RESEARCH

Analysis of a pitfall in congenital adrenal hyperplasia newborn screening: evidence of maternal use of corticoids detected on dried blood spot

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Abstract

Neonatal screening for congenital adrenal hyperplasia (CAH) faces many specific challenges. It must be done using a performant analytical approach that combines sensitivity and specificity to capture the potential causes of mortality during the first week of life, such as salt wasting and glucocorticoid deficiency. Here, we confirm that maternal inhaled corticosteroid intake during pregnancy is a possible cause of missed CAH diagnosis. Thanks to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis, we were able to quantify endogenous steroid metabolites and also detect the presence of exogenous steroids in the dried blood spot of a newborn. Adding LC-MS/MS analysis as second-tier test, especially one that includes both 17-hydroxyprogesterone and 21-deoxycortisol measurements, would probably improve CAH diagnosis. In familial neonatal screening one could also look for maternal corticosteroid therapies that are hidden to prevent false-negative tests.

Introduction

The newborn screening program (NBS) for congenital adrenal hyperplasia (CAH) presents a number of issues in the context of the prevention of life-threatening salt wasting crises and subsequent collapse. This neonatal period is critical for boys and girls whose signs of virilization at birth are mild, or that may be missed. The conversion of 17-hydroxyprogesterone (17-OHP) to 11-deoxycortisol and of progesterone to 11-deoxycorticosterone, which are precursors of cortisol and aldosterone, respectively, is promoted by 21-hydroxylase (1). Because 17-OHP is present at high levels in the serum of newborns with 21-hydroxylase

deficiency, it is possible to detect and quantify it from dried blood spot (DBS) samples (2, 3). In France, CAH prevalence is close to 1/14,500 patients and around 750,000 newborns are tested each year. On the one hand, the main pitfall of CAH screening performed by fluoro-immunoassay (FIA) is that it leads to many cases of false-positive results (4, 5). Some countries have estimated that 100 neonates would be unnecessarily examined for every case of CAH detected (6, 7). In our NBS, we reported that 87.0 and 78.2% of results were false positive for the Ile-de France (IdF) region in 2019 and 2020, respectively, with positive predictive values (PPVs) of

12.0 and 16.1%, respectively. Different factors could explain such a low PPV: (i) cross-reactive detection of 17-OHP with its steroid precursors and their sulphated conjugates, which are present in the first 48 h after birth, and longer in preterm neonates (8, 9); (ii) increased 17-OHP levels due to illness, stress, and biological variation (8, 10); (iii) the presence of high levels of delta-5 steroids, such as pregnenolone (Preg) and 17-hydroxypregnenolone (17OHPreg) in premature newborns due to immature adrenal function. These factors make it difficult to define a predictive threshold (8, 9) and result in anxiety for the parents during the recall process. For preterm babies, the cut-off value of the FIA has to be adapted to gestational age and weight (9, 11). Some countries use LC-MS/MS as a second-tier approach for DBS analysis related to CAH to reduce false positives , although France has not proposed this yet (5, 12). On the other hand, no study has clearly quantified the number of false-negative results and subsequent missed CAH diagnosis (13). In this study, we report on an unsuspected CAH patient who was initially ruled out after FIA exploration through the NBS program and was eventually positively diagnosed thanks to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), which allowed the quantitation of endogenous steroid metabolites and the identification of exogenous glucocorticoid treatment on a single DBS.

Materials and methods

Regular newborn screening algorithm for CAH

In the French NBS program, DBS is performed at 3 days of age (day 3) for newborns. CAH is suspected in full-term babies if their 17-OHP level is above 25 nmol/L. 17-OHP levels measured from a DBS are performed using FIA. On day 10 or 11, if the 17-OHP concentration is above the threshold of 25 nmol/L, newborns undergo a clinical evaluation by a pediatrician and a control serum is analyzed using LC-MS/MS. CAH is ruled out if the serum concentration of 17-OHP is below 15 nmol/L and the 21-deoxycortisol (21-DF) concentration is below 0.6 nmol/L. The study was approved by the french national newborn screening program and a non-objection certificate has been obtained from all newborn's relatives after full explanation of the purpose and nature of all procedures used.

