JIM
JIM 2025; 2 (3): e1005
DOI: 10.61012_20258_1005
Category: *Original Article*

# THE EFFECTS OF NEONATAL NUTRITION ON 17-A-HYDROXYPROGESTERONE LEVELS IN CLASSIC CONGENITAL ADRENAL HYPERPLASIA SCREENING: A RETROSPECTIVE STUDY

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***ABSTRACT* – Objective:** Newborn screening (NBS) on dried blood spots (DBS) is mandatory under Italian law (No. 167/2016), despite a fair heterogeneity in the panel of diseases being monitored. Classical Congenital Adrenal Hyperplasia (CAH) has been recently included in the NBS program of the Abruzzo region, providing screening tests to over 16,000 newborns to date. According to our workflow, 445 (2.73%) second-tier tests (2-TT) were performed, 7 (0.04%) newborns required further attention, and 4 (0.02%) positive cases were found. Our NBS lab has already set customized cutoffs based on gestational weeks; however, other factors, such as different types of nutrition, could influence 17-α-hydroxyprogesterone levels (17-OH).

**Materials and Methods:** We retrospectively evaluated the CAH screening performed on DBS specimens collected within the first 120 hours (colostrum production time) after birth. We compared 17-OHP and associated markers levels between newborns who consumed formula milk and those who received colostrum. The analysis focused on 17-OHP levels in 6,384 infants, of which 439 were formula-fed while 5,945 breast-fed.

**Results:** 17-OHP levels showed statistically significant differences ($p$<0.0001) depending on the different types of nutrition, as well as some markers quantified by the second-tier test (2-TT). Then, we set customized cut-off values for 17-OHP according to the types of nutrition. As a preliminary approach, we analyzed steroids in two infant formulas and three colostrum samples, observing the absence of cortisol in formula milk.

**Conclusions:** These results lay the foundation to remodulate the current cutoffs in order to reduce 2-TTs, thus optimizing our analytical performance.

**Keywords:** Classical congenital adrenal hyperplasia screening, 17-α-hydroxyprogesterone, Newborns feeding, Second-tier tests, Customized cutoff.

## INTRODUCTION

Newborn screening (NBS) on dried blood spot (DBS) is a preventive medicine program, mandatory under Italian law (No. 167/2016), aimed at the early identification of inborn errors of metabolism (IEMs). The Abruzzo region has launched a pilot project in 2022 to monitor additional diseases, including severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID), L-amino acid aromatic decarboxylase deficiency (AADC), spinal muscular atrophy (SMA), Fabry disease, Gaucher disease, mucopolysaccharidosis type I and classic salt-wasting form of congenital adrenal hyperplasia (CAH)[1].

CAH encompasses a group of genetic autosomal recessive diseases stemming from defects in one of the enzymes or factors involved in cortisol biosynthesis: 21-hydroxylase (21-OH), 11β-hydroxylase (11β-OH), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase, 17-20 lyase (17α-OH; 17-20 lyase), P450 oxidoreductase (POR), steroidogenic acute regulatory protein (StAR) and cholesterol desmolase (P450-SCC)[2].

At the pathophysiological level, CAH typically leads to reduced levels of glucocorticoids and mineralocorticoids, alongside increased androgen levels. These alterations often necessitate the implementation of hormone replacement therapy.

The most prevalent and severe form of CAH is associated with the deficiency of 21-OH, which plays a key role in the synthesis of cortisol and aldosterone[3]. This condition can appear in two variants: the classic form, which is more critical and appears in the neonatal period, and the non-classic form, which is milder and has a later onset. The classic form of CAH can be categorized into Simple Virilizing form (SV) and Salt-Wasting form (SW)[4]. SV is a moderate condition in which there is a cortisol synthesis deficiency, whereas aldosterone synthesis is unchanged. In females, this disease manifests at birth with the presence of genital ambiguity; in males, it is typically diagnosed between 3 and 6 years of age, presenting with precocious puberty and an abrupt acceleration in growth rate. SW represents a more serious disease characterized by a concurrent defect in the synthesis of both cortisol and aldosterone. The lack of aldosterone leads to an increased excretion of sodium, resulting in hyperreninemia, hyponatremia, and hypovolemia. Furthermore, excessive androgen levels *in utero* cause virilization of the genitalia, which is more pronounced in females.

NBS for classic SW form of CAH involves quantifying 17-OHP levels using an immunoassay test. Differentiated cutoffs can be employed based on gestational weeks (GW), as several studies[5] indicated an improved correlation of gestational age with 17-OHP values: in this regard, gestational age is more directly associated with the development of the hypothalamic-pituitary-adrenal (HPA) axis.

