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Article

The Association of Gestational Age, Birth Weight, and Age at Heel Prick with Biomarkers of Newborn Screening in The Netherlands

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Abstract

The Dutch nationwide newborn screening (NBS) programme screens for 28 disorders using biomarkers measured in heel prick samples, preferably collected between the third and seventh day of life. This study aimed to assess the association of gestational age (GA), birth weight (BW), and age at heel prick with biomarker values within the Dutch NBS programme. Retrospective analysis of 1,479,708 screening results (2010–2020) for 20 biomarkers was performed. Statistical analyses consisted of multivariable linear analyses, ANOVA, and Scheffé's post hoc test. Multivariable linear analyses showed moderate to strong associations of adult and foetal haemoglobin (HbA and HbF), 17-hydroxyprogesterone (17-OHP), and thyroxine (T4) with GA, BW, and/or age at heel prick ($R \geq 0.30$). HbA and HbF were similarly influenced by both GA and age at heel prick, whereas 17-OHP and T4 were predominantly affected by GA. BW contributed minimally beyond GA, likely due to their biological correlation. For the other biomarkers, a less pronounced association was found. The findings indicate that current cut-off values for HbA, haemoglobin Bart's 17-OHP, and T4 in the Dutch NBS programme may require re-evaluation, particularly for extremely premature newborns and for samples taken outside the preferred age window. Incorporating these findings, in combination with studies that include clinical outcomes, might improve the accuracy and predictive value of future NBS programmes.

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Keywords: dried blood spot; newborn; newborn screening; premature; gestational age; birth weight; hemoglobinopathies; congenital hypothyroidism; congenital adrenal hyperplasia

1. Introduction

The nationwide newborn screening (NBS) programme in the Netherlands was initiated in 1974 with screening for phenylketonuria (PKU). The programme currently encompasses 28 disorders (Table S1 (Supplementary Materials)) [1]. The optimal screening time window for each disorder is determined by the timing of disease onset, the maturation of the newborn's metabolism, and/or the clearance of metabolites transplacentally received from the mother, and differs between premature and term newborns [2]. Furthermore, several reports have demonstrated that gestational age (GA), birth weight (BW) and/or age (time between birth and heel prick) affect the concentrations of biomarkers analysed in NBS protocols [3–10].

Currently, four out of 28 Dutch NBS protocols take GA, BW and/or age at heel prick into account [11]. The oldest protocol, for congenital hypothyroidism (CH), uses thyroxine (T4) as the primary biomarker, followed by thyroid-stimulating hormone (TSH) and T4-binding globulin (TBG) in the 20% and 5% lowest T4 cases, respectively [12]. This protocol enables detection of both primary and central CH, characterised by low T4 and high TSH, and low T4, a low T4/TBG ratio, and normal TSH concentration, respectively [12]. Due to the high rate of false-positive referrals in premature newborns caused by hypothyroxinaemia associated with prematurity [13], "the premature rule" was introduced in 1982 [13,14]. This rule states that newborns born at GA of ≤ 36 weeks and with BW of ≤ 2500 g should be referred for screening based solely on elevated TSH concentrations. This limits screening to primary CH in this group of newborns. "The premature rule" was later extended to children aged ≥ 60 days at the time of the heel prick [14].

The protocol for congenital adrenal hyperplasia (CAH), introduced in 2002, employs four GA-dependent cut-off values (COVs) for 17-hydroxyprogesterone (17-OHP), with BW used if GA is unknown: ≥ 200 nmol/L if GA ≤ 33 + 0/7 weeks, ≥ 105 nmol/L if GA ≥ 33 + 1/7 weeks, ≥ 55 nmol/L if GA ≥ 35 + 1/7 weeks, and ≥ 25 nmol/L if GA ≥ 36 + 1/7 weeks [11].

In 2009, GA-dependent COVs for adult haemoglobin (HbA) were introduced for beta-thalassemia major (βTM) screening to reduce the risk of false-negatives, without increasing the number of false-positives among pre-term newborns: instead of a single COV for all newborns, referral will only take place when HbA is ≤ 1% if GA ≤ 32 weeks or ≤ 2.5% if GA > 32 weeks in the absence of other haemoglobin variants [11].

The NBS protocol for severe combined immunodeficiency (SCID), implemented in 2021, uses a GA, BW and age-adjusted protocol, with a second heel prick performed at corrected GA > 36 weeks for premature newborns with low T cell receptor excision circle (*TREC*) counts [11].

Improvements in perinatal care [15] have increased survival and NBS participation among very premature newborns [16], raising questions about the current applicability of GA- and BW-dependent COVs, most of which predate these changes. Furthermore, with the exception of CH, Dutch protocols provide no guidance on the validity of COVs outside the heel prick window of 73–168 h post-birth.

In a recent Dutch study, data from heel prick samples taken between 3 and 183 days after birth were analysed to evaluate the effect of perinatal factors, with a focus on metabolic disorders screened by mass spectrometry [10]. The authors suggested that NBS performance could be improved by integrating additional variables into the screening protocols used for metabolic screening. However, they analysed only a limited set of the

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biomarkers used in the Dutch NBS, and the impact of heel prick blood collection before the preferred age or in the last month of the eligible timeframe was not assessed.

The objective of this current study was to examine the association of GA, BW, and age at heel prick with the concentrations of biomarkers included in the Dutch NBS, in order to assess the potential impact on NBS protocols.

2. Materials and Methods

2.1. Study Population

For this retrospective study, pseudonymised data from all dried blood spot (DBS) samples acquired as part of the Dutch NBS programme between 1 January 2010 and 31 December 2020 were extracted from the nationwide information system called Praeventis (Table 1 and Table S2 (Supplementary Materials)). These samples included both negative and positive screening results. Data on final diagnosis were unknown. For the purpose of this study, the requested data were obtained from Praeventis following approval by the data request committee ('Commissie Dataverzoeken Praeventis en COVID-vaccinatie Informatie- en MonitoringsSysteem (CIMS)').

Table 1. Summary of characteristics of the entire dataset composed of screens performed in the Netherlands between 1 January 2010 and 31 December 2020. More detailed information is provided in Table S2 (Supplementary Materials). (SD = standard deviation).

Number of heel prick cards	Total	1,486,453
	Included	1,479,708 (99.55%)
Gestational age (weeks)	Mean	39 + 2/7
	SD	1 + 6/7
	Minimum	23 + 3/7
	Maximum	43 + 6/7
Birth weight (grams)	Mean	3410
	SD	575
	Minimum	390
	Maximum	8000
Age at heel prick (days)	Mean	5.5
	SD	11
	Minimum	0
	Maximum	215

2.2. DBS Collection and Laboratory Analyses

In the Netherlands, including the Caribbean islands of Bonaire, St. Eustatius, and Saba, NBS is offered to all newborns. Blood samples are obtained by heel prick and collected on filter paper, preferably between 72 and 96 h after birth, but no later than 168 h [17]. However, there are certain exceptions to this general rule. Firstly, when a newborn requires a blood transfusion, it is allowed to perform a heel prick between 49 and 72 h after birth, prior to the blood transfusion, or even within 48 h after birth in order to prevent a repeat heel prick after 90 days for haemoglobinopathies (HbPs). Secondly, in Bonaire, St. Eustatius, and Saba, a delayed heel prick is frequently observed in children born outside the island. This is because the heel prick is only offered when they return to their home island. Finally, NBS is offered to children up to six months of age. In most cases, this last exception applies to immigrants or refugees. GA, BW, date and time of birth, date and time of the heel prick, and date and time of blood transfusion, if applicable, are registered on the heel prick card.

