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Twenty Years of Neonatal Screening for Congenital Adrenal Hyperplasia in North-Eastern Italy: Role of Liquid Chromatography-Tandem Mass Spectrometry as a Second-Tier Test

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Congenital adrenal hyperplasia · 17-Hydroxyprogesterone · Newborn screening · Liquid chromatography-tandem mass spectroscopy

Abstract
Background: Newborn screening for congenital adrenal hyperplasia (CAH) based on 17-hydroxyprogesterone (17-OHP) concentration in dried blood spots has been taking place in North-Eastern Italy since 2001. Since 2017, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been introduced, for the first time in Italy, as a second-tier test. ***Aims:*** Our study aims to evaluate, on the one hand, the effectiveness of the newborn screening for CAH after 20 years of testing and, on the other, the impact that the introduction of the second-tier test had on the diagnostic accuracy of the screening program. ***Methods:*** Since 2001 dried blood spots taken from newborns have been screened with a time-resolved fluoroimmunoassay for 17-OHP determination. Over the years, the cut-off levels of 17-OHP were adjusted according to gestational age. Since 2017, a second-tier test in LC-MS/MS was introduced for samples displaying fluoroimmunoassay 17-OHP exceeding the cut-off. ***Results:*** In total, 862,521 newborns have been screened over a period of 20 years. The total incidence of 21-hydroxylase deficiency (21-OHD) was 1:25,368, moreover, a case of 11-β-hydroxylase deficiency was identified. All these diagnoses were genetically confirmed. The sensitivity and specificity of the screening program were 97% and 99.4%, respectively. The use of LC-MS/MS as a second-tier test significantly reduced the recall rate and increased the positive predictive value. ***Conclusions:*** Screening for CAH is useful in the neonatal diagnosis of a classic form of 21-OHD, allowing a precocious treatment of affected children. The introduction of an LC-MS/MS second-tier reduced the recall rate, avoiding unnecessary blood withdrawal and medical evaluations and preventing stress to families. Furthermore, it helped identify rarer forms of CAH.

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Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder due to enzyme deficiencies in the adrenal steroidogenesis pathway, leading to impaired cortisol biosynthesis, and intermediate precursors' accumulation, namely 17-hydroxyprogesterone (17-OHP) [1]. More than 90% of cases are due to 21-hydroxylase deficiency (21-OHD) caused by mutations in the corresponding gene, CYP21A1 [2–7]. Other rarer forms of CAH include 11β-hydroxylase (up to 8% of cases), 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase/17,20-lyase, P450 oxidoreductase, and steroidogenic acute regulatory protein deficiencies [1, 6]. The 21-OHD clinical spectrum mostly depends on the severity of the enzyme deficiency and the sex of the individual. Two clinical forms of the disease have been recognized: a classic form, distinguished in salt-wasting (SW) and simple virilizing (SV) variants, and a non-classic (NC), late-onset form. The worldwide incidence of the classic form ranges from 1:14,000 to 1:18,000 live births [4, 5], whereas the NC form has a prevalence of 1:1,000 in the white population [8].

If unrecognized and untreated, the SW form can cause a life-threatening salt crisis with shock, dehydration, severe hyponatremia, and hyperkalemia [9]. Moreover, the accumulating precursor 17-OHP is diverted to androgen synthesis, leading to varying degrees of virilization of the external genitalia, more evident in females, with consequent difficulties in the sex assignment at birth. When a higher residual activity is present, the SV form develops, with virilization of external genitalia not associated to SW crisis. The life-threatening salt loss crisis usually occurs within the second week of life, hence early diagnosis through newborn screening reduces the mortality rate, while allowing proper treatment and correct sex assignment.

