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TRIAGEM NEONATAL PÚBLICA PARA HIPERPLASIA ADRENAL CONGÊNITA NO RIO GRANDE DO SUL: DA IMPLANTAÇÃO À CARACTERIZAÇÃO CLÍNICO-LABORATORIAL

Porto Alegre

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LISTA DE ABREVIATURAS

17-OHP	17 hydroxyprogesterone
21-OH	21 Hydroxilase
ABS	Antley-Bixler Syndrome (ABS)
AR	Argentina
BR	Brazil
C-CAH	Classical congenital adrenal hyperplasia
CAH	Congenital adrenal hyperplasia
CT	Computed tomography
EFBN1	Ephrin-B1
FGFR	Fibroblast Growth Factor Receptor
g	Grams
HAC	Hiperplasia adrenal congênita
HAC-PS	Hiperplasia adrenal congênita perdedora de sal
HAC-VS	Hiperplasia adrenal congênita virilizante simples
HC	Hydrocortisone
MF	Malformation
MLPA	Multiplex ligation-probe Amplification
MRI	Magnetic resonance
NC-CAH	Non-classic congenital adrenal hyperplasia
ng/mL	Nanogram/mililiter
PCR	Polymerase chain reaction
pg/mL	Picogram/mililiter
POR	P450 oxidoreductase
PORD	*POR* deficiency
PPV	Positive predictive value
RS	Rio Grande do Sul
SD	Standard deviation
SEM	Standard error of the mean

SNaPshot	Single Nucleotide Primer Extension
SPSS	Statistical Package for the Social Sciences
SUS	Sistema Único de Saúde
SV-CAH	Simple virilizing congenital adrenal hyperplasia
SW-CAH	Salt-wasting congenital adrenal hyperplasia
TWIST1	Twist-related protein 1
WT	Wild type

RESUMO

A hiperplasia adrenal congênita (HAC) é um grupo de doenças hereditárias causadas por uma deficiência em uma das enzimas necessárias para a síntese de cortisol no cortex adrenal. Mais de 95% de todos os casos de HAC são devidos a 21-Hidroxilase (21-OHD). Existem 3 formas principais, duas com manifestações clínicas no período neonatal, a forma mais grave perdedora de sal (HAC-PS) e a forma virilizante simples (HAC-VS). Além da perda salina, o excesso de andrógenos leva à virilização de recém nascidas femininas. As formas neonatais são chamadas de clássicas, atividade enzimática da 21-OH bastante reduzida, de < 2% na HAC-PS e de 2-10% na HAC-VS. A forma parcial de início tardio é chamada de HAC não clássica (HAC-NC) e a principal manifestação na infância é a adrenarca precoce. Nesta forma a atividade da 21-OH é de 20-60%. Os programas de triagem para HAC visam, principalmente, ao diagnóstico precoce da forma clássica perdedora de sal, mais grave e potencialmente letal.

No Brasil, a triagem pública é realizada no Estado de Goiás desde 1997 e em Santa Catarina desde 2001. No Rio Grande do Sul (RS) foi implantada em maio de 2014 na fase IV do Programa Nacional de Triagem Neonatal. A inclusão da HAC trouxe consigo muitos desafios e a exigência de um fluxo de triagem e diagnóstico bem estruturados. O diagnóstico precoce é crucial para prevenir o óbito de lactentes por insuficiência adrenal. Dosa-se, em papel-filtro, a 17OH progesterona (17-OHP). Elevações podem ocorrer em recém-nascidos sem HAC (falso-positivos), devido a situações de estresse perinatal e por prematuridade. Após avaliação dos dados do primeiro ano de triagem para HAC neste estudo, a mediana da idade da coleta nos casos diagnosticados foi de 8 dias (4.25-15.75). Dos 8 casos diagnosticados de maio de 2014 a abril de 2015, 6 casos com forma perdedora de sal (incluindo 1 caso de óbito

por coleta tardia do TP aos 38 dias de vida). A incidência encontrada em nosso meio no primeiro ano foi de 1:13.551 casos. Com a estratégia do uso de pontos de corte estratificados pelo peso de nascimento[18], o índice total de resultados positivos em nosso meio foi de 0,5% da amostra avaliada ("n" total de 514 bebês), sendo mais frequente em recém nascidos com menos de 2000g de peso ao nascer. Além da confirmação clínica e laboratorial, o genótipo é importante, além de confirmar, para estabelecer gravidade da doença e também para ratificar o diagnostico dos falsos positivos na ausência de uma mutação do gene *CYP21A2*. Um dos casos confirmados de HAC-PS foi associado a múltiplas malformações e craniossinostose severa, suscitando a hipótese de um a associação com defeito de *FGFR2*. A correlação genótipo-fenótipo na avaliação dos casos em dois anos da triagem alcançou um alto nível de concordância de 87%. Diagnosticada, portanto, de forma assertiva a HAC forma clássica, institui-se a terapia glicocorticóide para as formas virilizante simples e acrescenta-se mineralocorticóide para as formas perdedoras de sal.

A triagem neonatal é um importante programa de saúde populacional e visa ao diagnóstico precoce de uma patologia com potencial risco à vida pela perda de sal, além de permitir adequada atribuição de sexo nas meninas com virilização genital e à saúde da criança. Estabelecer os fluxos adequados de triagem e manejo, além de ampliar o conhecimento sobre a HAC, com o reconhecimento dos desfechos e tratamentos adequados é essencial para minimizar as possíveis complicações nesta população de maior vulnerabilidade.

Palavras-Chave: Triagem Neonatal - Hiperplasia Adrenal Congênita - 17 OH progesterona - Genótipo-Fenótipo

ABSTRACT

Congenital adrenal hyperplasia (CAH) is a group of inherited diseases caused by a deficiency in one of the enzymes required for the cortisol synthesis by the adrenal cortex. More than 95% of all CAH cases are due to 21-hydroxylase (21-OHD). There are 3 forms, two with neonatal clinical manifestation: salt-wasting CAH (SW-CAH\) and the simple virilizing form (HAC-VS). In addition to salt loss, androgens excess lead to the virilization of female newborn. Neonatal forms are defined as classical CAH. The 21-OHD enzymatic activity in SW-CAH is less than <2% and in the SV-CAH 2-10%. A late-onset form, with partial enzymatic defect (20-60%) is called non-classical HAC (NC-CAH) and the main manifestation in childhood is early adrenarche.

In Brazil, public health screening has been conducted in the State of Goiás since 1997 and in te Sate of Santa Catarina since 2001. In Rio Grande do Sul (RS) it was implemented in May 2014, in phase IV of the National Neonatal Screening Program. The inclusion of CAH in the local screening program brought many challenges and the need of a well structured screening and diagnosis flowchart. Early diagnosis is crucial to prevent infant death due to adrenal insufficiency. Around de world, the screening programs for CAH main purpose is the early diagnosis of the more severe classical forms, especially SW-CAH. The cortisol precursor 17OH progesterone (17-OHP) is the main disease marker and is measured on filter paper. Elevations may occur in infants without CAH (false positive) due to perinatal stress and prematurity. Of newborns screened in the first year, median age of collection in diagnosed cases was 8 days (4.25-15.75) and 8 patients were diagnosed with CAH (four males, four females). The incidence of CAH in the state was 1:13,551. Six cases were identified as classic salt-wasting CAH and two were cases of virilizing CAH. The overall rate of positive results was 0.5%

(n = 514 infants). The number of false positive results was higher among newborns with birth weight < 2,000 g.

In addition to clinical and laboratory confirmation, the genotype is important to confirme 21-OH deficiency, to establish disease severity and also in the absence of a mutation of the *CYP21A2* gene to more precise exclude the diagnosis of suspected false positives. One of the confirmed cases of SW-CAH was associated with multiple malformations and severe craniosynostosis, raising the hypothesis of an association with *FGFR2* mutation. A high genotype-phenotype correlation of 87% was found in the cases after two years of screening. Once the classic CAH is diagnosed, glucocorticoid therapy is instituted and mineralocorticoid is added for SW-CAH.

CAH neonatal screening is an important population health program and aims to the early diagnosis of a pathology with a potential risk due to salt loss crisis. The early detection of cases also allows to adequate sex assignment in girls with genital virilization. Establishing adequate screening flows, proper diagnosis and management, in addition to increase knowledge about the disease, with the appropriate recognition of outcomes and treatments is essential to minimize complications in this population of greater vulnerability.

Keywords: Neonatal Screening - Congenital Adrenal Hyperplasia - 17 OH progesterone - Genotype-Phenotype

CAPÍTULO 1

Triagem Neonatal da Hiperplasia Adrenal Congênita

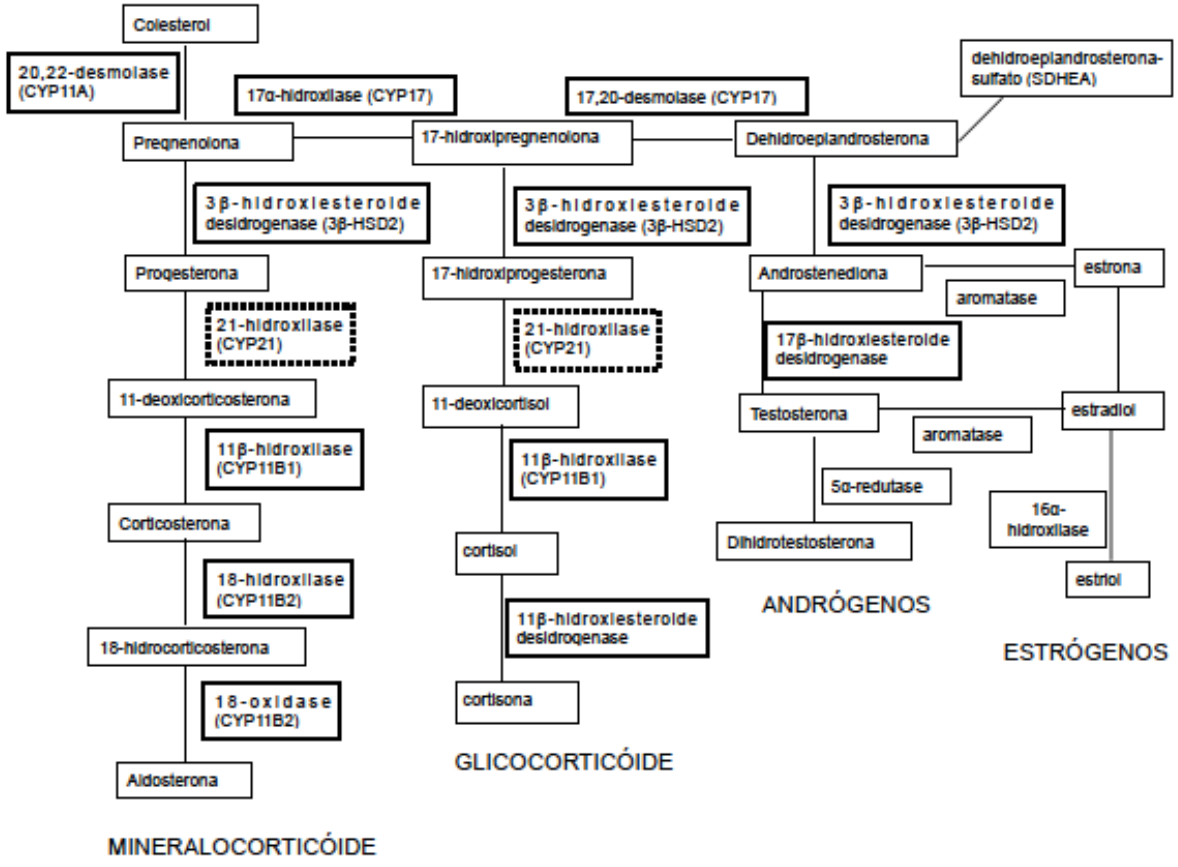
1. Hiperplasia adrenal congênita

A hiperplasia adrenal congênita (HAC) é patologia decorrente de defeitos da via enzmática de síntese de cortisol, aldosterona e hormônios androgênicos a partir do colesterol (figura 1)[1]. Está associada a uma significante morbidade e mortalidade em crianças e adultos afetados[2]. Tem herança autossômica recessiva e o defeito é decorrente, em cerca de 90%, de mutações no gene *CYP21A2*[3]. As mutações nesse gene podem levar à deficiência na produção da enzima 21-hidroxilase (21-OH). Esse defeito provoca um bloqueio em uma rota metabólica envolvida na síntese do cortisol (glicocorticóide) e aldosterona (mineralocorticóide), levando ao acúmulo de metabólitos precursores, dentre os quais está a 17-hidroxiprogesterona (17-OHP), principal marcador da doença. Em conseqüência, a hipófise passa a produzir grandes quantidades de ACTH, estimulando exageradamente a glândula adrenal a produzir precursores esteróides com ação virilizante[4]. Em 2/3 dos casos, ocorre a forma clássica da doença, com desequilíbrio hidro-eletrolítico (perda de sal) que acomete, tanto meninos, quanto meninas, e cujos sintomas iniciam nas duas primeiras semanas de vida, levando a graves conseqüências clínicas e ao óbito se não tratada a tempo, justificando, portanto, o diagnóstico precoce[5]. Em meninas, a exposição intra-útero de excesso de andrógeno pode determinar virilização em graus variados e ambiguidade genital, classificados segundo a escala de Prader[6] de 1-5, sendo 1 o grau mais leve com aumento isolado do clitóris e 5 o grau mais severo, com fusão labio-escrotal completa e uretra peniana (figura 2)[6] .

2. Epidemiologia

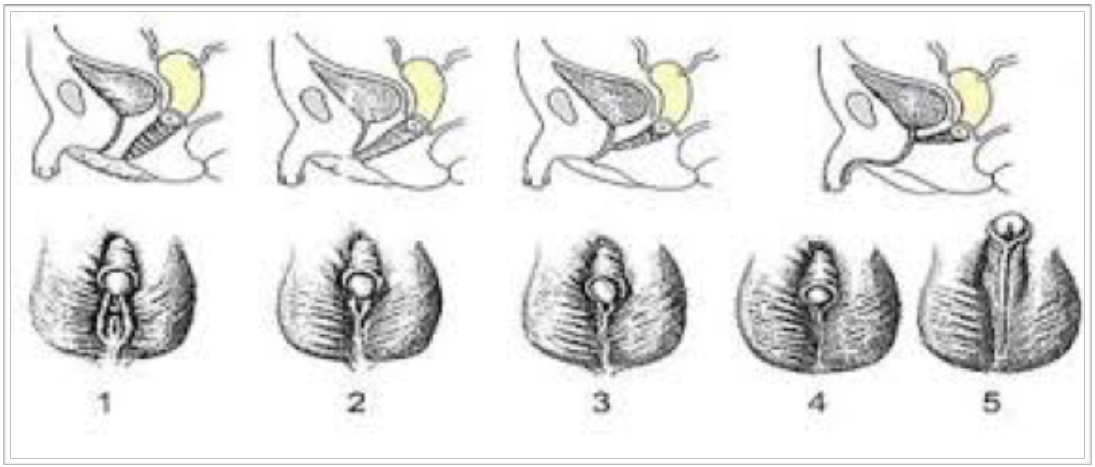
A incidência geral para HAC descrita na literatura é de 1/15-16.000 nascidos vivos[5]. No Brasil, a incidência encontrada em Minas Gerais foi de 1/20.000 nascidos vivos no período de 2007 a 2008[7]. Em Goiás, a incidência foi de 1/10.000[8]. A prevalência da forma não clássica é estimada em 1/1000 indivíduos[9].

Figura 1. Esteroidogênese da glândula adrenal[1]



Adaptado da referência 1

Figura 2. Escala de Virilização de Prader[6]



Prader 1: aumento isolado do clitóris

Prader 2: aumento do clitóris e intróito vaginal em forma de funil (abertura uretral e vaginal distintas)

Prader 3: aumento do clitóris e intróito vaginal profundo, pseudo seio-urogenital e fusão labioescrotal parcial

Prader 4: clitóris fálico com abertura urogenital em forma de fenda na base do falo

Prader 5: fusão labioescrotal completa e uretra peniana

Adaptado da referência 6

3. Formas Clínicas da Hiperplasia adrenal congênita

Existem quatro formas clínicas reconhecidas da HAC, as clássicas perdedora de sal (HAC-PS) e virilizante simples (HAC-VS) e forma não-clássica (HAC-NC) de aparecimento tardio. O principal objetivo da triagem da HAC é identificar o recém-nascido com risco de ser afetado pelas formas clássicas graves HAC-PS e HAC-VS[3,5,10,11]. Contudo, casos com níveis de 17OHP neonatal persistentemente elevados e sem clínica franca são suspeitos de HAC-NC e podem ser diagnosticados no período neonatal e em lactentes, identificados nos estudos de biologia molecular. As características clínicas das formas clássica e não clássica estão descritas na tabela 1[11].

Tabela 1. Características clínicas das formas clássica e não clássica de Hiperplasia Adrenal Congênita[11]

Característica	***HAC Clássica***	***HAC Não Clássica***
Virilização Pré-Natal	Meninas	Ausente
Virilização Pós-Natal	Meninas e Meninos	Variável
Perda de Sal	~75% dos afetados	Ausente
Deficiência de Cortisol	~100% dos afetados	Rara

Adaptado da referência 11

3. 1. Hiperplasia adrenal congênita Perdedora de Sal

A forma PS representa 75% dos casos clássicos, sendo a forma mais grave de HAC[3]. Nesse grupo, a 21-OH pode estar totalmente inativa ou com menos de 2% de sua atividade catalítica[3,11]. Devido à deficiência na síntese de cortisol não ocorre o *feedback* negativo e há um hiperestímulo do ACTH, com aumento da síntese de precursores e desvio para a rota de produção de andrógenos. As consequências clínicas relacionadas à deficiência de cortisol e de aldosterona são a hiponatremia e hipercalemia, desidratação, acidres metabólica e choque hipovolêmico[3]. As consequências clínicas do aumento dos andrógenos é a virilização em ambos os sexos, com ambiguidade genital nas meninas (figura 2 e tabela 1)[3,6,11]. O tratamento em ambos os sexos é realizado com glicocorticóide, que suprime CRH e ACTH e mineralocorticóide para repor o déficit de aldosterona e evitar a perda de sal[3,11].

3. 2. Hiperplasia adrenal congênita Virilizante Simples

A forma VS representa em torno de 25% dos casos clássicos de HAC. Nesse grupo, a atividade da 21OH é de 2 a 10%, permitindo apenas uma baixa produção de cortisol, que também não é suficiente para o *feedback* negativo no hipotálamo e hipófise. Da mesma forma que ocorre na HAC-PS, há um aumento da biossíntese de esteroides adrenais e a produção de andrógenos fica elevada. A quantidade sintetizada desses andrógenos é variável entre os indivíduos, com diferentes graus de virilização da genitália externa (figura 2)[1,3,11]. Em meninas, a virilização é mais facilmente identificada, pois leva a quadros de ambiguidade genital[3]. No entanto, em meninos esse diagnóstico clínico do aumento peniano e da hiperpigmentação genital nem sempre é identificado. Muitos estudos têm relatado uma menor proporção de meninos afetados por HAC que de meninas. O principal motivo para essa disparidade entre os gêneros é o subdiagnóstico de meninos com HAC-VS[12]. O tratamento, com glicocorticóide reduz os níveis de esteroides sexuais e seus danos são minimizados. Quando a virilização em meninas é muito grave, é necessária também uma intervenção cirúrgica para o restabelecimento da genitália normal[3,10].

