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Second-Tier Testing for 21-Hydroxylase Deficiency in the Netherlands: A Newborn Screening Pilot Study

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Abbreviations: 17-OHP, 17-hydroxyprogesterone; 21-DF, 21-deoxycortisol; 21-OHD, 21-hydroxylase deficiency; CAH, congenital adrenal hyperplasia; DBS, dried blood spot(s); GA, gestational age; IQR, interquartile range; LC-MS/MS, liquid chromatography–tandem mass spectrometry; NBS, newborn screening; NC, non-classic; PPV, positive predictive value; SV, simple-virilizing; SW, salt-wasting.

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Abstract

Context: Newborn screening (NBS) for classic congenital adrenal hyperplasia (CAH) consists of 17-hydroxyprogesterone (17-OHP) measurement with gestational age–adjusted cutoffs. A second heel puncture (HP) is performed in newborns with inconclusive results to reduce false positives.

Objective: We assessed the accuracy and turnaround time of the current CAH NBS algorithm in comparison with alternative algorithms by performing a second-tier 21-deoxycortisol (21-DF) pilot study.

Methods: Dried blood spots (DBS) of newborns with inconclusive and positive 17-OHP (immunoassay) first HP results were sent from regional NBS laboratories to the Amsterdam UMC Endocrine Laboratory. In 2017-2019, 21-DF concentrations were analyzed by LC-MS/MS in parallel with routine NBS. Diagnoses were confirmed by mutation analysis.

Results: A total of 328 DBS were analyzed; 37 newborns had confirmed classic CAH, 33 were false-positive and 258 were categorized as negative in the second HP following the current algorithm. With second-tier testing, all 37 confirmed CAH had elevated 21-DF, while all 33 false positives and 253/258 second-HP negatives had undetectable 21-DF. The elevated 21-DF of the other 5 newborns may be NBS false negatives or second-tier false positives. Adding the second-tier results to inconclusive first HPs reduced the number of false positives to 11 and prevented all 286 second HPs. Adding the second tier to both positive and inconclusive first HPs eliminated all false positives but delayed referral for 31 CAH patients (1-4 days).

Conclusion: Application of the second-tier 21-DF measurement to inconclusive first HPs improved our CAH NBS by reducing false positives, abolishing the second HP, and thereby shortening referral time.

Key Words: neonatal screening, CAH, second tier, 21deoxycortisol

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders of adrenal steroid synthesis. Ninety-five percent of CAH cases are caused by 21-hydroxylase deficiency (21-OHD) due to mutations in the *CYP21A2*-gene. In the remaining 5% of cases, other enzyme deficiencies are the cause of CAH, with 11β-hydroxylase deficiency as the most common one. In classic CAH due to 21-OHD, the conversion of 17-hydroxyprogesterone (17-OHP) to 11-deoxycortisol is limited, leading to deficient production of cortisol and in 75% of cases also of aldosterone, and a concomitant increase of precursor steroids, notably 17-OHP. Patients with aldosterone deficiency are classified as the salt-wasting (SW) phenotype and are at risk of life-threatening hyponatremia, hyperkalemia, and acidosis. Furthermore, hypocortisolism may predispose to life-threatening hypoglycemia. The lack of cortisol also leads to an increased secretion of adrenocorticotropic hormone (ACTH) by the pituitary gland. This in turn causes stimulation and hyperplasia of the adrenal cortex, which subsequently results in the excessive production of precursor steroids, which shunt into the nonaffected androgen pathway. Other clinical phenotypes include simple-virilizing (SV) CAH, with a residual enzymatic activity of 1% to 2 % and sufficient aldosterone levels to maintain sodium balance, and the mildest form,

non-classic (NC) CAH, with generally normal cortisol, but still slightly increased androgen production (1). The occurrence of life-threatening salt-wasting and hypoglycemia, the availability of a screening test (measurement of 17-OHP) and possibility of lifesaving in-hospital treatment led to the inclusion of the disorder in newborn screening (NBS) programs (1-4).