17-OHP measurement by fluoro-immunoassays from DBS

A solid phase, time-resolved FIA (GSP® Neonatal 17alpha-OH-Progesterone kit, Perkin Elmer, Wallac Oy, Turku, Finland) was routinely used. Single 3.2 mm Ø DBS from newborns, external calibrators (0–300 nmol/L (100 ng/mL)), and control samples were punched in microtitration plates coated with anti-rabbit IgG. The assay was based on the competitive reaction between Europium-labeled 17-OHP and 17-OHP from the DBS for a limited number of binding sites on 17-OHP polyclonal rabbit antibodies. After the incubation and washing steps, a DELFIA inducer dissociated europium ions from the labeled 17-OHP into a solution where they formed strong chelators with components of the DELFIA inducer. The fluorescence was then measured and was found to be inversely proportional to the sample 17-OHP blood concentration. The coefficients of variation were 8.9% inter-assay and 5.7% intra-assay.

LC-MS/MS endogenous steroid profile and exogenous glucocorticoids analysis

Steroid extraction from serum

A panel of steroid species was measured in serum by LC-MS/MS, as described elsewhere (14). Briefly, a mixture of the deuterated internal standard (IS) (150 µL containing 17-OHP-d8, testosterone-d4, D4-androstenedione (D4A)-d5, and 21-deoxycortisol (21-DF)-d8)) was added to 150 µL of serum. The solution was mixed and left standing for 5 min, and then loaded into an Isolute SLE+0.4 mL cartridge (Biotage, Uppsala, Sweden). The samples were adsorbed for 5 min before elution of the steroids through the addition of 2 × 0.9 mL methylene chloride. Both elution solutions containing the non-conjugated steroids were mixed and evaporated until they become dry. They were reconstituted in 150 µL of methanol/water (50/50, volume-to-volume ratio).

Steroid extraction from DBS

The extraction protocol of steroids from DBS was adapted from de Hora *et al.* (15) and Lacey *et al.* (4). In brief, the whole blood was eluted from a 4.8 mm Ø DBS by a methanol/water (50/50, v/v) solution containing the same deuterium-labeled IS mixture as was used in serum (17-OHP-d8, testosterone-d4, D4A-d5, and 21-DF-d8). Endogenous and exogenous steroids were extracted into diethyl ether, which was subsequently evaporated. The residue was dissolved in an LC mobile phase.

LC-MS/MS quantification method

The quantification method was adapted from (5). Briefly, the extracted endogenous and exogenous steroids from both serum and DBS were separated by high-performance LC

using a Shimadzu Nexera XR system (Shimazu France, Marne la Vallée, France) and a Core-Shell C18 column (Kinetex, 2.6 µm, 100 Å, 100×2.1 mm, Phenomenex, Le Pecq, France). The detection was performed using LC-MS/MS (Qtrap 6500, AB Sciex, Foster City, CA, USA). Upon collection, spectra were analyzed using MultiQuant Software (AB Sciex, version 3.0). The steroid profile included 21-DF, D4A, testosterone, and 17-OHP. The potential presence of exogenous glucosteroids in DBS was detected using the multiple reaction monitoring (MRM) fragmentation transitions m/z 435 → 415 and m/z 393 → 373 for triamcinolone acetonide and betamethasone, respectively ([16]).