3. 3. Hiperplasia adrenal congênita Não Clássica

A atividade da 21-OH na forma NC é em torno de 20 a 60%, o que permite uma taxa de biossíntese de cortisol suficiente para não acometer os graves danos descritos nas formas clássicas HAC-PS e HAC-VS[2,3,11]. No entanto, a quantidade sintetizada de cortisol também não é satisfatória para o correto controle do *feedback* negativo e leva ao hiperandrogenismo[1]. Esse excesso de andrógenos provoca manifestações clínicas tardias. Ao longo da infância as manifestações são a pubarca precoce e crescimento acelerado a ponto de poder comprometer a altura final normal do indivíduo devido a fusão epifisária precoce e na adolescência quadros graves de acne e infertilidade. Em mulheres pode acarretar também hirsutismo e

irregularidade menstrual[3,11]. O tratamento dos pacientes com HAC-NC é realizado apenas quando sintomáticos através da reposição de glicocorticóides[3,10].

5. **Triagem para Hiperplasia Adrenal Congênita**

O diagnóstico precoce é crucial para prevenir o óbito de lactentes por insuficiência adrenal. Os programas de triagem para HAC visam, principalmente, ao diagnóstico precoce da forma perdedora de sal, mais grave e potencialmente letal[5].

A triagem neonatal para HAC foi inicialmente realizada no Alaska no final da década de 70, com uma alta frequência de casos, 1/490 nascidos vivos[13]. Desde então, outros países passaram a implantar a triagem para HAC[14]. No Brasil, estados como Goiás e Santa Catarina acumulam experiências de mais de 10 anos na triagem neonatal da doença[15,16]. Dados disponíveis da incidência no Brasil variam de aproximadamente 1:10.000 a próximo de 1:20.000 [15-17].

Dosa-se, em papel-filtro, o metabólito 17-OHP por imunofluorescência. O que se observa, contudo, é que elevações desse metabólito podem ocorrer em recém-nascidos sem HAC (falso-positivos), devido a situações de estresse que acometem as mães e os recém-nascidos no período perinatal[18]. Os bebês prematuros sem HAC também podem apresentar elevados níveis de 17OHP decorrentes de maiores concentrações de esteróides conjugados e relativa imaturidade renal com função excretora insuficiente. Um teste negativo, por sua vez, não exclui a possibilidade do bebê ter a deficiência da enzima 21-hidroxilase e o diagnóstico molecular pode ser útil[8]. Falsos negativos também podem ocorrer pelo uso materno de corticóide no final da gestação[19]. O Ministério da Saúde sugere, através de seu grupo de assessoramento técnico, que uma das estratégias para minimizar o número de falsos-positivos seja uso de pontos de corte extratificados pelo peso ao nascer (tabela 2 e fluxograma 1)[18,20]. Uma vez diag-

nosticada a HAC forma clássica pela triagem neonatal, institui-se a terapia glicocorticóide para as formas virilizante simples e acrescenta-se mineralocorticóide para as formas perdedoras de sal[3,9,10].

Tabela 2. Níveis de 17 OHP (ng/mL) ajustados para peso de nascimento[18]

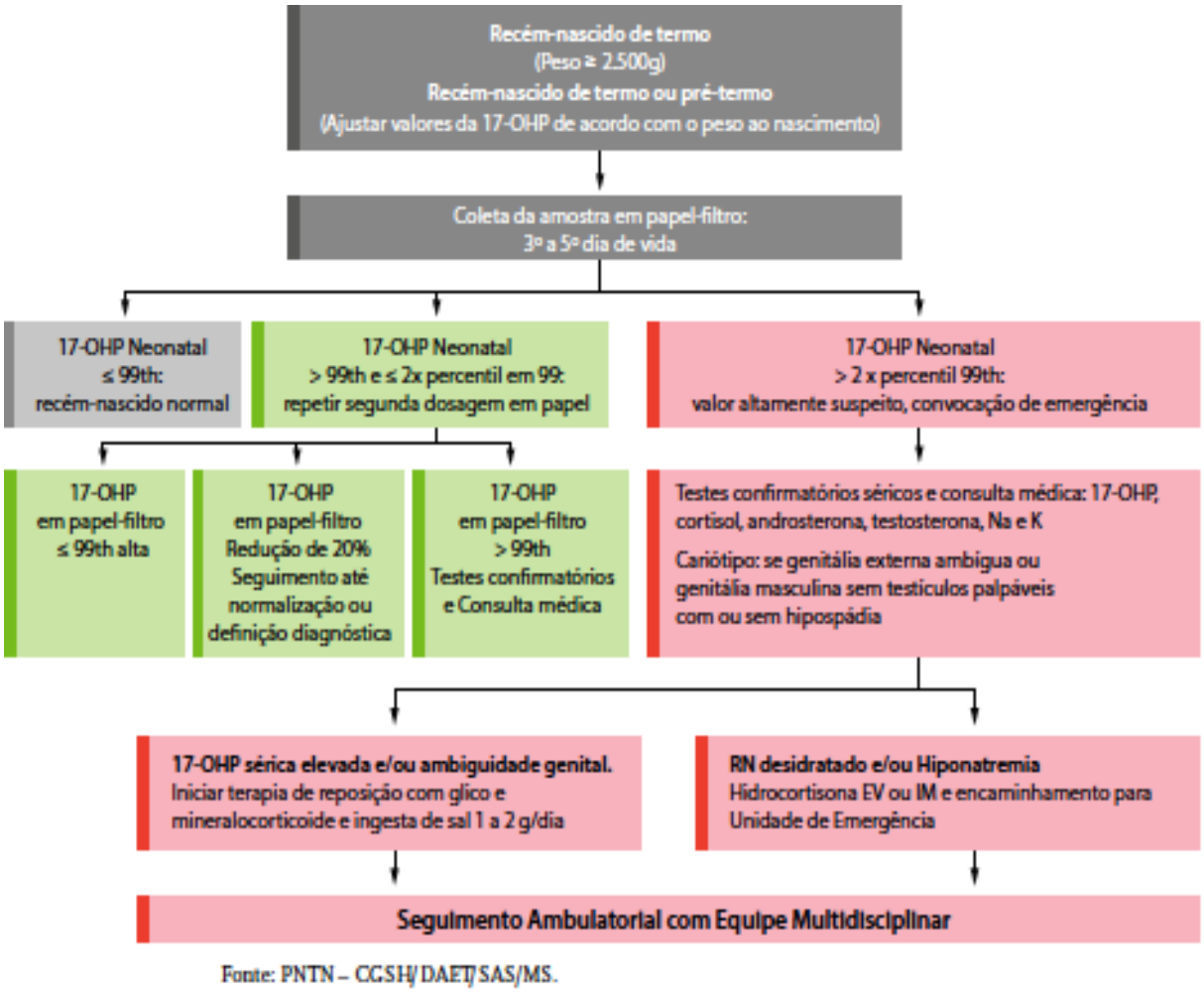
Faixa de peso ao nascer	>P99 corte para 2ª amostra	2X >P99 corte para consulta
< 1,500 g	110	220
1,501 – 2,000 g	43	86
2,001 – 2,500 g	28	56
> 2,501 g	15	30

Adaptado da referência 18

Em linhas gerais, para estimar a gravidade e prioridade de atendimento de pacientes suspeitos de HAC pela triagem neonatal, propõe-se o fluxo de encaminhamento prioritário de HAC, conforme descrito no fluxograma 2[21].

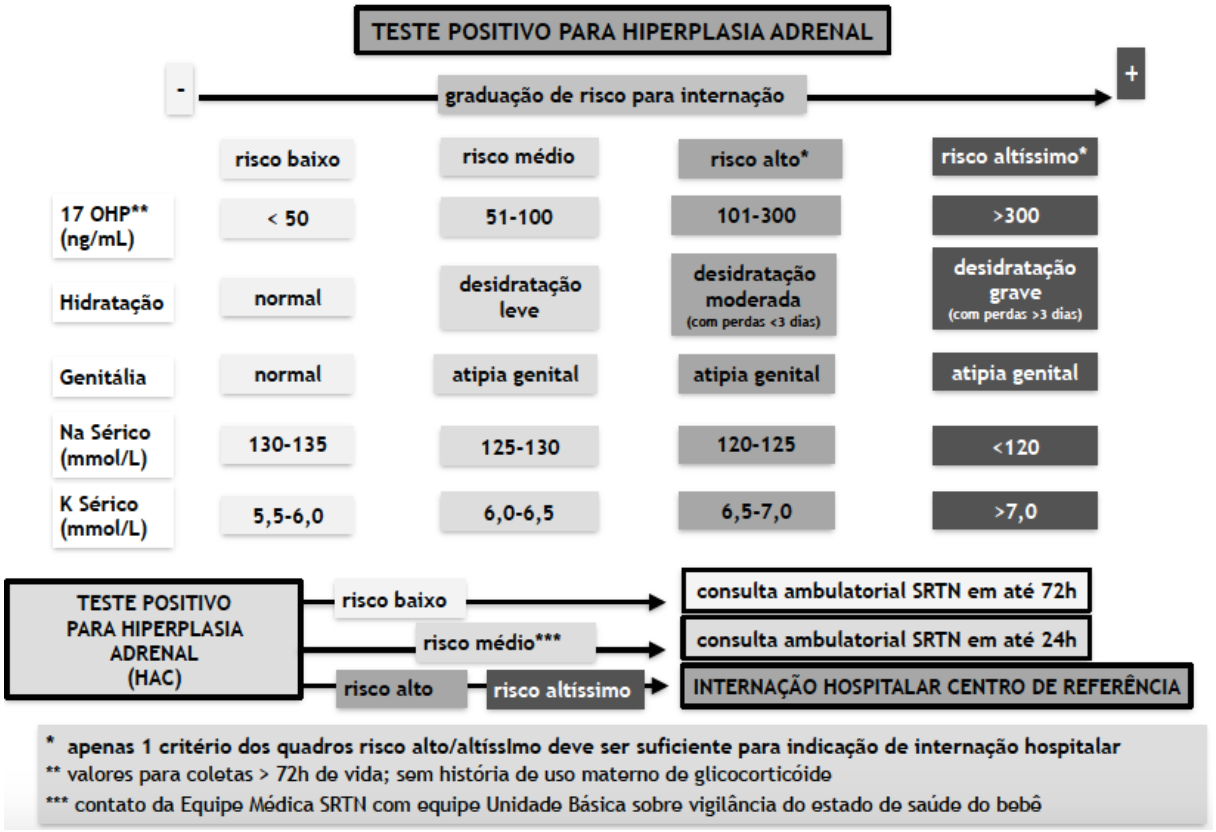
Tem sido sugerido que análises moleculares do gene *CYP21A2* podem melhorar a especificidade da triagem neonatal, ajudando também no manejo clínico da doença[8].

Fluxograma 1. Fluxo de Triagem Neonatal para Hiperplasia Adrenal Congênita por Deficiência de 21 Hidroxilase



Fonte: Araújo JPB & Goldbeck AS. Triagem neonatal: hiperplasia adrenal congênita. Ministério da Saúde, 2015[20].

Fluxograma 2. Fluxo de encaminhamento prioritário de atendimento a pacientes com Hiperplasia Adrenal Congênita



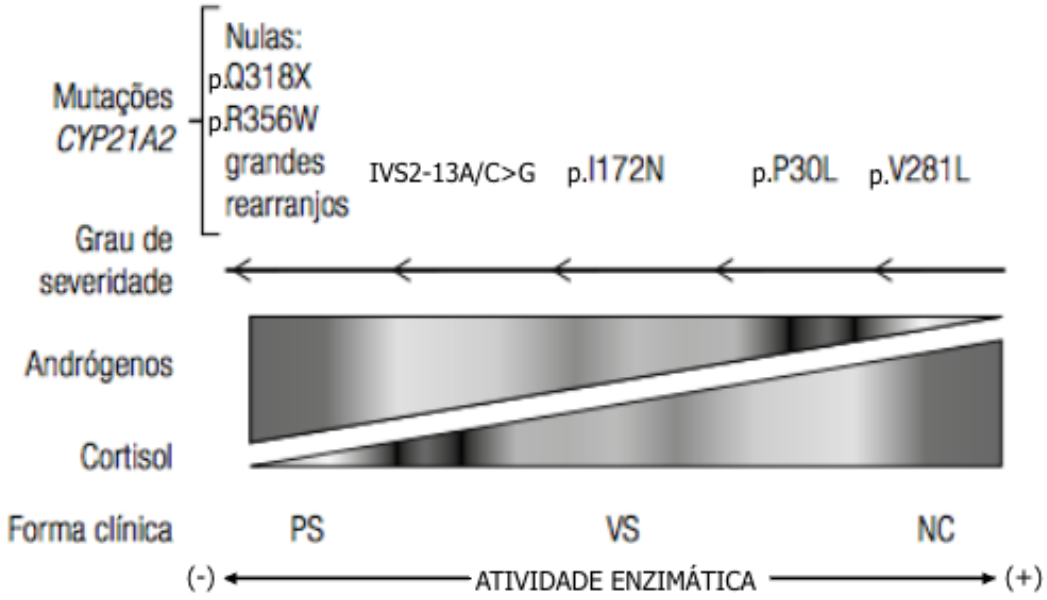
Fonte: Kopacek C. A Triagem neonatal da hiperplasia adrenal congênita no SUS, Bol Cient Ped, 2015[21]

Estudos moleculares do gene CYP21A2

A mutações do gene *CYP21A2* podem ser categorizadas em quatro grupos de acordo com as atividade enzimática residual da 21OH e está associada aos três fenótipos clínicos[2,4,11,22]. No grupo "Nulo" não existe atividade enzimática. O grupo "A" tem uma actividade residual mínima da 21OH (< 2%). Em ambos os grupos (Nulo e A), em homozigose ou heterozigose composta, o fenótipo correspondente é a HAC clássica perdedora de sal. O grupo "B" é com-

posto por mutações que permitem 2-10% de atividade da enzima e a biossíntese de aldosterona está preservada. O fenótipo é comumente associado à forma de HAC-VS. No grupo "C" atividade enzimática é de 20-60% e o fenótipo está associado com a forma NC. A figura 3 demonstra a atividade da enzima da 21OH, o espectro clínico da doença e as principais mutações (genótipo) associadas a cada fenótipo[23].

Figura 3. Correlação genótipo e fenótipo na deficiência da 21 Hidroxilase



Adaptado da referência 24

Histórico da Implantação da TN para HAC no RS

O início da TN no Sistema Único de Saúde (SUS) para HAC em nosso Estado do RS ocorreu em maio de 2014, seguindo as recomendações e o fluxo do Programa Nacional de Triagem Neonatal (PNTN) e do Ministério da Saúde[21]. A cobertura dos exames no SUS em nosso meio é cerca de 75%. A fase IV do PNTN trouxe um conceito novo, o da urgência no

diagnóstico dos casos confirmados. Após avaliação dos dados do primeiro ano de triagem para HAC, a mediana da idade da coleta nos casos diagnosticados foi de 8 dias (4.25-15.75). Dos 8 casos diagnosticados de maio de 2014 a abril de 2015, 6 casos com forma perdedora de sal (incluindo 1 caso de óbito por coleta tardia do TP aos 38 dias de vida). A incidência encontrada em nosso meio no primeiro ano foi de 1:13.551 casos. Com a estratégia do uso de pontos de corte estratificados pelo peso de nascimento[18], o índice total de resultados positivos em nosso meio foi de 0,5% da amostra avaliada ("n" total de 514 bebês), sendo mais frequente em recém nascidos com menos de 2000g de peso ao nascer, conforme descrito no capítulo 2 (Kopacek C, Castro SM, Prado MJ, Silva, CMD, Beltrão LA, Spritzer PM. ***Neonatal screening for congenital adrenal hyperplasia in Southern Brazil: a population-based study with 108,409 infants;*** *submitted BMC Pediatrics, 2016).* Segundo o Manual de Triagem para Hiperplasia Adrenal Congênita do Ministério da Saúde[20], usando-se o percentil 99 como ponto de corte para a 17-OHP, níveis ideais de reconvocação devem ser de 0,2%, mas aceitável até 1%. Dados referentes aos primeiros dois anos da triagem neonatal da HAC no RS estão compilados no capítulo 3 (Kopacek, C; Prado, MJ; da Silva, CMD; Castro, SM; Beltrão, LA; Vargas PR; Grandi, T; Rossetti, MLR; Spritzer, PM. ***Clinical and molecular profile of 132 newborns with confirmed or suspicious Congenital Adrenal Hyperplasia in the first two years of a public screening program implementation;*** formato de submissão para o Jornal de Pediatria*, 2016*). Dentre os desafios trazidos ela triagem da HAC, um caso em especial, associado a múltiplas malformações teve destaque, pela complexidade e gravidade dos achados clínicos. O estudo e relato deste caso foi o tema do capítulo 4 (Cristiane Kopacek, Liana Capelo Costa, Mayara Jorgens Prado, Rafael Fabiano Machado Rosa, Luciana Amorim Beltrão, Claudia Dornelles, Simone Martins de Castro, Poli Mara Spritzer, Gil Guerra Junior, Maricilda Palan-

di de Mello. ***Severe craniosynostosis syndrome associated to salt wasting congenital adrenal hyperplasia;*** *submitted Hormone Research in Pediatrics/Novel Insights from clinical experience, 2016*).

Relevância

A triagem neonatal é um importante programa de saúde populacional e visa ao diagnóstico precoce de patologias com potencial risco à vida e à saúde da criança. É importante, neste contexto, ampliar o conhecimento sobre as estratégias de triagem da HAC, diagnostico e seguimento de uma patologia que envolve alta morbimortalidade, mas também está associada a um alto índice de resultados falsos positivos e geram às famílias e profissionais de saúde dúvidas e anseios sobre a saúde dos recém-nascidos. É fundamental o conhecimento e o uso dos pontos de corte estratificados por peso de nascimento e a avaliação clínica criteriosa daqueles nascidos com história de prematuridade e/ou internação em UTIs neonatais. Ainda, o reconhecimento dos casos triados como positivos para HAC, a diferenciação entre falsos-positivos e casos verdadeiros, com auxílio de técnicas moleculares, a predição de seus desfechos e tratamentos adequados são essenciais para minimizar as possíveis complicações nesta população de maior vulnerabilidade.

Referências bibliográficas

1. Turcu AF & Auchus RJ. Adrenal steroidogenesis and congenital adrenal hyperplasia. Endocrinol Metab Clin North Am 2015;44:275-296.
2. Krone N & Arlt W. Genetics of congenital adrenal hyperplasia. Best Pract Res Clin Endocrinol Metab 2009; 23:181-192.
3. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Endocr Rev. 2000;21(3):245-91.
4. Miller WL & Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocr Rev 2011;32(1):81-151. Erratum in: Endocr Rev 2011;32(4):579.
5. Pang S & Clark A. Congenital adrenal hyperplasia due to 21-hydroxilase deficiency: newborn screening and its relationship to the diagnosis and treatment of disorder. Screening 1993; 2:105-139.
6. Prader A. Der Genitalbefund beim Pseudohermaphroditismus Femininus des kongenitalen Adrenogenitalen Syndromes. Helv Paediatr Acta 1954; 3: 231-248.
7. Barra CB, Silva IN, Pezzuti IL, Januário JN. Triagem neonatal para hiperplasia adrenal congênita. Rev Assoc Med Bras 2012; 58(4):459-464.
8. Silveira EL, Elnecave RH, dos Santos EP, Moura V, Pinto EM, van der Linden Nader I, Mendonca BB, Bachega TA. Molecular analysis of CYP21A2 can optimize the follow-up of positive results in newborn screening for congenital adrenal hyperplasia. Clin Genet 2009; 76:503-510.
9. Trakakis E, Basios G, Trompoukis P, Labos G, Grammatikakis I, Kassanos D. An update to 21-hydroxylase deficient congenital adrenal hyperplasia. Gynecol Endocrinol 2009;3:1– 9.