In 2002, CAH was included in the Dutch NBS program. The CAH NBS is based on a 17-OHP measurement in dried blood spots (DBS) on filter paper using an immunoassay (5). The cutoff values of 17-OHP are based on gestational age (GA) or birth weight (6). The algorithm has been refined by using a second heel puncture in case of an inconclusive first heel puncture (see Fig. 1), preventing false-positive referrals, at the cost of delaying the time until diagnosis. Despite this sophisticated algorithm, a substantial number of children are still referred with a false-positive NBS result due to the nonspecificity of the 17-OHP measurement. In a recent evaluation of the Dutch CAH NBS in the period 2002–2013, the positive predictive value (PPV) was 24.7% (7). 17-OHP levels are increased in prematurity or illness (1, 8, 9), but the measured concentrations can also be falsely elevated because of crossreactivity in the immunoassay (10, 11). Therefore, alternative methods such as the addition of a second-tier measurement have been described to improve the specificity. In CAH due to 21-OHD, the unaffected enzyme 11β-hydroxylase catalyzes the conversion of 17-OHP into 21-deoxycortisol (21-DF) (12). In healthy persons, the 21-DF concentration is undetectable, but in 21-OHD, the 21-DF is increased due to the increased conversion of elevated 17-OHP. 21-DF is a specific and promising biochemical marker for 21-OHD but is unsuitable as a primary marker in the NBS program due to the long analysis time. Therefore, 21-DF has been suggested as a useful second-tier marker for CAH (13). Second-tier profiling of 21-DF and other steroid precursors by liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been introduced in other NBS programs, resulting in significant improvement of the PPV (14, 15). However, less favorable results have also been reported; for example, Sarafoglou et al did not show a reduction of false-positive cases (16). In the Netherlands, reference intervals for steroids measured in DBS based on GA have recently been determined and 21-DF was confirmed as the only specific marker for 21-OHD, correctly identifying 8 genetically confirmed patients in a cohort of 92 screened neonates with positive NBS results (17). Implementation of 21-DF as a second-tier marker in all inconclusive first heel puncture results could remove the need for a second heel puncture. Moreover, application of the second-tier test to all positive and inconclusive first heel puncture results could potentially eliminate the false-positive referrals caused by the 17-OHP measurement, although an increased turnaround time due to the execution of the second-tier test could delay referral.

With the current prospective pilot study, we aimed to (1) assess the accuracy of the current routine CAH NBS algorithm, and compare this to the accuracy of alternative algorithms with the addition of 21-DF as a second-tier test in either inconclusive or both positive and inconclusive first heel punctures; and (2) establish the turnaround time of the second-tier test in the CAH NBS practice in the Netherlands.

Methods

Newborn Screening Procedures

In the Netherlands, NBS samples are collected on filter paper type S&S 903 (Schleicher & Schuell, Dassel, Germany) between 72 and 168 hours after birth and then sent by regular mail to 1 of the 5 regional NBS laboratories, following the standard procedure. NBS is performed on the day of receipt. 17-OHP in the DBS is measured by the GSP Neonatal 17α-OH-progesterone immunoassay (Perkin Elmer, MA, USA) according to the manufacturers protocol. NBS results are interpreted with the use of different cutoff values for 4 GA groups expressed in weeks + days (≤ 33 + 0; 33 + 1 to 35 + 0; 35 + 1 to 36 + 0; and ≥ 36 + 1). Figure 1 shows the cutoff values leading to a positive or inconclusive result. If GA is unknown, birth weight is used. In principle, the aim of this NBS algorithm is to detect classic CAH (SW and SV forms) and not the newborns with milder NC CAH (classified as false-positive). A positive result leads to immediate referral to a pediatric endocrinologist. In case of an inconclusive result, a second heel puncture has to be performed 7 to 9 days later (or 14 to 16 days later in cases where GA ≤ 33 + 0). A second inconclusive result leads to referral. After referral, the pediatric endocrinologist performs diagnostic tests according to the national protocol. The diagnosis is always confirmed by *CYP21A2* mutation analysis. Based on this algorithm, 3 groups of newborns can be defined in this study: group 1, true-positive referrals, defined as patients with a confirmed CAH diagnosis at referral; group 2, false-positive referrals, defined as newborns without the diagnosis CAH after additional diagnostic tests at referral (caused by either a positive first heel puncture or an inconclusive first heel puncture with referral based on the second heel puncture); and group 3, NBS negatives, defined as newborns with a negative second heel puncture after an inconclusive first heel puncture. Group 3 is not referred to a pediatric endocrinologist and assumed CAH NBS negative. The clinical evaluation and the definitive