To further reduce the recall rate and the false positive rate, second-tier tests (2-TTs) can be performed[6] to evaluate the steroid profile by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). In case of positivity at 2-TT, a molecular confirmatory test for *CYP21A2* is required[7], and further biochemical diagnostic evaluations such as plasma steroid profiling, electrolyte panel, and plasma renin activity or direct renin test are performed.

In our laboratory, we have screened 16,323 newborns for CAH to date. Following positivity, 445 (2.73%) 2-TTs were performed using different cutoffs according to GW, 7 (0.04%) newborns required further attention, and 4 (0.02%) positive cases were found. Given the low number of confirmed positive cases despite the routinely high use of 2-TTs, we wondered whether external factors[8] – such as variations in newborn nutrition – could influence 17-OHP levels during the first days of life.

In fact, it is important to emphasize that the composition of breast milk is not static and varies considerably during the different phases of lactation. Colostrum is the first type of milk produced in the first 5 days after birth; it is more viscous and concentrated than mature milk and, unlike formula milk, it is rich in bioactive compounds[9]. Moreover, colostrum has relatively low concentrations of lactose compared to mature milk, indicating that its primary functions are trophic rather than nutritional[10]. This is why it may have different effects on the adrenal gland than formula milk.

Hence, we conducted a retrospective evaluation of the overall screening workflow for CAH in the Abruzzo region, considering DBS specimens collected from newborns between 48 hours (minimum basal sampling required for NBS) and 120 hours after birth (the typical colostrum production period). Specifically, we compared the levels of 17-OHP and other associated markers (e.g., cortisol) between infants fed with formula milk and those who received colostrum, highlighting the necessity of setting personalized cutoffs to support the interpretation of the NBS results.

## MATERIALS AND METHODS

### First-Tier Test for CAH

This retrospective study was performed on data from 16,323 samples analyzed by the Neonatal Screening Center of the Abruzzo Region, located at the Center for Advanced Studies and Technology (CAST), "G. d'Annunzio" University, during the period spanning from June 2022 to May 2024. The study protocol (No. 0000992) was approved on 7 October 2021 by the Ethics Committee of G. d'Annunzio University of Chieti-Pescara, and informed consent was obtained from parents or legal guardians for the use of their child's blood samples, previously collected during routine newborn screening and clinical practice.

DBS specimens were collected within the first 120 hours of life on Ahlstrom 266 filter paper.

According to our protocols, in specific neonatal populations, it is possible to schedule additional sampling at 14 or 28 days after birth. These include infants classified as: preterm, low birth weight, transfused, receiving parenteral nutrition, admitted to intensive care unit (ICU), or under corticosteroid treatments.

The first-tier test measures 17-OHP levels on DBS through a time-resolved immunofluorescence assay, which relies on the GSP Neonatal 17-OHP kit (Revvity, Turku, Finland, OY), using the GSP Instrument (IVD) (Revvity, Turku, Finland, OY). The kit is based on a competitive binding immunoassay principle. Europium-labeled 17-OHP competes with the 17-OHP in the sample for a fixed number of binding sites on polyclonal anti-17-OHP antibodies (derived from rabbits). A second antibody, specific for rabbit IgG, is immobilized on a solid phase. This provides an effective separation of the bound antibody-antigen complex from the free antigen. The DELFIA inducer (Revvity, Turku, Finland, OY) dissociates europium ions from the labeled antigen, leading to the formation of highly fluorescent chelates. The fluorescence intensity, measured for each well, is inversely proportional to 17-OHP concentration in the sample (Figure 1A).

As previously described, we set up customized cutoffs for 17-OHP based on GW: 65 ng/mL for preterm infants with GW <34, 35 ng/mL for newborns with 34< GW <36, and 11 ng/mL for full-term newborns with GW >37.

### Second-Tier Test for CAH

The 2-TT consists of the simultaneous quantification of 21-deoxycortisol (21-DC), 11-deoxycortisol (11-DC), 4-androstene-3,17-dione (A4), 17-OHP, and cortisol (CORT) on DBS by LC-MS/MS (Figure 1B and C).

The method is fully described by Ferrante et al[8]. 250 µL of working solution – 95:5 (v/v) methanol/water – with internal standards (ISs) are added to two 3.2 mm diameter punches from DBS samples, calibrators, and Quality Controls (QCs). After incubation for 30 minutes at 700 rpm, supernatants are dried, and the residues are reconstituted with 100 µL of 60:40 (v/v) water 0.05 mM ammonium fluoride ($NH_4$)/methanol (Figure 1B).