Since 1977 newborn screening programs for CAH based on 17-OHP determination have been widely implemented [10]. Nowadays, newborn screening for CAH is routinely performed in at least 30 countries around the world through 17-OHP fluoroimmunoassay determination on blood spots taken at birth [5]. Despite the clinical usefulness of CAH screening, the positive predictive value (PPV) remains low due to a large number of false positives, especially in preterms, low birth weight (BW) newborns, sick or stressed infants [11, 12]. Moreover, the screening procedure does not allow to identify babies with NC 21-OHD, since basal serum 17-OHP levels at birth are often in the normal range in these patients [13]. To partially address these drawbacks, several different screening programs decided to adjust their cut-off values for 17-OHP based on BW and/or on gestational age (GA); in doing so, they, however, achieved only limited improvement in terms of recall rate reduction [2, 14, 15]. The turning occurred in the early 2000s, with the introduction of a more specific test than the 17-OHP fluoroimmunoassay as a second-tier test [16]. This assay is based on the simultaneous determination of a steroid profile by liquid chromatography-tandem mass spectrometry (LC-MS/MS) carried out on the same blood spot taken at birth and displaying a fluoroimmunoassay 17-OHP above a set threshold [17–23]. Currently, this method is implemented in different European [24, 25] and international screening programs [18, 26]. In North-Eastern Italy, newborn screening for CAH has been taking place since 2001 [14], and a second-tier test in LC-MS/MS was routinely introduced in 2017, for the first time in Italy. Our study aims are to evaluate, on the one hand, the effectiveness of the newborn screening for 21-OHD in North-Eastern Italy and, on the other, the impact that the introduction of the second-tier test had on the diagnostic accuracy of the screening program.

Materials and Methods

Subjects and Newborn Screening Workflow

In Italy, newborn screening for CAH is not mandatory and is performed only in some regions after obtaining parents' informed consent. At the University Hospital of Verona (Italy) newborn screening for 21-OHD began in 2001 and concerned the neonatal population from North-Eastern Italy. During this time, only 0.5‰ of the families did not consent to screen their baby.

Blood samples are collected by a heel prick and absorbed on a Guthrie filter card. Time of blood collection changed over the years, according to normative prescriptions or to scientific evidences. Initially, the blood sample was collected at 49–96 h of life. Between 2009 and 2013, blood collection was anticipated to 36–48 h of life, whereas in 2016 a national prescription recommended blood collection for all newborn screening programs between 48 and 72 h of life (Table 1). Concomitantly with the CAH screening, other screening tests are performed in our center in order to investigate different metabolic disorders and congenital hypothyroidism. These diseases require retesting at 2 and 4 weeks of age for all infants with GA <37 weeks or with BW <2,500 g.

All blood samples were initially sent to our center by mail, but since 2010 a 5 days/week service of couriers has been purposely appointed; it turned into a 6 days/week service in May 2016. Mother and infant demographic information are electronically collected along with GA, gender, BW, therapy, and nutritional information.

From 2001 to 2017, all specimens have been routinely analyzed only by a time-resolved fluoroimmunoassay method for 17-OHP determination (with Perkin Elmer Inc. DELFIA® Neonatal 17α-OH-progesterone kit and then, from 2014, with Perkin Elmer

Inc. GSP Neonatal 17α-OH-progesterone kit). The assay is based on the competitive reaction between europium-labeled 17-OHP and sample 17-OHP for a limited amount of binding sites on 17-OHP specific polyclonal antibodies derived from a rabbit. A second antibody, directed against rabbit IgG, is coated to the solid phase. The fluorescence of each sample is inversely proportional to the concentration of 17-OHP in the sample. Results were available after one working day. The cut-off level for 17-OHP was based on GA [14] and was annually reviewed, taking into account the population distribution and 17-OHP levels of detected true positive cases (Table 1). A serum test for 17-OHP and electrolytes determination was requested for all cases displaying at least two out of three screening values exceeding the chosen cut-off, since 17-OHP results above the cut-off were routinely retested in duplicate. Upon reporting to the birth hospital, the infant was recalled and visited by a pediatric endocrinologist to confirm or exclude the diagnosis.