Group 1. True-positive identified at referral (n=37)

GA category (weeks+days)	17-OHP (nmol/L blood) 1st heel puncture				
	0-24	25-54	55-104	105-199	≥ 200
≤ 33+0					0
33+1 - 35+0				0	0
35+1 - 36+0			0	0	1
≥ 36+1		6	8	7	15

Group 2. False-positive identified at referral (n=33)

GA category (weeks+days)	17-OHP (nmol/L blood) 1st heel puncture				
	0-24	25-54	55-104	105-199	≥ 200
≤ 33+0					2
33+1 - 35+0				0	1
35+1 - 36+0			3	3	0
≥ 36+1		17	5	2	0

Group 3. Negative 2nd HP results (n=258)

GA category (weeks+days)	17-OHP (nmol/L blood) 1st heel puncture				
	0-24	25-54	55-104	105-199	≥ 200
≤ 33+0					68
33+1 - 35+0				12	
35+1 - 36+0			12		
≥ 36+1		166			

Figure 1. Dutch CAH NBS algorithm and distribution of first heel puncture 17-OHP results of cases in each GA-based category, divided into 3 groups; 1) true-positive referrals, 2) false-positive referrals, and 3) NBS negatives (newborns with a negative second heel puncture). 17-OHP concentrations are interpreted based on GA. White fields correspond to a negative result, light-gray fields to an inconclusive result leading to a second heel puncture, and gray fields to a positive result with immediate referral. This algorithm leads to identification of 3 groups: Group 1, patients with the diagnosis CAH confirmed at referral by additional diagnostic tests; group 2, false-positive referrals, defined as newborns without the diagnosis CAH after additional diagnostic tests (caused by either a positive first heel puncture or an inconclusive first heel puncture with referral based on the second heel puncture); and group 3, NBS negatives, defined as newborns with inconclusive first heel puncture results but negative second heel puncture results. Abbreviations: 17-OHP, 17-hydroxyprogesterone; CAH, congenital adrenal hyperplasia; GA, gestational age; HP, heel puncture; NBS, newborn screening.

diagnosis are documented in the national NBS registry (www.neorah.nl). In case a newborn with CAH is missed by NBS, this is reported as a false-negative result to the Dutch Pediatric Surveillance System (www.nsck.nl) and also documented in the national NBS registry.

Study Procedures

From January 1, 2017, until December 31, 2019, DBS of all neonates with inconclusive and positive first heel puncture results were sent from the regional laboratories to the Endocrine Laboratory of the Amsterdam UMC, location AMC (Amsterdam, the Netherlands), 1 of the 5 NBS laboratories. Here, 21-DF concentrations were analyzed by LC/MS-MS using an Acquity UPLC connected to a Xevo TQS mass spectrometer (both Waters, MA, USA). As described in a previous study, the lower limit of quantitation of 21-DF is 1.0 nmol/L blood (17). In the current study, a 21-DF level of ≥ 1 nmol/L blood was considered elevated and thus as a positive second-tier result. To study turnaround time, during the year 2019, all laboratories sent these DBS by regular mail on all working days as soon as the initial NBS result was known. Subsequently, the Endocrine Laboratory was notified so immediate steroid-profiling could be organized for the next day. All newborns with positive NBS results were referred as usual, without considering the second-tier results. Also, second heel punctures were drawn after inconclusive results, following the Dutch NBS protocol, parallel to the second-tier analyses. A schematic overview of the CAH NBS and second tier study protocol is given in Fig. 2.