The DBS cards are sent via regular post to one of the designated regional screening laboratories, which conducts the laboratory analyses in accordance with the instructions of the manufacturer and nationwide screening protocols. Table S3 (Supplementary Materials) summarises the commercial assays that are and have been used during the study period to acquire the biomarker concentrations/activities.

2.3. Dataset and Criteria

Data were received as TXT files per year, each containing the specific biomarker values per NBS condition, GA in days, BW in grams, and age at heel prick in days and in hours (the day and time of birth is 0) of the children screened in that specific year. The dataset was created using SPSS (IBM; version 29.0.1.0).

The following exclusion criteria were applied to the entire dataset:

1. Repeat samples: Only the first heel prick sample per child was registered to avoid duplication.
2. Samples obtained within 24 h after a blood transfusion, or cases where HbA was 50–70% with age at heel prick < 25 days, or HbA ≥ 70% with age at heel prick < 90 days; these findings suggest an unreported blood transfusion.
3. Age at heel prick ≥ 216 days (seven months).
4. GA < 161 days (23 weeks) and ≥ 308 days (44 weeks).

In addition, the following exclusion criteria were applied for selected biomarkers.

5. C3, methylmalonic acid (MMA), methylcitric acid (MCA), and C0/(C16 + C18), as these markers are used to screen for disorders included in the Dutch programme after 2019; for these disorders, the variables were already investigated in recent validation studies.
6. TSH, TBG, pancreatitis-associated protein (PAP), and total galactose (TGAL), because these are subsequent tier markers and concentrations are only determined in a selection of samples.
7. 17-OHP, T4 and immunoreactive trypsinogen (IRT): Results obtained before 1 January 2013, as the current assay was introduced in 2011 and 2012 in the Dutch laboratories.
8. Galactose-1-phosphate uridyltransferase (GALT): Results obtained before 1 July 2014 due to a difference in analytical units (percentage versus absolute values).
9. Amino acids, acylcarnitines, succinylacetone (SA): Results obtained before 1 January 2018, because the current assay was introduced in 2018.

Values outside the measuring range were recorded in Praeventis as below the lower or above the upper limit of quantification (LLOQ and ULOQ, respectively). In the dataset, values below the LLOQ were replaced by $\sqrt{0.5} \times$ LLOQ, and values above the ULOQ were recorded without the 'greater than' symbol. For a brief period following the transition to the current assay, the LLOQ for SA was not updated from 0.1 to 0.01 µmol/L. Therefore, 10,141 samples had a registered result of < 0.1 µmol/L, which was replaced as if the result was below the official LLOQ for SA (0.00707 µmol/L, calculated as $\sqrt{0.5} \times 0.01$).

C5 was excluded from the analysis because over 65% of the results were below the LLOQ. Consequently, the number of results was insufficient to perform statistical analysis.

Between 1 October 2008 and 1 October 2021, biotinidase (biot) activity, the screening marker for biotinidase deficiency, was expressed as a percentage of the daily mean. Two different assays were used during this period, which showed comparable results (unpublished data). All values were transformed to a percentage of the daily mean, allowing data from both assays to be combined for this study.

2.4. Statistical Analyses

All statistical analyses were performed using SPSS. The normality of the distribution of the biomarkers was assessed by histograms and normal-probability plots. While the majority of histograms exhibited slight skewness or truncation at the limit of detection/quantification, overall, the data were consistent with a normal distribution. The application of the (log)transformation did not result in improved normal distributions. Therefore, it was determined that statistical analyses appropriate for normally distributed data could be employed. The collinearity between GA and BW was analysed with a Pearson Correlation. To analyse the correlation between GA (days), BW (grams), and age at heel prick (hours), a multivariable linear regression analysis was performed for each biomarker. If the multivariable linear regression analysis resulted in an R value of at least 0.30, it was assumed that the correlation was sufficiently robust to continue investigation of the correlation [18]. These biomarkers were categorised, with each category representing a similar size of the variable. This resulted in 21 categories for GA (per week), 76 categories for BW (per 100 g), and 215 categories for age at heel prick (per day). An ANOVA test was performed to test whether the distribution of values for each biomarker was equal across the various categories of the variables. For HbA and foetal haemoglobin (HbF), the ANOVA analyses were performed on subsets of the dataset, as it is known that, starting just before birth, the production of HbF is gradually replaced by the production of HbA. Using a subset allowed the influence of GA or age at heel prick to be evaluated with minimal interference from the other variable. The subsets contained either results from DBS cards taken 72 to 119 h after birth or data from children born at GA 38 weeks + 0 days to 40 weeks + 6 days. The Scheffé's post hoc test was performed to determine the significant differences between the various categories, and to assess, if applicable, the point at which differences between categories are no longer significant or where significant differences appear, i.e., whether a stable level had been reached or had been lost. This type of post hoc is used because of the large number of categories and the unequal sample sizes. Scheffé's post hoc test in SPSS can only be applied with a maximum of 50 categories. Consequently, a subset of BW and age categories was evaluated for T4 and 17-OHP, based on the categories of interest that emerged from the means plot resulting from the ANOVA test.

3. Results

The dataset comprised the screening results for 28 biomarkers, as well as the three variables GA, BW, and age at heel prick, from 1,486,453 heel prick cards that were received by one of the screening laboratories for the Dutch NBS programme between 1 January 2010 and 31 December 2020. Table 1 summarises the characteristics of the dataset. In this study, the data for 1,479,708 screened children were included for analysis. Of these, 0.28% (n = 4145) were born extremely pre-term (GA < 28 weeks), 0.72% (n = 10,652) very preterm (between 28 and 32 weeks GA), 6.82% (n = 100,913) moderately or late pre-term (between 32 and 37 weeks GA), 78.26% (n = 1,157,988) were born at term (between 37 and 41 weeks GA), 13.03% (n = 192,870) were born late term (42 weeks GA) and 0.45% (n = 6639) were born post-term (GA > 42 weeks). The mean GA was 39 weeks and 2 days. Of the samples, 1.445% (n = 21,388) had their heel prick performed before the preferred age (within 72 h/day 3 of life), 39.109% (n = 578,694) had their heel prick performed at the preferred age (between 73 and 96 h/day 3 of life), 56.875% (n = 841,582) had their heel prick performed within the allowed time after birth (between 97 and 168 h/day 4 to 6 of life), and 2.571% (n = 38,044) had their heel prick performed late (beyond 168 h/7 days of life). The mean age at heel prick was 5.5 days (standard deviation (SD) 11 days).

The results of 6745 DBS cards (0.45%) were excluded based on the aforementioned exclusion criteria 1–4. As previously mentioned, C5 was not analysed. Together with

exclusion criteria 5–9, this resulted in a total of 20 usable biomarkers for testing: 17-OHP, T4, GALT, IRT, biot, HbA, HbF, haemoglobin Bart's (Hb Bart's), phenylalanine (phe), leucine (leu), valine (val), SA and eight acylcarnitines (C0, C2, C5DC, C5OH, C8, C10, C14:1 and C16OH). Table 2 summarises the number of data points included in the analysis of each tested biomarker.