20 µL of sample is injected into the ion source of the LC-MS/MS system, consisting of an ACQUITY™ UPLC™ I-Class system (Waters Co., Milford, MA, USA), comprised of a Binary Solvent Manager (BSM) and a Sample Manager with Flow-Through Needle (SM-FTN) coupled to a Xevo® TQ-S micro mass spectrometer (Waters Corporation, Milford, MA, USA). The system operates in positive electrospray ionization ($ESI^+$) mode. The run time is 2.50 minutes, injection-to-injection. Chromatographic separation of the analytes is achieved using a thermostated CORTECS C18 column [2.7 µM, 50 mm × 2.1 mm (Waters Co., Milford, MA, USA)], employing a CORTECS C18 2.7 µM VanGuard pre-column (Waters Co., Milford, MA, USA). Mobile phases are composed of 60% water (A) with 0.05 mM ammonium fluoride ($NH_4$) and 40% methanol (B). Flow rate is 1.0 mL/min. LC gradient and parameters referring to multiple reaction monitoring (MRM) functions for the detection of CORT, 21-DC, 11-DC, A4, 17-OHP are shown in Table 1 and Table 2, respectively. Figure 1C shows the chromatographic profile of the quantified analytes by 2-TT.

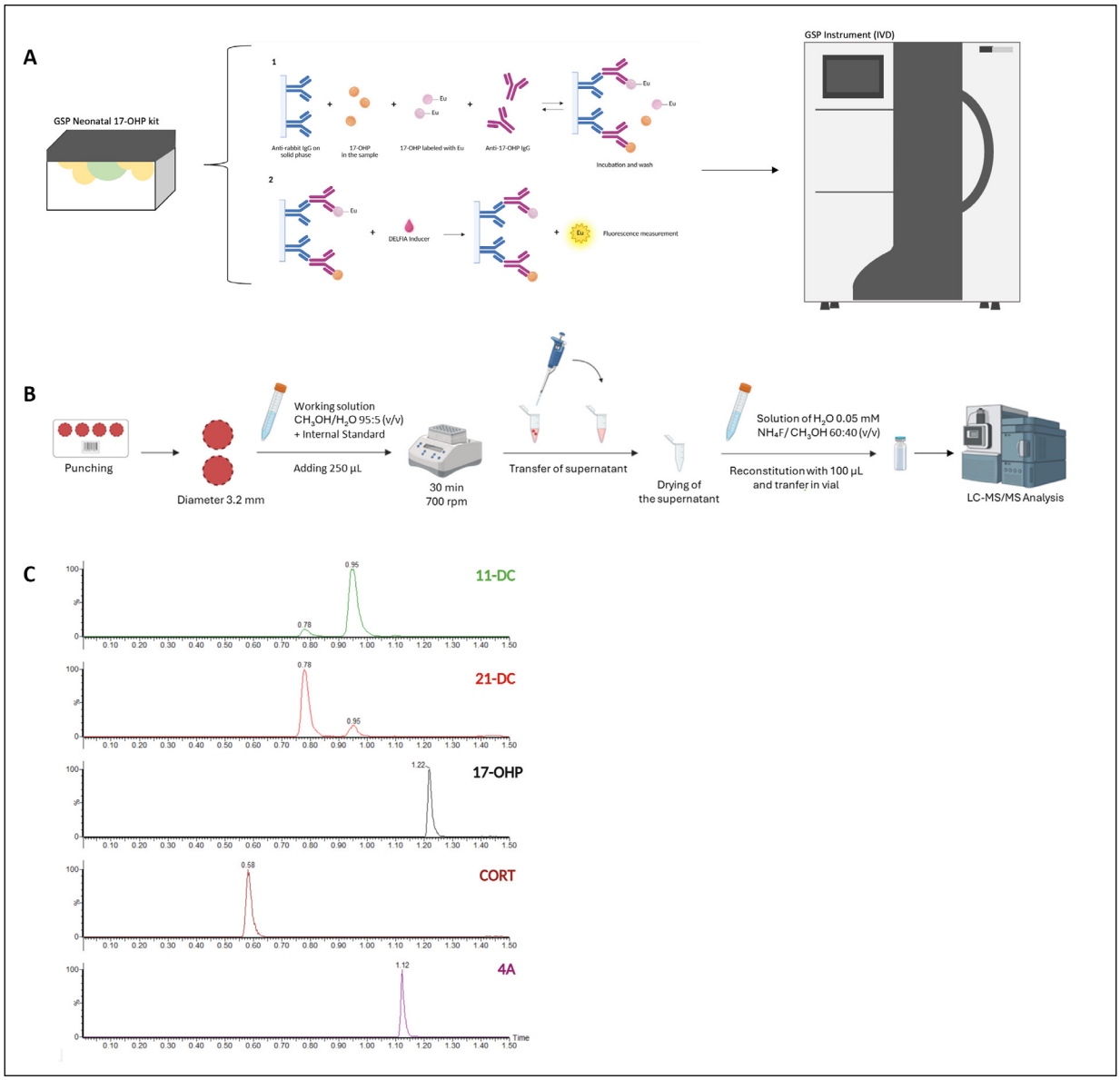


**Figure 1. A**, First-tier test for measuring 17-OHP levels on DBS by DELFIA through GSP Neonatal 17-OHP kit (Revvity) using the GSP Instrument (Revvity). **B**, DBS sample preparation and extraction for 2-TT trough LC-MS/MS. **C**, Chromatographic separation of CORT, 21-DC, 11-DC, A4, 17-OHP on DBS.

**Table 1.** Timetable of gradient elution for the detection of CORT, 21-DC, 11-DC, A4, and 17-OHP.

| No. | Time (min) | Flow rate (mL/min) | %A | %B | Curve |
|---|---|---|---|---|---|
| **1** | Initial | 1.000 | 60.0 | 40.0 | Initial |
