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Determination of 17-Hydroxyprogesterone Cut-Off Values Adjusted for Gestational Age for Neonatal Screening of Congenital Adrenal Hyperplasia

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Abstract

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder caused by a deficiency of enzymes involved in cortisol biosynthesis. More than 90% of the cases are due to 21-hydroxilase deficiency. This leads to the accumulation of 17-hydroxyprogesterone (17OHP) and allows diagnosis of this disorder in newborn screening (NS) programs. Premature newborns typically have higher levels than full-term newborns, making the use of age-appropriate cut-off values important for accurate interpretation. The aim of our study was to update the gestational age-adjusted cut-off values for 17OHP, the methodology currently used in Argentina by the jurisdictions participating in the National Program for the Strengthening of Early Detection of Congenital Diseases. Cut-off limits were obtained using percentiles of the distribution of 17OHP values for each gestational age on 53,230 dry blood spot (DBS) samples. Sensitivity, specificity, false positive rate, positive predictive value, and incidence were calculated. We successfully established gestational age-specific cut-off values for 17OHP. The analysis showed a sensitivity of 100%, specificity of 99.52%, a false positive rate of 0.49%, and a positive predictive value of 1.54%. The incidence of CAH was 1:13,308 live births. These findings underscore the importance of using population-appropriate cut-off limits. Such harmonization enables comparability among regional neonatal screening programs with the added benefit of decreasing the rate of recalls and associated costs.

Keywords: Newborn screening; Congenital adrenal hyperplasia; cut-off values; 17 hydroxyprogesterone; recall rate

1. Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder characterized by deficient activity of adrenal steroidogenic enzymes, leading to impaired or no cortisol synthesis.

The absence of negative feedback results in an increase in adrenocorticotropin levels, leading to adrenal hyperplasia. The clinical presentation of CAH is highly variable depending on the enzyme involved and the degree of residual enzyme activity. The most common form of CAH (90%) is caused by pathogenic variants in the CYP21A2 gene, which encodes the enzyme 21 hydroxylase. This enzyme catalyzes the conversion of 17-hydroxyprogesterone (17OHP) to 11-deoxycortisol and of progesterone to deoxycorticosterone, a precursor of aldosterone. The classic forms occur in the neonatal period and are associated with severe enzyme deficiency, including salt-wasting (SW) forms and the less severe simple virilizing (SV) forms [1]. A cardinal feature is ambiguous genitalia in girls,

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whereas boys may be overlooked. If the disorder is not recognized and treated during neonatal period, the severe form can be fatal due to an SW crisis [2,3]. Additionally, both sexes may exhibit accelerated postnatal growth and signs of sexual precocity. Severe forms can be rapidly fatal; therefore, early diagnosis and intervention are critical to preventing neonatal death. In CAH, 17OHP accumulates, enabling diagnosis through Newborn Screening (NS) programs.

In Argentina, National Law 26,279 (2007) establishes the mandatory diagnosis and treatment of the following conditions: Phenylketonuria, Congenital Hypothyroidism, Cystic Fibrosis of the Pancreas, Biotinidase Deficiency, Galactosemia, Congenital Adrenal Hyperplasia, Retinopathy of Prematurity, Chagas disease, and Syphilis. Neonatal HSC screening has been adopted worldwide, with more than 50% coverage in at least 49 countries [4].

The incidence of CAH reported by Ministry of Health of Argentina, DISAPENIA (Dirección de Salud Perinatal Niñez y Adolescencias) is 1:15,260 newborns. Data from the public sector show that 401 cases were detected among 6,119,126 newborns screened between 2006 and 2023 (unpublished data). Based on neonatal screening and national registries, the worldwide incidence in most studies ranges from 1:14,000 to 1:18,000 [5–7].

The positive predictive value of the CAH screening has been reported to be generally low. To address this, various strategies have been adopted. The main challenge occurs in preterm infants, who tend to have elevated levels of 17OHP compared to full-term infants [8]. This is mainly due to natural overproduction and cross-reactivity with structurally related steroids produced by the fetal adrenal gland. A widely used work model is the stratification of cut-off values according to gestational age (GE) and/or birth weight to avoid an excessive recall rate [9–12]. The aim of this study was to reassess the gestational age–adjusted cut-off values for 17OHP, the methodology used by the National Program for the Strengthening of Early Detection of Congenital Diseases in the provincial NS programs of Argentina, and to show our experience with its application in a population of the Patagonian region of the Argentine Republic.