3.1. Multivariable Linear Regression Analysis

In order to assess the degree of influence of GA, BW, and age at heel prick, the 20 biomarkers were analysed using multivariable linear regression. All analyses resulted in a $p < 0.001$ (Table 2), indicating that a model combining the variables GA, BW, and age at heel prick can predict the dependent variable (biomarker value). Due to the large sample size, low p values were to be expected. BW had minimal impact on most biomarkers within the model. This can be attributed to the biological correlation between GA and BW, as Pearson's correlation coefficient revealed a strong positive correlation between GA and BW ($r = 0.662$, $p < 0.001$). Of the 20 analysed biomarkers, the multivariable linear regression analysis of HbA, HbF, 17-OHP and T4 resulted in an R value of at least 0.30, indicating that the regression model explains at least a reasonable proportion of the variance. For HbA and HbF, the standardised coefficient beta (β) for GA and age at heel prick were comparable. This finding indicates that both variables influence the biomarker value in a comparable manner. For 17-OHP and T4, the β for GA was evidently higher than for the other variables. This suggests that GA had the greatest impact on 17-OHP and T4 concentrations. Within the remaining sixteen biomarkers with the initial exclusion criterium of $R < 0.30$, some might be interesting to study in the future because of the higher β; C0 has the most deviating β for BW (−0.200), GALT, C14:1 and C5DC have deviating β for GA (−0.207, −0.167 and −0.151, respectively), and C5OH has mildly deviating β for both GA as well as age (0.113 and 0.116, respectively).

3.2. Comparison of Mean Biomarker Concentration/Activity over Categories Based on Gestational Age, Birth Weight, or Age at Heel Prick

An ANOVA analysis was performed to assess the effect of the studied variables—GA, BW, and age at heel prick—on HbA, HbF, 17-OHP, and T4, the biomarkers with an R value ≥ 0.30 in the multivariable linear regression analysis. The two subsets used for the analysis of HbA and HbF consisted of 923,928 newborns who underwent a heel prick between 72 and 119 h after birth, and 888,084 children born at GA between 38 + 0/7 weeks and 40 + 6/7 weeks. Figure 1 shows the mean concentration/activity and standard deviation for each category of HbA (Figure 1A–C), HbF (Figure 1D–F), 17-OHP (Figure 1G–I), and T4 (Figure 1J–L).

Table 2. Results of multiple linear regression analyses performed in SPSS. This table shows the number of samples per biomarker, mean biomarker concentration or activity and standard deviation (SD), the correlation coefficient R and the adjusted R^2. Of the three independent variables, gestational age (GA), birth weight (BW) and time between birth and heel prick (Age), the standardised coefficient beta (β) and the 95% confidence interval (CI) of the unstandardised coefficient beta (B) are shown. Where a value of 0.00 for the adjusted R^2 or 0.000 for the 95% CI of B is shown, this value is not actually zero; no further decimal places are presented for reasons of readability.

			Multiple Variables		*GA (Days)*		*BW (Grams)*		*Age (Hours)*	
Biomarker	**Mean (SD)**	**N**	**R ****	**Adj. R^2**	**St. β ***	**95% CI Unst. B**	**st. β ***	**95% CI Unst. B**	**St. β ***	**95% CI Unst. B**
HbA (%)	17.2 (6.9)	1,296,422	0.66	0.43	0.455	[0.240, 0.242]	0.023	[0.000, 0.000]	0.501	[0.017, 0.017]
HbF (%)	57.7 (6.3)	1,296,422	0.57	0.33	−0.407	[−0.198, −0.196]	0.028	[0.000, 0.000]	−0.457	[-0.014, -0.014]
17-OHP (nmol/L)	5 (6)	1,290,029	0.51	0.26	−0.498	[−0.238, −0.236]	−0.020	[0.000, 0.000]	−0.032	[-0.001, -0.001]
T4 (nmol/L)	89 (21)	1,291,772	0.31	0.09	0.213	[0.337, 0.344]	0.108	[0.004, 0.004]	−0.083	[-0.010, -0.010]
Biot (%)	100 (21) ***	1,291,662	0.20	0.04	0.109	[0.105, 0.112]	0.004	[0.004, 0.004]	0.015	[0.014, 0.015]
Hb Bart's (%)	3.2 (1.0)	1,296,424	0.19	0.04	−0.145	[−0011, −0.011]	−0.056	[0.000, 0.000]	0.031	[0.000, 0.000]
C0 (µmol/L)	18.9 (7.6)	476,336	0.19	0.04	0.070	[0.039, 0.043]	−0.200	[−0.003, −0.003]	0.093	[0.004, 0.004]
C14:1 (µmol/L)	0.06 (0.02)	476,371	0.18	0.03	−0.167	[0.000, 0.000]	0.017	[0.000, 0.000]	−0.095	[0.000, 0.000]
GALT (U/dL)	12.1 (3.7)	960,252	0.17	0.03	−0.207	[−0.061, −0.060]	0.066	[0.000, 0.000]	0.025	[0.001, 0.001]
C5OH (µmol/L)	0.2 (0.1)	476,372	0.16	0.03	0.113	[0.001, 0.001]	−0.005	[0.000, 0.000]	0.116	[0.000, 0.000]
C5DC (µmol/L)	0.06 (0.02)	476,376	0.13	0.02	−0.151	[0.000, 0.000]	0.086	[0.000, 0.000]	−0.063	[0.000, 0.000]
Phe (µmol/L)	44 (11)	476,362	0.13	0.02	−0.136	[−0.123, −0.117]	0.017	[0.000, 0.000]	−0.030	[-0.002, -0.002]
C2 (µmol/L)	16.6 (6.6)	476,372	0.11	0.01	0.019	[0.008, 0.011]	−0.091	[−0.001, −0.001]	−0.069	[-0.003, -0.003]
Leu (µmol/L)	142 (36)	476,368	0.10	0.01	0.101	[0.273, 0.293]	−0.018	[−0.001, −0.001]	0.044	[0.009, 0.010]
Val (µmol/L)	129 (31)	476,367	0.08	0.01	0.072	[0.164, 0.181]	0.000	[0.000, 0.000]	0.030	[0.005, 0.006]
IRT (µg/L)	20 (11)	1,290,654	0.06	0.00	0.003	[0.001, 0.005]	−0.054	[−0.001, −0.001]	−0.037	[−0.002, −0.002]
C8 (µmol/L)	0.03 (0.05)	476,373	0.05	0.00	−0.032	[0.000, 0.000]	−0.018	[0.000, 0.000]	0.003	[0.000, 0.000]
SA (µmol/L)	0.12 (0.08)	466,280	0.04	0.00	−0.030	[0.000, 0.000]	−0.013	[0.000, 0.000]	−0.015	[0.000, 0.000]
C16OH (µmol/L)	0.01 (0.01)	476,372	0.04	0.00	−0.020	[0.000, 0.000]	0.026	[0.000, 0.000]	−0.034	[0.000, 0.000]
C10 (µmol/L)	0.05 (0.02)	476,371	0.02	0.00	0.010	[0.000, 0.000]	−0.023	[0.000, 0.000]	0.014	[0.000, 0.000]

* Coefficients were significant ($p < 0.001$), with the exception of GA in IRT ($p = 0.002$), BW in C5OH ($p = 0.005$), Val ($p = 0.850$) and age at heel prick in C8 ($p = 0.053$). ** For all biomarkers, the result was significant ($p < 0.001$). *** Biot analytical unit is a percentage of the daily mean. This will per definition result in an overall mean of 100%.