In October 2017, we introduced a second-tier test, based on LC-MS/MS steroid profile evaluation that was performed on the same blood spot taken at birth and displaying a 17-OHP above the set cut-off. Newborns were subsequently recalled whenever the second-tier test proved positive, except for the newborns with a 17-OHP fluoroimmunoassay >114 nmol/L which were promptly recalled. The 2nd tier analysis provides the simultaneous determination of 17-OHP, cortisol, 11-deoxycortisol (11-DC), Δ4-androstenedione, and 21-deoxycortisol (21-DC). The 2nd tier algorithm for screening recalls is reported in Figure 1. In particular, in addition to 17-OHP concentration, we also took into consideration the ratio between the sum of the 17-OHP (nmol/L) plus Δ4-androstenedione concentrations (nmol/L) divided by the cortisol concentration (nmol/L) as well as the levels of 11-DC and 21-DC.

Second-Tier Test

Chemicals and Reagents

17-OHP, d8-17-OHP, 11-DC, 21-DC, Δ4-androstenedione, cortisol were purchased from Merck Life Science S.r.l. (Milano, Italy). d2-11-DC, d8-21-DC, d7-androstenedione were obtained from CDN Isotopes Inc. (Quebec, Canada), and d3-cortisol from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Methanol recommended for UPLC-MS/MS was acquired from Merck Life Science S.r.l. Milli-*Q* water is taken from a Milli-Q Direct System (Merck Millipore Darmstadt, Germany).

Calibrators and Controls

Dried blood spots for calibration and quality-control monitoring were obtained from the Newborn Screening Quality Assurance Program of the Centers for Disease Control and Prevention (Atlanta, GA, USA).

Assay Procedures

Two 3.2-mm blood spots were punched out from filter cards (Panthera Puncher; Perkin Elmer Inc.) The spots were placed into a flat bottom microplate. In total, 200 µL of extraction solution (90:10 methanol:water) containing deuterate internal standard for each analyte were added (d8-17-OHP, d2-11-DC, d8-21-DC, d7-androstenedione, d3-cortisol). The plate was first covered and agitated for 50 min at 120 rpm in a microplate shaker (Trinest; Perkin Elmer Inc.) and then centrifuged at 2,000 rpm for 2 min. The extract was transferred to a new microplate and dried out under gentle nitrogen flow using an SPE-Dry 96 Nitrogen Evaporator (35 min with an upper manifold set at 50°C). The dry residue was re-

Table 1. Characteristic of screening and variation of cut-off during the years

2001: first-tier test by time-resolved fluoroimmunoassay method

2008: antibody change

2017: second-tier test by LC/MS/MS

Withdrawal time: 2001: 49–96 h; 2009: 36–48 h; 2017: 48–72 h

17-OHP cut-off level, nM blood

17-OHP to second-tier testing, nM blood

Years	2001–2003	2004–2009	2010	Mar 2011	Jul 2011	Sep 2011	2012–2014	2015–2017	Nov 2017	2019	2020
GA											
All	≥30								≥23	≥41	
≥37 wk		≥40									≥16
<37 wk		≥72									≥47
≥36 wk			≥60		≥26	≥43 (male) ≥64 (female)					
<36 wk			≥100		≥43	≥85					
≥37 wk				≥22			≥26	≥23			
33 wk ≤ GA ≤ 36 wk								≥51			
32 wk ≤ GA ≤ 36 wk				≥43			≥50				
≤32 wk								≥84			
≤31 wk				≥70			≥80				

The cut-off reevaluation is performed at least every 6 months, taking into account both the neonatal percentiles distribution and the observation of false negative, false positive, and true positive cases.