We successfully established gestational age-stratified cut-off values for 17OHP in CAH screening.

2. Materials and Methods

Design:

A total of 58,911 unselected dry blood spot (DBS) samples received between June 2020 and March 2025 from the provinces of Chubut, Rio Negro, Santa Cruz, and Tierra del Fuego, were included. The 17OHP results were analyzed using preliminary cut-off values adjusted for GE, calculated based on percentiles of 17OHP levels in the study population (Table 1).

Table 1. Preliminary cut-off values of 17OHP (ng/mL serum).

Stage	Gestational age group (weeks)	Sample size	Preliminary Cut off (ng/mL serum)
1	≤ 34	58	162.2
	35	78	99.2
	36	146	77.2
	37	330	69.3
	>37	3296	55.8
2	≤ 34	490	148.3
	35	335	99.9
	36	756	87
	37	1829	71.8

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	>37	18,079	53.2

Stage 1: calculated on 7542 samples from June/2020 to October/2020 (data not published), used from 2020 to 2022.
Stage 2: calculated on 21,489 samples from October/2020 to September /2022 [13], used from 2022 to 2025.

Inclusion criteria:

(1) First sample collected between 36 hours and 7 days of life, and (2) 17OHP value within the quantification range (0.9 – 230 or 245 ng/mL serum depending on the batch). Of the 58,911 neonatal screening samples collected between June 2020 and March 2025, only 53,230 met the inclusion criteria.

Methodology:

Blood samples were collected on Whatman 903 filter paper. 17OHP was quantified using a commercial enzyme immunoassay kit (competitive ELISA), Elizen Neonatal 17OHP Screening (Zentech, Belgium), provided by the National Ministry of Health. The Evolis ELISA Microplate System (Bio-Rad) and the ChroMate Microplate Reader (Awareness Technology) were used for analysis.

Samples with results outside the cut-off values defined by the Elizen Neonatal 17OHP Screening kit were retested using the DELFIA Neonatal 17 α-OH-Progesterone kit (Revvity, Finland).

Positive cases were confirmed by quantification of 17OHP in serum using radioimmunoassay (RIA), performed before and after extraction with ethyl ether.

Clinical evaluation for diagnostic confirmation was performed by neonatologists and/or pediatric endocrinologists in each patient's province of origin.

Molecular studies:

The analysis of molecular variants of the CYP21A2 gene was performed using DBS samples collected on NS filter paper samples. Commercial extraction columns (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany) were used for DNA extraction according to the manufacturer's recommendations with some modifications. For Sanger sequencing of the entire coding region and flanking intronic regions of the CYP21A2 gene, two overlapping PCR fragments were specifically amplified (Promoter-exon 6 and exon 3-region 3´UTR). After enzymatic purification with ExoSAP-IT, the fragments were used as templates for direct sequencing using BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, California, United States) on an ABI PRISM 3130 Genetic Analyzer capillary DNA Sequencer (Applied Biosystems, California, United States) [14].

Detection of macrodeletions/macroconversions was performed using Multiplex Ligation-dependent Probe Amplification (MLPA) with the MRC Holland P050-CAH version C1 kit (Amsterdam, the Netherlands).

Statistical analysis:

Statistical analyses were performed using Medcalc Version 13.1.2.0 and InfoStat Version 2020e software. Minimum and maximum, mean, median, standard deviation (SD), and the 95th, 97.5th, and 99th percentiles were calculated for each GE group and ANOVA and Scheffé test was performed to evaluate differences between them. Sensitivity, specificity, false positive rate, and positive predictive value were determined. Comparison of proportions was performed to evaluate differences between incidences.

3. Results

The distribution of the results of the statistical parameters calculated for 17OHP values across gestational age groups are shown in Table 2. We observed that the mean 17OHP concentration for each group decreased with increasing gestational age, while the standard deviation was wider in groups with a lower gestational age. Cut-off values were selected using lower percentiles for groups with smaller sample sizes to ensure a greater safety margin.

Accordingly, the 95th percentile was used for newborns < 32 weeks of gestation; the 97.5th percentile for those of 32, 33, 34, and 35 weeks, and the 99th percentile for those of 36, 37, and >37 weeks. Applying ANOVA (F-ratio 917.199; $p < 0.001$; data not shown) and the Scheffé test for all

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pairwise comparisons, we found statistically significant differences between gestational age groups, supporting the validity of stratifying the population in this manner.

