order to minimize these problems, cutoff values for 17OHP were established for NBS according to birthweight or gestational age (11-14).

In Sao Paulo, APAE (Associação de Pais e Amigos dos Excepcionais) is one of Reference Centers for Neonatal Screening in the state. In 2009, it started a pilot CAH newborn screening program, which has screened 73,427 live births. To measure neonatal 17OHP levels, APAE – Sao Paulo has been using the Auto DELFIA-B024-112 immunofluorometric assay, the same methodology used by the most private centers in Brazil, and for which there are no data regarding multitiered weight-adjusted neonatal 17OHP levels.

Considering the incidence of CAH in Brazil, it is expected that it will be included in the National Newborn Screening Program. In order to improve the CAH-NBS efficiency and to decrease the rate of false-positive results, we retrospectively evaluated a multitiered weight-adjusted strategy for neonatal 17OHP levels using the Auto DELFIA- B024-112 immunofluorometric assay.

PATIENTS

We retrospectively analyzed clinical data of all NBS results of samples sent to the APAE – Sao Paulo from June 2009 to December 2010.

According to the APAE guidelines, heel prick capillary blood was collected from newborns between 2 and 7 days of age on S&S 903 filter paper, and dried samples were shipped daily to APAE Laboratory. A total of 73,427 samples were evaluated: 51% from females, 48% males. For 1% of the samples, sex data were not available on the filter paper.

Suspicion of CAH was based on neonatal (N) 17OHP level ≥ 20 ng/mL (serum equivalence) and, in these cases, a second sample was immediately requested. In the case of the presence of a second positive result (N17OHP ≥ 20 ng/mL), newborns were called back to perform confirmatory tests, and submitted to a complete clinical evaluation in a Pediatric Endocrinology Center.

Diagnosis of classical forms was established by increased neonatal and serum 17OHP levels, and all females presented external genitalia virilization, graded according to Prader score (15). Patients with the salt-wasting form also presented hyponatremic dehydration.

Simple false-positives, i.e., those newborns with normal N17OHP levels in the second sample, were not submitted to any further investigations, whereas asymptomatic newborns with persistent slight or moderate increase of 17OHP levels were submitted to strict clinical and biochemical follow-up until the normalization of serum 17OHP levels. These latter children were classified as having uncertain diagnoses.

SUBJECTS AND METHODS

Filter paper hormonal assays were performed using the time-resolved, solid-phase fluoroimmunoassay 17OHP neonatal kit (B024-112 – Auto DELFIA – Neonatal 17OH-progesterone – Perkin Elmer Life and Analytical Sciences, Wallac Oy, Turku – Finland). N17OHP concentrations were expressed as serum equivalence (conversion rate: 1 nmol/L = 0.33 ng/mL in whole blood, and 0.73 ng/mL in serum equivalence). Quality assurance of the analyses was carried out by the Newborn Screening Quality Assurance Program – Centers for Disease Control and Prevention, Atlanta, USA.

N17OHP levels were correlated with birthweight, which was calculated on the basis of data reported in the filter paper of each infant. N17OHP levels (serum equivalence) were grouped according to birthweight into four categories, as follows: G1 birthweight < 1,500 g, G2 between 1,501 and 2,000 g, G3 between 2,000 and 2,500 g and G4 > 2,500 g.

All hormonal confirmatory tests, including serum 17OHP levels, were performed by independent private laboratories.

Statistical analysis

Numerical data are presented as means \pm SD (standard deviation), medians, ranges and percentages. As 17OHP levels presented a non-Gaussian distribution (Kolmogorov-Smirnov test, $p < 0.05$) in all groups, different N17OHP cutoff values were determined for each birthweight group using the 97.5th, 99th, 99.5th and 99.8th percentiles, and the number of cases that were called back, which would result from different putative cutoff levels for each group, were recalculated.