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3.2.1. HbA

As expected, based on the β in Table 2, comparison of the categories demonstrated an increase in mean HbA with an increase in GA, BW, or age (Figure 1). In all categories, mean HbA exceeded the COV of 2.5%, but for the categories with a low GA or BW, mean HbA approached the used COVs. In addition to the lower mean HbA, these lower categories also showed greater variability (higher standard deviation), resulting in a broader range of HbA percentages. For GA 30 weeks, the mean (M) minus the SD reached below the COV of 2.5% (M = 6.5%, SD = 4.7, n = 1633), and for GA 29 weeks even below the COV of 1.0% (M = 6.8%, SD = 6.2, n = 1305). This indicates that a newborn with a GA ≤ 29 weeks might have an increased risk of HbA below the COV and the following referral for βTM. Up to 36 weeks GA, mean HbA per week of GA was comparable between categories. Analysis showed that the mean HbA increased more markedly from 37 weeks GA, as indicated by the formation of very small homogeneous subsets. The mean HbA at the time of the heel prick varied considerably between categories, ranging from 14.3% HbA one day after birth (M = 14.3%, SD = 4.6, n = 12) to 77.3% HbA at day 185 after birth (M = 77.3%, SD = 4.0, n = 10).

3.2.2. HbF

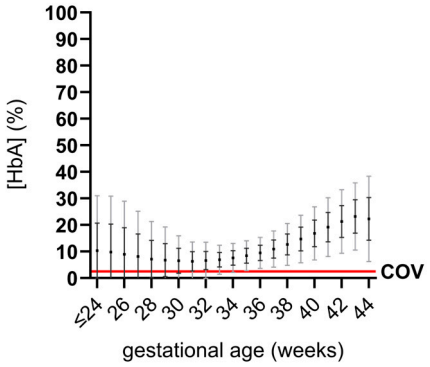
In the Dutch NBS, no COVs were implemented on the percentage of HbF. As the production of HbF is gradually replaced by the production of HbA around birth, the graphs in Figure 1 demonstrate the reverse pattern to that observed for HbA. As the GA, BW, and age at heel prick increased, so did the decrease in HbF. As depicted in Figure 1F, the mean HbF demonstrated the most pronounced decline in categories based on age at heel prick; from 58.5% at day 2 (M = 58.5%, SD = 5.1, n = 55) to 7.0% at day 183 (M = 7.0%, SD = 3.6, n = 19).

3.2.3. 17-OHP

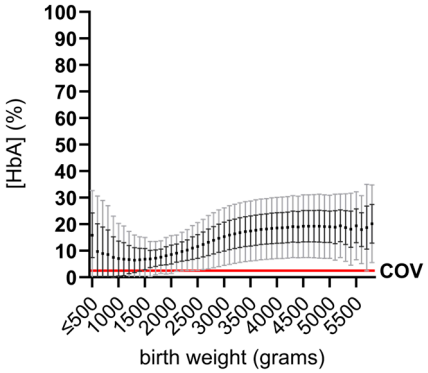
In accordance with the β from Table 2, the mean 17-OHP concentration decreased as GA and BW increased. When comparing categories, the highest mean 17-OHP concentration was observed in newborns with a GA ≤ 24 + 0/7 weeks (M = 81 nmol/L blood, SD = 62, n = 48), and in those with a BW between 601 and 700 g (M = 62 nmol/L blood, SD = 53, n = 382). The mean 17-OHP stabilised from the GA category 37 + 1/7 and 38 + 0/7 weeks (M = 5.7 nmol/L blood, SD = 3.5, n = 109,823) or from the BW category between 2601 and 2700 g (M = 6.0 nmol/L blood, SD = 5.4, n = 26,185), as indicated by the formation of larger homogeneous subsets in the post hoc test. For the effect of age at heel prick, the mean 17-OHP concentration was highest on day 1 (M = 20.8 nmol/L blood, SD = 14.0, n = 26) and declined rapidly by day 5 after birth (M = 4.6 nmol/L blood, SD = 5.3, n = 421,134). There was no significant difference from day 5 onwards.

All means for GA and BW categories were below the GA- or BW-dependent COV applied in the Dutch NBS (Figure 1). However, the most significant difference across means was observed for the categories below GA 33 weeks and below BW 2100 g, which currently constitutes the lowest category with one COV in the Dutch NBS protocol. The mean concentrations plus 2 SD for the categories below GA 26 weeks reach above the currently applied COV (200 nmol/L blood); 40 of 898 (4.5%) newborns with GA ≤ 26 weeks had a deviant 17-OHP concentration, where 0.07% of the entire dataset had a deviant 17-OHP according to the associated COV. The mean 17-OHP concentration for each age at heel prick category was below the lowest COV (25 nmol/L blood), as visualised in Figure 1I.