Table 2. Distribution of 17OHP results (ng/mL serum) and selection of cut-off values for each gestational age group.

Gestational age group (weeks)	Sample size	Min	Max	Mean	Median	SD	P95	P97.5	P99	Selected cut-off
<32	329	6.7	508.9	104.7	85.1	75.6	235	245	355.2	235
32	159	5.5	245	73.7	64.1	50.9	178.6	206.4	235	206.4
33	209	2.8	271.9	59.7	45.9	46.5	147.7	194.9	221.3	194.9
34	434	4.3	247.3	53	40.4	41.2	141.7	181.3	206.6	181.3
35	886	3.2	235	40.3	32.2	30.9	94.7	127.4	166.9	127.4
36	1871	0.9	195.8	31.7	27.1	21.2	70.5	85.4	117.2	117.2
37	4355	0.9	289.9	26.9	23.6	17.2	54.5	65.8	83.2	83.2
>37	44,983	0.9	240	20.7	19.1	10.9	38.9	45.3	55	55
All	53,226	0.9	508.9	23	19.9	17.2	45.8	58.5	85	

During the study period, four cases of CAH were detected and confirmed. Two premature twins (patients 1 and 2) and two full-term patients (patients 3 and 4) (Table 3). All patients had 17OHP levels in the screening sample exceeding the upper limit of the assay calibration curve. Serum 17OHP quantification of the twin patients was performed at the Endocrinology Laboratory of Garrahan Hospital, while testing for patients 3 and 4 was conducted in a private laboratory.

Table 3. CAH patients .

Patient	**1**	**2**	**3**	**4**
Sex	male	male	male	female
Genitalia	intrascrotal testis	intrascrotal testis	intrascrotal testis	atypical
Birth weight (g)	2808	2500	3005	3610
Gestational age (weeks)	35	35	39	38
17OHP NBS (ng/mL)	221.8	>235	>235	>235
17OHP serum[1] (ng/mL)	184.1	100.5	>25	n/a
17OHP extracted[2] (ng/mL)	> 106	50.9	>25	60.9
***CYP21A2* gene variants**				
Allele 1	c.518T>A p.(Ile173Asn)	c.518T>A p.(Ile173Asn)	*TNXA/TNXB*-CH1	c.1448_1449delinsC p.(Arg483Profs*58)
Allele 2	*CYP21A1P/CYP21A2*- CH1	*CYP21A1P/CYP21A2*- CH1	*CYP21A1P/CYP21A2*- CH1	*CYP21A1P/CYP21A2*- CH1

Normal values of 17OHP were established between the 2.5th and the 97.5th percentile range. 1.17OHP non extracted males: 1.8 to 17.8 ng/mL. 2. 17OHP extracted females and males: 0.12 to 3.8 ng/mL [15]. n/a: not available. *CYP21A2 RefSeq: NG_007941.3. CYP21A1P/CYP21A2-CH1: chimera with junction site in exon 3. TNXA-TNXB-CH1: chimera characterized by CYP21A2 gene deletion and a 120 bp deletion in exon and intron 35 of the TNXB gene.*

Molecular study:

The presence of pathogenic variants on both alleles was confirmed in all four cases.

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Patients 1 and 2 were compound heterozygotes for the most frequent point pathogenic variant associated with the classic SV form, p. (Ile173Asn), found in 37.3% of SV alleles in our population, and for a CYP21A1P/CYP21A2-CH1 macroconversion with a junction site in exon 3. In our cohort, 87.2% of patients with this genotype presented with the classic SV form, while 12.8% had the SW form.

Patient 3 was a compound heterozygote for a CYP21A1P/CYP21A2-CH1 macrodeletion with a junction site in exon 3 and a larger macrodeletion TNXA/TNXB-CH1 characterized by complete deletion of the CYP21A2 gene and a 120 bp deletion spanning exon and intron 35 of the TNXB gene. This genotype is associated with a contiguous gene deletion syndrome known as CAH-X. Most patients with this genotype present with the classic SW form (100% in our cohort) [16] and the hypermobility type of Ehlers-Danlos syndrome due to haploinsufficiency of the TNXB gene [17]. Early diagnosis of CAH-X is important to ensure long-term follow-up by specialists focused on preventing musculoskeletal manifestations and related complications.

Patient 4 was a compound heterozygote for the point pathogenic variant p.(Arg483Profs*58) and a CYP21A1P/CYP21A2-CH1 macroconversion with a junction site in exon 3. This genotype is associated, in most patients, with the SW clinical form.