| **2** | 0.10 | 1.000 | 60.0 | 40.0 | 6 |
| **3** | 0.25 | 1.000 | 55.0 | 45.0 | 6 |
| **4** | 0.75 | 1.000 | 55.0 | 45.0 | 6 |
| **5** | 0.90 | 1.000 | 37.5 | 62.5 | 6 |
| **6** | 1.15 | 1.000 | 5.0 | 95.0 | 11 |
| **7** | 1.40 | 1.000 | 60 | 40 | 11 |

**Table 2.** Multiple Reaction Monitoring (MRM) functions and settings for the detection of CORT, 21-DC, 11-DC, A4, and 17-OHP.

| MRM Function | Time Window (min) | Analyte | Dwell time (s) | Transitions (m/z) | Cone (V) | Collision Energy (CE) |
|---|---|---|---|---|---|---|
| **1** | 0-2.50 | A4 (Quant.) | 0.010 | 287.30 > 97.10 | 50.0 | 20.0 |
| **2** | 0-2.50 | A4 (Qual.) | 0.010 | 287.30 > 109.10 | 50.0 | 20.0 |
| **3** | 0-2.50 | A4 D7 IS | 0.010 | 294.30 > 100.00 | 50.0 | 20.0 |
| **4** | 0-2.50 | 17-OHP (Quant.) | 0.010 | 331.30 > 97.10 | 50.0 | 20.0 |
| **5** | 0-2.50 | 17-OHP (Qual.) | 0.010 | 331.30 > 109.10 | 50.0 | 20.0 |
| **6** | 0-2.50 | 17-OHP D8 IS | 0.010 | 339.30 > 100.10 | 50.0 | 20.0 |
| **7** | 0-2.50 | 11-DC (Quant.) | 0.010 | 347.30 > 97.10 | 50.0 | 22.0 |
| **8** | 0-2.50 | 11-DC (Qual.) | 0.010 | 347.30 > 109.10 | 50.0 | 22.0 |
| **9** | 0-2.50 | 11-DC D2 IS | 0.010 | 349.30 > 97.00 | 50.0 | 22.0 |
| **10** | 0-2.50 | 21-DC (Quant.) | 0.010 | 347.30 > 311.10 | 50.0 | 14.0 |
| **11** | 0-2.50 | 21-DC (Qual.) | 0.010 | 347.30 > 121.20 | 50.0 | 22.0 |
| **12** | 0-2.50 | 21-DC D8 IS | 0.010 | 355.30 > 319.30 | 50.0 | 14.0 |
| **13** | 0-2.50 | CORT (Quant.) | 0.010 | 363.30 > 121.10 | 50.0 | 22.0 |
| **14** | 0-2.50 | CORT (Qual.) | 0.010 | 363.30 > 91.10 | 50.0 | 50.0 |
| **15** | 0-2.50 | CORT D3 IS | 0.010 | 366.30 > 121.00 | 50.0 | 22.0 |