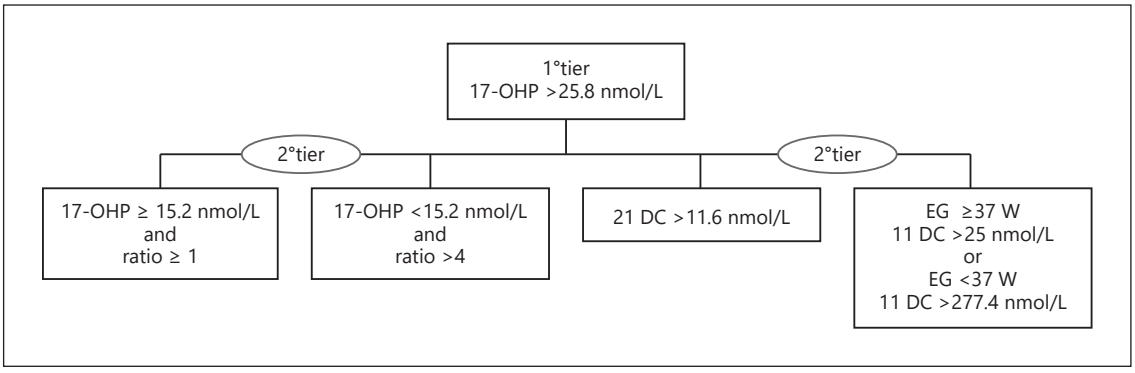

Fig. 1. Algorithm for screening recalls in our center. At the 1° tier, the 17-OHP level used as cut-off is the same for term and preterm newborns. At the two-tier test, a newborn is recalled if one of the criteria is met. 17-OHP concentration nmol/L (17-OHP), RATIO = ([17-OHP + Δ4-androstenedione]/cortisol), 11-DC nmol/L, and 21-DC nmol/L. 11-DC, 11-deoxycortisol; 21-DC, 21-deoxycortisol.

constituted with 50-μL solution of 50:50 methanol:water. The plate was covered and gently orbital agitated for 30 min at 120 rpm.

A Waters Acquity UPLC system was used for chromatographic separation. Cortisol, Δ4-androstenedione, and 17-OHP were chromatographically resolved using a reverse-phase column (Waters Acquity UPLC BEH C18, 130 Å 1.7 μm, 2.1 × 50 mm, and 0.2 μm in-line precolumn filter). The LC separation was performed using a linear gradient from 65% of mobile phase A (methanol:water 50:50) and 35% of mobile phase B (100% methanol) to 1% of mobile phase A and 99% of mobile phase B, over 5 min time interval with a constant flow rate of 0.4 mL/min. The column temperature was maintained at 50°C throughout the separation (modified from [27]).

Detection and quantitation were achieved by tandem MS/MS using a Waters Xevo TQDetector, equipped with an electrospray source operating in positive ionization mode. The samples were analyzed in multiple reaction monitoring modes. For the monitored transitions, see Table 2. Data acquisition was performed using MassLynx software and analyzed with TargetLynx software.

Statistical Analysis

Statistical analysis was performed using SPSS 25.0 for Windows and STATA v.14. Normal distribution was assessed by the Kolmogorov-Smirnov test. Comparisons between groups were performed using Student's *t* test or the Mann-Whitney U test, whenever appropriate. Rates were compared by χ^2 or Fisher's exact test (2-sided), whenever appropriate. Data are expressed as frequency, median plus range, or mean ± standard deviation, as appropriate. Statistical significance was reached as p values <0.05, and all tests were two-sided.

Results

From 2001 to the end of December 2020, 862,521 newborns (49.1% females and 50.9% males) were screened. In 33 of them, a diagnosis of a classic form of 21-OHD was clinically established and successively genetically confirmed. To date, we have been notified of one false-negative case: a male child clinically identified at the age of 5 years for a significant bone age advancement and a precocious pubarche. ACTH test and hormonal levels have raised the suspicion of CAH and the genetic analysis confirmed a mild form of 21-OHD, clinically identifiable as an SV form. Therefore, the overall incidence of a classic form of 21-OHD was 1:25,368, the diagnostic sensitivity of the screening program is 97%, and its specificity 99.4%. Among affected children (50% females, 50% males), 63.6% presented an SW form, the remaining 36.4% an SV form. During these 20 years, we identified at least 6 NC forms of 21-OHD.