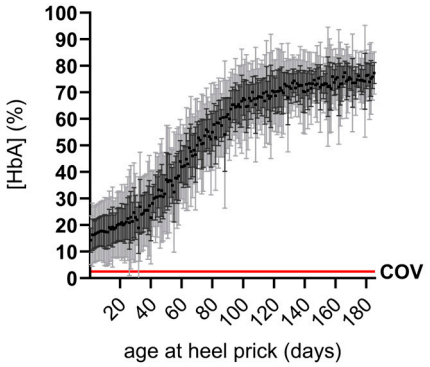
HbA



(**A**) gestational age categories

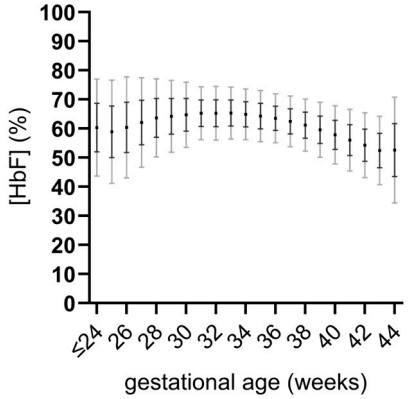


(**B**) birth weight categories

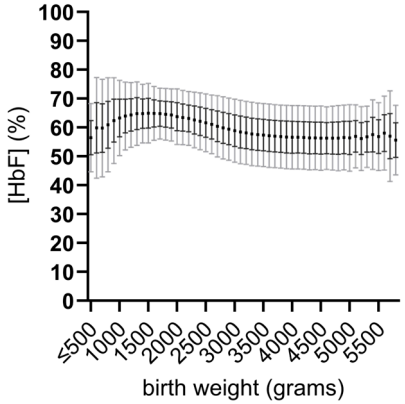


(**C**) age at heel prick categories

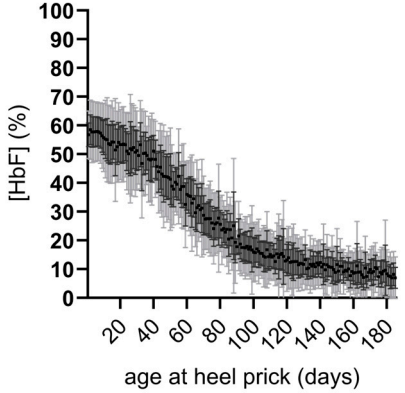
HbF



(**D**) gestational age categories

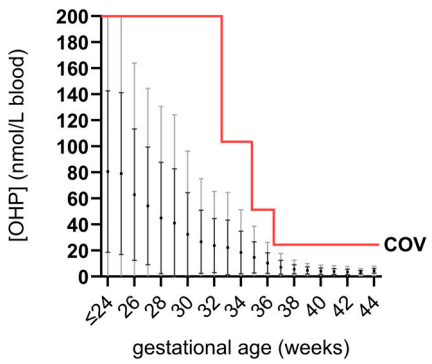


(**E**) birth weight categories

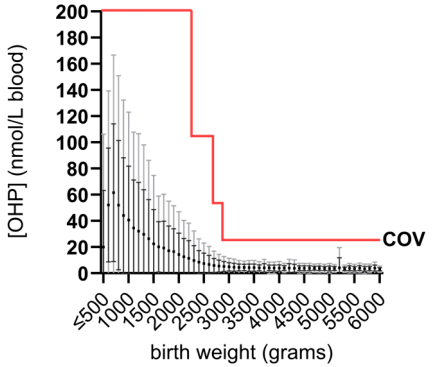


(**F**) age at heel prick categories

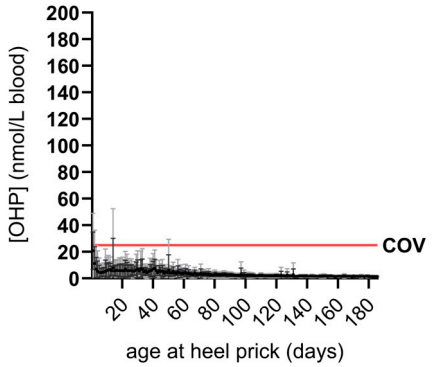
17-OHP



(**G**) gestational age categories

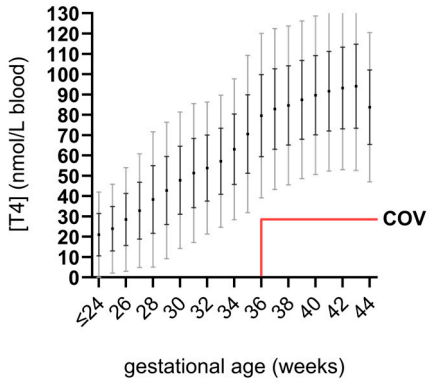


(**H**) birth weight categories

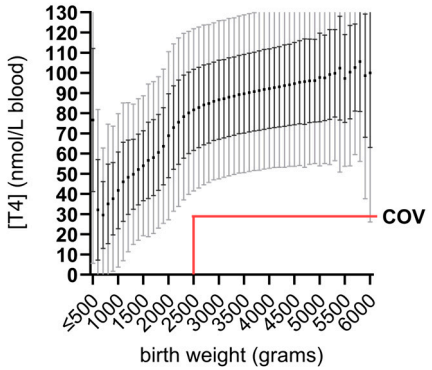


(**I**) age at heel prick categories

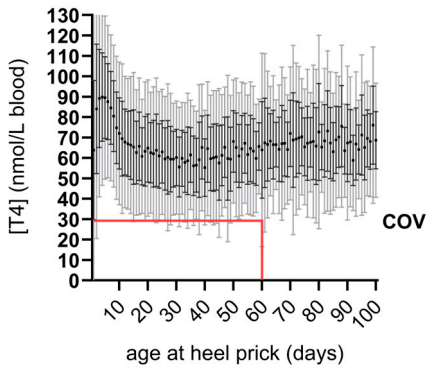
T4



(**J**) gestational age categories



(**K**) birth weight categories



(**L**) age at heel prick categories

Figure 1. Panels of mean concentrations of HbA (**A**–**C**), HbF (**D**–**F**), 17-OHP (**G**–**I**) and T4 (**J**–**L**) per category of independent variable, including 1 (dark grey) and 2 (light grey) standard deviations. Gestational age was categorised per week. On the X-as, only full weeks are indicated. If the X-as shows 36 weeks, this means the mean of samples with a gestational age between 35 weeks + 1/7 and 36 weeks + 0/7 is depicted. Birth weight was categorised per 100 g. If the X-as indicates 2100, this means the mean of samples with a birth weight between 2001 and 2100 g is depicted. Postpartum age was categorised per day, with day of birth being day 0. If the heel prick sample was taken on day 3, this corresponds to 73–96 h after birth. Only categories with at least 10 samples are included in the graphs. For HbA and HbF, selected data are shown; for the diagrams depicting the effect of gestational age or birth weight, only data from heel prick samples taken in the preferred window (between 72 and 119 h) are shown; for diagrams depicting the effect of age at heel prick, only data from samples of children born 38 weeks +

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0/7 − 40 weeks + 6/7 GA are shown. For T4, diagram L stops at 100 days after birth. T4 concentration is used for referral if heel prick is taken within 60 days after birth. More categories on the X-as reduced the visibility of the effect of age in the 60-day window. Cut-off values (COVs), if applicable, are indicated with a red line in the diagrams.

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3.2.4. T4

The mean T4 concentration gradually increased from 21 nmol/L blood for newborns with a GA ≤ 24 weeks (M = 21 nmol/L blood, SD = 10, n = 48) to 94 nmol/L blood for those with a GA between 42 + 1/7 and 43 + 0/7 weeks (M = 94 nmol/L blood, SD = 21, n = 5720) (Figure 1). A comparable yet more gradual increase was observed across BW categories, from 30 nmol T4/L blood in newborns weighing between 601 and 700 g (M = 30 nmol/L blood, SD = 17, n = 382) to 106 nmol/L blood in newborns weighing between 5701 and 5800 g (M = 106 nmol/L blood, SD = 25, n = 28). Initially, the mean T4 per age at heel prick increased from 64 nmol/L blood at day 1 (within the first 24 h after birth; M = 64 nmol/L blood, SD = 34, n = 25) to 90 nmol/L blood at day 4 (M = 90 nmol/L blood, SD = 22, n = 507,892) but then declined. As of day 15 (M = 65 nmol/L blood, SD = 18, n = 201) onward, the mean concentration minus 2 SD for certain categories fell below the COV; from that age, 43 of 6997 (0.62%) children had a T4 below the COV, where 0.28% had a deviant T4 concentration. From day 9 (M = 75 nmol/L blood, SD = 18, n = 2240) onward, analysis did not demonstrate a significant difference between subsequent categories, indicating that mean T4 stabilised from day 9.

4. Discussion

Within the Dutch NBS programme, the screening protocols for CH, CAH, βTM and SCID utilise COVs that are dependent on the GA, BW and/or the age at heel prick [11]. The initial three protocols have been in use for many years, yet no evaluation has been conducted. In this study, we examined the correlation of different biomarkers within the Dutch NBS with GA, BW, and age at heel prick. Results of multivariable regression analysis showed a correlation of these variables with the HbA, HbF, 17-OHP, and T4 values in NBS DBSs.

HbA is used as a biomarker in screening for βTM, as affected children present with a marked reduction in HbA production, or even an inability to produce HbA at all [19–21]. The findings of this study demonstrate that a model incorporating GA, BW and age at heel prick explains only 43% (R^2) of the variance in HbA percentage and show that HbA levels increase with advancing gestational and postnatal age. The observed association with birth weight most likely reflects the greater developmental maturity of higher-birthweight infants rather than an independent effect of birth weight on haemoglobin production. The Dutch programme currently utilises two GA-dependent COVs. A COV of 1% applies for samples of extremely or very premature children (GA ≤ 32 weeks), while a COV of 2.5% is applied for children born at ≥32 weeks and 1 day [11]. Children with a screening result below the GA-appropriate COV will be referred for suspected βTM. The findings of this study demonstrate that an increase in GA or BW is associated with an increase in HbA. As illustrated in Figure 1A, in cases of extreme prematurity, the range of HbA falls below the applied COV. This increases the probability of a false-positive result in newborns with a very low GA. The current screening protocol adapts for GA only, but our findings indicate that age at heel prick has an even greater influence on HbA percentage. It is essential to consider age at heel prick, as these findings show that HbA levels increase physiologically during the first months of life; consequently, a three-month-old infant has substantially higher HbA levels than a four-day-old newborn. Delayed collection of the heel prick sample may therefore result in HbA levels exceeding the COV, which could increase the risk of false-negative NBS results. Taken together, these findings suggest that the current NBS protocol for βTM screening would benefit from further evaluation. The objective of such an evaluation could be to ascertain whether further distinction of the COVs might improve the positive predictive values (PPVs) in children with low GA, while carefully assessing the potential impact of the risk of a false-negative result in older children.