### Milk Steroid Profile Evaluation

The steroid profile was evaluated in two different infant formulas and three colostrum samples (in analytical triplicate), donated as leftover material during the first days of lactation. The milk steroid profile is determined by LC-MS/MS quantifying CORT, 11-DC, 4-A, 17-OHP, testosterone, progesterone, corticosterone and aldosterone using the CHS™ MSMS Steroids Kit, Revvity® (Revvity, Turku, Finland, OY), following the same protocol previously described[11]. The details for quantitative determination of steroid profile in milk samples by UP-LC-MS/MS are fully described in Supplementary Materials, where Supplementary Table 1 and Supplementary Table 2 detail specifically chromatographic and mass spectrometer parameters.

### Statistical Analysis

Univariate statistical analysis by *t*-test was performed using GraphPad Prism (GraphPad software, Inc., Boston, MA, USA) to assess whether differences in 17-OHP and other associated markers (e.g., cortisol) levels between breast-fed and formula-fed newborns were statistically significant. $p<0.05$ was considered statistically significant. Outliers were identified with the ROUT method employing a false discovery rate of 1%. Additionally, we set customized cutoffs at the 99.5$^{th}$ percentile for colostrum-fed infants and formula-fed infants.

## RESULTS

### The Type of Nutrition Affects Steroid Profiles on DBS

To assess the influence of the type of nutrition on 17-OHP levels, we retrospectively analyzed data from 6,384 full-term, normal-weight newborns within the first 120 hours of life, representing 39.11% of the total population screened for CAH. According to our experience, in our NBS laboratory, 16,323 newborns were screened for CAH from June 2022. Following the positivity to the first-tier test, 445 (2.73%) 2-TTs were performed using different cutoffs according to GW. Only 7/445 (0.04%) newborns were referred to clinical attention and diagnostic confirmation, and 4 (0.02%) positive cases were found to date (Figure 2).

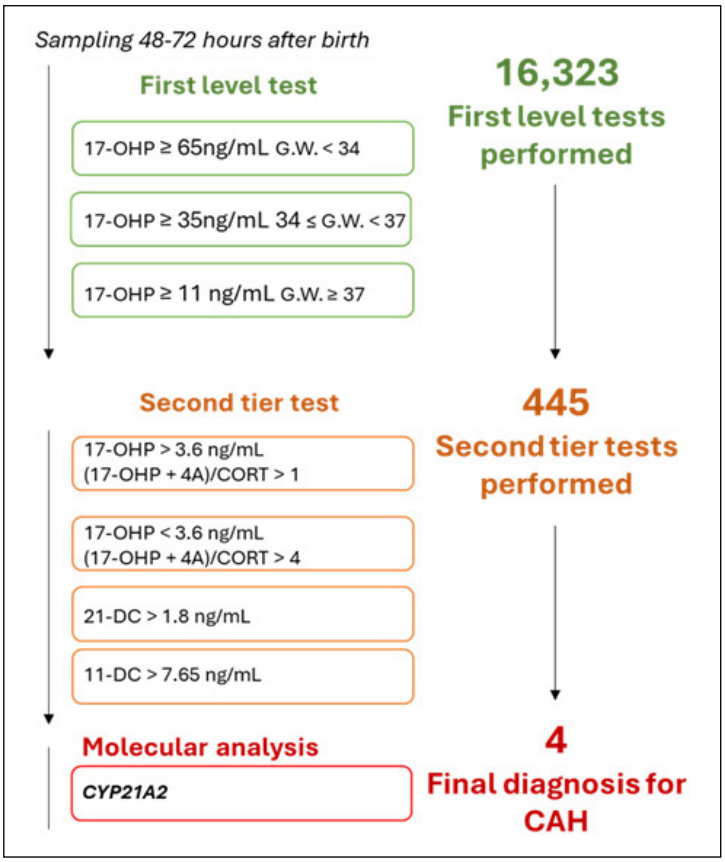


**Figure 2.** NBS data overview for CAH in the Abruzzo region (Italy).

The results from the first-tier test highlighted statistically significant differences ($p$<0.0001) between 17-OHP levels measured on DBS between breast-fed newborns (n=5,945) and those who received formula milk (n=439) (Figure 3A). Herein, 9.34% of formula-fed newborns (n=41) and 1.51% of breast-fed infants (n=90) required 2-TT due to elevated 17-OHP levels based on our cutoff criteria. Even among the subpopulation that underwent 2-TT, 17-OHP levels measured at the first-tier test remained significantly higher in formula-fed newborns compared to breast-fed infants ($p$-value=0.0320), consistent with the previously observed trend (Figure 3B).