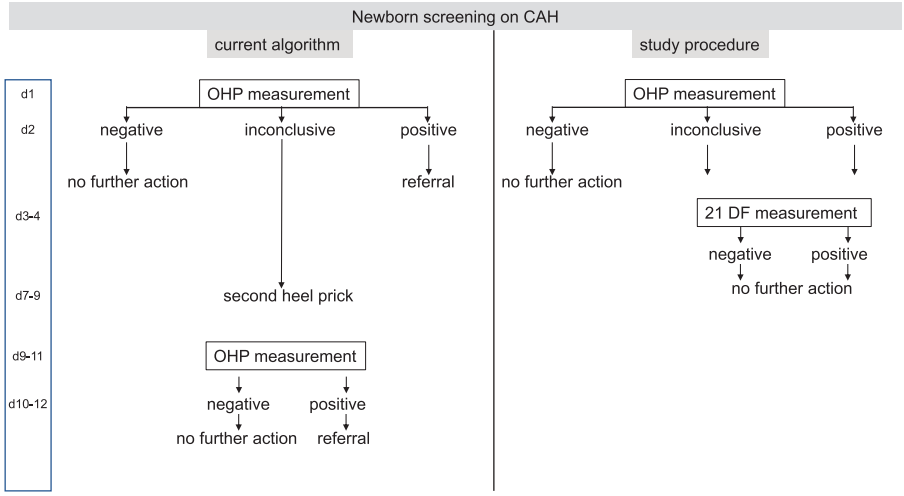

Figure 2. A schematic overview of the routine CAH NBS and the study procedure. Days are given at the vertical axis. Day 1 is the day the heel prick has been received at the screening laboratory and the measurement has been performed. Abbreviations: 21-DF, 21-deoxycortisol; CAH, congenital adrenal hyperplasia; OHP, 17-hydroxyprogesterone.

Data Collection and Exclusion Criteria

Permission for conducting this research project was given by the Dutch Research Working Group for the Neonatal Heel Prick Screening Program (WOHNS). Clinical information of all newborns who received second-tier profiling, including confirmation of the diagnosis by mutation analysis, was retrieved pseudo-anonymously from the national NBS registry, upon completion of the registration with assistance of the attending pediatric endocrinologists. Newborns who died before diagnosis or were treated for CAH prior to heel puncture were excluded from the main analyses.

Study Outcomes and Data Analyses

During the 3-year study period, the NBS 17-OHP results and the second-tier results in the 3 groups were collected. The second-tier turnaround time was calculated as the time from routine first heel puncture result in the regional laboratory until second-tier result in the Endocrine Laboratory of the Amsterdam UMC. The true-positive, false-positive and second heel puncture rates of the routine NBS algorithm were determined and compared with 2 alternative algorithms: the routine first heel puncture with addition of the second-tier test to either (1) the inconclusive; or (2) both the positive and inconclusive first heel puncture results. Analyte concentrations and turnaround time are reported as median and interquartile range (IQR).

Results

Routine Newborn Screening Results

Between January 1, 2017 and December 31, 2019, 350 newborns had an inconclusive or positive first heel puncture result. Four newborns could not be included due to missing NBS data (refusal for scientific research or no available heel puncture material [patient deceased]). Twelve newborns were not referred despite their NBS results, because they died shortly after the heel prick was taken and before NBS procedures could be finalized. The cause of death is unknown but prematurity may play the most important role, as 10 newborns were born between 24 and 28 weeks of pregnancy. These newborns, and 6 newborns diagnosed with CAH who were already treated at the time of NBS sampling, were excluded. Ultimately, we included 328 eligible newborns (Fig. 1). Seventy newborns were referred to the pediatric endocrinologist based on 17-OHP results of the first (n = 42) or second (n = 28) heel puncture. In 37 newborns, the diagnosis of classic CAH was confirmed based on diagnostic workup (group 1; 31 detected by the first and 6 by the second heel puncture). None of the patients received glucocorticoids before delivery. In 33 newborns, the diagnosis was not confirmed at referral (group 2: false positives; 11 referred via the first and 22 via the second heel puncture; 9 newborns were premature). Furthermore, 258 newborns had an inconclusive first heel puncture and a negative second heel puncture result and were therefore not referred (group 3: NBS negatives).

Second-Tier 21-DF Results

Second-tier 21-DF and NBS 17-OHP concentrations in the 3 groups are reported in Table 1. All newborns in group 1 had blood 21-DF concentrations ≥1 nmol/L, whereas all newborns in group 2 had undetectable 21-DF concentrations (< 1 nmol/L blood). The 21-DF concentrations in 253 out of 258 newborns of group 3 were undetectable. The remaining 5 newborns had elevated 21-DF concentrations (see Table 2

Table 1. NBS 17-OHP concentrations of positive and inconclusive first heel puncture results in the Dutch CAH NBS from 2017 – 2019, and second-tier 21-DF results measured for the pilot study