The second-tier test helped to identify 1 male baby affected by 11-β-hydroxylase deficiency. The presence of elevated 11-DC on screening test suggested the diagnosis, hormonal pattern on serum was compatible with the screening suspect, and molecular analysis gave a final confirmation. In all true positive cases, the diagnosis was established within 10 days of life and an appropriate treatment was promptly instituted, preventing the life-threatening consequences of a diagnostic delay. All of them were born at term, except for a female baby born at 35 weeks of GA.

The overall mean recall rate during these 20 years of screening is 0.63%. In particular, in the first 15 years, despite the GA adjustments, the recall rate remained almost constant, and only upon the introduction of LC-MS/MS 2nd tier, we observed a significant reduction (Fig. 2).

To specifically evaluate the impact of the LC-MS/MS second-tier tests on the accuracy of screening diagnostic, we compared two-time intervals of 31 months each: the

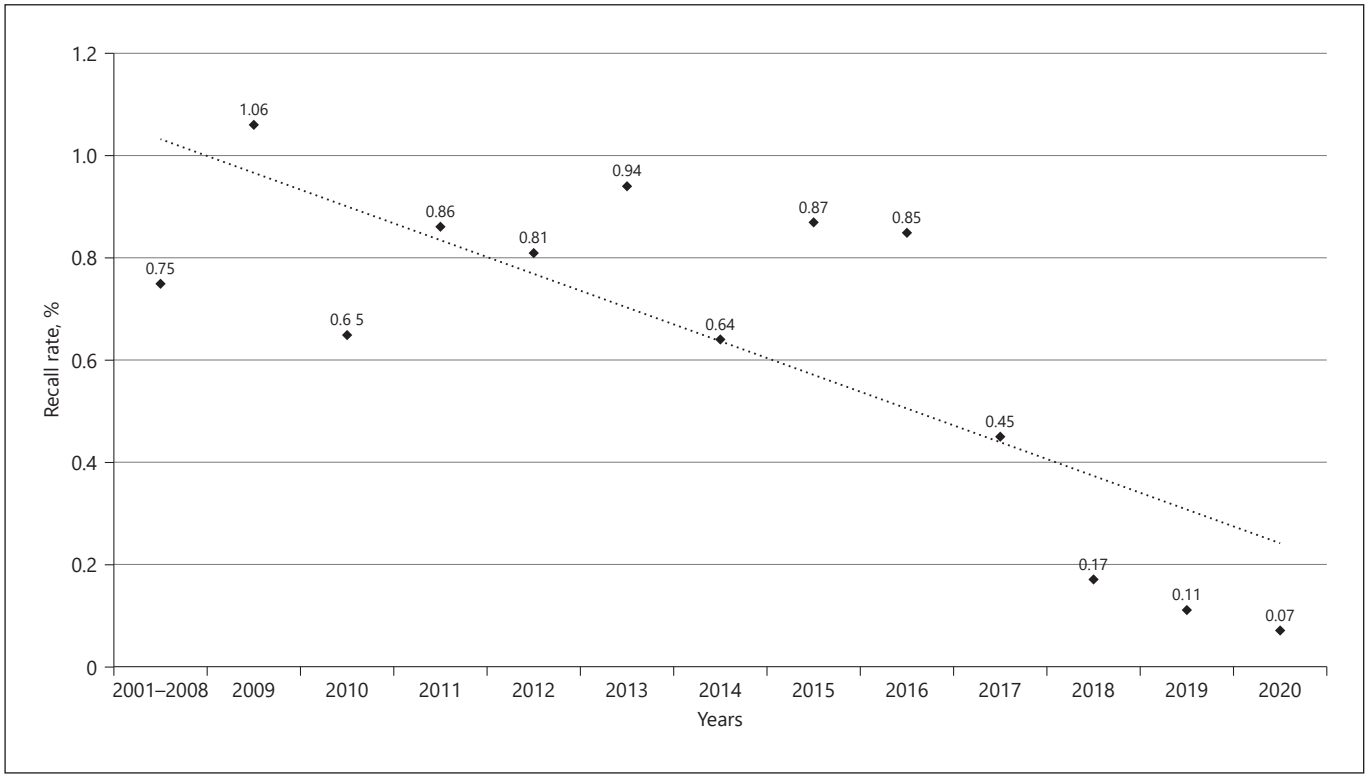

Fig. 2. Representation of the recall rate in the various years of screening activities. The trend line is shown as dashed.