The screening program achieved a sensitivity of 100%, specificity of 99.5%, a false positive rate of 0.49%, and a positive predictive value of 1.5%. When evaluating preterm group, less than and equal to 36 weeks separately from the group of full-term newborns, the specificity was 97.2% and 99.7%, respectively. The recall rate decreased with increasing gestational age (Table 4).

Table 4. False positives/negatives, true positives/negatives and percentage recall rate distributed according to gestational age.

Gestational age group (weeks)	Sample size	False positives	True positives	False negatives	True negatives	Recall rate %
≤32	488	24	0	0	464	4.9
33	209	9	0	0	200	4.3
34	434	14	0	0	420	3.2
35	888	29	2	0	857	3.5
36	1871	35	0	0	1836	1.9
37	4355	53	0	0	4302	1.2
>37	44,985	92	2	0	44,891	0.2
Total	53,230	256	4	0	52,970	0.5

The resulting incidence of CAH was 1:13,308, with no significant difference from the national incidence 1:15,260 (p=0.9276).

4. Discussion

NS cut-off values are calculated to ensure the detection of all true positive cases, minimizing false negatives as much as possible. However, this approach results in a high rate of false positives, leading to a high rate of recalls to confirm or rule out the initial findings. It is well known that recalls can cause considerable uncertainty and distress in families, in addition to the costs associated with repeat sampling and confirmatory testing [18]. NS laboratories continuously work to reduce the recall rate without compromising the sensitivity of the screening method.

Gestational age–stratified cut-off points are an important tool in improving the performance of CAH screening. Based on our results, we were able to establish cut-off values for gestational ages from 32 to 37 weeks. Combination of the different percentiles increased the specificity [19]. This

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approach significantly reduced the recall rate to confirm elevated 17OHP levels, while ensuring the detection of all true positive cases.

In our experience, the use of these cut-off values is applicable to second samples, calculating the corrected gestational age as the sum of the GE and the age at the time of sampling [20].

When compared with the preliminary cut-off values, no significant differences were observed, suggesting that the percentiles established in the initial population were appropriate for implementation.

Four cases of CAH were detected in 53,230 newborns, resulting in an incidence of 1:13,308, with no significant difference from 1:15,260 reported by the National Program for the Strengthening of the Early Detection of Congenital Diseases. Possibly due to the inclusion of twin siblings. Although we cannot rule out the possibility of false negatives in the study period, no cases of undiagnosed CAH were reported in the Patagonian provinces served by our laboratory.

A specificity greater than 98% is considered adequate for the methodology used. However, when analyzed separately in the preterm and term groups, the proportion of false positive cases remains higher in premature newborns, even when cut-off values adjusted for gestational age are used. Although the positive predictive value obtained was low, it is comparable to values reported in other screening programs. The implementation of a second sample collection can improve the positive predictive value, but this approach increases costs and may be logistically challenging in some regions [21,22]. According to the Endocrine Society Clinical Practice Guideline published in 2018, to improve the positive predictive value of screening, the use of a second-tier test in neonatal screening laboratories is recommended, preferably using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [7] . This technique enables measurement of an extended steroid profile and evaluate its ratios [23–25]. However, access to this methodology remains limited in NS programs in Latin American countries.

On the other hand, the guidelines also propose genotyping as an alternative strategy to increase specificity. Several studies have suggested that molecular analysis performed directly on DBS samples may be a useful adjunct to hormonal measurements [14,26–28]. However, no large-scale studies have evaluated its efficacy as a second-tier test. When comparing the two methodologies, genotyping is more expensive and requires more processing time per sample. Nevertheless, it offers the additional benefit of providing valuable information for genetic counseling.

Finally, given the conditions of NS for CAH in our setting, we emphasize the importance of establishing adequate cut-off limits for the population studied. Each laboratory is responsible for the definition and continuous evaluation of these values. However, the standardization of the diagnostic algorithm and the harmonization of the cut-off limits across laboratories will enable comparability of results among different regional screening programs.

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Abbreviations

The following abbreviations are used in this manuscript:

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CAH	Congenital Adrenal Hyperplasia
17OHP	17-hydroxyprogesterone
NS	Newborn screening
DBS	Dry blood spot
SW	Salt-wasting
SV	Simple-virilizing
GE	gestational age
MPLA	Multiplex Ligation-dependent Probe Amplification
SD	Standard deviation

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