	N	NBS				LC-MS/MS	
		17-OHP – 1st HP		17-OHP – 2nd HP		2nd tier 21-DF	
		Median	IQR	Median	IQR	Median	IQR
Total	**328**						
Referrals	70						
Based on 1st HP	42	161	81-300			15	2-46
Based on 2nd HP	28	39	33-52	35	29-59	<1	<1-4
1) True positives	37						
Based on 1st HP	31	264	98-300			25	11-55
Based on 2nd HP	6	43	35-49	52	31-68	10	6-13
2) False positives	33						
Based on 1st HP	11	81	62-130			<1	<1-<1
Based on 2nd HP	22	39	32-53	35	29-53	<1	<1-<1
3) Negatives following 2nd HP	258	35	27-201	14	10-20	<1	<1-<1
Positive 21-DF	5	28	27-29	19	15-22	6	5-11

17-OHP and 21-DF are reported in nmol/L blood. The lower limit of quantitation of 21-DF is 1 nmol/L blood. The second-tier analyses were performed on the DBS of the first heel puncture.
This table provides an overview of the measurements in groups 1 & 2, divided in subgroups based on first or second heel puncture–based referral, and group 3, with a separate row for 5 screen-negatives with a detectable 21-DF concentration. For a full definition of the groups, we refer to the methods section or Fig. 1. CAH patients who received treatment at time of NBS sampling (n = 6) and newborns deceased before referral (n = 12) were excluded.
Abbreviations: 17-OHP, 17-hydroxyprogesterone; 21-DF, 21-deoxycortisol; CAH, congenital adrenal hyperplasia; DBS, dried blood spot; HP, heel puncture; IQR, interquartile range; presented as the first and third quartile; LC-MS/MS, liquid chromatography–tandem mass spectrometry; NBS, newborn screening.

Table 2. NBS results and second-tier analysis of 5 non-referred newborns due a negative second heel puncture 17-OHP results, with detectable 21-DF concentrations, in the period 2017-2019

Sample	NBS 17-OHP – 1st HP	NBS 17-OHP – 2nd HP	GA (weeks + days)	Gestational weight (g)	2nd-tier 21-DF (nmol/L)
#1	28	15	39 + 4	4070	3.5
#2	27	24	39	3060	4.6
#3	37	22	42	3760	5.7
#4	25	7	38 + 2	3875	11
#5	29	19	41 + 5	4430	18

The second-tier analyses were performed on the DBS of the first heel puncture.
Abbreviations: 17-OHP,17-hydroxyprogesterone; 21-DF, 21-deoxycortisol; DBS, dried blood spot; GA, gestational age; HP, heel puncture; NBS, newborn screening.

for individual values). Assessment of these 5 newborns could not be initiated within the scope of the study.

CAH Patients Detected by Newborn Screening

The diagnoses of all 37 classic CAH patients in group 1 were confirmed by mutation analysis (33 SW and 4 SV CAH) as reported in Table 3. One NC CAH patient was routinely categorized as a false-positive result.

Turnaround Time and Logistics

In the pilot-year 2019, DBS material of 73 newborns with a positive or inconclusive 17-OHP result in the first heel puncture was sent by regular mail to the Endocrine Laboratory of the Amsterdam UMC for second-tier testing. The median turnaround time was 2 working days (IQR, 1-2; range, 1-4 days). Factors influencing the turnaround time were the availability of the postal service on the same or the next day, day-to-day differences in the delivery timeslot by the postal service, and delay within our institute.

Routine Newborn Screening Algorithm and Alternative Algorithms Compared

Replacing the routine NBS algorithm by an alternative algorithm, consisting of a routine first heel puncture with addition of the second-tier 21-DF to the inconclusive first heel

Table 3. *CYP21A2* mutation analysis and clinical phenotype of genetically confirmed CAH patients, untreated at time of NBS sampling, in the NBS period 2017-2019