Our results show that mean HbF declines with increasing GA, BW, and age at heel prick. Analogous to HbA, the association of BW likely reflects the greater developmental maturity of higher-birth-weight infants. Currently, no COV applies for HbF, and therefore the effect of the variables on the HbF percentage is not relevant for the Dutch NBS. However, HbF reflects gamma-globin chain production [22,23]. In the case of haemoglobin H (HbH) disease, excess gamma-globin chains form the tetramer Hb Bart's, which is the biomarker used for the screening of HbH disease [22,23]. As the production of gamma-globin chains declines with age, Hb Bart's production also decreases [22,23]. Consequently, Hb Bart's becomes progressively less suitable as a biomarker in older infants. The multiple linear regression analysis of Hb Bart's yielded a low R (0.19), indicating a weak predictive model with only 4% of the variance explained by the model, but with a β for GA of −0.145. This might be caused by the limited number of samples with an elevated Hb Bart's and factors such as haemoglobin degradation due to high temperature or humidity during transport, which can artificially increase the HPLC peak containing Hb Bart's [24,25]. The Dutch NBS protocol employs a COV of 18% Hb Bart's for the screening of HbH disease [11]. As gamma-globin production physiologically declines with increasing age, both HbF and Hb Bart's levels decrease. Therefore, applying the current protocol to older children might increase the risk of false-negative results in HbH disease screening. Based on the results of this study, an evaluation of the feasibility and performance of DBS-based HbH disease screening when the heel prick is performed outside of the recommended timeframe is advised.

The current Dutch NBS protocol for CAH utilises a two-tier approach. In the first tier, 17-OHP concentration is determined, with four different COVs applied depending on the GA or BW [11]. A moderately elevated 17-OHP value leads to a 21-deoxycortisol (21DOCL) measurement as a second-tier test. However, if the 17-OHP concentration exceeds the upper COV, the child will be referred directly without second-tier testing [11,26]. The highest COV within the protocol is used for premature newborns with a GA ≤ 33 weeks and will always require a positive second-tier determination of 21DOCL before referral to a paediatric endocrinologist takes place. This study demonstrates that, in a multiple linear regression analysis, only 26% (R^2) of the variance in 17-OHP values is explained by a model including GA, BW, and age at heel prick, but also shows a substantial difference in the mean 17-OHP across individual weeks of GA in this specific subgroup. Olthof et al. previously reported a relatively high number of positive first-tier results (high 17-OHP concentrations) in heel prick blood of premature newborns, leading to second-tier analyses [26]. These combined observations suggest that adding an additional COV for extremely premature newborns, on top of the current COVs, might lower the number of second-tier analyses and might improve the PPV of the screening for CAH. Further research, which should include the final diagnosis, should be performed to determine the precise nature of this additional COV. We therefore recommend investigating the implementation of an additional GA and BW category, along with an associated COV for 17-OHP, and assessing its impact on the PPV.

The Dutch NBS for CH has been designed to detect both primary and central CH. T4 is the primary biomarker, and TSH is determined if T4 is among the lowest 20% of the samples in a given run. To reduce the number of false-positive referrals, for instance due to TBG deficiency, TBG is determined if T4 is among the lowest 5% of concentrations. A moderately decreased T4 in combination with moderately elevated TSH and/or decreased T4/TBG ratio leads to the request of a repeat DBS sample. Newborns are referred for diagnostic evaluation when T4 is very low with normal TBG concentration, when TSH is high, or when, in a repeat DBS sample, T4 is low in combination with a decreased T4/TBG ratio, and/or TSH is moderately elevated. According to the "premature rule", children who are born at GA ≤ 36 + 0/7 weeks, in combination with a BW ≤ 2500 g, are referred

based solely on elevated TSH concentrations. The same rule is also applied if the heel prick takes place 60 days after birth or later. However, the results obtained in this study demonstrate that T4 concentrations reach maximum values on the fourth day of life and then rapidly decrease until the ninth day. No significant differences were observed between the mean T4 concentrations of the days thereafter; a slower decline was observed until the end of the fourth week of life, and subsequently, a gradual stabilisation of the values was observed. This finding suggests that the "premature rule" can not only be applied to heel prick samples taken at age 60 days or later, but potentially already earlier in the first months of life in order to prevent false-positive referrals based on low T4 concentrations. However, this would mean that a larger part of the screened newborns would not be screened for central CH, as the Dutch NBS intends to do. Alternatively, an age-specific T4 COV for older children could be applied, which might help to maintain detection of central CH while reducing the number of false-positive referrals. However, the feasibility of such adaptations of the screening protocol and the potential impact on screening sensitivity and specificity require additional analysis.

For all other biomarkers analysed in this study, multiple linear regression analysis yielded an R below 0.30, with a maximum of 4% biomarker variance explained by the model. This indicates that models incorporating GA, BW, and age at heel prick offer limited predictive value for these biomarkers. In the recent publication by Meijer et al., it was shown that the C0 concentration was affected by GA, BW, and age at heel prick [10]. Notwithstanding the low R, our study demonstrates comparable results, although the effects were smaller. The β of the variables BW and age from the multiple linear regression analysis exhibited higher values than those observed for the majority of the other biomarkers (Table 2). Although the R for C14:1, C5OH, C5DC, and GALT were below 0.30, the notable β suggests that GA, BW, and/or age at heel prick influence these biomarkers. In the case of C14:1, Meijer et al. observed effects of GA and age that were similar to our results, but Meijer concluded that these were of lesser relevance. For C5OH and C5DC, similar yet negligible effects were noted by Meijer et al. [10]. The potential impact of GA on the GALT concentration in heel prick blood may be an interesting topic in future evaluations.

Our study had some limitations. One such limitation is the potential for data entry errors, given that information on gestational age, birth weight, date of birth, date of heel prick, and blood transfusion history is manually entered into the NBS programme's information system. Obvious inconsistencies, such as an extremely high birth weight for a very premature newborn or NBS results for an 8-year-old child, were identified and removed during data cleaning. However, we cannot rule out the possibility that some less apparent errors remained in the dataset. Given the size of the dataset, which contains nearly 1,500,000 records, we believe that remaining errors are unlikely to have had a substantial influence on our results. Nevertheless, unregistered blood transfusions could potentially have led to higher HbA, which may have increased SD values in categories with a limited sample size. Additionally, sex was not part of our dataset. From previous studies, we know that female newborns have statistically higher T4 values than male newborns [27], while male newborns have statistically higher 17-OHP values than female newborns at 13 to 15 days after birth [28]. However, for both biomarkers, the researchers concluded in their publication that there was no need for sex-specific reference intervals. Also, for the metabolic biomarkers, sex was not found to significantly affect biomarker values [10]. Therefore, we do not expect that inclusion of sex would have affected our study results significantly.