Table 2. Characteristic of analytes used in tandem MS/MS

Analyte	Parent ion, *m/z*	Daughter ion, *m/z*	Cone voltage, V	Collision energy, V
d8-17-OHP	339	100	35	25
17-OHP	331	97	30	22
d2-11-DC	349	97	28	22
11-DC	347	97	28	27
d8-21-DC	355	319	26	17
21-DC	347	311	26	17
d7-androstenedione	294	100	25	25
Androstenedione	287	97	30	20
d3-cortisol	366	121	35	26
Cortisol	363	121	35	26

first running from March 2015 to October 2017, and the second from October 2017 to April 2020. We found a significant reduction of recall rate thanks to the second-tier test both in preterm newborns (from 3.99% to 1.64%; $p < 0.05$), and in term babies (from 0.3% to 0.01%; $p < 0.05$) (Table 3).

In the first time interval, the PPV was 0.24%, 0.44%, and 0% for full-terms and preterms, respectively. Upon the introduction of the second-tier test, the PPV increased to 2.54%, 20% in term babies, and 0.93% in preterms (Table 3). Hence, PPV improved significantly only for term infants ($p < 0.01$). The negative predictive value for full-term and preterm infants combined was 100%. No false-negative results were identified in these two intervals.

We found a significant correlation between the levels of 17-OHP measured by LC-MS/MS and fluoroimmuno-

Table 3. Recall rate in the two-time intervals, total and differentiated by sex and GA

Period	Patients	Sex	Screened patients	Recalled	Confirmed cases	PPV, %	Recall rate, %
From Mar 2015 to Oct 2017	Preterm	Males	5,161	225	0	0	4.36
		Females	4,836	174	0	0	3.60
		Total	**9,997**	**399**	**0**	**0**	**3.99**
	Full-term	Males	61,820	303	1	0.33	0.49
		Females	60,627	148	1	0.68	0.24
		Total	**122,447**	**451**	**2**	**0.44**	**0.37**
	Total		**132,444**	**850**	**2**	**0.24**	**0.64**
From Oct 2017 to Apr 2020	Preterm	Males	3,515	66	0	0	1.88
		Females	3,088	42	1	2.38	1.36
		Total	**6,603**	**108**	**1**	**0.93**	**1.64**
	Full-term	Males	47,490	9	1	11.11	0.02
		Females	45,425	1	1	100	0
		Total	**42,915**	**10**	**2**	**20.0**	**0.01**
	Total		**99,518**	**118**	**3**	**2.54**	**0.12**

assay, although the former were significantly lower than the latter, and this is likely due to antibody cross-reactivity with other steroids in fluoroimmunoassay method. Moreover, the levels both of the fluoroimmunoassay and the LC-MS/MS 17-OHP decreased with GA. In addition to the recall rate reduction, the use of LC-MS/MS allowed us to define specific cut-offs for other steroids analyzed (11-DC, 21-DC, and the ratio between the sum of the 17-OHP plus Δ4-androstenedione concentrations divided by the cortisol concentration) (Fig. 1), which can be useful for the identification of other, rarer, deficiencies such as 11β-hydroxylase deficiency.

Discussion

Our data confirm the invaluability of newborn screening for precocious diagnosis of a classic form of 21-OHD. The implementation of LC-MS/MS second-tier test has greatly reduced the recall rate and improved the PPV. To our knowledge, since 2001 the newborn screening failed to detect only 1 male child with SV form, whereas no infants died of CAH.