Results

Recently, we experienced a missed diagnosis in the last female newborn of a consanguineous family from Mali that already had five children, including a teenage boy suffering from CAH. The previous five pregnancies had been closely monitored using SRY maternal analysis and trophoblast molecular analysis for *CYP21A2* mutations. For the current pregnancy, the parents had refused any amniocentesis because the COVID-19 pandemic lockdown had secluded the father in Mali. On day 3, the neonatal screening for 17-OHP by DBS was 11.1 nmol/L (N <25 nmol/L). On the same DBS on day 3, the LC-MS/MS assay measured the concentration of serum 17-OHP at 12.8 nmol/L, whereas the concentration of 21-DF was 17.9 nmol/L. The female newborn weight gain was not sufficient, and she was thus recalled on day 13 (Table 1). The pediatrician noticed she had a minor labioscrotal fusion, with no clitoral enlargement, and two separate vaginal and urethral orifices. She weighed 3476 g, had sunken eyes, and did not experience vomiting. Her blood pressure was 80/60 mmHg. At that age, the serum 17-OHP at 121.0 nmol/L (N < 7 nmol/L) was undoubtedly high. Hydrocortisone treatment was started, and genetic mutation analysis showed that she had the same CYP21A2 variant as her elder brother. She was homozygous for the p.(Ile77Thr) mutation in exon 2 of the *CYP21A2* gene. This mutation is responsible for the classical virilizing form of CAH. To understand why she passed the usual neonatal screening (Table 1) without a discriminant test, we questioned the mother regarding her medication. The mother happened to be on nasal spray corticosteroids (triamcinolone) throughout the pregnancy and during the neonatal period while she was breastfeeding. This significantly lowered all neonatal adrenal steroids (Table 1) except 21-DF as a result. Subsequently, LC-MS/MS showed, on the same newborn DBS sampled on day 3, a specific peak corresponding to the

Table 1 Biological profile of the misdiagnosed index CAH patient case.

	Usual values	Birth	Day 3	Day 13
Weight (g)		3400	N/A	3476
DBS FIA				
17 hydroxyprogesterone (nmol/L)	<25		11.1	
DBS LC-MS/MS				
17 hydroxyprogesterone (nmol/L)			9.6	
21 deoxycortisol (nmol/L)			1.7	
Testosterone (nmol/L)			0.6	
Serum LC-MS/MS				
17 OHP (nmol/L)			12.8	121.0
Testosterone (nmol/L)			0.4	1.0
Delta 4-androstenedione (nmol/L)			4.0	9.4
21 deoxycortisol (nmol/L)			17.9	52.0
Plasma ionogram				
Sodium (mmol/L)	132–142		N/A	137
Potassium (mmol/L)	3.6–5.7		N/A	5.2
Chloride (mmol/L)	98–106		N/A	99

DBS, dried blood spot; FIA, fluoro-immunoassay; LC-MS/MS, liquid chromatography tandem mass spectrometry.

triamcinolone molecule present in the nasal spray used by the mother (Fig. 1).

Discussion

Neonatal screening for CAH presents numerous challenges, especially because of the life-threatening nature of salt wasting and glucocorticoid deficiency in the first week of life. The analytical approach must have satisfactory maximum sensitivity and satisfy the true PPV criteria.

We identified a potentially underestimated cause of missed CAH diagnosis, a maternal inhaled corticosteroid intake during pregnancy. Indeed, oral and inhaled corticosteroids are often described in the medication taken by pregnant women, whereas nasal nebulization is less often reported because it is mistakenly thought to have no side effects (https://www.accessdata.fda.gov/drugsatfda_docs/label/2008/020468s024lbl.pdf; [17]). Here, we describe a case report in which triamcinolone acetonide taken by the mother was revealed to be present in the same newborn DBS as is used in the NBS program.