Next, we evaluated the influence of the type of nutrition on all markers quantified by 2-TT, analyzing data from the 131 full-term, normal-weight newborns who required it. 21-DC levels were below the limit of quantification (LOQ) in all samples, while A4 had comparable values between groups. Notably, we observed statistically significant differences in quantified levels of 17-OHP ($p$=0.0125), CORT ($p$=0.0048), 11-DC ($p$<0.0001), and (17-OHP + A4)/CORT ratio ($p$<0.0001) (Figure 3C). Dot plots display higher levels of 17-OHP and 11-DC, as well as the ratio (17-OHP + A4)/CORT, in formula-fed infants compared to breast-fed infants, while CORT shows the opposite trend.

## Customized Cutoffs for 17-OHP According to the Type of Nutrition

Based on the impact of the feeding type on all marker levels, we set customized cutoff values for 17-OHP according to either breastfeeding or artificial nutrition. Based on the 99.5$^{th}$ percentile, we established feeding-customized 17-OHP cutoff values of 12.33 ng/mL for breast-fed infants and 18.61 ng/mL for formula-fed newborns.

Retrospective application of these personalized cutoffs would have yielded only 38 2-TTs (29.8% of the total 2-TTs performed).

## Steroid Profile of Natural and Artificial Milk

As a preliminary approach, the characterization of the steroid profile of two formula milks and three colostrum samples showed a statistically significant difference in CORT levels ($p$=0.0004), highlighting a complete absence of CORT in formula milks (Figure 4). The other steroids showed no significant differences or were under LOQ.