During a 20-year-long activity of newborn screening, the incidence of a classic form of 21-OHD identified in North-Eastern Italy was 1:25,368 and remained constant throughout the years [14]. An incidence close to ours was detected in New Zealand [28], whereas in the USA and in Europe 21-OHD incidence ranges from 1:11,000 to 1:18,000 live births [4, 29]. A recent paper reports a mean prevalence of 1:16,825 in the USA, with a wide range throughout the States, from 1:9,941 to 1:28,661 live births [30]. The reasons behind such an inconsistency are still matter of debate. First, premature babies affected by SW might die before the pathology is recognized, since in these babies the screening test is sometimes performed with a certain time delay as a consequence of their critical conditions. Second, premature babies often present an elevated 17-OHP due to life-threatening conditions, such as septicemia or respiratory distress [31–33]. Furthermore, we must not forget that these variations might be an effect of genetic variation in different populations or related to different screening methodologies.

While the incidence remained constant, the implementation of LC-MS/MS mostly impacted on the recall rate, significantly improving the PPV. It is well known that false positives, mostly preterms, affect greatly the CAH newborn screening procedures based only on fluoroimmunoassay 17-OHP determination [12, 34, 35]. Previous studies recommend a recall rate between 0.5% and 1%, suggesting the adjustment of the 17-OHP cut-off level either in relation to GA or to BW [36]. We tried to adjust the cut-off threshold in relation to GA [14], but a significant reduction in recall rate, especially between

preterm newborns, was only achieved in 2017, when we routinely implemented the LC-MS/MS second-tier test in the workflow. Furthermore, scientific literature has acknowledged the importance of LC-MS/MS in improving the specificity of CAH newborn screening [4, 17–19, 21, 37] and in reducing both the overall economic costs, since it makes confirmatory tests less frequently needed [38], and the emotional burden bore by families when a newborn is recalled for further diagnostic ascertainments. In addition, two-tier testing strategy has the advantage of reducing the duration of follow-up in newborns with positive CAH screening [25]. On this basis, the 2018 Endocrine Society Clinical Practice Guidelines [4] have officially endorsed the practice of two-tier testing with the twofold aim to increase the specificity and PPV and to identify rarer forms of CAH [26, 27, 39]. Thanks to this assay, we have decreased the false positive rate of more than 81% and have identified a neonate affected by 11-β-hydroxylase.

The specificity improvement is particularly evident in term babies, whose PPV has increased from 0.44% to 20%, whereas, in preterm infants, the PPV remained low despite cut-off adjustments based on the GA during the first time interval, and the subsequent use of LC-MS/MS. This finding is of particular relevance in relation to the ongoing debate about the role of CAH screening in preterm newborns, especially at the light of the recommendations given by Coulm et al. [12], which suggests to discontinue newborn screening for preterm neonates. Their PPV between preterm babies was, in fact, 0.4%. According to their conclusions, most preterm neonates are subject to careful pediatric care; consequently, an incipient SW adrenal crisis can be readily recognized and appropriately treated [12]. On the contrary, the Swedish Group of Newborn Screening for CAH considers screening of preterm newborns justified, nevertheless their PPV for preterms was only 1.4%, because of early detection of patients, since, in their view, the second sample can be easily obtained from admitted infants [35]. A compromising solution comes from the Minnesota State newborn screening program, which suggests to rescreen premature infants at 2 and 4 weeks of life. This proposal has been endorsed by the Endocrine Society because turned out very useful in reducing false positives, even more than the implementation of second-tier test strategy [40]. Such strategy might even allow the diagnosis of mild form of 21-OHD. Indisputably, however, this screening strategy appears more expensive. Notably, the rescreening at 2 or 4 weeks of life allows neither to identify earlier life-threatening shocks in babies that are constantly monitored in the neonatal intensive unit nor to achieve a faster correct sex assignment, namely the primary objectives of newborn screening for CAH. A possible solution might be to delay the execution of neonatal screening in premature babies after 72–96 h of life. Nevertheless, the simultaneous execution of newborn screening for other metabolic disorders makes it impossible to postpone the neonatal screening at the indicated time. An alternative might be to screen the CAH in preterm babies with GA <34 weeks only at 15 days of life, concomitantly with the second screening test, which in many newborn screening procedures is mandatory for all premature babies (e.g., TSH for congenital hypothyroidism) [41]. In this way, the screening would still be performed before discharge and, in-line with the French proposal, the continuous monitoring of premature babies during hospitalization would allow to diagnose the disease and intervene promptly before the patient suffers a shock.