As other countries are doing, we are planning to improve our NBS by adding LC-MS/MS as a second-tier test. In our French NBS center, we consider, at present, a first-tier immunoassay with a threshold level for 17-OHP concentration of 25 nmol/L in a DBS. If the concentration of 17-OHP measured by FIA is greater than 25 nmol/L but less
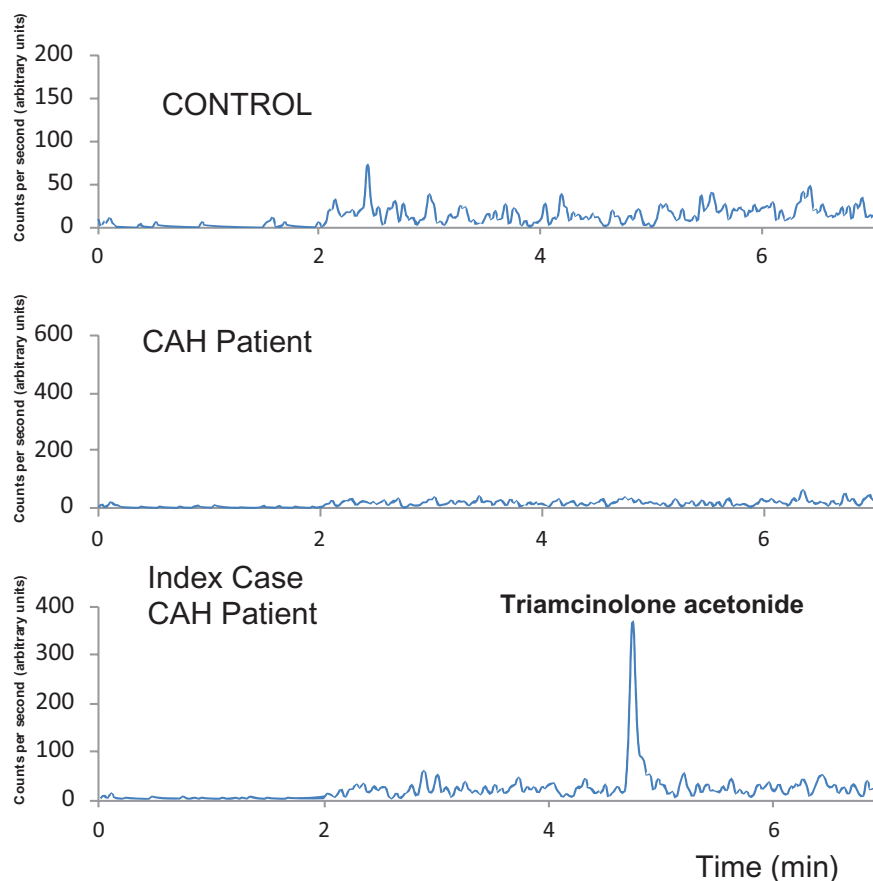
Figure 1
Identification of a peak corresponding to maternal intake of triamcinolone in the index case newborn dried blood spot compared to control (CTRL) and to CAH patient newborn blood spots (MRM fragmentation transition m/z 435 → 415).

than 40 nmol/L (with a term >36 weeks), a second control DBS is requested. In total, if we consider the time required to resample the newborn and a second shipment by mail, it often takes more than 2 days. As a new recommendation, we propose that, for all DBS samples presenting an FIA 17-OHP concentration over this threshold and those from newborns with a familial history of CAH, a steroid DBS should be profiled by LC-MS/MS as previously described (18). This NBS algorithm, which would include GSP for immunoassay analysis, followed by an NBS extraction and analysis by LC-MS/MS as a second-tier, can certainly seem longer. However, if the complete duration of care management is calculated, it ultimately reduces the time needed to identify suspicious cases. Indeed, if immunoassay is coupled with LC-MS/MS as a second-tier test, the time saved is at least 48 hours (including resampling and shipment). In many jurisdictions, LC-MS/MS 17-OHP quantitation is now a standard second-tier approach for DBS analysis related to CAH to reduce false positives. Moreover, the current FIA screening method looks for only the 17-OHP level, whereas with LCMS/MS we have an additional level of information for other steroid species, such as 21-DF (19, 20). We present here a proof of concept that LC-MS/MS steroid profiling

(including at least 17-OHP, 21-DF, testosterone, and D4-androstendione) may be useful for detecting not only false positive, as described elsewhere (9, 10) but also false negative CAH cases.

Adding 21-DF measurements could be particularly helpful because it is only elevated in patients with *CYP21A2* mutations (20). 21-DF is already being evaluated in children with 21-hydroxylase deficiency as an excellent and specific marker of this disease (21). For years, 17-OHP has been used and is still being used by clinicians for CAH exploration. However, in several recent studies, we and others have shown the implication of 21-deoxysteroid metabolites in the diagnosis of late-onset CAH and 21-DF was found to be elevated in patients with 21-hydroxylase deficiency compared with controls (14, 22, 23, 24, 25, 26). Elevated 21-DF quantified by LC-MS/MS has been shown to increase the sensitivity of newborn screening for CAH (5). The question of substituting the 17-OHP with the 21-DF assay has been raised. Recently, Miller *et al.* urged clinical investigators, commercial reference laboratories, and newborn screening programs to consider replacing 17-OHP with 21-deoxycortisol as the analyte of choice for studies of 21-OH deficiency (27). The (17-OHP+21-DF)/cortisol ratio has also been proposed

because it could discriminate CAH newborns from normal full-term or premature neonates (5, 28, 29).