10. Speiser PW, Azziz R, Baskin LS, Ghizzoni L, Hensle TW, Merke DP, et al. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2010;95(9):4133-60.

11. Nimkarn S, Gangishetti PK, Yau M, New MI. 21-Hydroxylase-Deficient Congenital Adrenal Hyperplasia.GeneReviews® [Internet] https://www.ncbi.nlm.nih.gov/books/NBK1171/ Initial Posting: February 26, 2002; Last Update: February 4, 2016.

12. Therrell BJ, Berenbaum S, Manter-Kapanke V, Simmank, J, Korman, K, Prentice, L, Gonzalez, J, Gunn, S. Results of screening 1.9 million Texas newborns for 21-hydroxylase-deficient congenital adrenal hyperplasia. *Pediatrics*. 1998;101(4):583-590.

13. Pang S, Hotchkiss J, Drash AL, Levine LS, New MI. Microfilter paper method for 17 alpha-hydroxyprogesterone radioimmunoassay: its application for rapid screening for congenital adrenal hyperplasia. J Clin Endocrinol Metab. 1977;45:1003-100.

14. Pang S, Murphey W, Levine LS. Spence DA, Leon A, LaFranchi S, Surve AS & New MI A pilot newborn screening for congenital adrenal hyperplasia in Alaska. J Clin Endocr Metab 1982; 55: 413-419.

15. Silveira EL, dos Santos EP, Bachega TA, van der Linden Nader I, Gross JL, Elnecave RH. The actual incidence of congenital adrenal hyperplasia in Brazil may not be as high as inferred: an estimate based on a public neonatal screening program in the state of Goiás. J Pediatr Endocrinol Metab 2008;21(5):455-60.

16. Nascimento ML, Cristiano AN, Campos Td, Ohira M, Cechinel E, Simoni G, Lee Jv, Linhares RM, Silva PC. Ten-year evaluation of a Neonatal Screening Program for congenital adrenal hyperplasia. Arq Bras Endocrinol Metabol. 2014;58(7):765-71.

17. Pezzuti IL, Barra CB, Mantovani RM, Januário JN, Silva IN. A three-year follow-up of congenital adrenal hyperplasia newborn screening. J Pediatr (Rio J) 2014;90(3) 400-407.

18. Hayashi G, Faure C , Brondi MF, Vallejos C, Soares D, Oliveira E, Brito VN, Mendonça BB, Bachega TASS. Weight-adjusted neonatal 17OH-progesterone cutoff levels improve the efficiency of newborn screening for congenital adrenal hyperplasia. *Arq Bras Endocrinol Metab* 2011; 55(8): 632-637.

19. Gatelais F, Berthelot J, Beringue F, Descamps P, Bonneau D, Limal JM, Coutant R. Effect of single and multiple courses of prenatal corticosteroids on 17hydroxyprogesterone levels: implication for neonatal screening of congenital adrenal hyperplasia. Pediatr Res. 2004;56(5):701-5.

20. Araújo JPB & Goldbeck AS. Triagem neonatal: hiperplasia adrenal congênita. Ministério da Saúde, Secretaria de Atenção à Saúde, Departamento de Atenção Especializada e Temática. – Brasília: Ministério da Saúde, 2015. 44p. : il. Editora MS – OS 2015/0241 ISBN 978-85-334-2262-9. Printed in Brazil.

21. Kopacek C. Triagem neonatal da hiperplasia adrenal congênita no SUS. Bol Cient Pediatr. 2015;04(3):59-64

22. Bachega T, Billerbeck AE, Parente EB; Lemos-Marini S, Baptista MT, Mello MP, Guerra Jr G, Kuperman H, Setian N, Damiani D, Torres N, Castro M, Mendonça BB. Estudo multicêntrico de pacientes brasileiros com deficiência da 21-hidroxilase: correlação do genótipo com o fenótipo. Arq Bras Endocrinol Metab 2004; 48(5):697-704

23. Costa-Barbosa FA, Telles-Silveira M, Kater CE. Hiperplasia adrenal congênita em mulheres adultas: manejo de antigos e novos desafios. Arq Bras Endocrinol Metab. 2014;58(2):124-31

CAPÍTULO 2

ARTIGO 1

Neonatal screening for congenital adrenal hyperplasia in Southern Brazil: a population based study with 108,409 infants

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ABSTRACT

Background: Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder associated with inborn errors of steroid metabolism. 21-hydroxylase enzyme deficiency occurs in 90% to 95% of all cases of CAH, with accumulation of 17 hydroxyprogesterone (17- OHP). Early diagnosis of CAH based on newborn screening is possible before the development of symptoms and allows proper treatment, correct sex assignment, and reduced mortality rates. This study describes the results obtained in the first year of a public CAH screening program in the state of Rio Grande do Sul, Brazil. Methodology: We reviewed the screening database in search of babies with suspected CAH, that is, altered birth-weight adjusted 17-OHP values at screening. The following data were analyzed for this population: screening 17-OHP values, retest 17-OHP values, serum 17-OHP values for those with confirmed CAH on retest, maternal and newborn data, and family history of CAH. For the screening program, 17-OHP levels are determined on dried blood spots obtained in filter paper with GSP solid phase time-resolved immunofluorescence. Results: Of 108,409 newborns screened, 8 were diagnosed with CAH (four males, four females). The incidence of CAH in the state was 1:13,551. Six cases were identified as classic salt-wasting CAH and two were cases of virilizing CAH. The positive predictive value (PPV) of the initial screening (before diagnostic confirmation) was 1.6%. The overall rate of false positive results was 0.47%. The number of false positive results was higher among newborns with birth weight < 2,000 g. Conclusion: The present results support the need for CAH screening by the public health care system in the state, and show that the strategy adopted is adequate. PPV and false positive results were similar to those reported for other states of Brazil with similar ethnic backgrounds.

Keywords: congenital adrenal hyperplasia; incidence; neonatal screening; mass screening; 21-amino-17-hydroxyprogesterone.

BACKGROUND

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder associated with inborn errors of steroid metabolism caused by deficiency of enzymes involved in the biosynthesis of cortisol from cholesterol [1]. 21-hydroxylase deficiency occurs in 90% to 95% of all cases of CAH and is related to mutations in the CYP21A2 gene [1,2]. In the presence of 21-hydroxylase deficiency, 17 hydroxyprogesterone (17-OHP) accumulates and is diverted to androgen synthesis with virilizing effects [1,2]. Mineralocorticoid synthesis may or may not be reduced, depending on the extent to which 21-hydroxylase activity is impaired [1,3].

Three clinical forms of CAH have been recognized: two classic forms, salt-wasting CAH (SW) and simple virilizing CAH (SV), and non-classic, late onset CAH (NC). SW is the most prevalent, occurring in around 75% of newborns with a diagnosis of CAH (1). Considering that the salt loss crisis is critical and starts in the second week of life, early diagnosis of classic forms of CAH based on newborn screening is desirable even before the beginning of symptoms. This allows proper treatment, correct sex assignment, and reduced mortality rates [2,4,5]. CAH occurs in about 1 of every 10,000 to 18,000 live births in the general population, and is more common in Caucasians [1]. Incidence varies according to ethnicity and geographical region [1,6]. In addition, 17-OHP levels in neonates are affected by factors such as gestational age at birth, birth weight, and age at the time of 17-OHP testing [7-11]. Perinatal stress has been associated with high values of 17-OHP on screening [8,12], while maternal use of corticosteroids towards the end of pregnancy and early sample collection seem to reduce these values [10,13]. Reference 17-OHP values for diagnosis of CAH in full term newborns vary from 15 to 40 ng/mL among different laboratories.

Because of the many factors impacting the outcome of CAH screening, the stratification of 17-OHP values according to birth weight is recommended in order to decrease false positive results [8,10,14]. A high rate of false positive results translates into increased health care cost and distress for families [15-17].

Even though screening for CAH has been available through the public health care system for many years in some Brazilian states [10,16,18,19], only in May 2014 was it introduced in the southernmost state of Rio Grande do Sul. Therefore, the aims of the present study were to summarize the results of the first year of CAH newborn screening in this population, to determine the incidence of CAH in the state, and to estimate the rate of false positive results in the local screening program.

METHODS

Design and population

A population-based study was conducted with newborns included in the first year of a public CAH screening program in the state of Rio Grande do Sul, Brazil (May 2014 to April 2015). For the screening program, dried blood samples (heel prick test) are collected 2 to 40 days after birth. Babies with positive screening are retested. Participation is open to public and private primary care facilities, health care units, hospitals, and maternity hospitals. The study population corresponded to about three-fourths of the live newborns in the state during this period. The other 25% of newborn babies are screened in private outpatient services, and data from this population are not freely available.

In the present study, we reviewed the screening database in search of babies with suspected CAH, that is, altered 17-OHP values at screening. The following data were analyzed for this population: screening 17-OHP values, retest 17-OHP values, serum 17-OHP

values (for those with suspected CAH on screening and retest), maternal and newborn data, and family history of CAH. Figure 1 describes the screening strategy.

The study protocol was approved by the Research Ethics Committee at Hospital Materno Infantil Presidente Vargas, and meets the guidelines and norms regulating research involving human beings.

Blood collection and 17-OHP measurements

Dried blood spots were obtained using filter paper (S & S 903). 17-OHP was measured with the GSP solid phase [time-resolved] immunofluorescence assay (Neonatal 17-OHP kit - PerkinElmer, Turku, Finland). The linearity range for serum 17-OHP concentration was 0.9 ng/mL to 229 ng/mL.

The reference 17-OHP values used in the present study are those recommended by the Brazilian National CAH Screening Program [20], which were based on a pilot study with the population of the state of São Paulo [10]. Four birth weight tiers were established: tier 1, birth weight ≤ 1,500 g; tier 2, birth weight 1,501 to 2,000 g; tier 3, birth weight 2,001 to 2,500 g; and tier 4, birth weight > 2,500 g. For each tier, the 99th percentile (P99) 17-OHP cut-off levels to diagnose CAH were 110.4, 43.0, 28.2 and 15.1 ng/mL respectively. In the pilot study, newborns from mothers with informed corticosteroid use late in pregnancy were called for a second collection after 15 days of life. This record was added to the filter paper in order to minimize the risk of false negative results [13]. For the present study, early (<48h) samples collected for 17-OHP determinations were excluded. In the Rio Grande do Sul screening program, CAH screening is based on samples collected between the 2nd and 40th post-natal days. Samples from 0-1 days and/or without weight information were excluded from this

analysis, but these babies were called for immediate new collection in the valid period and with correct weight information.

Classic CAH (SW and SV) was diagnosed by increased 17-OHP on screening, confirmed by dried blood spot retest and further clinical evaluation showing virilized external genitalia in girls and salt-wasting signs in both sexes and serum/dried blood spot 17-OHP measurement.

Statistical analysis

Descriptive data were expressed as mean ± standard deviation (SD) or median and 25-75 interquartile range. Categorical variables are reported as frequencies (%). Log10 transformation was used to normalize the distribution of non-Gaussian variables and Student's t test was used for comparisons between two groups. Categorical variables were compared using Fisher's exact test. Generalized estimating equations (GEE) were used to estimate the interaction between birth weight tier and the difference (delta) between 17-OHP levels at screening and retest, followed by Bonferroni test. All analyses were performed using the Statistical Package for the Social Sciences 22.0 (SPSS, Armonk, NY, USA). Data were considered to be significant at $p < 0.05$.

RESULTS

Of the 108,409 total samples obtained at the initial screening, 104,737 were collected between the 3rd and 40th post-natal days, and included in the present analysis, corresponding to 98.4% of the total. Of these, 83,424 (77%) were collected at age 3-7 days. Most retest samples were collected around the second or third week of life [median 17 (14.0 – 21.0) days]. Eight newborns were diagnosed with CAH (four males, four females). None of the four

females had a clinical diagnosis of CAH prior to the screening: the first female presented genital ambiguity of unknown etiology; the second was initially considered as a male; and in the other two females, clitoromegaly was not recognized. Two deaths occurred, one due to complications associated with several malformations and the other due to hyponatremia and metabolic acidosis. In this child, screening was not performed until 38 days of life.

The incidence of CAH in the state was 1:13,551. Six cases were identified as classic salt-wasting CAH and two were cases of virilizing CAH. Figure 2 shows the incidence of CAH in the state and in the other Brazilian states.

During this first year, 514 infants (0.47% of the total screened population) had 17-OHP levels that were higher than the reference cut-off levels (>P99 or 2 times P99 for each birth weight tier) on the screening test. Of these 514 infants, 21 died before retest from various causes, of which extreme prematurity was the most frequent (mean weight 1.413,4 ± 970,4) and 376 (73%) had normal 17-OHP levels on retest. The remaining 117 infants with suspected CAH at retest were examined by a pediatrician and underwent serum or dried blood measurement of 17-OHP. CAH diagnosis was confirmed in eight infants. One of them initiated treatment before the second sample collection. Clinical and laboratory assessment of the other 109 patients (0.1% of the total population) was negative, and the patients were considered to be FP.

The estimated positive predictive value (PPV) of the initial screening test was 1.6%. Table 1 shows the rates of altered 17-OHP values at the initial screening according to birth weight tier in the general population screened until 40 days of age.

Median age was similar for CAH cases and false positive at the initial screening (n=493) [8 (4.25- 15.75) and 5 (4.0-6.0) days P=0.199] and at retest (n=492) [20.0 (17-20.0) and 17 (14-21) days, P=0.205]. Median 17-OHP values at initial screening were significantly

different between CAH cases and false positive [446.50 ng/mL (72.60 – 501.25) and 25.80 (17.4 – 41.8) ng/mL; p=0.001]. The same was true for the retest, with a 17-OHP median of 435 ng/mL (209 – 521) and 8.30 (5.86– 12.60) ng/mL (p< 0.001) respectively.

Table 2 shows 17-OHP values at the initial screening and retest according to birth weight tier. At the initial screening test as wells as at the retest, 17-OHP values were progressively lower with increasing weight. Delta 17-OHP levels (retest minus screening value) were also significantly different in each tier compared to the others.

Regarding the 117 infants who underwent further clinical and laboratory evaluation of CAH, 61.5% (n=72) were in birth weight tier 4 (> 2,500g), vs. 7.7% (n=9) in tier 1, 13.6% (n=16) in tier 2, and 17% (n=20) in tier 3. No CAH case was diagnosed in tier 1 or 2, with birth weight < 2,000g. The prevalence of maternal complications, such as gestational diabetes, maternal hypertension, or maternal infection was similar in the case and false positive groups. The frequency of neonatal complications (hypoglycemia, jaundice, sepsis, ventilation, oxygen therapy, diarrhea, vomiting) was also similar between these two groups. Comparison of the clinical and laboratory data obtained for cases and babies with false positive results are presented in Table 3. Significant differences were observed between the groups, with higher prematurity rate, lower gestational age, and lower weight in false positive patients. In turn, consanguinity and dehydration were more frequent in CAH cases. Also, lower levels of sodium, higher levels of potassium and higher serum levels of 17-OHP were detected in CAH patients, as was to be expected.

DISCUSSION

Early diagnosis of CAH is crucial to prevent infant death due to adrenal insufficiency. In the present study, the first year of a CAH screening program provided by the public health

care system in the state of Rio Grande do Sul, Brazil was assessed. The program successfully screened a high proportion of newborns (98.4%) between the 2nd and 40th post-natal days, and 80% of the valid samples were screened at the ideal moment, that is, between the 3rd and 7th post-natal days [2,4,10].

The incidence of CAH in the state of Rio Grande do Sul detected by the screening program, 1:13,551, was similar to that reported for other populations [1]. It was also very close to the incidence of 1:14,972 reported for the only adjacent Brazilian state, in which a similar, predominantly Caucasian population is found [19]. In contrast, other Brazilian states had a lower incidence of CAH [16,17]. Ethnicity and geographic factors are known to affect the incidence of CAH [1,6]. Thus, in a country such as Brazil, covering a large territory, with a racially mixed population, different ratios are to be expected. According to the latest Brazilian census, of 2010, 78% of the population in the South is white, in contrast to 42% in the Midwest and 55% in the Southeast [21]. Regarding confirmed CAH cases, the inability to diagnose the disease even in the presence of genital atypia has been reported in other Brazilian studies [10], and reinforces the need for universal newborn screening for CAH in Brazil. In this sense, improving time to test, transport time to the laboratory, and time to result is still a challenge that must be overcome. In turn, the 15 day-interval to retest seems to be adequate in most cases, since these are premature newborns, hospitalized in intensive care units, born from mothers who may have received corticoids during the final pregnancy days for improving fetal lung maturation.

Since 1977, when Pang et al. [22] described a microfilter paper assay for determination of 17-OHP levels in newborns, neonatal screening has been available for CAH due to 21-hydroxylase deficiency. Later, an immunofluorimetric assay was introduced, which is currently the most widely used technique worldwide [2,23,24]. More recent studies suggest

a higher specificity and better sensitivity for mass spectrometry, especially when used as a second tier test [25-27]. In contrast, immunofluorimetric methods are less expensive, require a smaller blood spot, and are still widely available and recommended [2,10,24]. Also, mass spectrometry does not completely eliminate false positive results, especially in preterm infants [14,27] .

In our sample, PPV (1.6%) and false positive rate were similar to those of previous reports [10,24,28]. False positive results are a long-standing concern of CAH neonatal screening programs [7,9-11,23,27,29]. In the past two decades, a decrease in false positive rates has been noted [10,11,23,29,30], possibly as a result of both improved 17-OHP detection methods and adjustment of diagnostic cut-off points to birth weight [7,10]. Adjustment of diagnostic levels of 17-OHP according to birth weight tiers [7,9,10,19] has been proposed as a useful strategy to minimize false positive. However, it is also important to recognize other possible factors associated with an increased 17-OHP level in newborns. Indeed, studies have shown that low birth weight, premature or critically ill infants may have elevated 17-OHP levels per se, without a link to 21-hydroxylase deficiency [8,12,31]. Possible explanations for the transient elevation in 17-OHP levels in these patients are immature hepatic function, leading to a decrease in the metabolic clearance of 17-OHP; increase in stress-induced production of 17-OHP, especially if the sample is collected in the first 24 hours of life; or immaturity of the adrenal glands [31,32]. Low birth weight, premature, and critically ill infants should be monitored in relation to 17-OHP concentrations, with a second sample collected on a later occasion to prevent false diagnoses and waste of resources.