LC-MS/MS second-tier test could be of particular diagnostic relevance since severe preterm infants often present an immature adrenal function [33]. Several studies have demonstrated that babies born prior to 29 weeks often present decreased activity of the 11-β-hydroxylase and 21-hydroxylase [42, 43]. Consequently, the accumulation of steroid precursors as 17-OHP and 17-hydroxypregnenolone leads to a distribution of steroid concentrations that can actually mimic patterns observed in children affected by 21-OHD [33]. From this point of view, the use of LC-MS/MS may show higher levels of 17-OHP independently of the diagnosis of CAH, as a consequence of an adrenal insufficiency due to prematurity [26].

During these 20 years of screening, we rarely identified NC forms of 21-OHD, and this is for several reasons. First of all, the newborn screening is meant to detect classical forms, in order to prevent salt crisis and sex miss-assignments; cut-off levels were thus set accordingly [35]. Second, in the NC forms 17-OHP levels often increase at later time, after the screening test has already been carried out [33, 44, 45]. Third, children with positive screening and slight increases in serum 17-OHP are not always submitted to cosyntropin stimulation test to confirm the diagnosis, and in some cases, the results of this test are not communicated to the screening center.

The present study has some limitations: we changed several times the cut-off of first-tier fluoroimmunoassay 17-OHP, and there were also some changes in the reference ranges and in the second-tier steroid profiling analytes during the time intervals under the survey. The possible effects are difficult to quantify but in principle, it should not reasonably affect the overall performance.

In conclusion, the incidence of classic CAH in North-Eastern Italy is lower than previously described in literature but constant throughout the 20 years of screening program. Screening for CAH proved to be useful in the neonatal diagnosis of a classic form of 21-OHD, allowing a precocious treatment of affected children. The use of LC-MS/MS as a second-tier test allowed a significant reduction of recall rate and an improvement of PPV, primarily for term babies, preventing patients from unnecessary blood withdrawal and further medical evaluations and reducing stress to families. Moreover, it was useful in identifying rare forms of CAH as 11-β-hydroxylase deficiency, as happened in one of our newborns.

As a possible future perspective and to further improve the PPV among preterm infants, it would be useful to investigate the possibility of carrying out a heel prick withdrawal at 15 days of life in extremely premature babies, when a mandatory blood spot collection is scheduled for other newborn screening disorders. This would be a valuable alternative to the blood withdrawal at birth. On this point, more specific evidence-based studies and expert consensus are needed.

Statement of Ethics

The study was conducted in compliance with the terms of the Helsinki II Declaration. The Institutional Ethics Committee of the provinces of Verona and Rovigo, Italy, took note of the study and approved it in order to publish the results (approval number 3406). Written informed consent was obtained from the parents of each affected patient.

Conflict of Interest Statement

The authors declare that there are no competing interests or conflicts of interest that could be perceived as prejudicing the impartiality of the affirmation reported.

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Author Contributions

All the authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Moreover, all authors read and approved the final manuscript. In particular: P.C. and M.C. conceived of the study, contributed to the preparation, and critical review of the manuscript; L.P. and R.G. wrote the manuscript; F.T., M.V., and S.L. participated in the design of the study and contributed to the critical review of the manuscript; and F.A. and G.P. conceived the study and participated in its coordination.

Data Availability Statement

All data analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author (C.P.).

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