Sample	Mutation analysis		Clinical phenotype
	Allele 1	Allele 2	
#1	c0.293-13A/C > G	c0.518T > A	SW
#2	deletion/conversion	unspecified	SW
#3	deletion/conversion	c0.518T > A	SW
#4	deletion/conversion	deletion/conversion	SW
#5	deletion/conversion	unspecified	SW
#6	c0.844G > T	deletion/conversion	SV
#7	c0.844G > T	deletion/conversion	SV
#8	c0.518T > A	c0.1069C > T	SW
#9	c0.293-13A/C > G	c0.1069C > T	SW
#10	deletion/conversion	unspecified	SW
#11	c0.844G > T	deletion/conversion	SV
#12	c0.949C > T	c0.955C > T	SW
#13	c0.293-13A/C > G	deletion/conversion	SW
#14	c0.293-13A/C > G	c0.518T > A	SW
#15	deletion/conversion	deletion/conversion	SW
#16	c0.923dup	c0.293-13A/C > G	SV
#17	c.(?_-107)_447+?del	c.(?_-107)_447+?del	SW
#18	deletion/conversion	deletion/conversion	SW
#19	c0.293-13C > G	c.(?_-107)_447+?del	SW
#20*	c0.844G > T; c0.923dup; c0.955C > T; c0.1069C > T; deletion/conversion		SW
#21	unspecified	unspecified	SW
#22	c0.293-13C > G	c0.293-13C > G	SW
#23	c0.518T > A	c0.518T > A	SW
#24	deletion/conversion	c0.293-13A/C > G	SW
#25**	c0.293-13C > G	c0.905C > A	NC
#26	c0.332_339del	c0.332_339del	SW
#27	c0.518T > A	c.(?_-107)_939+?del	SW
#28	unspecified	unspecified	SW
#29	unspecified	unspecified	SW
#30	c0.293-13C > G	c0.293-13C > G	SW
#31	c0.293-13A/C > G	c0.293-13A/C > G	SW
#32	c0.710T > A	unspecified	SW
#33	deletion/conversion	c0.1069C > T	SW
#34	c0.293-13A/C > G	c0.518T > A	SW
#35	deletion/conversion	unspecified	SW
#36*	c0.844G > T; c0.923dup; c0.955C > T; c. 1069C > T		SW
#37*	c0.332_339del; c0.844G > T; c0.923dup; c0.955C > T; c0.1069C > T		SW
#38	c0.293-13C > G	c.(?_-107)_447+?del	SW

Deletion/conversion: confirmed, but not further described. Unspecified: genetic confirmation of CAH, but mutation not further specified.
Abbreviations: CAH, congenital adrenal hyperplasia; NBS, newborn screening; NC, non-classic CAH; SV, simple-virilizing CAH; SW, salt-wasting CAH.
*Carrier analyses not yet performed. ** NC CAH patient, categorized as a false-positive NBS result.

puncture results, led to detection of all 37 CAH patients and reduction of false positives from 33 to 11. Addition of the second-tier test to both the inconclusive and positive first heel punctures reduced false positives to zero. In both alternative algorithms, a second heel puncture was no longer required, leading to a shorter time until diagnosis in 6 newborns. Five routinely nonreferred newborns became screen-positive due to an elevated 21-DF measured in their first heel puncture DBS. Clinical assessment of these newborns could not be initiated within the scope of this study. Use of the second-tier test led to a 1- to 4-day increase of the turnaround time to 5 to 11 days (Fig. 3).

Discussion

The aim of this 3-year pilot study was to assess the accuracy and turnaround time of the NBS second-tier marker 21-DF in a large cohort of newborns who screened positive or inconclusive for CAH by the routine 17-OHP–based NBS algorithm, and to establish whether implementation of this second-tier test could remove the need of the second heel puncture and eliminate the false-positive referrals caused by the current NBS algorithm. A time interval of 1 year within this pilot was reserved to establish the turnaround time from the first heel puncture result until the 21-DF second-tier result was known. We showed that addition of the second-tier test increased the turnaround time by 1 to 4 working days (IQR, 1-2 days). We also showed that in 3 years of NBS for CAH, 37 patients were detected at referral by the current NBS algorithm and by the second-tier test. The algorithm currently used in the Netherlands (GA-specific cutoff values and second heel punctures (6)) has kept the number of false-positive referrals at an acceptable level (PPV = 37 true positives / [37 true-positive + 33 false-positive referrals] = 53%). We demonstrated that introduction of 21-DF as a second-tier test to the inconclusive first heel punctures reduced the number of false positives to 11 and shortened the time to referral of 6 CAH patients by a week, because they were referred after the first rather than a second heel puncture. Furthermore, with this alternative algorithm, all 286 second heel punctures would no longer be necessary. Therefore, we conclude that the stressful impact of a second heel puncture could be eliminated for all newborns and their families by using 21-DF as a second-tier test. Alternatively, adding the second tier to both inconclusive and positive first heel