We found an association between low birth weight and false positive results. The highest rate of false positive (4.0%) was found in the group with birth weight of 1,500-2,000 g (tier 2), in which no cases of CAH were finally detected (Table 1). We speculate that

survival is more likely in tier 2 newborns as compared to those in tier 1 (< 1,500g). We also recorded a higher rate of false positive results in preterm versus term infants (Table 3). Moreover, the gestational age of false positive babies was significantly lower than that of CAH cases. While a high correlation exists between birth weight and gestational age, one study suggests that gestational age-related 17-OHP cutoff levels improve CAH screening [9]. Nevertheless, birth weight data is more easily assessed than gestational age. Coulm et al. reported a PPV of 0.4% for CAH screening in pre-term infants, a value that is lower than that observed for term infants. Another study [33] suggests a correction factor for prematurity and weight, but does not use stratified cut-offs, which complicates the analysis of PPV. Interestingly, we observed that even if above the diagnostic cut-off point for the birth weight tier, 17-OHP values of false positive infants were significantly lower than those of CAH cases in both the initial screening and retest. Other studies have reported similar findings [10,19], which might be explained by a more severe clinical status, since many of these false positive infants required intensive care [12].

Consanguinity was an important factor in this population, present in 25% of CAH cases but absent in false positive cases. Thus, adding information about consanguinity to the initial screening might support CAH diagnosis in the presence of high 17-OHP levels. Limitations of this study are its retrospective nature, which prevented the analysis of factors related to false positive results, and the lack of proper information on initial screening regarding prenatal use of glucocorticoid, which might affect 17-OHP levels. Prospective studies with adequate design are required for these analyses.

CONCLUSION

The screening of CAH remains a challenge, and the implementation of an adequate screening flow makes population programs more assertive. In addition to the 17-OHP dosing method, diagnostic 17-OHP cut-offs stratified by birth weight, collection of samples at specific time points, and performance of retests even in the absence of clinical suspicion of CAH or confounding factors, such as prematurity and critical illness, greatly contribute to decrease false positive rates.

The present results support the need for CAH screening by the public health care system, and show that the strategy adopted is adequate, despite the initial screening of some infants after the 7th post-natal day. Future prospective studies may be useful to establish specific strategies for preterm groups, lower weight newborns, and ICU patients, and to improve effectiveness and PPV in all weight tiers.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Research Ethics Committee at Hospital Materno Infantil Presidente Vargas, and meets the guidelines and norms regulating research involving human beings. Approval of consent was waived.

Consent for publication

Not applicable.

Availability of data and material

All data analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CK, MJP and LAB have made substantial contributions acquisition, analysis and interpretation of data. CK, SMC and PMS conceived the design of the study. CK, SMS, CMDS and PMS have been involved in drafting the manuscript or revising it critically for important intellectual content. SMS and PMS have given final approval of the version to be published. All authors read and approved the final manuscript.

References

1. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Endocr Rev. 2000;21(3):245-91.

2. Speiser PW, Azziz R, Baskin LS, Ghizzoni L, Hensle TW, Merke DP, et al. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2010;95(9):4133-60.

3. Bachega TA, Billerbeck AE, Parente EB, Lemos-Marini SH, Baptista MT, Mello MP, Guerra G Jr, Kuperman H, Setian N, Damiani D, Torres N, Castro Md, Mendonça BB. [Multicentric study of Brazilian patients with 21-hydroxylase deficiency: a genotypephenotype correlation]. Arq Bras Endocrinol Metabol. 2004;48(5):697-704.

4. Joint LWPES/ESPE CAH Working Group. Consensus statement on 21-hydroxylase deficiency from the Lawson Wilkins Pediatric Endocrine Society and the European Society for Paediatric Endocrinology. J Clin Endocrinol Metab. 2002;87(9):4048-53.

5. Speiser, PW. Prenatal and neonatal diagnosis and treatment of congenital adrenal hyperplasia. Horm Res. 2007;68 (5): 90-2.

6. Pang SY, Wallace MA, Hofman L, Thuline HC, Dorche C, Lyon IC, Dobbins RH, Kling S, Fujieda K, Suwa S. Worldwide experience in newborn screening for classical congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Pediatrics. 1988;81(6):866-74.

7. Allen DB, Hoffman GL, Fitzpatrick P, Laessig R, Maby S, Slyper A. Improved precision of newborn screening for congenital adrenal hyperplasia using weight-adjusted criteria for 17-hydroxyprogesterone levels. J Pediatr. 1997;130(1):128-33.

8. Nordenström A1, Wedell A, Hagenfeldt L, Marcus C, Larsson A. Neonatal screening for congenital adrenal hyperplasia: 17-hydroxyprogesterone levels and CYP21 genotypes in preterm infants. Pediatrics. 200;108(4):E68.

9. Olgemöller B, Roscher AA, Liebl B, Fingerhut R. Screening for congenital adrenal hyperplasia: adjustment of 17-hydroxyprogesterone cut-off values to both age and birth weight markedly improves the predictive value. J Clin Endocrinol Metab. 2003;88(12):5790-4.

10. Hayashi G, Faure C, Brondi MF, Vallejos C, Soares D, Oliveira E, Brito VN, Mendonca BB, Bachega TA. Weight-adjusted neonatal 17OH-progesterone cutoff levels improve the efficiency of newborn screening for congenital adrenal hyperplasia. Arq Bras Endocrinol Metabol. 2011;55(8):632-7.

11. Tsuji A, Konishi K, Hasegawa S, Anazawa A, Onishi T, Ono M, Morio T, Kitagawa T, Kashimada K. Newborn screening for congenital adrenal hyperplasia in Tokyo, Japan from 1989 to 2013: a retrospective population-based study. BMC Pediatr. 2015;15;15(1):209.

12. Pauwels G, Allegaert NK, Régal L, Meulemans A. Risk factors for elevated levels of 17-hydroxyprogesterone during neonatal intensive care unit admission. Acta Clin Belg. 2012;67(2):88-93.

13. Gatelais F, Berthelot J, Beringue F, Descamps P, Bonneau D, Limal JM, Coutant R. Effect of single and multiple courses of prenatal corticosteroids on 17 hydroxyprogesterone levels: implication for neonatal screening of congenital adrenal hyperplasia. Pediatr Res. 2004;56(5): 701-5.

14. Sarafoglou K, Banks K, Gaviglio A, Hietala A, McCann M, Thomas W. Comparison of one-tier and two-tier newborn screening metrics for congenital adrenal hyperplasia. Pediatrics. 2012;130(5):e1261-8. doi: 10.1542/peds.2012-1219. Epub 2012 Oct 15.

15. Yoo BK, Grosse SD. The cost effectiveness of screening newborns for congenital adrenal hyperplasia. Public Health Genomics. 2009;12(2):67-72. doi: 10.1159/000156115. Epub 2008 Sep

16. Barra CB, Silva IN, Pezzuti IL, Januário JN. Neonatal screening for congenital adrenal hyperplasia. Rev Assoc Med Bras. 2012;58(4):459-64.

17. Pezzuti IL, Barra CB, Mantovani RM, Januário JN, Silva IN. A three-year follow-up of congenital adrenal hyperplasia newborn screening. J Pediatr (Rio J). 2014;90(3):300-407.

18. Silveira EL, dos Santos EP, Bachega TA, van der Linden Nader I, Gross JL, Elnecave RH. The actual incidence of congenital adrenal hyperplasia in Brazil may not be as high as inferred--an estimate based on a public neonatal screening program in the state of Goiás. J Pediatr Endocrinol Metab. 2008;21(5):455-60.

19. Nascimento ML, Cristiano AN, Campos Td, Ohira M, Cechinel E, Simoni G, Lee Jv, Linhares RM, Silva PC. Ten-year evaluation of a Neonatal Screening Program for congenital adrenal hyperplasia. Arq Bras Endocrinol Metabol. 2014;58(7):765-71.

20. Triagem neonatal: hiperplasia adrenal congênita / Ministério da Saúde, Secretaria de Atenção à Saúde, Departamento de Atenção Especializada e Temática. – Brasília: Ministério da Saúde, 2015. 44 p. : il. Editora MS – OS 2015/0241 ISBN 978-85-334-2262- 9. Printed in Brazil.

21. IBGE. Censo Demográfico 2010. Available at: http://www. censo2010.ibge.gov.br

22. Pang S, Hotchkiss J, Drash AL, Levine LS, New MI. Microfilter paper method for 17 alpha-hydroxyprogesterone radioimmunoassay: its application for rapid screening for congenital adrenal hyperplasia. J Clin Endocrinol Metab. 1977;45:1003-100.

23. Cartigny-Maciejewski M, Guilley N, Vanderbecken S, Gondé S, Stuckens C, Ponté C, Weill J, Farriaux JP, Paux E. Neonatal screening of congenital adrenal hyperplasia due to 21-hydroxylase deficiency: Lille experience 1980-1996]. Arch Pediatr. 1999;6(2):151-8.

24. White PC. Neonatal screening for congenital adrenal hyperplasia. Nat Rev Endocrinol. 2009;5(9): 490-8. doi: 10.1038/nrendo.2009.148.

25. Lacey JM, Minutti CZ, Magera MJ, Tauscher AL, Casetta B, McCann M, Lymp J, Hahn SH, Rinaldo P, Matern D. Improved specificity of newborn screening for congenital adrenal hyperplasia by second-tier steroid profiling using tandem mass spectrometry. Clin Chem. 2004;50(3):621-5.

26. Schwarz E, Liu A, Randall H, Haslip C, Keune F, Murray M, et al. Use of steroid profiling by UPLC-MS/MS as a second tier test in newborn screening for congenital adrenal hyperplasia: the Utah experience. Pediatr Res. 2009;66:230-5.

27. Tajima T, Fukushi M. Neonatal mass screening for 21-hydroxylase deficiency. Clin Pediatr Endocrinol. 2016;25(1):1-8. doi: 10.1297/cpe.25.1. Epub 2016 Jan 30.

28. Coulm B, Coste J, Tardy V, Ecosse E, Roussey M, Morel Y, et al. DHCSF Study Group Efficiency of neonatal screening for congenital adrenal hyperplasia due to 21-hydroxylase deficiency in children born in mainland France between 1996 and 2003. Arch Pediatr Adolesc Med 2012;166: 113-20.

29. Kwon C, Farrell PM. The Magnitude and Challenge of False-Positive Newborn Screening Test Results. Arch Pediatr Adolesc Med. 2000;154(7):714-8. doi:10.1001/archpedi. 154.7.714.

30. Gidlof S, Wedell A, Guthenberg C, von Dobeln U, Nordenstrom A. Nationwide Neonatal Screening for Congenital Adrenal Hyperplasia in Sweden: A 26-Year Longitudinal Prospective Population-Based Study. JAMA Pediatr. 2014;168(6):567-74.

31. Alves CA, Junior VB, Toralles MB. Neonatal screening for congenital adrenal hyperplasia: considerations regarding the transient rise of the 17- hydroxyprogesterone. Braz J Promotion (RBPS). 2006;19(4):203-8.

32. Nomura S Immature adrenal steroidogenesis in preterm infants. Early Hum Dev. 1997; 49:225-33.

33. Lee JE, Moon Y, Lee MH, Jun YH, Oh KI, Choi JW. Corrected 17-alpha hydroxyprogesterone values adjusted by a scoring system for screening congenital adrenal hyperplasia in premature infants. Ann Clin Lab Sci. 2008;38(3):235-40.

34. Silveira EL, Elnecave RH, dos Santos EP, Moura V, Pinto EM, van der Linden Nader I, Mendonca BB, Bachega TA. Molecular analysis of CYP21A2 can optimize the follow- up of positive results in newborn screening for congenital adrenal hyperplasia. Clin Genet. 2009;76(6):503-10. doi: 10.1111/j.1399-0004.2009.01274.x

Figure legends

Figure 1. Flow diagram of newborn screening for congenital adrenal hyperplasia

Adapted from references 15 and 19; P99 (99th percentile) 17-OHP cutoff points to diagnose CAH: birth weight ≤ 1500 g: 110.4 ng/mL; birth weight 1,501 to 2,000 g: 43.0 ng/mL; birth weight 2,001 to 2,500 g: 28.2 ng/mL; and birth weight weight > 2500 g: 15.1 ng/mL [9].

Figure 2. Reported incidence of CAH at neonatal screening in different states of Brazil

GO = State of Goiás [17]; MG = State of Minas Gerais [16], [21]; SP = State of São Paulo [9]; SC = State of Santa Catarina); RS = State of Rio Grande do Sul.

Table 1. Rate of altered 17-OHP results on initial CAH screening[a] stratified by birth weight tier in the general population tested until 40 days of age in the state of Rio Grande do Sul, Brazil

Birth weight tier	n	17 OHP (>P99 or 2 times P99)
		n (%)
< 1,500 g	1,071	35 (3.3%)
1,501 – 2,000 g	1,773	71 (4.0%)
2,001 – 2,500 g	6,462	106 (1.6%)
> 2,501 g	95,431	302 (0.3%)
Total	104,737	514 (0.5%)

[a]17-OHP diagnostic cut-off levels: birth weight ≤ 1500 g: 110.4 ng/mL; birth weight 1,501 to 2,000 g: 43.0 ng/mL; birth weight 2,001 to 2,500 g: 28.2 ng/mL; and birth weight weight > 2500 g: 15.1 ng/mL

Table 2. Median 17-OHP levels in infants with suspected congenital adrenal hyperplasia on newborn screening and retest according to birth weight tier

Sample[#]	Birth weight tier				p
	≤ 1,500g n=23	1,501-2,000g n=67	2,001-2,500g n=105	≥2,501g n=298[§]	
Screening (median ng/mL [P25-75])	154 (120 to 208)[a]	53.6 (47.0 to 64.7)[b]	33.6 (29.9 to 41.9)[c]	18.8 (16.0 to 23.4)[d]	<0.001
Retest (median ng/mL [P25-75])	48.1 (21.9 to 96.5)[a]	12.7 (10.1 to 20.5)[b]	8.1 (6.4 to 12.3)[c]	7.3 (5.1 to 10.6)[d]	<0.001
Δ Samples	-98.6 (-172.5 to -67.0)[a]	-38.9 (-47.2 to -33.2)[b]	-24.9 (-31.5 to -21.2)[c]	-11.8 (-15.6 to -7.95)[d]	<0.001

[#]17-OHP diagnostic cut-off levels: birth weight ≤ 1500 g: 110.4 ng/mL; birth weight 1,501 to 2,000 g: 43 ng/mL; birth weight 2,001 to 2,500 g: 28.2 ng/mL; and birth weight weight > 2500 g: 15.1 ng/mL; [§]n=297 on retest

Δ Samples: difference between 17-OHP at retest and screening.

Values are expressed as median and interquartile range; different superscript letters indicate statistical difference by GEE test.

Table 3. Family history, maternal, perinatal, newborn and laboratory data of newborns diagnosed with congenital adrenal hyperplasia vs. false positive newborns

Variables	**CAH cases (n=8)**	**False positives (n=109)**	**p**
Maternal data			
Caesarean delivery (n [%])	4/8 (50.0)	42/70 (60.0)	0.496
Newborn data			
ICU care (n [%])	4/8 (50.0)	71/98 (72.4)	0.281
Preterm (n [%])	2/8 (25.0)	59/109 (54.1)	0.004
Birth weight (n [%])	2,940 ± 570.34 (n=8)	2,496 ± 761.63 (n=109)	0.110
Gestational age (week)	38.0 ± 1.9 (n=8)	34.8 ± 3.2 (n=72)	0.007
Dehydration (n [%])	5/8 (62.5)	3/76 (3.9)	<0.001
Na (nmol/L)[a]	122.25 ± 10.15 (n=8)	136.56 ± 2.28 (n=54)	0.005
K (nmol/L)[a]	6.17 ± 1.21 (n=8)	5.31 ± 0.67 (n=54)	0.004
Serum 17-OHP (ng/mL) (Md [P25-P75])	25.6 (12.8 – 285) (n=3)	12.5 (7.4 – 17.8) (n=45)	0.006
Family data			
Family history (n [%])	3/8 (37.5)	9/67 (13.4)	0.196
Consanguinity (n [%])	2/8 (25.0)	0/109 (0%)	<0.001

Figure 1. Flow diagram of newborn screening for congenital adrenal hyperplasia

Adapted from references 15 and 19; P99 (99th percentile) 17-OHP cutoff points to diagnose CAH: birth weight ≤ 1500 g: 110.4 ng/mL; birth weight 1,501 to 2,000 g: 43.0 ng/mL; birth weight 2,001 to 2,500 g: 28.2 ng/mL; and birth weight weight > 2500 g: 15.1 ng/mL [9].

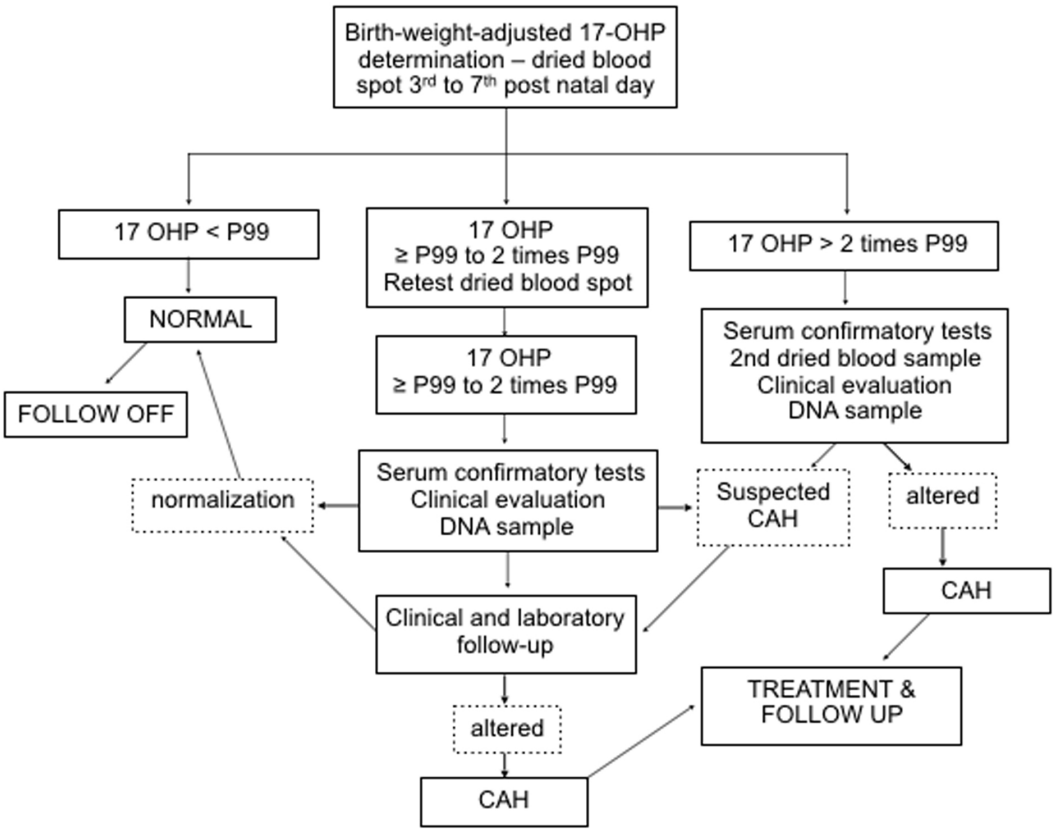
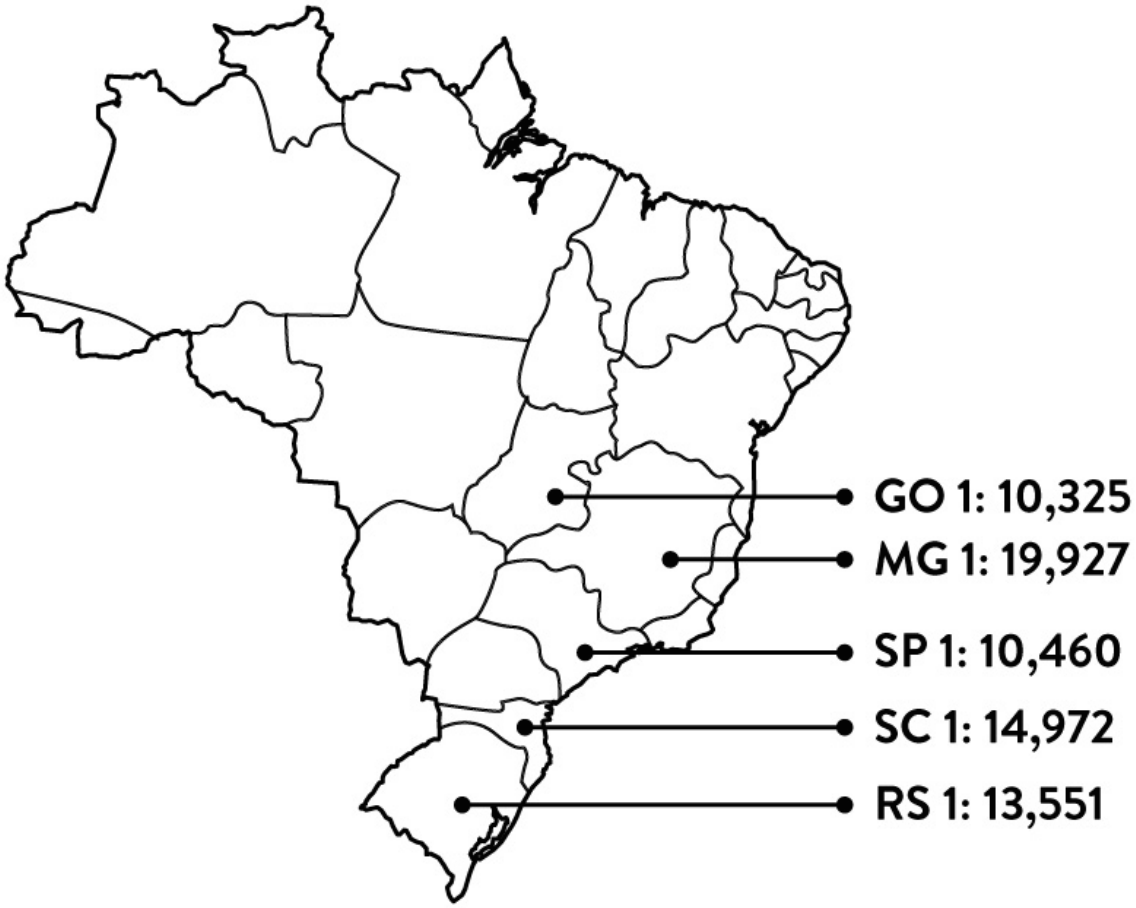