Sensitivity, specificity, positive and negative predictive values were defined as follows: sensitivity indicated the proportion of CAH newborns that had a positive result (ratio of true positive cases to the sum of true positive and false negative cases). Specificity indicated the proportion of normal newborns that had a negative result (ratio of true negative cases to the sum of true negative and false positive cases). Positive predictive values indicated the probability that a newborn with a positive result had CAH (ratio of true positive cases to the sum of true positive and false positive cases). Negative predictive values indicated the probability that a newborn with a negative result did not have CAH (ratio of true negative cases to the sum of true negative and false negative cases). CAH incidence was estimated as the number of newborns with confirmed CAH divided by the total number of screened newborns.

All statistical analyses were performed using the SigmaStat 3.5 for Windows. Statistical significance was determined by P value < 0.05 .

RESULTS

During the 18 months of this pilot program, 73,427 newborns were screened for CAH. To determine adequate N17OHP cutoff values for each birth weight group, data of 5,787 newborns were excluded because samples were collected after the 7th day of life, mainly due to the presence of illness and/or blood transfusions associated with prematurity (mean of 13 days after birth, range from 8 to 60 days). Data from 67,640 newborns were analyzed: 229 from the G1 birthweight group; 702 from G2; 3,882 from G3; and 62,827 from G4.

Using the original N17OHP cutoff level ≥ 20 ng/mL for all birthweight groups, 391 (0.6%) out of 67,640 newborns presented positive results. After a second sample collection, 353/391 tests presented normal levels and were classified as true false-positive results, 32 asymptomatic cases remained with slightly increased N17OHP levels (hyper-17OHPemia) and were maintained in follow-up. The remaining six newborns were affected by CAH.

True false-positive results made up 0.52% of tests; mean N17OHP levels was 28.5 ng/mL (± 10.7), and ranged from 20 to 113.7 ng/mL. As expected, low birthweight newborns made up 69% of this group (Table 1). In the group with hyper-17OHPemia, mean N17OHP levels in the first and second samples were 39 (± 28) ng/mL and 34.5 (± 20) ng/mL, respectively.

Table 1. Percentage of false-positive results using different N17OHP cutoff values

Birthweight group	≥ 20 ng/mL	97.5 th percentile	99 th percentile	99.5 th percentile	99.8 th percentile
< 1,500 n = 229	28%	2.6%	1.3%	0.9%	0.4%
1,501-2,000 n = 702	12%	2.6%	1.3%	0.6%	0.3%
2,001-2,500 n = 3,882	3%	2.5%	1%	0.5%	0.2%
> 2,500 n = 62,827	0.2%	2.5%	1%	0.5%	0.2%
Total 67,640	5.2%	2.5%	1%	0.5%	0.2%

CAH was diagnosed in six cases from those samples that were collected between 2 and 7 days after birth, and in another one from a sample collected later on (13th day of life). All these 7 newborns belonged to the full-term group (G4). One female presented the simple virilizing form, and the remaining newborns (1 female and 5 males), the salt-wasting form. The two females presented external genitalia virilization at birth (Prader III); however, in the salt-wasting female, the ambiguous genitalia was recognized only after the NBS result. N17OHP levels in the simple virilizing patient was 52 ng/mL, and those of salt-wasting newborns, except for one of them, were substantially increased (> 120 ng/mL). One salt-wasting patient presented moderately increased N17OHP levels (31 ng/mL), but her sample was collected 46 hours after birth (Table 2). Among all 73,247 newborns, the incidence of CAH was 1:10,460 live births.

In this pilot program, which used the cutoff value ≥ 20 ng/mL for N17OHP, independent of birthweight, the positive predictive value of an abnormal test was only 1.5%. N17OHP levels were retrospectively evaluated in four-tiered, weight-adjusted criteria in this screened cohort. N17OHP levels are described in table 3, in serum equivalence, using the 97.5th, 99th, 99.5th and 99.8th percentiles as cutoff levels.

False-positive rates decreased substantially in pre-term groups (G1-G3) in all percentiles; however, rates of the 97.5th and 99th percentiles are still inadequate for a public NBS (Table 1). Using the N17OHP values of 99.5 and 99.8th percentiles as cutoff levels, 8.7% and 5.5% of the preterms, respectively, remained false-positive. Regarding full-term newborns (G4), only the value of the 99.8th percentile presented a lower false-positive rate.