The 17-OHP concentrations in this study were measured using a fluoroimmunoassay. This assay exhibits known cross-reactivity with 17-OHP steroid precursors, such as 17α-hydroxypregnenolone [29,30]. In premature neonates, the immature hypothalamic–pituitary–adrenal axis leads to the accumulation of these precursors, resulting in elevated

17-OHP concentrations during NBS. Although mass spectrometry provides greater specificity [30,31], it is not routinely employed in NBS. Implementation of a second-tier measurement of 17-OHP using mass spectrometry, together with the determination of 21DOCL, may help reduce the number of false-positive referrals as reported by other programmes [32–34].

Although the present dataset included all referred children, the final diagnosis was not included in the dataset. Consequently, it was not possible to suggest alternative categorisations and associated COVs, or to determine the subsequent effect on screening performance. Additionally, true patient biomarker values may represent outliers and could disproportionately affect the mean, particularly in categories with small sample sizes. However, given the low prevalence of the disorders within the NBS programme and the size of the dataset used, the impact of true patient outliers on the overall results is likely to be minimal. Follow-up studies should include the final diagnosis to assess the real effect of alternative COVs on screening performance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijns12040104/s1. Table S1: Overview of disorders included in the Dutch newborn screening programme as of 1-10-2024; Table S2: Detailed information on the characteristics of the entire dataset composed of screens performed in the Netherlands between 1 January 2010 and 31 December 2020; Table S3: Overview of used assays and instruments in the newborn screening laboratories in the Netherlands.

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Institutional Review Board Statement: The study was conducted in accordance with the principles of the Declaration of Helsinki. According to Dutch law (Medical Research Involving Human Subjects Act, WMO), the research described in this manuscript does not require formal review by a Medical Ethics Review Committee (METC). Approval for use of pseudonymised data was obtained from the local data request committee (request number 2021-021), 19 May 2022.

Informed Consent Statement: Informed consent for the anonymous use of data obtained from the newborn screening in the Netherlands was obtained at the time of heel prick.

Data Availability Statement: Data presented in this study are not publicly available due to privacy restrictions and access can only be requested from the appropriate committees within the Dutch programme, as described in Section 2.1. Procedures are described on https://www.pns.nl/hielprik/professionals/opvragen-data-en-bloed (in Dutch, accessed on 01 August 2026).

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Abbreviations

The following abbreviations are used in this manuscript:

17-OHP	17-hydroxyprogesterone
21DOCL	21-deoxycortisol
B	unstandardised coefficient beta
β	standardised coefficient beta
Biot	biotinidase
βTM	beta-thalassemia major
BW	birth weight
C0	free carnitine
C2	acetylcarnitine
C3	propionylcarnitine
C5	isovalerylcarnitine
C5DC	glutarylcarnitine
C5OH	3-hydroxyisovalerylcarnitine
C8	octanoylcarnitine
C10	decanoylcarnitine
C14:1	tetradecanoylcarnitine
C16	palmitoylcarnitine
C16OH	3-hydroxypalmitoylcarnitine
C18	octadecanoylcarnitine
CAH	congenital adrenal hyperplasia
CH	congenital hypothyroidism
CI	confidence interval
CIMS	COVID-vaccinatie Informatie- en MonitoringsSysteem
COV	cut-off value
DBS	dried blood spot
GA	gestational age
GALT	galactose-1-phosphate uridyltransferase
HbA	adult haemoglobin
Hb Bart's	haemoglobin Bart's
HbF	foetal haemoglobin
HbH disease	haemoglobin H disease
HbP	haemoglobinopathy
IRT	immunoreactive trypsinogen
Leu	leucine
LLOQ	lower limit of quantification
M	mean
MMA	methylmalonic acid
MCA	methylcitric acid
NBS	newborn screening
PAP	pancreatitis-associated protein
Phe	phenylalanine
PKU	phenylketonuria
PPV	positive predictive value
SA	succinylacetone
SCID	severe combined immunodeficiency
SD	standard deviation
T4	thyroxine
TBG	thyroxine-binding globulin
TGAL	total galactose
TREC	T cell receptor excision circles
TSH	thyroid-stimulating hormone

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ULOQ	upper limit of quantification
Val	valine

References

1. Aandoeningen. Available online: https://draaiboekhielprikscreening.rivm.nl/aandoeningen (accessed on 5 October 2026).
2. CLSI. *Newborn Screening for Preterm, Low Birth Weight, and Sick Newborns, in CLSI Guideline NBS03*; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2019.
3. Vermette, L.; Washburn, J.; Klug, T. Analysis of the Effect of Demographic Variables on Lysosomal Enzyme Activities in the Missouri Newborn Screening Program. *Int. J. Neonatal Screen.* **2025**, *11*, 48.
4. Gruñeiro de Papendieck, L.; Prieto, L.; Chiesa, A.; Bengolea, S.; Bergadá, C. Congenital adrenal hyperplasia and early newborn screening: 17 alpha-hydroxyprogesterone (17 alpha-OHP) during the first days of life. *J. Med. Screen.* **1998**, *5*, 24–26.
5. van der Kamp, H.J.; Oudshoorn, C.G.M.; Elvers, L.H.; van Baarle, M.; Otten, B.J.; Wit, J.M.; Verkerk, P.H. Cutoff Levels of 17-α-Hydroxyprogesterone in Neonatal Screening for Congenital Adrenal Hyperplasia Should Be Based on Gestational Age Rather Than on Birth Weight. *J. Clin. Endocrinol. Metab.* **2005**, *90*, 3904–3907.
6. Blom, M.; Pico-Knijnenburg, I.; Sijne-van Veen, M.; Boelen, A.; Bredius, R.G.M.; van der Burg, M.; Schielen, P.C.J.I. An evaluation of the TREC assay with regard to the integration of SCID screening into the Dutch newborn screening program. *Clin. Immunol.* **2017**, *180*, 106–110.
7. VanVleck, N.; Wolf, B.; Seeterlin, M.; Monaghan, K.G.; Stanley, E.; Hawkins, H.; Taffe, B. Improved Identification of Partial Biotinidase Deficiency by Newborn Screening Using Age-Related Enzyme Activity Cutoffs: Reduction of the False-Positive Rate. *Int. J. Neonatal Screen.* **2015**, *1*, 45–56.
8. Vernooij-van Langen, A.M.M.; Loeber, J.G.; Elvers, L.H.; Triepels, R.H.; Roefs, J.; Gille, J.J.P.; Reijntjens, S.; Dompeling, E.; Dankert-Roelse, J.E. The influence of sex, gestational age, birth weight, blood transfusion, and timing of the heel prick on the pancreatitis-associated protein concentration in newborn screening for cystic fibrosis. *J. Inherit. Metab. Dis.* **2013**, *36*, 147–154.
9. Crefcoeur, L.L.; de Sain-van der Velden, M.G.M.; Ferdinandusse, S.; Langeveld, M.; Maase, R.E.; Vaz, F.M.; Visser, G.; Wanders, R.J.A.; Wijburg, F.A.; Verschoof-Puite, R.K.; et al. Neonatal carnitine concentrations in relation to gestational age and weight. *JIMD Rep.* **2020**, *56*, 95–104.
10. Meijer, N.W.F.; Maase, R.E.; Hall, P.L.; Visser, W.F.; Koop, K.; Bosch, A.M.; Heiner-Fokkema, M.R.; de Sain-van der Velden, M.G.M.; the CLIR-NBS Group. Reducing False Positives in Newborn Screening: The Role of Perinatal Factors in the Dutch NBS Program. *Metabolites* **2025**, *15*, 634.
11. Afkapgrenzen en beslissingscriteria van de Neonatale Hielprikscreening. Available online: https://draaiboekhielprikscreening.rivm.nl/proces/de-uitslag/afkapgrenzen (accessed on 5 October 2026).
12. Léger, J.; Olivieri, A.; Donaldson, M.; Torresani, T.; Krude, H.; van Vliet, G.; Polak, M.; Butler, G. On behalf of ESPE-PES-SLEP-JSPE-APEG-APPES-ISPAE, and the Congenital Hyporhyroidism Consensus Conference Group. European Society for Paediatric Endocrinology consensus guidelines on screening, diagnosis, and management of congenital hypothyroidism. *Horm. Res. Paediatr.* **2014**, *81*, 80–103.
13. Boelen, A.; Heijboer, A.C. Newborn screening and the screening laboratory: Past, present and future. *Eur. Thyroid J.* **2025**, *14*, e240325.
14. Verkerk, P.H.; van Trotsenburg, A.S.; Hoorweg-Nijman, J.J.; Oostdijk, W.; van Tijn, D.A.; Kempers, M.J.; van den Akker, E.L.; Loeber, J.G.; Elvers, L.H.; Vulsma, T. Neonatal screening for congenital hypothyroidism: More than 30 years of experience in the Netherlands. *Ned. Tijdschr. Geneeskd.* **2014**, *158*, A6564.
15. Perinataal Beleid Bij Extreme Vroeggeboorte. Available online: https://richtlijnendatabase.nl/richtlijn/perinataal_beleid_bij_extreme_vroeggeboorte/startpagina_-_perinataal_beleid_bij_extreme_vroeggeboorte.html (accessed on 10 August 2026).
16. Klumper, J.; Ravelli, A.C.J.; Roos, C.; Abu-Hanna, A.; Oudijk, M.A. Trends in preterm birth in the Netherlands in 2011–2019: A population-based study among singletons and multiples. *Acta Obstet. Gynecol. Scand.* **2024**, *103*, 449–458.
17. Tijdigheid. Available online: https://draaiboekhielprikscreening.rivm.nl/tijdigheid (accessed on 5 October 2026).
18. Akoglu, H. User's guide to correlation coefficients. *Turk. J. Emerg. Med.* **2018**, *18*, 91–93.
19. Allaf, B.; Pondarre, C.; Allali, S.; De Montalembert, M.; Arnaud, C.; Barrey, C.; Benkerrou, M.; Benhaim, P.; Bensaid, P.; Brousse, V.; et al. Appropriate thresholds for accurate screening for β-thalassemias in the newborn period: Results from a French center for newborn screening. *Clin. Chem. Lab. Med.* **2020**, *59*, 209–216.