Figure 2. Reported incidence of CAH at neonatal screening in different states of Brazil

GO = State of Goiás [17]; MG = State of Minas Gerais [16], [21]; SP = State of São Paulo [9]; SC = State of Santa Catarina); RS = State of Rio Grande do Sul.



CAPÍTULO 3

ARTIGO 2

Clinical and molecular profile of 132 newborns with confirmed or suspicious congenital adrenal hyperplasia detected in the first two years of a public screening program implementation

Short Title: Congenital Adrenal Hyperplasia molecular diagnosis

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None of the authors have conflicts of interest

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ABSTRACT

Objective: To describe the results obtained in a neonatal screening program after its implementation and to assess the clinical and molecular profiles of confirmed and suspicious congenital adrenal hyperplasia (CAH) cases.

Methods: A cross-sectional study was conducted. Newborns with suspected disease due to high 17-OHP levels and adjusted for birth weight were selected. Classical CAH (salt wasting and simple virilizing forms) was diagnosed by an increase in 17-OHP levels as confirmed in the retest, clinical evaluation, and genotype determined by polymerase chain reaction, SnapShot, and Multiplex ligation-dependent probe amplification.

Results: After 24 months, 15 classic congenital adrenal hyperplasia cases were diagnosed from a total of 217,965 newborns, with an estimated incidence of 1:14,531. From a 132 patients sample, seven non-classical and 14 heterozygous patients were screened for *CYP21A2* mutations, and 96 patients presented false positives with wild type *CYP21A2*. On retest, increased 17-OHP levels were found in classical CAH patients and showed significant correlation with genotype-related classical CAH. The most frequent mutation was IVS2-13A/C>G followed by gene deletion or rearrangement events in classical CAH. In non-classical and heterozygous diseases, p.V281L was the most common mutation.

Conclusions: The results underscore the effectiveness of CAH neonatal screening in the public health system and indicate that the adopted strategy was appropriate. The second sample collection along with genotyping of suspected cases helped to properly diagnose both severe and milder CAH cases and delineate them from false positive patients.

BACKGROUND

Congenital adrenal hyperplasia (CAH) is characterized by impairment in the metabolic pathway involved in the synthesis of cortisol and aldosterone (1). Autosomal recessive genetic inheritance is caused by mutations in the *CYP21A2* gene in approximately 90% of all cases, leading to 21-hydroxylase deficiency and causing accumulation of precursor metabolite (2). The main disease marker in 21-hydroxylase (21-OH) deficiency is 17-hydroxyprogesterone (17-OHP)(2). There are three recognized clinical forms of CAH: 1) classical salt-wasting (SW); 2) simple virilizing (SV); and 3) late onset form, termed nonclassical- (NC) CAH (1,3). The main purpose of neonatal screening is to identify newborns at risk of being affected by the two classical forms, SW and SV (4,5). However, asymptomatic cases with persistently elevated 17-OHP levels in neonatal screening are suspect for NC-CAH and may be further identified by molecular diagnosis (6,7).

The overall incidence for CAH described in the literature is 1:15,000 in live newborns (4). The prevalence of NC-CAH is estimated at 1:1,000 individuals (1). In 2/3 cases of the classical forms, salt loss affects both genders, and symptoms begin in the second week of life, thus reinforcing the importance of an early diagnosis (5). In girls, intrauterine androgen excess exposure causes different degrees of virilization and genital ambiguity as defined by the Prader Scale (8).

Increases in 17-OHP have been observed in newborns without HAC (false positives) due to stressful situations and premature birth (9,10). In turn, false negative cases can occur as a result of maternal use of corticosteroids at the end of pregnancy (11,12). In order to minimize the number of false positives, cutoffs stratified by both Brazilian and international birthweight categories have been established (9–11).

Molecular analysis of the *CYP21A2* gene can improve neonatal screening specificity and can also help in the clinical management of the disease (7,13). The main *CYP21A2* mutations have been categorized into four groups (Null, A, B, and C) according to residual 21-OH enzymatic activity, which allows for the determination of the expected phenotype (2,6,14,15). Both Null and A are associated with SW-CAH, group B with the SV-CAH, and group C with NC-CAH. The point mutations IVS2-13A/C>G, p.I172N and p.V281L are the most common related do SW-CAH, SV-CAH and NC-CAH, respectively (2,6,13–15).

In Brazil, states such as Goiás, Santa Catarina, and São Paulo have accumulated over 10 years experience in neonatal CAH screening (9,14,16,17). Therefore, the aim of this study had several points: 1) to describe the results 24 months after a CAH Neonatal Screening in a public health program in Southern Brazil was implemented and 2) to assess the clinical profiles of confirmed and suspicious CAH cases in addition to molecular genotyping of these cases as a complementary tool to improve CAH diagnosis.

METHODS

Design and population

A cross-sectional study was conducted with newborns included in the first two years of the public CAH screening program implementation in the state of Rio Grande do Sul, Brazil.

The database of babies with suspected CAH, which was based on altered 17-OHP values from a 2014 to 2016 screening, were revised. Classical CAH (SW and SV) was diagnosed by increased 17-OHP levels detected at screening, confirmed by retest and/or clinical evaluation showing virilized external genitalia in girls and salt-wasting signs in both sexes, followed

by genotype studies. False positives were characterized by lower 17-OHP levels on retest and by the wild type (WT) genotype.

The study protocol was approved by the Research Ethics Committee at Hospital Materno Infantil Presidente Vargas and by Ethics Committee of Fundação Estadual de Produção e Pesquisa em Saúde (N○ 341.289/June 8, 2013), and all parents gave written informed consent.

Blood collection and 17-OHP measurements

Dried blood spots were obtained using filter paper (S & S 903) from the 3rd to 40th post-natal days. The GSP solid phase (time-resolved) immunofluorescence assay (Neonatal 17-OHP kit; PerkinElmer, Turku, Finland) was used to measure 17-OHP.

The reference 17-OHP values used in the present study were those recommended by the Brazilian National CAH Screening Program, which was based on a previous Brazilian study (9,11). Cut-off levels of 17-OHP used to diagnose CAH were 110.4, 43.0, 28.2, and 15.1 ng/mL for four sets of respective birth weight tiers: 1) birth weight ≤1,500 g; 2) birth weight 1,501–2,000 g; 3) birth weight 2,001–2,500 g; and 4) birth weight >2,500 g. Newborns subject to their mother's corticosteroid use at the end of pregnancy as recorded on filter paper were called for a second collection after 15 days of life (11,12). A second sample was also obtained in these patients around the second to third week of life (median 17 [14.0–21.0] days) according to previous data (Kopacek, BMC Pediatrics 2016, submitted) (18).

Mutation analysis of the *CYP21A2* gene

Genotyping was performed in 132 subjects. Blood sample was collected at the time of retest. A multiplex minisequencing assay was chosen to analyze point mutations in *CYP21A2*

according to Prado (19). Twelve mutations were selected consisting of the 10 most common worldwide mutations derived from *CYP21A1P* (p.Q318X, p.R356W, p.Leu307fs(InsT), p.V236E, IVS2-13A/C>G, p.I172N, p.P30L, p.P453S, p.V281L, p.G110EfsX21 (E3Δ8bp) and other two mutations detected in the Brazilian population (p.R408C, "Null" group and p.H62L, "C" group) (14,20). Amplification of *CYP21A2* was performed using an allele specific polymerase chain reaction (PCR) described by Krone et al. (21). A multiplex minisequencing reaction was performed with SNaPshot Multiplex Ready Reaction reagents (Applied Biosystems, Foster City, California, USA) according to the manufacturer's protocol. This technique was performed in all of the study subjects. Large deletions, rearrangements, and gene conversions were assessed for patients with SW or SV that was suspicious after detection by MLPA-SALSA MLPA probemix P050-C1 CAH kit (MRC-Holland; Amsterdam, The Netherlands) according to the manufacturer's recommendations. Direct sequencing of *CYP21A2* was used to characterize mutations that were present in the multiplex minisequencing panel or in the MLPA-probed CAH suspicious patients. This assay was also performed to confirm all point mutations found by multiplex minisequencing.

Statistical analysis

Results were expressed as mean ± standard deviation (SD) or frequencies (expressed as percentages [%]). Comparisons among groups were analyzed by univariate analysis of variance (ANOVA) followed by Tukey test. Covariance ANOVA (ANCOVA) was used for 17-OHP adjustments by birth weight, and data was expressed as mean ± standard error of the mean (SEM). A partial correlation was estimated between 17-OHP and electrolytes. Analyses were performed using the Statistical Package for the Social Sciences Version 18.0. (Chicago: SPSS Inc). Data was considered to be significant at $p < 0.05$.

RESULTS

From a total of 217,965 samples obtained at the 2-year screening, 15 classic CAH cases were diagnosed with an estimated incidence of 1:14,531. Nine newborns were females and six were males, and 80% (n=12) were clinically characterized as SW-CAH patients and the remainder as SV patients. Table 1 describes clinical data, 17-OHP and electrolyte values, and phenotype-genotype evaluation of these cases. Three girls (cases 3, 5, and 11) had mild virilization (Prader I classification) and were not clinically characterized as presenting with clitoromegaly previous to CAH screening. Another girl (case 2) was sex assigned as a boy and the other one (case 10) was registered with a neutral name, suitable for either sex due to genital ambiguity. In the other four girls (cases 4, 12,13, and 14) genital ambiguity was identified, but CAH was not diagnosed prior to screening. Among the males, case 6 and 15 presented with penis enlargement and the other four had normal male genitalia.

Ten of the 15 cases (66.6%) were born at birth weight >2,500g and no cases weighed < 2,000g. Mothers of three cases (5, 8, and 10) were using glucocorticoids at the end of their pregnancies. In two of them, a net increase in 17-OHP levels in the second sample was observed. In a third infant (case 8), the onset of corticotherapy due to salt loss preceded the possibility to obtain a second sample. Four infants (26.6%) had consanguineous parents.

Table 1 also describes the clinical profile, 17-OHP values, and phenotype-genotype evaluation of NC-CAH and heterozygous cases that were detected during screening. Only one heterozygous patient (case 31) presented low birth weight related to extreme prematurity and had an increased 17-OHP value in the second sample that was associated with clinical worsening in the intensive neonatal unit. This patient was followed in a tertiary hospital far from the screening center. Normal electrolyte values and no other clinical signs of CAH were reported by this hospital. A third sample was collected after clinical improvement and showed a

considerable decrease in 17 OHP (17 ng/mL). In this patient, genotype was crucial for diagnosis elucidation. Another three patients with NC-CAH (16, 17, and 22) presented higher 17-OHP values in the second sample and a history of maternal corticosteroid use.

Hyponatremia and hyperkalemia were not observed in NC-CAH and heterozygous cases. Among initial suspected cases of CAH, one patient with transient genital hyperpigmentation (patient 16) was diagnosed as NC-CAH, and three girls with transient clitoromegaly were later identified as heterozygous cases. In these cases, spontaneous regression of clitoromegaly was seen during the follow-up through the first six months of life. Parental consanguinity was found in two (14.3%) of heterozygous patients.

Tables 1 and 2 show the genotype distribution according to the enzyme activity groups (Null, A, B, and C), heterozygous, and WT in the newborn population. Genotype-phenotype agreement was observed in 13 (86.6%) cases. Among the cases with classical CAH, IVS2-13A/C>G was the most frequent mutation (33% in homozygosity and 20% in compound heterozygosity), followed by p.I172N, deletion, and gene conversion, each seen in 20% of the classical CAH cases. Seven patients presented with non-classical mutations (group C), and the other 14 heterozygous patients had the wild allele detected during neonatal screening. Among the whole sample, p.V281L was the most frequent mutation in NC-CAH patients (57% heterozygous and 28.5% homozygous) and also in six heterozygous patients (43%) (Table 2). A new allele IVS_T was described by Prado in a heterozygous girl born at term with birth weight of 2,905g (sample 1: 17 OHP 18.2 ng/mL and sample 2: 35.0 ng/mL) and in a boy born late-premature with birth weight of 2,540g (sample 1: 17-OHP 25.3 ng/mL and sample 2: 15.9 ng/mL) with positive screening (19). Surprisingly, the clitoromegaly identified in the girl during the first examination resolved spontaneously during the follow up period.

Both had normal electrolyte profiles and did not show any other symptoms associated with CAH during the 6-month follow-up.

Levels of 17-OHP, adjusted for birth weight, were analyzed according to the groups (1=classical CAH; 2=NC CAH; 3= heterozygous; 4=WT) in samples 1 and 2 (Figure 1A and B, respectively). Significantly higher 17-OHP levels were observed in classical CAH compared to other groups in samples 1 and 2. Figure 2 shows 17-OHP levels, adjusted for birth weight, in samples 1 and 2, classified according to the mutation groups. In sample 1, individuals with mutation groups null and A had significantly higher levels of 17-OHP and in sample 2, mutation groups null, A, and B maintained higher 17-OHP levels, which were statistically different from group C (NC-CAH), heterozygous, and WT patients.

Table 1 also shows electrolyte values in the different groups. Classical CAH cases had significantly lower Na^+ and higher K^+ levels. A strong inverse correlation between adjusted-for-birthweight 17-OHP and Na^+ ($r = -0.795$ for 17-OHP Sample 1 and $r = -0.740$, for Sample 2, $p < 0.05$) and positive correlation with K^+ was found ($r = 0.494$ for 17-OHP Sample 1 and $r = 0.531$ for Sample 2, $p < 0.05$).

DISCUSSION

In the present study, the 2-year CAH neonatal screening program using significantly higher levels of the marker, 17-OHP, properly allowed for diagnosing of 15 classical CAH newborns. In addition, the retest successfully discriminated between cases and disease severity in concordance with the corresponding genotype investigation.

The screening program was highly effective in detecting some cases that would not be clinically recognized before the screening. In this sense, mild genital atypia (cases 3, 4, and 11) and increased penis size in boys (cases 6 and 15) was not detected before specialized

evaluation. Even in those with ambiguous genitalia, CAH was not diagnosed before screening and incorrect sex assignment was made in two cases (2 and 10). Indeed, we observed that >50% of the females had an incorrect clinical evaluation of the virilization features, which was similarly identified in previous studies (9,15, and 22). This reinforces the importance of universal newborn screening for hyperplasia in Brazil.

We previously reported data from the first year of screening, in which we found that most of the false positives were newborns <2,000g (18). A second sample showing lower 17-OHP levels in assymptomatic cases is usually sufficient to elucidate false positive cases, especially in premature and low birth weight infants. Also, as previously reported, consanguinity rates are higher among CAH cases (18, 23). Thus, this additional information can be useful in distinguishing between actual cases and false positives. Similarly, information about maternal corticoid use at the end of gestation is relevant. In this case, the collection of a second sample after 15 days of the newborn's life helps to diagnose cases such as that of patient 11, who presentd with mild virilization not previously recognized and further SW-CAH development (22).

In neonatal screening, high 17-OHP levels may be present in premature and critically ill newborns. For this reason, screening stratified by birth weight has been widely recommended (9–11). In addition, the genotype has been recommended to improve the diagnosis of neonatal screening accuracy, discriminate between actual cases and false positives, clarify borderline cases, and eventually allow carriers of the NC forms to be diagnosed (7,13,15). High 17-OHP values corresponded to the severity of each genotype group (14,15,22). Interestingly, when we stratified 17-OHP levels according to mutation group severity in the second sample, a better differentiation of SV forms (group B mutations) from NC (group C mutations), heterozygous, and WT was seen. These last three groups have significantly lower 17-

OHP levels, justifying the strategy of retesting these patients and when there is still doubt, performing genotype analysis (22).

The efficacy of the screening program can also be demonstrated when severe forms such as carriers of group null mutations are identified (15). Deletion and large gene conversions characterizing group null were observed in 20% of our sample, which is in agreement to a recent, large genotype-phenotype correlation study (24). The group of null/null mutations were observed in 26.67% of our sample (4/15 patients) and null/A characterizing SW-CAH in another three patients. In patient 11, there were no clear phenotype-genotype correlations as a result of the manifestation of a mild SW-CAH phenotype. When group null/B compound heterozygous mutation is present, including p.I172N in one allelle, SV-CAH is expected in most cases (6,15). This observation has been reported by other groups (24,25), including a very rare association of p.I172N mutation with a NC-CAH (24). The salt loss associated with p.I172N is more common when a second more severe mutation is associated in heterozygosity (25,26).