	Routine NBS	**Alternative algorithms**	
	Based on a 1st and 2nd HP	Routine 1st HP + 2nd-tier in inconclusive 1st HP	Routine 1st HP + 2nd-tier in positive and inconclusive 1st HP
Total referred	70	53	42
Routine 2nd HP	286	0	0
True-positives	**37**	**37**	**37**
Based on positive 1st HP	31	31	-
Based on inconclusive 1st HP + positive 2nd HP	6	-	-
Based on 2nd-tier 21-DF	-	6	37
False-positives	**33**	**11**	**0**
Based on positive 1st HP	11	11	-
Based on inconclusive 1st HP + positive 2nd HP	22	-	-
Based on 2nd-tier 21-DF	-	0	0
Routine NBS negatives with positive 21-DF	**-**	**5**	**5**
Turnaround time			
Positive 1st HP newborns	4 – 7 days	4 – 7 days	-
Inconclusive 1st HPs newborns	11 – 16 (23*) days	-	-
1st HPs + 2nd-tier newborns	-	5 – 11 days	5 – 11 days

Figure 3. Routine NBS referral numbers and turnaround time compared with 2 applications of 21-DF as a second-tier test. To calculate referral numbers of the alternative algorithms, the second-tier test always overruled the applicable routine NBS first heel puncture result. Turnaround time of the second-tier test is monitored as the time (in days) from the first heel puncture result until the second-tier result is known. Total turnaround time is a sum of this and the established turnaround times in our NBS program (described in detail in the methods section). Abbreviations: 21-DF, 21-deoxycortisol; CAH, congenital adrenal hyperplasia; HP, heel puncture; NBS, newborn screening.

punctures successfully eliminated all false-positive referrals. The downside of this alternative, however, was the delayed referral of 29 SW and 2 SV CAH patients due to the increased turnaround time. The benefit of an additional reduction of 11 false-positive referrals does not, in our opinion, outweigh the risk of a potentially lethal neonatal course. Remarkably, in 5 of 258 newborns with an inconclusive first heel puncture, but negative second heel puncture, the second-tier test resulted in a positive 21-DF. As these newborns have not been reported as false-negative CAH patients by the pediatricians, they are unlikely to have life-threatening SW CAH, but could have mild SV or even NC CAH, as 21-DF has also been shown to be a reliable diagnostic marker for NC CAH (18). However, newborns with the milder form of CAH, the NC CAH, are classified as false-positive in our NBS algorithm, as our aim is to detect only classic CAH. On the other hand, 1 newborn, with the confirmed diagnosis of NC CAH identified by our NBS because of increased OHP concentrations, had an undetectable 21-DF. Therefore, use of the second-tier test would have prevented the referral of this newborn included in the false-positive group.

The results of our promising second-tier are similar to a study reporting a specificity and PPV of 100% (13). However, definitive accuracy numbers in our population are to be awaited, as clinical assessment of 5 non-referred newborns with an elevated 21-DF could not be initiated within the scope of this study. At this point, it is impossible to determine whether they have CAH or whether we are dealing with false positives due to 21-DF. To our knowledge, no studies have reported the appearance of positive 21-DF results in newborns with negative 17-OHP results. While in our national NBS registry in 2017–2019 no false-negatives have been reported, the risk of missed cases should still be considered, as the registration is not perfect. During the preparation of this manuscript, 2 patients screened in the study period were reported false-negative due to a negative first heel puncture 17-OHP result. For these children, 21-DF as a second-tier test could not have led to their identification, but retrospective analysis of their 21-DF might contribute to understanding why they were missed. Higher false-negative rates caused by second-tier testing have been reported but this could be related to early blood sampling during the first 2 days of life, although negative second-tier results have also been reported when the 17-OHP result was positive (16, 19). Although currently not the target of CAH NBS in the Netherlands, with the introduction of LC-MS/MS second-tier testing, the rare 11β-hydroxylase deficiency could be detected too by measuring 11-deoxycortisol and other steroids. Results show, however, that 17-OHP in the primary immunoassay could be negative in these patients (20).

In conclusion, we report the promising application of second-tier 21-DF measurement using LC-MS/MS in the Dutch NBS for CAH. This method improved the NBS accuracy by reducing false-positive referrals, removed the need for a second heel puncture, and shortened the time to referral in case of an inconclusive first heel puncture, while maintaining current NBS sensitivity.

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