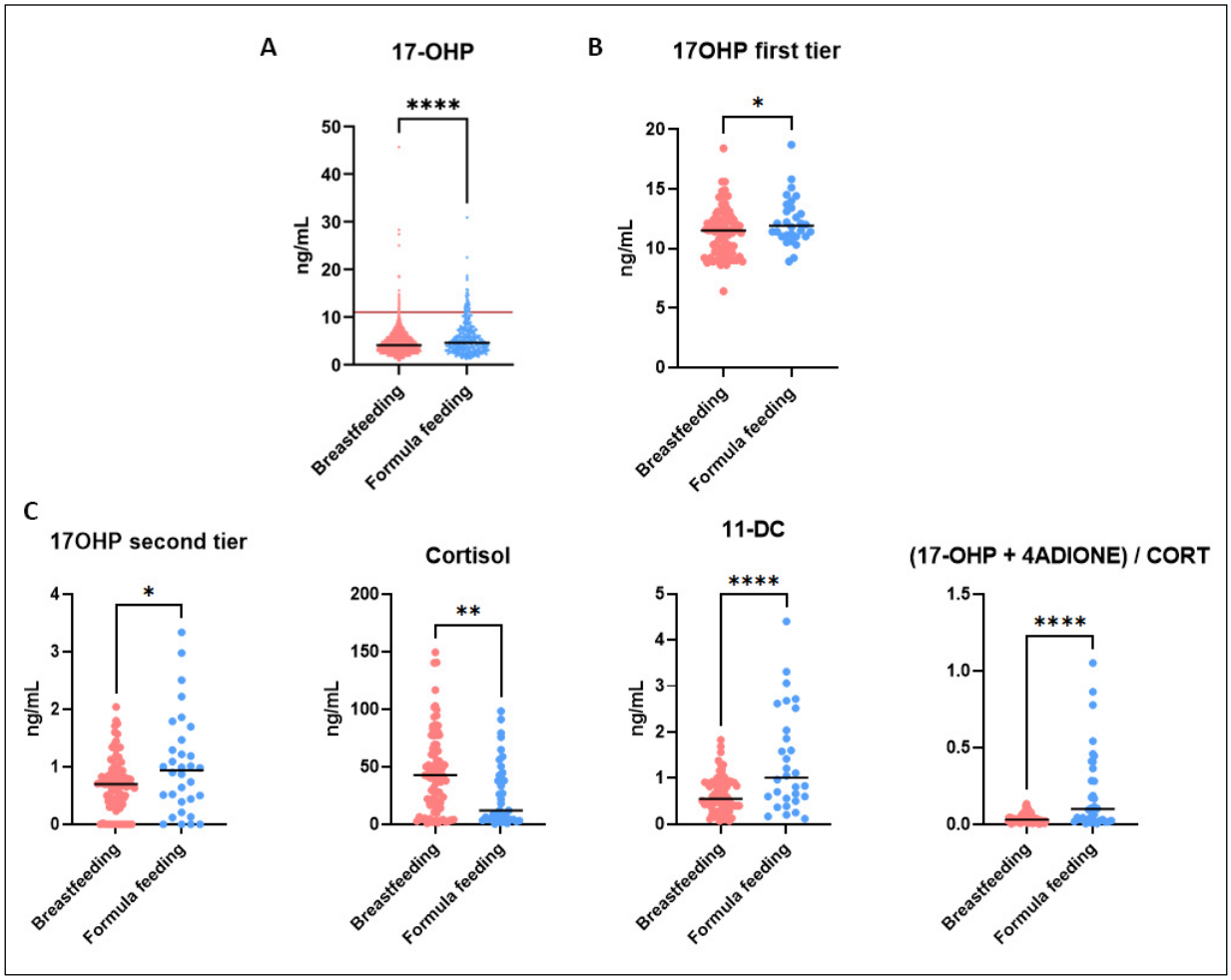


**Figure 3. A**, Dots represent the entire Abruzzo neonatal population screened for 17-OHP from June 2022. Data at the first-tier test for formula-fed and breast-fed infants are reported as concentration in (ng/mL). The red line indicates the cutoff for full-term newborns. **B**, Dots represent the newborns who required 2-TT for increased 17-OHP concentration (ng/mL) at the first-tier test, divided into formula-fed and breast-fed. The red line indicates the cutoff for full-term newborns. **C**, 17-OHP, CORT, 11-DC concentrations and (17-OHP + A4)/CORT ratio for formula-fed and breast-fed newborns quantified by 2-TT. *$p$<0.05, **$p$<0.01, ****$p$<0.0001.

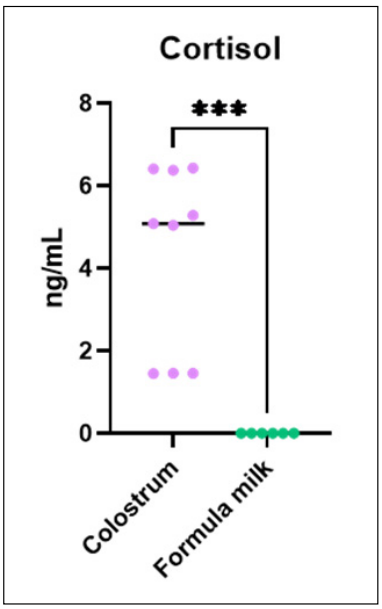


**Figure 4.** Cortisol levels in infant formulas and colostrums quantified by LC-MS/MS. ***$p$<0.01.

## DISCUSSION

Since 2022, thanks to a pilot project, the Abruzzo region has been providing screening for CAH, a disorder caused by defective adrenal steroid hormone synthesis. The most common cause of CAH is 21-OH deficiency[11]. The investigation is focused on the SW form of CAH, which presents in the neonatal period, and it is characterized by impaired synthesis of cortisol and aldosterone, resulting in an excessive sodium excretion, hyperreninemia, hyponatremia, hypovolemia, and genitalia virilization in females[5]. Our protocols ensure reliable quantification of 17-OHP and other associated markers through a workflow that includes first- and 2-TT testing on DBS, as well as plasma steroid profiling. According to this strategy, we analyzed 16,323 samples and performed 445 2-TTs (2.73%), of which only four were positive cases (0.02%). The overall effectiveness of the screening program for CAH has already been improved by setting cutoffs based on gestational age and recalling preterm infants and newborns undergoing special treatments for clinical demands at 14 or 28 days after birth. However, to further reduce the high incidence of 2-TT, we asked whether environmental factors and clinical variables could somehow influence 17-OHP levels in newborns, as already observed for propionyl-carnitine[1].

Therefore, we retrospectively analyzed data from over 6,000 full-term, normal-weight newborns within the first 120 hours of life, which is the typical colostrum production time[9] to compare 17-OHP levels between breast- and formula-fed infants. It is well known that colostrum presents bioactive compounds compared to formula milk[9]. In fact, cytokines and growth factors in colostrum could play an essential role in infant immune maturation[12], including insulin-like growth factor 1 and 2 (IGF-1, IGF-2) and epidermal growth factor (EGF), which contribute to the development of intestinal mucosa. Moreover, colostrum contains hormones, immunoglobulins (i.e., IgA, IgG, IgM), some specific antibodies against bacteria, viruses and fungi, such as *E. coli*, *Salmonella*, respiratory syncytial virus, *Candida*, *Streptococcus*, *Staphylococcus*, *Cryptosporidium* and *H. pylori*. It also has more proteins, minerals and fat-soluble vitamins (A, E and K) than normal breast milk[13]. Hence, the primary colostrum function is trophic rather than nutritional[10]: in this context, colostrum could stimulate adrenal gland function differently in breast-fed infants compared to those receiving formula milk.