In Brazil, a study by Bachega et al. determined the frequency of point mutations in 130 patients with classical and non-classical forms of CAH and correlated genotype with phenotype (27). The most frequent mutations were IVS2-13A/C>G in 55% of the alleles of the patients with the SW form, p.I172N was found in 42% of the SV-CAH, and p.V281L in 70% of the individuals with NC-CAH. The frequency of IVS2-13A/C>G mutation in our sample was, therefore, not surprising for the Brazilian population (53% in homo and/or heterozygosity in the classical cases). In a Brazilian sample from São Paulo, Carvalho D et al. recently found 21% in allele frequency of IVS2-13A/C>G mutations (14). In a larger Argentine cohort study, IVS2-13A/C>G corresponded to 20% of mutations in homozygosity (28). Brazil and Argentina (AR) show very similar rates of this mutation (14,28). The State of Rio Grande do Sul is near AR and the population shares common ethnic origins. This group also

describes the association of IVS2-13A/C>G alleles to SV forms, which may explain the relative non-concordant phenotype-genotype in case 3 (28). However, our data show excellent genotype-phenotype agreement, well above the close to 50% reported by New MI et a in a large number of individuals (24).

A new variant T in IVS2-13A/C>G was identified in two patients. The T allele was observed in heterozygous cases with the benign C allele as previously described (19). This variant was detected by the multiplex mini sequencing assay with locally-developed primers and was confirmed by MLPA. Because the female patient presented clitoromegaly at birth, even in heterozygous cases, this finding cannot be overlooked and requires further genetic studies. The description of three cases with transient clitoromegaly in our sample is worthy of additional study. It has been reported that some heterozygous patients had higher androgen levels, especially carriers of p.V281L mutation with premature adrenarche (1,29). It is expected that NC-CAH and heterozygous patients are asymptomatic at birth, but we hypothesized that possible transient androgen elevations may occur. Further studies to clarify this phenomenon may use expanded genotyping with MLPA and *CYP21A2* sequencing in determining for whom only SNaPshot be performed in order to exclude other mutations. In our sample, a better understanding of this new variant can provide more specific information.

One of the strengths of this study originated from the fact that diagnoses and follow-up examinations were performed by the same pediatric endocrinologist. Therefore, adequate clinical assessment of patients made in this study are suitable to clinical and molecular characterization of this sample in CAH neonatal screening. Another strength was the fact that data was collected from a population screening program, which favored the evaluation of a large number of individuals. In this context, our study was the first neonatal screening program/

genotyping by the use of SnapShot Mini Sequencing for the most common point mutations and confirmed *by CYP21A2* sequencing.

In conclusion, the results of this study underscore the effectiveness of the screening program in detecting CAH cases and excluding suspicious cases based on 17-OHP level cut-offs linked to birth weight stratification. In addition, the second sample collection, together with the genotyping of suspected samples, helped to properly diagnose both severe WS/SV-CAH cases and milder SV cases in addition to differentiating between classical CAH cases and false positive patients (WT). The present results also indicated that genotyping is a valuable and complementary diagnostic tool for neonatal screening. An additional benefit appears to be that it provides information on disease severity, allows for genetic counseling in severe cases, and avoids over-treating the late onset NC-HAC and the false positive patients.

References

1. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21- hydroxylase deficiency. Endocr Rev. 2000;21(3):245-91.

2. Krone N, Arlt W. Genetics of congenital adrenal hyperplasia. Best Pract Res Clin Endocrinol Metab. 2009; 23:181-92.

3. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocr Rev. 2011;32(1):81-151. Erratum in: Endocr Rev. 2011;32(4):579.

4. Pang S & Clark A. Congenital adrenal hyperplasia due to 21-hydroxilase deficiency: newborn screening and its relationship to the diagnosis and treatment of disorder. Screening 1993; 2:105-139

5. Speiser PW, Azziz R, Baskin LS, Ghizzoni L, Hensle TW, Merke DP et al. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an endocrine society clinical practice guideline. J. Clin. Endocrinol. Metab. 2010; 95(9): 4133-60.

6. Bachega T, Billerbeck AE, Parente EB, Lemos-Marini S, Baptista MT, Mello MP, et al. Estudo multicêntrico de pacientes brasileiros com deficiência da 21-hidroxilase: correlação do genótipo com o fenótipo. Arq Bras Endocrinol Metab. 2004;48(5):697-704.

7. Silveira EL, Elnecave RH, dos Santos EP, Moura V, Pinto EM, van der Linden Nader I, et al. Molecular analysis of CYP21A2 can optimize the follow-up of positive results in newborn screening for congenital adrenal hyperplasia. Clin Genet. 2009; 76:50310.

8. Prader A. Der Genitalbefund beim Pseudohermaphroditismus Femininus des kongenitalen Adrenogenitalen Syndromes. Helv Paediatr Acta. 1954; 3:231-48.

9. Hayashi G, Faure C, Brondi MF, Vallejos C, Soares D, Oliveira E, et al. Weight-adjusted neonatal 17OH-progesterone cutoff levels improve the efficiency of newborn screening for congenital adrenal hyperplasia. Arq Bras Endocrinol Metab. 2011;55(8): 632-7.

10. Olgemöller B, Roscher AA, Liebl B, Fingerhut R. Screening for congenital adrenal hyperplasia: adjustment of 17-hydroxyprogesterone cut-off values to both age and birth weight markedly improves the predictive value. J Clin Endocrinol Metab. 2003;88(12):5790-4.

11. Araújo JPB & Goldbeck AS. Triagem neonatal: hiperplasia adrenal congênita / Ministério da Saúde, Secretaria de Atenção à Saúde, Departamento de Atenção Especializada e Temática. – Brasília: Ministério da Saúde, 2015. 44 p. : il. Editora MS – OS 2015/0241 ISBN 978-85-334-2262-9. Printed in Brazil.

12. Gatelais F, Berthelot J, Beringue F, Descamps P, Bonneau D, Limal JM, et al. Effect of single and multiple courses of prenatal corticosteroids on 17hydroxyprogesterone levels: implication for neonatal screening of congenital adrenal hyperplasia. Pediatr Res. 2004;56(5): 701-5.

13. Balsamo A, Baldazzi L, Menabò S, Cicognani A. Impact of molecular genetics on congenital adrenal hyperplasia management.Sex Dev. 2010 Sep;4(4-5):233-48. doi: 10.1159/000315959. Epub 2010 Jul 15.

14. Carvalho DF, Miranda MC, Gomes LG, Madureira, G, Marcondes, JAM, Billierbeck, AEC et al. Molecular *CYP21A2* Diagnosis in 480 Brazilian 433 Patients with Congenital Adrenal Hyperplasia Before Newborn Screening 434 Introduction. *Eur J Endocrinol*. 2016; (May):1-21

15. Falhammar H, Wedell A, Nordenström A. Biochemical and genetic diagnosis of 21-hydroxylase deficiency.Endocrine. 2015 Nov;50(2):306-14.

16. Silveira EL, dos Santos EP, Bachega TA, van der Linden Nader I, Gross JL, Elnecave RH. The actual incidence of congenital adrenal hyperplasia in Brazil may not be as high as inferred: an estimate based on a public neonatal screening program in the state of Goiás. J Pediatr Endocrinol Metab. 2008;21(5):455-60.

17. Nascimento ML, Cristiano AN, Campos Td, Ohira M, Cechinel E, Simoni G, et al. Ten-year evaluation of a Neonatal Screening Program for congenital adrenal hyperplasia. Arq Bras Endocrinol Metabol. 2014;58(7):765-71.

18. Kopacek C, Castro SM, Prado MJ, Silva, CMD, Beltrão LA, Spritzer PM. Neonatal screening for congenital adrenal hyperplasia in Southern Brazil: a population-based study with 108,409 infants; *submitted* BMC Pediatrics, 201*6*

19. Prado, MJ. 2016. Mutation detection of *CYP21A2* gene in congenital adrenal hyperplasia patients from southern Brazil. Masters Dissertation, Federal University of Rio Grande do Sul (UFRGS). Porto Alegre, Rio Grande do Sul, Brazil. 106 p.

20. Soardi FC, Barbaro M, Lau IF, Lemos-Marini SHV, Baptista MTM, Guerra G et al. Inhibition of *CYP21A2* enzyme activity caused by novel missense mutations identified in brazilian and scandinavian patients. *J Clin Endocrinol Metab*. 2008;93(6):2416-2420.

21. Krone N, Braun A, Weinert S, Peter, M, Roscher, AA, Partsch, CJ et al. Multiplex minisequencing of the 21-hydroxylase gene as a rapid strategy to confirm 356 congenital adrenal hyperplasia. *Clin Chem*. 2002;48(6):818-25.

22. Nordenstrom A, Thilen L, Hagenfeldt L, Larsson A, Wedell A. Genotyping is a valuable diagnostic complement to neonatal screening for congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency. J. Clin. Endocrinol. Metab 1999; 84(5): 1505–9

23. Vakili R, Baradaran-Heravi A, Barid-Fatehi B, Gholamin M, Ghaemi N, Abbaszadegan MR.

Molecular analysis of the CYP21 gene and prenatal diagnosis in families with 21-hydroxylase deficiency in northeastern Iran. Horm Res. 2005;63(3):119-24.

24. New MI, Abraham M, Gonzalez B, Dumic M, Razzaghy-Azar M, Chitayat D et al. Genotype-phenotype correlation in 1,507 families with congenital adrenal hyperplasia owing to 21-hydroxylase deficiency. Proc Natl Acad Sci U S A. 2013 Feb 12;110(7):2611-6.

25. Díez López I, Rodríguez Estévez A, González Molina E, Martínez Ayucar M, Rodríguez Pérez B, Ezquieta Zubicaray B. Síndrome suprarrenogenital *congénito* virilizante con *mutación* de novo I172N: estudio de un nuevo caso. An Pediatr (Barc). 2010;72(1):72–78

26. Frish H, Battelino T, Shober E, Baumgartner-Parzer S, Nowotny P, Vierhapper H. Salt wasting in simple virilizing conhenital adrenal hyperplasia. J Clin Endocrinol Metab. 2001;14:1649–55.

27. Bachega TASS, Billerbeck AEC, Madureira G, Marcondes, JAM, Longui, CA, Leite, MV et al. Molecular genotyping in Brazilian patients 446 with the classical and nonclassical forms of 21-hydroxylase deficiency. *J Clin* 447 *Endocrinol Metab*. 1998;83(12):4416-4419. 448

28. Marino R, Ramirez P, Galeano J, Perez Garrido N, Rocco C, Ciaccio M et al. Steroid 21-hydroxylase gene mutational spectrum in 454 Argentinean patients: genotype-phenotype correlation in a large cohort of patients with congenital adrenal hyperplasia. Clin Endocrinol (Oxf). 2011;75(4):427-35.

29. Neocleous V, Shammas C, Phedonos AP, Karaoli E, Kyriakou A, Toumba M et al. Genetic defects in the CYP21A2 gene in heterozygous girls with premature adrenarche and adolescent females with hyperandrogenemia. Georgian Med News. 2012 Sep;(210):40-7.

Figure legends

Figure 1. 17 OHP levels in the four groups (classical CAH, NC CAH, heterozygous, and wild type)

1=classical CAH; 2=NC CAH; 3= heterozygous; 4= Wild Type; A. 17 OHP levels in sample 1; B. 17 OHP levels in sample 2.

CAH=congenital adrenal hyperplasia; NC= non classical; * $p<0.05$ versus all other groups

Figure 2. 17-OHP levels according to genotype (mutation severity group, heterozygous and wild type); A. 17 OHP levels in sample 1; B. 17 OHP levels in sample 2.

Genotypes (1=Group Null; 2=Group A; 3=Group B; 4=Group C; 5=Heterozygous; 6=Wild Type)

* $p <0.05$ versus all other groups

∂ $p <0.05$ versus groups 1 and 2

List of abbreviations:

CAH: Congenital adrenal hyperplasia; 17-OHP: 17 hydroxyprogesterone; SW: salt-wasting; SV: simple virilizing and nonclassic; NC: nonclassic, late onset; WT: wild-type; SPSS: Statistical Package for the Social Sciences.

Table 1. Clinical aspects and genotypes in classical, non-classical, and heterozygous CAH patients

	Patient nº	SEX	Weight (g)	Maternal corticoid use	Consanguinity	17 OHP (ng/mL) Sample 1	17 OHP (ng/mL) Sample 2	[NA+] (nmol/L)	[K+] (nmol/L)	Virilization/ Prader Scale	CAH Phenotype	Genotype	Mutation Group	Phenotype/ Genotype correlation
CLASSICAL CAH	1	M	3,640	no	yes	105.0	283.0	133	5.91	normal	SW	IVS2-13A/C>G/ IVS2-13A/C>G	A/A	yes
	2	F	3,325	no	yes	432.0	759.0	108	5.4	Prader IV	SW	Del CYP21A2/ LGC	null/null	yes
	3	F	2,490	no	no	44.9	94.9	132	5.58	Prader I	SV	IVS2-13A/C>G/ IVS2-13A/C>G	A/A	no
	4	F	2,040	no	no	512.0	521.0	126	5.34	Prader III	SW	Del 30Kb/ IVS-2-13A/C>G	null/A	yes
	5	F	3,200	yes	no	61.8	209.0	132	5.0	Prader I	SV	Del CYP21A2/ p.I172N	null/B	yes
	6	M	3,450	no	no	733.0	-	120	5.7	penis enlargement	SW	Del CYP21A2/ CLUSTER E6	null/null	yes
	7	M	2,980	no	no	461.0	489.0	109	6.54	normal	SW	Del CYP21A2/ p.R356W	null/null	yes
	8	M	3,295	yes	yes	382.0	-	126	6.6	normal	SW	IVS2-13A/C>G/ IVS2-13A/C>G	A/A	yes
	9	M	2,395	no	no	469.0	435.0	118	8.9	normal	SW	Dup CYP21P +IVS2-13A/C>G / LGC	null/A	yes
	10	F	2,270	yes	no	37.0	354.0	129	5.9	Prader IV	SW	IVS2-13A/C>G/ IVS2-13A/C>G	A/A	yes
	11	F	2,890	no	no	34.2	133.0	130	5.3	Prader I	SW	LGC+p.I172N	null/B	no
	12	F	3,580	no	no	459.0	-	131	6.0	Prader IV	SW	IVS2-13A/C>G/ IVS2-13A/C>G	A/A	yes
	13	F	3,730	no	yes	515.0	-	-	-	Prader IV	SW	p.Q318X/ p.Q318X	null/null	yes

	14	F	3,500	no	no	65.2	313.0	125	7.4	Prader III	SW	Dup CYP21P+ insT/ IVS2-13A/ C>G	null/A	yes
	15	M	2,340	no	no	32.5	58.0	131	5.85	penis enlage- ment	SV	p.I172N/p.I172N	B/B	yes
	mean ±SD/% positi- ve		3,008 3 (± . 588)	20%	27%	289.57∂ (± 240.13)	331.72∂ (± 210.37)	125±8.3 0∂	6.10±1. 01∂					86,6%
NON CLASSICAL CAH	16	M	3,465	Yes	No	2.8	46.30	135	5.11	Normal*	NC	p.V281L/p.281L	C	Yes
	17	M	3,340	Yes	No	4.16	15.1	136	5.25	Normal	NC	p.V281L/p.281L	C	Yes
	18	M	2,508	No	No	31.2	5.00	135	4.84	Normal	NC	p.Q318X/p.281L	C	Yes
	19	F	2,660	No	No	38.2	47.2	135	5.3	Normal	NC	p.P453S/p.P453S	C	Yes
	20	F	2,988	No	No	15.2	18.3	131	5.82	Normal	NC	p.Q318X/ p.R356W/InsT/ p.V281L	C	Yes
	21	M	2,830	No	No	25.8	15.1	129**	4.67	Normal	NC	IVS2-13A/C>G / p.V281L	C	Yes
	22	F	2,630	Yes	No	15.1	22.1	135	5.4***	Normal	NC	p.R356W/ p.V281L	C	Yes
	mean ±SD/% positi- ve		2,917 3 (± . 366)	43%	0%	18.92 (± 13.39)	24.16 (±16.28)	134.5±1. 76	5.16+0. 40					100%
HE	23	F	3,680	Yes	No	14.5	15.4	133	5.6	Normal	HT	p.Q318X/WT	HT	Yes
	24	M	2,750	No	No	18.3	16.9	135	5.96	Normal	HT	p.R356W/WT	HT	Yes
	25	F	2,605	Yes	No	24.5	16.9	134	-	Normal	HT	p.Q318X/WT	HT	Yes
	26	M	2,345	Yes	No	6.1	17.7	135	5.3	Normal	HT	p.V281L/WT	HT	Yes
	27	M	2,570	No	Yes	31.0	11.5	133	6.34***	Normal	HT	p.V281L/WT	HT	Yes
	28	M	2,535	Yes	No	36.8	9.0	134	5.11	Normal	HT	p.Q318X/WT	HT	Yes
	29	M	2,600	No	No	50.9	10.9	137	5.4	Normal	HT	p.R356W/WT	HT	Yes
	30	M	2,550	Yes	Yes	25.8	16.2	131	5.18	Normal	HT	p.V281L/WT	HT	Yes

HETEROZYGOUS	31	M	1,215	No	No	30.1	127.0†	-	-	Normal	HT	p.V281L/WT	HT	Yes
	32	F	2,504 ¥	No	No	35.3	10.4	134	4.95	transient clitoromegaly	suspected CAH §	p.V281L/WT	HT	No
	33	F	3,150	No	No	15.4	16.10	135	4.3	Normal	HT	p.Q318X/WT	HT	Yes
	34	F	2,560 ¥	Yes	No	22.4	16.7	137	5.15	transient clitoromegaly*	suspected CAH §	p.V281L/ DUPCYP21P	HT	No
	35	M	2,750	No	No	25.3	15.9	136	5.29	Normal	HT	IVS_T/WT	HT	Yes
	36	F	2,905 ¥	No	No	18.20	35.0	139	4.8	transient clitoromegaly	suspected CAH §	IVS_T/WT	HT	No
	mean ±SD/% positive		2,623 (± 535)	43%	14%	25.33 (± 11.21)	23.97 (± 30.28)	134.85±2.07	5.18±0.43					79%

∂ p<0.05 Group Classical CAH *versus* others

* mild hyperpigmentation described on first clinical examination; ** Na normalized in reanalysis (laboratory error assumed); *** hemolysis described on laboratory result

F (Female); M (Male); NC (Non Classical); HT (Heterozigous); CAH (Congenital Adrenal Hiperplasia); LGC (Large Gene Conversion)

§ Classical CAH Suspected on first pediatric endocrinologist clinical evaluation, not confirmed in follow up

¥(Case 10: Premature, 35w; Case 12: Term, 38 w; Case 14: Term, 39w)