Table 2. Clinical data of affected newborns diagnosed by means of CAH newborn screening in APAE – Sao Paulo

Patient	Sex	Birthweight (g)	Sample collection (days after birth)	N17OHP (ng/mL)	Clinical form
1	F	3,340	3	52	SV
2	F	2,950	2*	31	SW
3	M	3,985	2	127	SW
4	M	3,095	2	396	SW
5	M	3,580	3	411	SW
6	M	3,650	3	480	SW
7	M	2,600	13 ^Δ	437	SW

SW: salt-wasting form; SV: simple virilizing form; *: samples were collected at 46 hours of life, Δ: later sample collection.

Table 3. Multitiered weight-adjusted neonatal 17OH-progesterone levels (ng/mL)

Birthweight	97.5 th	99 th	99.5 th	99.8 th
G1: < 1,500 g	81.2	110.4	134.5	160.2
G2: 1,501-2,000 g	36	43	53.7	55.2
G3: 2,001-2,500 g	21.8	28.2	31.8	40.6
G4: > 2,500 g	12.4	15.1	17.1	20

N17OHP levels in serum equivalence.

Sensitivity was 100% for both cutoff levels, and specificity was 99.3% and 99.5% for the cutoff levels of the 99.5th and 99.8th percentiles, respectively. Positive predictive values using the cutoff levels of the 99.5th and 99.8th percentiles were 1.5% and 2%, respectively, and negative predictive values were 99.3% and 99.5%, respectively.

DISCUSSION

CAH is a disease suited to newborn screening programs because it is common and potentially lethal. Although the Brazilian population presents different levels of miscegenation, the incidence of CAH in this series from Sao Paulo was similar to the one previously described in the state of Goias (5).

In this pilot program, and similar to literature findings, we demonstrated that CAH-NBS was efficient in detecting the salt-wasting form of the disorder, as well as affected males (1,6,7). We believed that the higher proportion of males in relation to females in this screened cohort is related to the small sample of affected subjects. Another study had similar results, and the proportion of males/females among CAH patients was normalized by increasing the sample size (16).

The efficiency of CAH-NBS is also evidenced by analyzing the characteristics of a cohort from Hospital das Clínicas-SP, which has been diagnosed without NBS in the same state. In this latter series, a lower proportion of salt-wasting patients was observed (55%), and an unbalanced sex ratio among salt wasters (5 females *versus* 1 male) was an indicator of excess mortality. Although the state of Sao Paulo is one of the most important medical centers in Brazil, in the unscreened cohort, genital ambiguity was not recognized at birth in 37% of females and 6 of them, due very late diagnosis, were raised as males (17). It is known that CAH-NBS does not prevent errors of sex assignment at birth, but allows early correction, as evidenced in the diagnosis of the salt-wasting female in the APAE-Sao Paulo cohort. These data indicate that, in Brazil, not only males, but also females should be submitted to CAH-NBS.

A common difficulty in CAH-NBS is the high rate of false-positive results observed mainly in preterm, low-birth-weight, or stressed newborns; generally, these cases present moderate increases in N17OHP levels. The reasons for higher N17OHP levels in preterms are related to immature adrenal function, lower 11-hydroxylase capacity, higher levels of glucuronic acid, and sulfate-conjugated steroid metabolites in the circulation (13,14,16). In order to minimize the aforementioned problems, NBS programs have established cutoff values for 17OHP levels according to birthweight or gestational age (11-14).

To the best of our knowledge, the state of Goias and most private laboratories from Brazil have been using AutoDELFIA immunofluorometric assays. A previous Brazilian study established different cutoff values for preterm and full-term newborns based on birthweight; however, both the kit and the filter paper used in that study were discontinued and, consequently, those levels could not be extrapolated to the actual methodology (18). Considering these data, in the present study, we retrospectively analyzed a multitiered weight-adjusted strategy for N17OHP levels using the current immunofluorometric assay available in Brazil (Auto DELFIA-B024-112 immunofluorometric assay®).