Notably, we found statistically significant differences for 17-OHP at either first- and 2-TT, but also for CORT, 11-DC and (17-OHP + A4)/CORT ratio. Therefore, we set customized cutoffs for 17-OHP based on the type of nutrition, with higher values for formula-fed infants than for those receiving natural milk. Interestingly, the retrospective application of these personalized cutoffs would have resulted in a third of the 2-TTs performed.

For a deeper comprehension of our findings, we compared the steroid profile between two different infant formulas and three colostrum samples. This preliminary approach showed a statistically significant difference in CORT levels, revealing the presence of CORT exclusively in colostrum.

Our preliminary results on the concentration of steroids in milk are consistent with what is reported in the literature. Unlike infant formulas, colostrum contains steroid hormones and their precursors. In particular, previous analyses have shown that cortisol is absent from infant formula[14]. Cortisol in breast milk could play a crucial role in infant development, as the transmission of glucocorticoids through milk can influence both behavior and brain maturation in newborns. Thus, breast milk helps shape the infant's phenotype through its biologically active components[15]. It has been demonstrated that the adrenal gland is regulated even in the prenatal phase, and maternal malnutrition exerts a broad suppressive effect on the steroidogenic enzymes of the offspring[16]. In this regard, it is important to emphasize that prenatal exposure to cortisol has both direct and long-term effects on the regulation of the HPA axis[17]. A recent study[18] indicates that both environmental and physiological factors modulate adrenal activity in newborns. Notably, an adrenal response to feeding has been observed, which could have implications for HPA axis development and future health outcomes.

For this reason, our results, although preliminary, lay the foundation for a possible remodulation of the cutoffs in CAH screening, taking into account the type of neonatal nutrition. This approach could contribute to reducing 2-TTs, saving time and costs while improving identification of true positives. However, these findings need to be validated in a larger population. Moreover, further studies on the compositional differences between formula milk and colostrum are essential, as current data in the literature are limited. Investigating these aspects would allow us to better understand the molecular mechanisms underlying these metabolic processes, which could regulate the adrenal gland function differently.

## CONCLUSIONS

Our study highlights a previously underexplored variable in the interpretation of 17-OHP levels in CAH screening: the type of neonatal nutrition. The use of nutrition-based cutoffs for 17-OHP could substantially reduce the number of 2-TTs, improving screening efficiency and reducing costs and time. Although these preliminary findings are promising, they require validation in larger cohorts. Further studies on the different compositions of breast milk and formulas are needed to understand their physiological impact to optimize screening strategies. Integrating environmental and clinical variables, such as neonatal nutrition, into CAH screening algorithms could represent a meaningful step forward in precision medicine.

### Artificial Intelligence-Assisted Technologies:

No artificial intelligence-assisted technologies were used in the production of this article.

### Authors' Contributions:

Study conception and design: M.L.T., S.Va., M.C.C.; data collection: G.D.C., L.N., R.P.; analysis: A.F., M.Z., D.S., S.Ve.; data interpretation: M.L.T., S.Va.; manuscript drafting: M.L.T., G.D.C.; manuscript editing: S.Va., M.C.C., D.F., V.C., S.D.V., I.C. All authors have read and agreed to the published version of the manuscript.

### Availability of Data and Material:

All data generated or analysed during this study are included in this published article or its Supplementary Material.

### Conflict of Interest:

The authors declare that they have no conflict of interest to disclose.

### Ethics Approval and Informed Consent:

The study was approved by the Ethics Committee of G. d'Annunzio University of Chieti-Pescara with protocol No. 0000992, on September 30, 2021, and informed consent was obtained from parents or legal guardians for the use of their child's blood samples, previously collected during routine newborn screening and clinical practice, within the project.

### Funding:

M.L.T. produced this publication while attending the PhD program in Biomolecular and Pharmaceutical Sciences at the University of Chieti, Cycle XXXVIII, with the support of a scholarship financed by the Ministerial Decree No. 351 of 9 April 2022, based on the NRRP-funded by the European Union-NextGenerationEU.

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