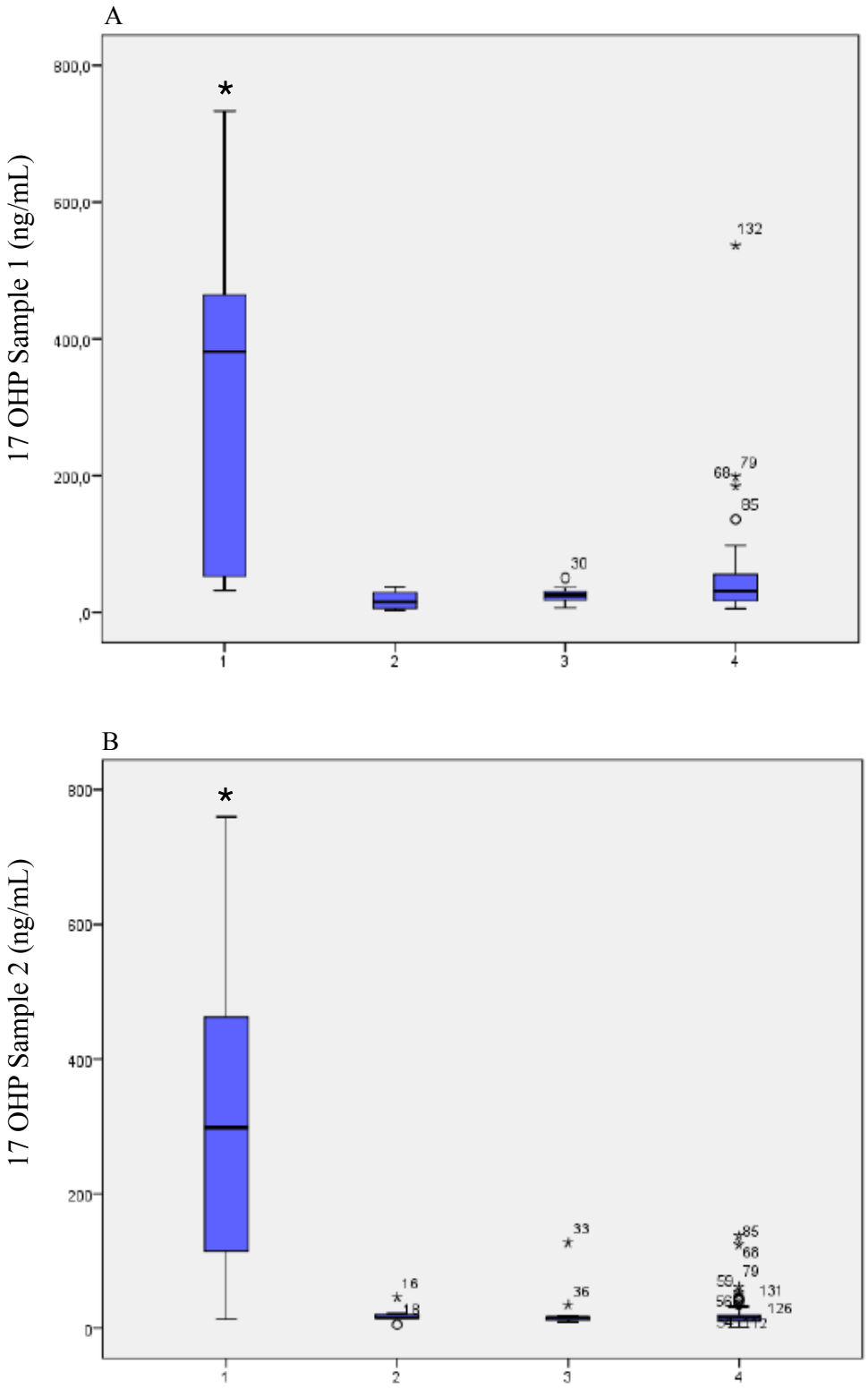
† Sample 3: 17.10 ng/mL

Table 2. Genotype frequency in classical CAH, non-classical, heterozygous and false positives patients

	Classical	CAH	Non Classical and	False Positives	
Total (n= 132)	**SW CAH (Mutation Group Null and A) (n=13)**	**SV CAH (Mutation Group B) (n=2)**	**NC CAH (Mutation Group c) (n=7)**	**Heterozigous (n=14)**	**WT (n=96)**
	Del CYP21A2 / Large gene conversion (n-1)	I172N /I172N (n=1)	IVS2-13A/C>G / V281L (n=1)	Q318X / WT (n=4)	
	DupCYP21P+InsT/IVS (n=1)	Del CYP21A2 / I172N (n=1)	P453S / P453S (n=1)	V281L / WT (n=5)	
	Del CYP21A2 / Cluster E6 (n=1)	IVS2-13A/C>G/IVS2-13A/C>G (n=1)*	Q319X; R357W; Leu307PhefsX6 / V281L (n=1)	R356W / WT (n=2)	
	Q318X / Q318X (n=2)		Q318X / V281L (n=1)	V281L/ DupCYP21P (n=1)	
	Del CYP21A2 / R356W (n=2)		R356W /V281L (n=1)	IVS_T/WT (n=2)	
	IVS2-13A/C>G / IVS2-13A/C>G (n=5)		V281L / V281L (n=2)		
	Del 30Kb / IVS2-13A/C>G (n=1)				
	DupCYP21P+IVS (n=1)				
	DupCYP21P+del30Kb (n=1)				
	Large gene conversion+I172N (n=1)*				
	* genotype-phenotype discordance				

CAH=Congenital Adrenal Hyperplasia; SW=Salt-Wasting; SV= Simple Virilizing; NC= Non-Classic; WT=Wild Type

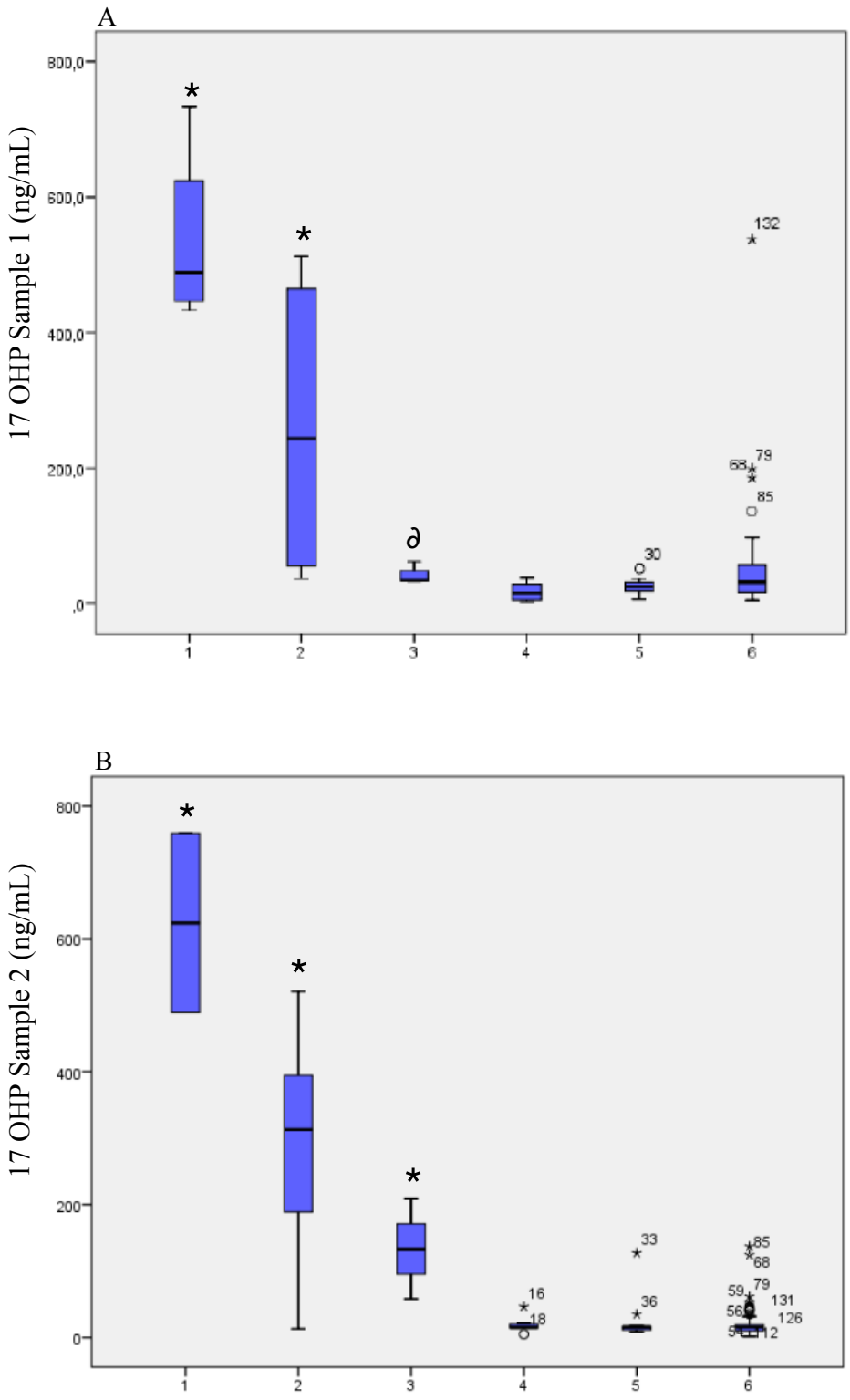
Figure 1. 17 OHP levels in sample 1 and 2 in the four groups (classical CAH, NC CAH, heterozygous and Wild Type)



1=classical CAH; 2=NC CAH; 3= heterozygous; 4= Wild Type

CAH=congenital adrenal hyperplasia; NC= non classical; * p<0.05 versus all other groups

Figure 2. 17 OHP levels in sample 1 and 2 according to genotype (mutation severity group, heterozygous and wild type)



Genotypes (1=Group Null; 2=Group A; 3=Group B; 4=Group C; 5=Heterozygous; 6=Wild Type)

* p<0.05 versus all other groups

∂ p<0.05 versus group 1 and 2

CAPÍTULO 4

ARTIGO 3

HORMONE RESEARCH IN PEDIATRICS

Novel Insights from clinical experience:

Severe craniosynostosis syndrome associated to salt wasting congenital adrenal hyperplasia

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Abstract

Craniosynostosis presents many challenges in etiology. One known form associated to steroidogenesis impairment is the Antley-Bixler Syndrome (ABS). ABS-phenotype without hormonal impairment is associated to *FGFR* mutations, whereas those with altered steroidogenesis related to P450 oxidoreductase deficiency, with mild to moderate 17OH progesterone (17-OHP) elevation and basal normal cortisol levels.

Case presentation: a term newborn, male phenotype, with a severe craniosynostosis, hand and feet malformation, presented early respiratory insufficiency and needed mechanic ventilation. At 15 days, there was clinical suspicion of adrenal insufficiency. Hydrocortisone was initiated and 3 days later also fludrocortisone, with further clinical improvement. At neonatal screening 17-OHP levels were 733 ng/mL. Salt-wasting congenital adrenal hyperplasia (CAH-SW) diagnosis was performed. At 42 days, patient was transferred to a tertiary complexity hospital. Cranial CT and MRI showed severe medium face hypoplasia, posterior fossa with Arnold-Chiari type 1 and cloverleaf skull, suggesting syndromic aspects. Early neurosurgical approach was performed.

Conclusion: A severe craniosynostosis syndrome with clinical aspects of *FGFR* mutations associated to SW-CAH in a different presentation than in ABS had never been described before. It may indicate an overlap of two rare conditions or possible interaction between *FGFR* and steroidogenesis.

Established facts

Antley Bixler phenotype and normal steroidogenesis is associated to *FGFR* mutations, whereas the same phenotype with steroidogenesis impairment is linked to P450 oxidoreductase deficiency.

Novel insights

A severe syndrome with clinical aspects of *FGFR* mutations craniosynostosis and classical salt-wasting CAH with *CYP21A2* mutation in a different clinical presentation than in ABS may indicate overlap of two rare conditions or possible interaction between *FGFR* and steroidogenesis.

Introduction

Craniosynostosis is defined as the premature fusion of the cranial sutures and presents many challenges in etiology (1). The overall prevalence of craniosynostosis has been estimated to be approximately 1 in 2,500 births. It is a very heterogeneous disease and its causes and presentation varies from isolated to syndrome-related condition (1). The Fibroblast Growth Factor Receptor (FGFR) family is associated with up to half of single gene disorders. The *FGFR2* gene is mutated in 32% of all genetic cases. Heterozygous mutations of *FGFR2* cause three classical craniosynostosis syndromes: Apert, Crouzon and Pfeiffer. Other genes commonly mutated in craniosynostosis are *FGFR3*, *TWIST1* and *EFNB1* (1,2). Some clinically non-syndromic synostosis (usually affecting the coronal suture) can be caused by single gene mutations in *FGFR3* (1). Much rarer, craniosynostosis are associated to P450 oxidoreductase (*POR*) gene mutation, leading to a condition called Antley-Bixler syndrome (ABS) (1-3). ABS-phenotype with normal steroidogenesis is associated to *FGFR* mutations (2), whereas those with ambiguous genitalia and altered steroidogenesis are due to POR deficiency (PORD), a rare cause of congenital adrenal hyperplasia (CAH) with mild to moderate 17 OH progesterone (17-OHP) elevation and basal normal cortisol levels (3, 4). CAH is most caused due to *CYP21A2* mutations, around 90%, and its incidence in general population is around 1:10,000 to 18,000 (5, 6). Other enzymatic impairments are much rarer. POR is in fact a co-enzyme to 21- hydroxylase and 17-hydroxylase. PORD is a disorder of steroidogenesis with a phenotypic spectrum ranging from cortisol deficiency at the milder end to classic ABS at the severe end (3, 4). While classic CYP21 deficiency male patients have normal genital development at birth, PORD is associated to poor masculinization and maternal virilization during pregnancy with an affected fetus (3, 4). Manifestations of ABS also include skeletal anomalies, renal anomalies and other associated malformations (4).

Case description and results

A term newborn, normal weight (3,450g), male phenotype, third child of a non consanguineous couple, was born with a severe craniosynostosis (turribrachycephalic skull shape), extreme ocular proptosis (unable to close eyelids) (Figure 1), hand and feet malformation (Figure 2). Mother referred normal gestation, discovered at 20 weeks, without virilization at any time and an obstetric ultrasound suggesting skull malformation. The newborn had Apgar score 3 and 7 and presented early respiratory insufficiency, evolving to mechanic ventilation and admitted at intensive neonatal unit care in a secondary medical center. At 15 days of life, there was clinical suspicion of adrenal insufficiency due to skin pigmentation and lowering of serum sodium. Hydrocortisone (HC) was initiated and 3 days later also fludrocortisone, with further normalization of electrolytes. Neonatal screening was collected after 1 dose of HC and 17 hydroxyprogesterone (17-OHP) levels from dried blood on filter paper were 733 ng/mL. Salt-wasting congenital adrenal hyperplasia (CAH) diagnosis was made. At 42 days of life, due to worsening of upper respiratory distress, patient was transferred to a tertiary complexity hospital. At the admission, signs of macrogenitossomia and cutaneous hyperpigmentation were seen (Figure 3. A). Hormones levels were according to clinical evaluation, with ACTH 839 pg/mL (normal <46) / 17-OHP 111.7 ng/mL (normal range 0.8-5.0). Patient improved clinical and laboratory parameters after glucocorticoid adjustment (Figure 3. B). No signs of radioulnar or other joint synostoses or skeletal fractures were clinically or radiologically observed in this patient. Computed tomography (CT scan) and magnetic resonance (MRI) showed intense demineralization compatible with craniolacunia (Figure 4), severe medium face hypoplasia, corpus callosum and septum pellucidum absence, posterior fossa with Arnold -Chiari type 1 and cloverleaf skull, suggesting Crouzon or Pfeiffer Syndrome aspects, according to careful radiological evaluation (Figure 5). To avoid compressive complications of craniosy-

nostosis, an early neurosurgical approach was performed, after careful evaluation by a multi-disciplinary team at three and a half months old. Patient evolves favorably after surgery but was continuously dependent on oxygen therapy and tracheostomy was required. He passed at 5 months and 20 days due to complications from respiratory infection, even with the use of appropriate doses of glucocorticoids in stress situations.

A further genetic analysis was performed with informed parental consent. By sequencing technique two mutations have been identified in Cluster 6 (p.I236N and p.V237E) homozygous and from molecular analysis, commercial kit Multiplex ligation-probe Amplification (MLPA) P050 CAH, a deletion of the *CYP21A2* gene in heterozygous was detected. The combination of these mutations is known and identified in patients with the classic form of salt-wasting CAH. Analysis of the *POR* gene was performed by PCR and a single heterozygous was found in intron 2, without pathogenic meaning. The *FGFR2* gene has not been studied so far.

Discussion

ABS, first described in 1975 by the authors that originated the syndrome name (7), was the first hypothesis outlined, since Miller and collaborators have described the association of cranyossinostosis and impaired steroidogenesis due to PORD in 2004 (8). The ABS represents the severe end of the spectrum of syndromic craniosynostosis. Many patients have choanal atresia and severe respiratory distress, often resulting in early death (9). Reardon and collaborators described an overlapping in ABS and Pfeiffer syndrome phenotypes, arising the hypothesis of digenic inheritance for ABS (8), although it became further clear that POR mechanisms also are involved in ABS phenotype (3,8). However, when differential diagnosis of syndromes with craniosynostosis (1,2,4) are compared (Table 1), the phenotypic aspect, the type of cranial malformation, the severity of craniosynostosis and malformations of hands and

feet refers to the diagnosis of Pfeiffer syndrome (2). But, in this case, the clinical and laboratory presentation besides the molecular confirmation of mutation in the *CYP21A2* gene confirms the diagnosis of classic salt-wasting CAH (10-12). In addition, according to the six domains presented by Krone and collaborators for the PORD diagnosis (4), the presented case fulfilled criteria in all, except in femoral arching (Table 2). And according to these authors, clinical presentation varies among PORD patient.

Interestingly, in 1977, Bixler, the same author that described ABS two years before, also described an unusual association of Saethre-Chotzen syndrome and CAH (13). This syndrome is typically caused by *TWIST1* mutations, but a family with phenotypic features of Saethre-Chotzen syndrome and normal TWIST1 sequence analysis had the *FGFR2* mutation, suggesting that the *TWIST1* and *FGFR* products may interact during development (14).

Since more cases have been studied by different groups (3,4,8,9), the overlap between *POR* and *FGFR2* mutations may exist. A severe craniosynostosis syndrome with clinical aspects of *FGFR2* mutations, more specifically, Pfeiffer syndrome phenotype associated to salt-wasting CAH with confirmed *CYP21A2* mutations in a different presentation than in ABS, had never been described before. According to Cragun & Hopkin (16) and confirmed by Miller (17), the ABS is not appropriate for this related condition, since it is clear that the severity and other dysmorphologic findings lead to a *FGFR2*-related craniosynostosis syndrome. It may indicate an overlap of two rare conditions (*CYP21A2* and *FGFR2* mutations) or possible interaction between *FGFR* products and steroidogenesis.

References

1. Johnson D, Wilkie AO. Craniosynostosis. Eur J Hum Genet. 2011 Apr;19(4):369-376.

2. Robin NH, Falk MJ, Haldeman-Englert CR. FGRR-Related Craniosynostosis Syndromes. In: Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, Bird TD, Fong CT, Mefford HC, Smith RJH, Stephens K, editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2016.1998 Oct 20 [updated 2011 Jun 7].

3. Flück CE, Tajima T, Pandey AV, Arlt W, Okuhara K, Verge CF, Jabs EW, et al: Mutant P450 oxidoreductase causes disordered steroidogenesis with and without Antley-Bixler syndrome. Nat Genet 2004; 36: 228–230.

4. Krone N, Reisch N, Idkowiak J, Dhir V, Ivison HE, Hughes BA, Rose IT, et al: Genotypephenotype analysis in congenital adrenal hyperplasia due to P450 oxidoreductase deficiency. J Clin Endocrinol Metab 2012; 97: E257–E267.

5. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Endocr Rev. 2000 Jun;21(3):245-291.

6. Speiser PW, Azziz R, Baskin LS, Ghizzoni L, Hensle TW, Merke DP, Meyer-Bahlburg HF, et al: Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 2010; 95: 4133–4160.

7. Antley R, Bixler D.Trapezoidocephaly, midfacial hypoplasia and cartilage abnormalities with multiple synostoses and skeletal fractures.Birth Defects Orig Artic Ser. 1975;11(2): 397-401.

8. Miller WL. P450 oxidoreductase deficiency. A new disorder of steroidogenesis with multiple clinical manifestations. Trends Endocrinol Metab 2004; 15: 311–315.

9. Reardon W, Smith A, Honour JW, Hindmarsh P, Das D, Rumsby G, Nelson I, Malcolm S, Adès L, Sillence D, Kumar D, DeLozier-Blanchet C, McKee S, Kelly T, McKeehan WL, Baraitser M, Winter RM. Evidence for digenic inheritance in some cases of Antley-Bixler syndrome? J Med Genet. 2000; 37(1):26-32.

10. Bachega TA, Billerbeck AE, Parente EB, Lemos-Marini SH, Baptista MT, Mello MP, Guerra G Jr, Kuperman H, Setian N, Damiani D, Torres N, Castro Md, Mendonça BB. Multicentric study of Brazilian patients with 21-hydroxylase deficiency: a genotype-phenotype correlation. Arq Bras Endocrinol Metabol. 2004 Oct;48(5): 697-704.

11. New MI, Abraham M, Gonzalez B, Dumic M, Razzaghy-Azar M, Chitayat D, Sun L, Zaidi M, Wilson RC, Yuen T. Genotype-phenotype correlation in 1,507 families with congenital adrenal hyperplasia owing to 21-hydroxylase deficiency. Proc Natl Acad Sci U S A 2013;110 (7):2611-2616.

12. Krone N, Rose IT, Willis DS, Hodson J, Wild SH, Doherty EJ, Hahner S, Parajes S, Stimson RH, Han TS, Carroll PV, Conway GS, Walker BR, MacDonald F, Ross RJ, Arlt W; United Kingdom Congenital adrenal Hyperplasia Adult Study Executive (CaHASE). Genotype-phenotype correlation in 153 adult patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency: analysis of the United Kingdom Congenital adrenal Hyperplasia Adult Study Executive (CaHASE) cohort. J Clin Endocrinol Metab. 2013; 98(2):E346-54.

13. Escobar V, Brandt IK, Bixler D. Unusual association of Saethre-Chotzen syndrome and congenital adrenal hyperplasia.Clin Genet. 1977 May;11(5):365-371.

14. Freitas EC, Nascimento SR, de Mello MP, Gil-da-Silva-Lopes VL. Q289P mutation in FGFR2 gene causes Saethre-Chotzen syndrome: some considerations about familial heterogeneity. Cleft Palate Craniofac J. 2006;43:142–147.

15. Huang N, Pandey AV, Agrawal V, Reardon W, Lapunzina PD, Mowat D, Jabs EW, Van Vliet G, Sack J, Flück CE, Miller WL (2005) Diversity and function of mutations in P450 oxidoreductase in patients with Antley-Bixler syndrome and disordered steroidogenesis. Am J Hum Genet 76:729–749

16. Cragun D, Hopkin RJ. Use of the term "Antley-Bixler syndrome": minimizing confusion.Am J Hum Genet. 2005 Aug;77(2):327-8; author reply 328-40.

17. Miller WL. “Antley-Bixler Syndrome”—A Reply to Cragun and Hopkin. Am. J. Hum. Genet. ; 77 (2):reply 328-330.

Legends of Figures

Figure 1: Craniosynostosis, turribrachycephaly and eyes proptosis

Figure 2: Hand an foot malformation

Figure 3: (A and B): Genital appearance before and after glucocorticoid adjustment

Figure 4: Early suture closing and bone porosity on CT

Figure 5 : Turribrachycephalic shape and ocular proptosis on MRI

Table 1. Differential diagnosis of syndromes with craniosynostosis

Syndrome	*FRGR* Mutations	Thumbs/Toes	Hand and Feet	Impaired Steroidogenesis
CROUZON	Yes	normal	normal	No
PFEIFFER	Yes	medial deviation	brachydactyly	No
ANTLEY BIXLER	Yes	arachnodactyly	arachnodactyly	No
PORD/ANTLEY BIXLER	No	arachnodactyly	arachnodactyly	Yes

Table 2. The six malformation domains for POR deficiency malformations

DOMAIN/ SCORE	Midface hypoplasia	Craniosynostosis	Hand and Feet MF	Large joints synostosis	Femoral arching	Additional MF
0	none	none	none	none	none	none
1	moderate (low implanted ears, pear shaped nose)	moderate	1	extension deficit	present	1 additional MF
2	severe (structures compression, proptosis)	severe (2 or more sutures, turrincephaly)	2	fixed synostosis, contracture of a large joint	neonatal fractures	2 or more additional MF
3	complicated by stenosis or choanal atresia, tracheostomy	complicated by hydrocephalus, need for ventriculoperitoneal shunt	3 or more	various joints contracture	-	-

MF= malformation

Figure 1: Craniosynostosis, turribrachycephaly and eyes proptosis

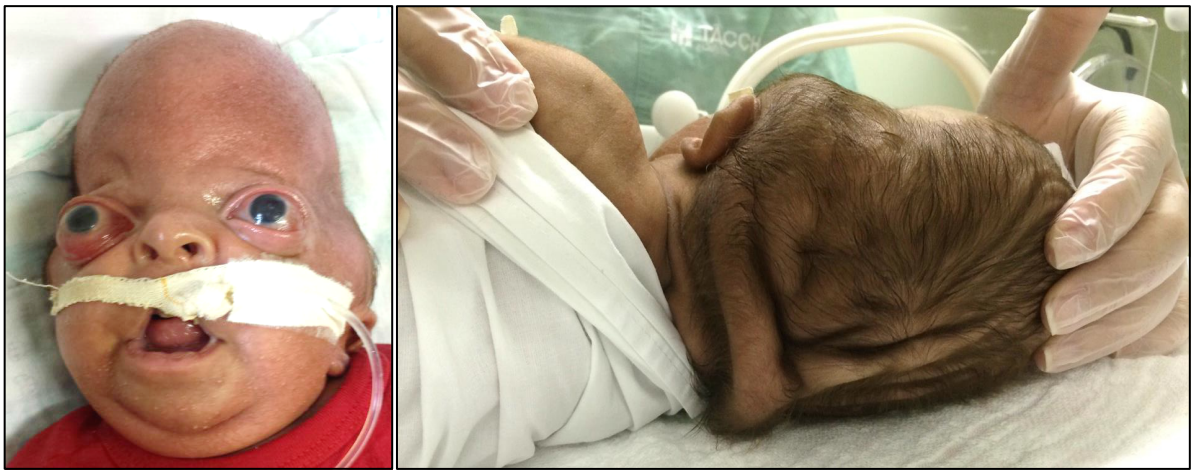


Figure 2: Hand an foot malformation

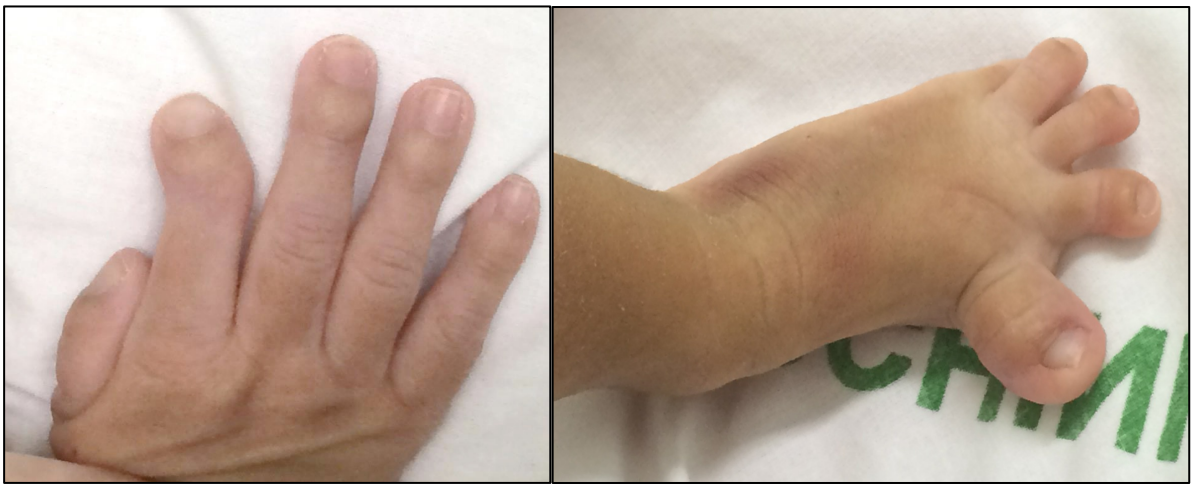


Figure 3: (A and B): Genital appearance before and after glucocorticoid adjustment

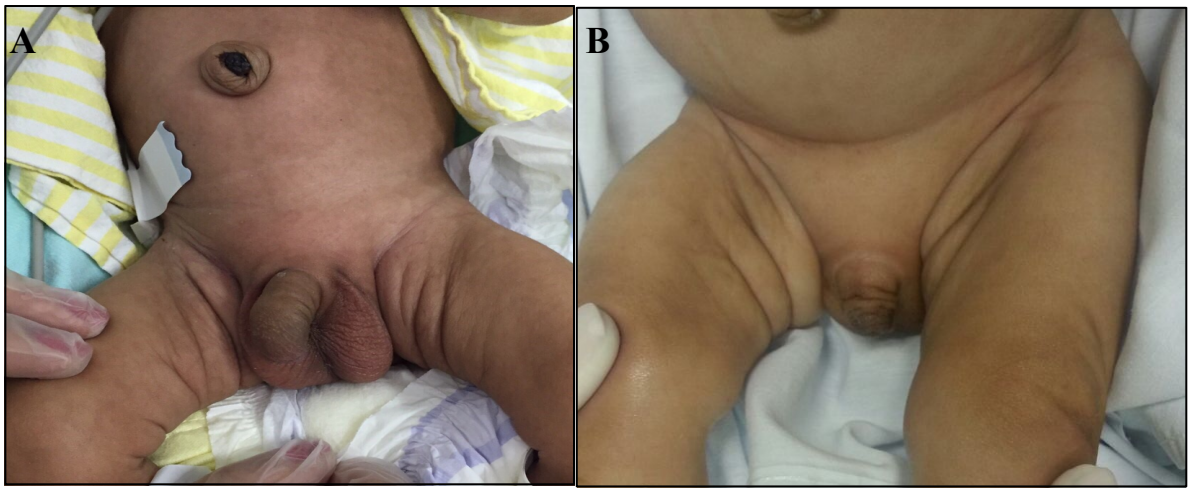


Figure 4: Early suture closing and bone porosity on CT

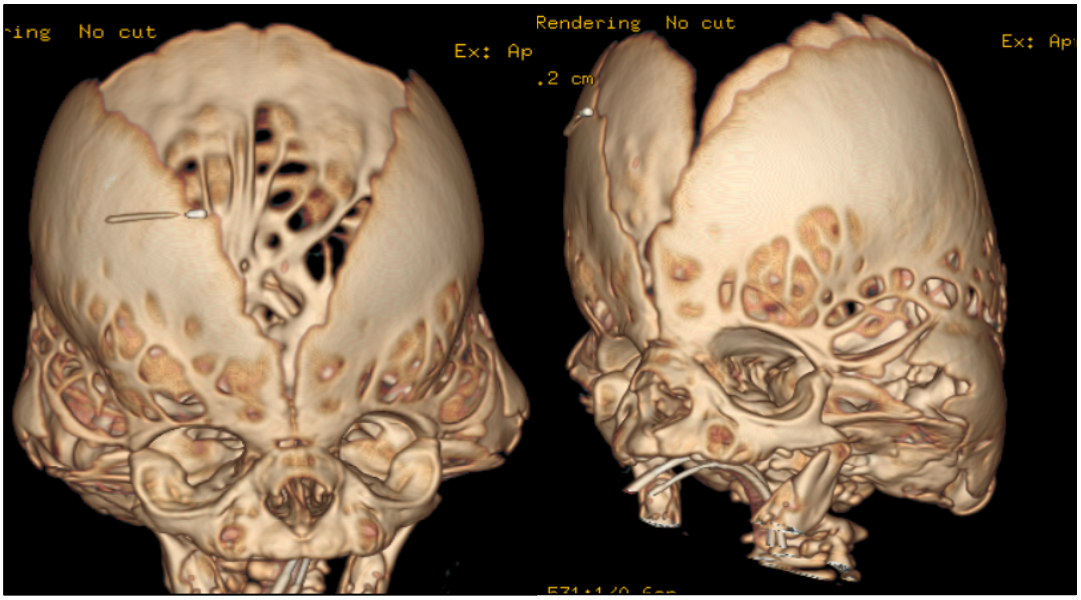
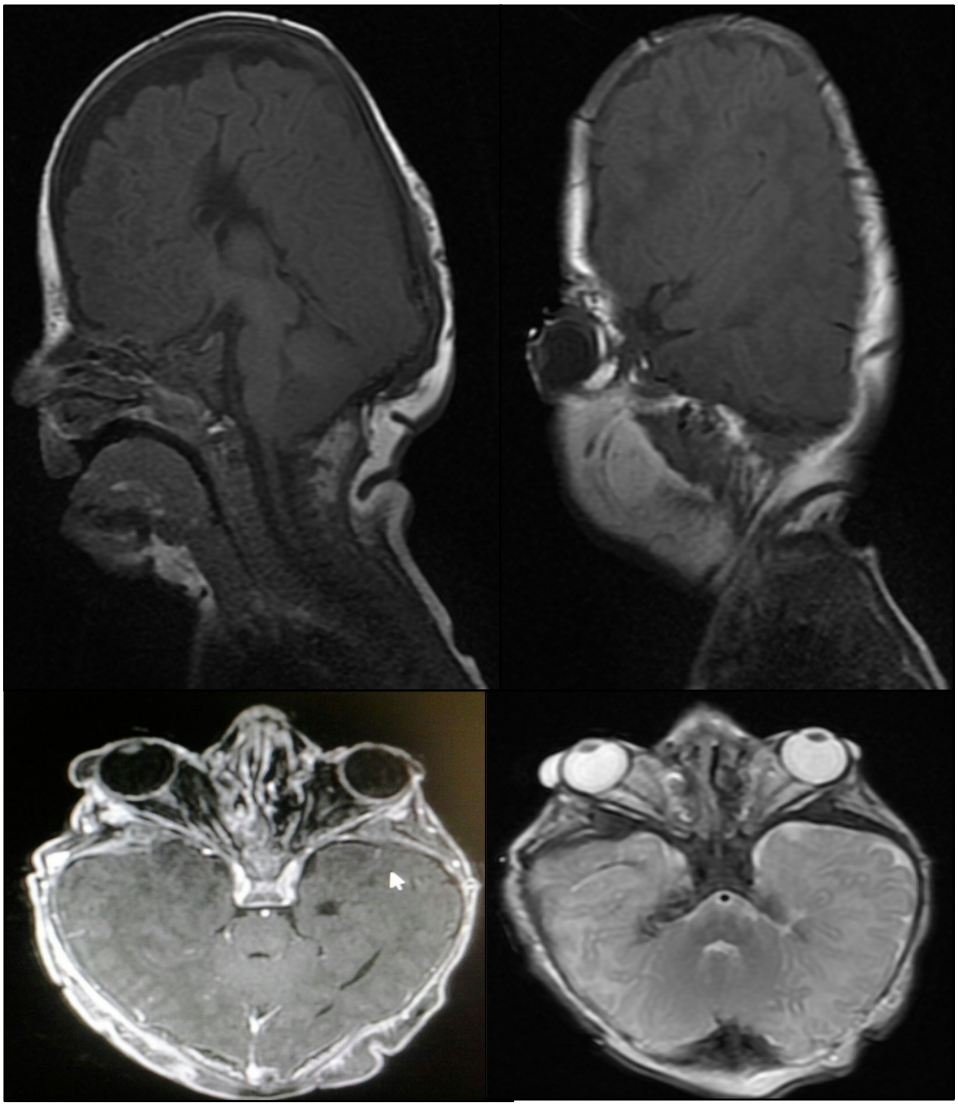


Figure 5 : Turribrachycephalic shape and ocular proptosis on MRI



CAPÍTULO 5

CONSIDERAÇÕES FINAIS

Participar do início de um programa de triagem de uma doença rara e complexa, como a Hiperplasia Adrenal Congênita, no contexto público, pode gerar grande ansiedade e desafio. Por outro lado é estimulante ter a possibilidade de acompanhar o seu andamento e perceber os seus frutos, quer nos diagnósticos realizados, quer nas cooperações técnicas, quer na gratidão das famílias atendidas.

É gratificante perceber que após 24 meses da Triagem da Hiperplasia os resultados são positivos e que o programa está alinhado com as estratégias do Programa Nacional de Triagem e do Ministério da Saúde. A incidência da doença encontrada no RS muito próxima de outros estados com população de origem semelhante, bem como em consonância com dados internacionais corrobora a eficácia da Triagem Neonatal da HAC em nosso meio. A estratégia de se adotar pontos de corte da 17 OH progesterona estratificada para o peso de nascimento é de inequívoca eficácia. Os dados apresentados ainda que um reteste para todos os bebês com primeiro screening alterado auxilia muito na diferenciação entre casos e falsos-positivos, especialmente para bebês prematuros e na faixa de peso abaixo de 2000g. Se ainda permanece alterada a segunda amostra, o genótipo é uma ferramenta adicional e assertiva, e a metodologia desenvolvida de Mini-sequenciamento Multiplex (SnapShot) muito adequada para um programa populacional. Em conjunto, essas estratégias permitem o diagnostico e tratamento assertivos, além de discriminar a severidade dos casos e evitar o tratamento indevido de falsos-positivos. Já o diagnóstico de formas não clássicas no período neonatal é controverso. Mas ainda assim, pode-se evitar hiper tratamentos também neste grupo de pacientes. E acom-

panha-los desde o nascimento pode acrescentar novas informações sobre o perfil hormonal e a história natural da doença nesta forma específica de HAC.

Os dados de incidência e genótipo são inéditos para a população do Rio Grande do Sul e para a comunidade científica nacional e internacional. Além disto, são os primeiros resultados brasileiros sobre HAC após a implementação da fase IV do Programa Nacional de Triagem Neonatal.

Contudo, alguns desafios ainda permanecem, como melhora no tempo de coleta da primeira amostra, mais informação sobre a doença e a importância da triagem neonatal para a comunidade, além de conhecimento médico mais difundido sobre o reconhecimento dos sintomas clínicos e acompanhamento próximo aos pacientes e familiares. Além disso, uma vez que o fluxograma de detecção da doença e a metodologia para o genótipo foi implantada com sucesso neste programa de triagem, pode servir como uma ferramenta adicional e mais amplamente disponível em casos selecionados após o reteste ou confirmação da doença clínica. E, certamente, outros frutos deste trabalho surgirão à medida que vai sendo aperfeiçoado e ampliado.