We chose birthweight categorization instead of gestational age to classify different degrees of prematurity, since it was thought that birthweight data presented on the filter paper was more accurate and reliable than the data on gestational age that the mothers had reported (12). For all preterm groups (G1-G3) there was a significant reduction of false-positive rates using N17OHP

values of the 97.5th, 99th, 99.5th and 99.8th percentiles as cutoff values. However, data of 97.5th and 99th percentiles are still inadequate for a public NBS.

After using the four multitiered weight-adjusted N17OHP levels, 8.7% and 5.5% of all preterm newborns from the present study still presented false-positive results using the 99.5th and 99.8th percentiles, respectively. Our data are similar to those from the literature; this issue remains unresolved even after adjustment of neonatal 17OHP levels according to gestational age, and the use of more sensitive methodologies, such as extraction procedures and tandem mass spectrometry (19). In Sweden, prematurity made up 43% of false-positive results after making use of gestational age-related cutoff values and an ether extraction methodology (14,20).

One limitation of our study was that the samples were of newborns that had been delivered in private hospitals in the state of Sao Paulo. Consequently, newborns with positive results in the second sample collection were followed up at different Pediatric Centers. We contacted all these centers and, to our knowledge, none of the newborns were affected by CAH. However, we cannot rule out the possibility that a small proportion of these males could have presented the simple virilizing form. Classically, N17OHP levels of newborns with hyper-17OHPemia present an overlap with those of true false-positive, and those of affected by simple virilizing and nonclassical forms. In a previous Brazilian study, which evaluated the Goias series, 3 out of 19 asymptomatic cases with persistently increased 17OHP levels were diagnosed by means of molecular analysis as having the simple virilizing form, and the other two, the nonclassical form. The three simple virilizing patients did not present evident hyperandrogenic signs in the first year of life (16).

Neonatal 17OHP values corresponding to 99.5th and 99.8th percentiles presented 100% sensitivity in identifying full-term newborns with the salt-wasting form, and the female newborn with simple virilizing form. Both cutoff levels presented high negative predictive values; however, only the cutoff of the 99.8th percentile presented higher positive predictive value (2%), which was still too low. In the Swedish program, the predictive value of a positive result for a full-term newborn is approximately 27%, but the methodology based on extraction procedures contributes to this higher positive predictive value (14,20).

Neonatal 17OHP levels of the salt-wasting patients were markedly increased, except for one, whose sample

was collected at 46 hours of life. We suppose that this earlier collection corroborated this lower value, and this hypothesis was based on reports that cited evidence that CAH-NBS diagnostic accuracy is poor on the first 2 days of life (7).

Considering that, in our state, newborn samples could be collected starting at 48 hours of life, and we suggest that, when CAH-NBS is implemented, N17OHP levels related to the 99.8th percentile are considered as abnormal results, and that they are treated as urgent, requiring a phone call to the responsible physician, recommending the immediate collection of a second sample. These cutoff levels should be reassessed after the diagnosis of a sufficient number of salt-wasting and simple virilizing newborns. Recently, after this study was over, we diagnosed the salt-wasting form in two preterm male newborns with birthweights of 1,800 g and 2,350 g, and their N17OHP levels were extremely increased, 380 and 330 ng/mL, respectively.

In conclusion, we confirmed the benefits of neonatal screening for newborns affected by the classic form of 21-OHD, particularly in males. The incidence of CAH in this series, as well as the increased morbidity and mortality, qualify this disorder as eligible for our National Newborn Screening Program. At the beginning of a CAH-NBS program, we suggest the use of 99.8th percentile of N17OHP values to reduce the rate of false-positive results, but these levels should be evaluated after the diagnosis of a significant number of affected newborns in all birthweight groups.

Acknowledgements: this research was partially supported by grants from FAPESP # 08/57616-5; Tânia A. S. S. Bachega and Berenice B. Mendonca received CNPq grants # 305117/2009-2 and # 301339/2008-9, respectively.

Disclosure: no potential conflict of interest relevant to this article was reported.

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