Moreover, we were able to detect, in the infant blood on day 3 of DBS using LC-MS/MS, a specific peak corresponding to a maternal chronic nasal inhaled treatment of triamcinolone. Prenatal maternal corticosteroid treatment has the ability to suppress cortisol response to a CRH test on day 7, as is shown in very low birth weight and premature infants; even one or two doses of betamethasone can have an impact this strong on adrenal infant functions (16). There are also many long-term issues linked to corticosteroid treatments, especially metabolic consequences such as insulin resistance, type 2 diabetes, and hypertension in adulthood (17). However, triamcinolone pharmacokinetics and pharmacodynamics studies have not been performed in newborn populations. It is therefore rather difficult to compare the glucocorticoid concentration with those of other studies or to evaluate if the concentration is substantial or not. In adults, after inhalation, triamcinolone bioavailability averaged 22% with maximum levels of 2.0 ng/mL observed after 2.1 h. The resulting systemic levels for this treatment also cause a significant decrease in the number of lymphocytes in the blood (30). In our case report, it should be mentioned that the mother was still breastfeeding the patient and was under nasal triamcinolone treatment while the DBS was performed. Our goal was then to confirm, using LC-MS/MS, the presence (or absence) of triamcinolone in patient blood to explain the low 17-OHP concentration in the DBS on day 3 (<25 nmol/L), probably a consequence of an exogenous blockage of the hypothalamic–pituitary–adrenal axis. This has implications not only for NBS but also for the development of further pharmaco-toxicological studies that identify precisely which drugs are able to pass through the placenta during pregnancy. Further studies concerning the DBS reference values for steroids in relation to newborn age must be performed before implementation in the NBS program.

On an important note, we should mention that, by definition, if a first-tier FIA test is a false negative, no LC-MS/MS second-tier test can be performed. Thus, without his family history with known affected siblings, this newborn's pathology would likely have been missed by the NBS. Technically, we could perform LC-MS/MS analysis including a steroid profile (i.e., 17-OHP, 21-DF, testosterone, D4A, and cortisol) along with exogenous glucosteroids in the same DBS as a first-tier test. Although this would be efficient to avoid both false-positive and false-negative results, it is currently unreasonable, at least in France, to perform such a steroid profile as a first-tier test due to medico-economic issues.

To reach a compromise, we propose a new algorithm as follows: a first-tier immunoassay for 17-OHP except in case of familial CAH and known glucocorticoid treatment, for which an LC-MS/MS steroid profile will be performed directly as a first-tier test. Assessment of corticoid intake should be reinforced by the use of a questionnaire concomitant to the DBS sampling. It should clearly mention the use of oral or inhaled corticosteroids. If doubt was to arise concerning a possible hidden treatment, qualitative screening of exogenous glucocorticoids could be achieved, by LC-MS/MS within the same DBS extract. As a second-tier test, LC-MS/MS profile will still be performed in serum according to the FIA 17-OHP DBS threshold.

Conclusion

We identified triamcinolone maternal treatment as the cause of our unflagged CAH case in a family at risk. The contribution of 21-DF is a great help, as it can detect authentic CAH patients. This favors the use of the LC-MS/MS method in the NBS program. It could enhance the analytical performance (sensitivity and specificity) both by limiting the high level of false-positive results and by reducing false-negative results, particularly to prevent misdiagnosed CAH cases. In conclusion, the LC-MS/MS analysis of both endogenous and exogenous steroid metabolites improves the screening specificity of CAH diagnosis and should be considered in NBS programs.

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