20. Bain, B.J.; Daniel, Y.; Henthorn, J.; de la Salle, B.; Hogan, A.; Roy, N.B.A.; Mooney, C.; Langabeer, L.; Rees, D.C. On behalf of the BSH Committee. Significant haemoglobinopathies: A guideline for screening and diagnosis. *Br. J. Haematol.* **2023**, *201*, 1047–1065.
21. Mantikou, E.; Harteveld, C.L.; Giordano, P.C. Newborn screening for hemoglobinopathies using capillary electrophoresis technology: Testing the Capillarys(R) Neonat Fast Hb device. *Clin. Biochem.* **2010**, *43*, 1345–1350.
22. Harteveld, C.L.; Higgs, D.R. A-thalassaemia. *Orphanet J. Rare Dis.* **2010**, *5*, 13.
23. Musallam, K.M.; Cappellini, M.D.; Coates, T.D.; Kuo, K.H.M.; Al-Samkari, H.; Sheth, S.; Viprakasit, V.; Taher, A.T. Alpha-thalassemia: A practical overview. *Blood Rev.* **2024**, *64*, 101165.
24. Adam, B.W.; Chafin, D.L.; De Jesús, V.R. Stabilities of hemoglobins A and S in dried blood spots stored under controlled conditions. *Clin. Biochem.* **2013**, *46*, 1089–1092.
25. Eastman, J.W.; Wong, R.; Liao, C.L.; Morales, D.R. Automated HPLC screening of newborns for sickle cell anemia and other hemoglobinopathies. *Clin. Chem.* **1996**, *42*, 704–710.
26. Olthof, A.; Bouva, M.J.; Claahsen-van der Grinten, H.L.; Westra, D.; Dekkers, E.; Heijboer, A.C.; Hannema, S.E.; Boelen, A. 21-deoxycortisol as a second-tier test in congenital adrenal hyperplasia newborn screening in The Netherlands: Two-year evaluation. *Arch. Dis. Child.* **2025**, *111*, 62–68.
27. Stroek, K.; Heijboer, A.C.; van Veen-Sijne, M.; Bosch, A.M.; van der Ploeg, C.P.B.; Zwaveling-Soonawala, N.; de Jonge, R.; van Trotsenburg, A.S.P.; Boelen, A. Improving the Dutch Newborn Screening for Central Congenital Hypothyroidism by Using 95% Reference Intervals for Thyroxine-Binding Globulin. *Eur. Thyroid J.* **2021**, *10*, 222–229.
28. Olthof, A.; Naafs, J.C.; Zwaveling-Soonawala, N.; Heinen, C.A.; Hannema, S.E.; Hillebrand, J.J.; Boelen, A.; van Trotsenburg, A.S.P.; Heijboer, A.C. Neonatal reference intervals for serum steroid hormone concentrations measured by LC-MS/MS. *Clin. Chem. Lab Med.* **2025**, *63*, 805–811.
29. Wong, T.; Shackleton, C.H.; Covey, T.R.; Ellis, G. Identification of the steroids in neonatal plasma that interfere with 17 alpha-hydroxyprogesterone radioimmunoassays. *Clin. Chem.* **1992**, *38*, 1830–1837.
30. Held, P.K.; Bird, I.M.; Heather, N.L. Newborn Screening for Congenital Adrenal Hyperplasia: Review of Factors Affecting Screening Accuracy. *J. Clin. Endocrinol. Metab.* **2020**, *6*, 67.
31. de Hora, M.R.; Heather, N.L.; Patel, T.; Bresnahan, L.G.; Webster, D.; Hofman, P.L. Measurement of 17-Hydroxyprogesterone by LCMSMS Improves Newborn Screening for CAH Due to 21-Hydroxylase Deficiency in New Zealand. *J. Clin. Endocrinol. Metab.* **2020**, *6*, 6.
32. de Hora, M.R.; Thorstensen, E.; Heather, N.L.; Albert, B.; Webster, D.; Hofman, P.L. Expanded Steroid Profiling Identifies Novel Newborn Screening Markers for Congenital Adrenal Hyperplasia. *J. Clin. Endocrinol. Metab.* **2026**, *111*, e462–e475.
33. Engström, K.; Zetterström, R.H.; Wedell, A.; Nordenström, A. Neonatal Screening for CAH in Sweden-Results of Implementing Second-Tier Testing. *J. Clin. Endocrinol. Metab.* **2026**, *12*, 29.
34. Gaudl, A.; Lehmicke, L.; Biemann, R.; Dittrich, J.; Ceglarek, U. Enhancing primary newborn screening efficiency for congenital adrenal hyperplasia with LC-MS/MS/MS. *Clin. Chim. Acta* **2026**, *578*, 120482.

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