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*Published in:*  
 Epigenomics

*DOI:*  
[10.2217/epi-2023-0119](https://doi.org/10.2217/epi-2023-0119)

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*Document Version*  
 Publisher's PDF, also known as Version of record

*Publication date:*  
 2023

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Klerk, D. H., Kooi, E. M. W., Bos, A. F., Hulscher, J. B. F., Verkaik-Schakel, R. N., & Plösch, T. (2023). Hypermethylation of CTDSPL2 prior to necrotizing enterocolitis onset. *Epigenomics*, 15(8), 479-486. <https://doi.org/10.2217/epi-2023-0119>

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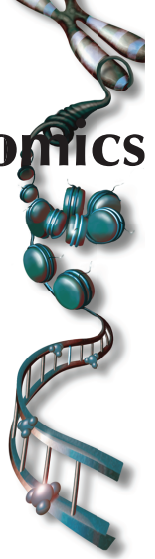
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# Hypermethylation of *CTDSPL2* prior to necrotizing enterocolitis onset

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**Background:** Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in preterm infants. Epigenetic changes in DNA methylation may be present prior to NEC onset. **Methods:** 24 preterm infants with NEC and 45 matched controls were included. Human DNA was isolated from stool samples and methylation of *CTDSPL2*, *HERC1*, *NXPE3* and *PTGDR* was measured using pyrosequencing. **Results:** *CTDSPL2* displayed a higher DNA methylation of 51% compared with 17% in controls, prior to NEC onset ( $p = 0.047$ ). **Discussion:** Noninvasive measurement of methylation in stool allows for comparison with healthy preterm controls. This potentially allows future biomarker or risk predictor use. The effect of *CTDSPL2* hypermethylation on gene expression remains unclear.

## Plain language summary:

**What is this article about?:** Necrotizing enterocolitis (NEC) is a common emergency condition affecting the gastrointestinal system of preterm infants. Epigenetic changes in DNA methylation may be present in infants before the onset of NEC. DNA methylation is a natural process that can help turn genes on or off, thereby affecting their function. This study focused on measuring the amount of DNA methylation in certain genes in preterm infants who developed NEC.

**What were the results?:** This study included 24 preterm infants with NEC and 45 matched healthy controls. The researchers isolated human DNA from stool samples, and the amount of DNA methylation of four specific genes was measured. They found that one of the genes, *CTDSPL2*, had significantly higher DNA methylation in infants who later developed NEC than in healthy infants.

**What do the results of the study mean?:** In this study, researchers found that *CTDSPL2* showed a higher level of DNA methylation in stool samples of infants who later developed NEC. The effect of this change remains unclear, but may affect the way cells grow and respond to injury or infection, which could contribute to the development of NEC. Measuring DNA methylation in stool samples provides a noninvasive method for identifying DNA methylation changes in preterm infants. Comparing the amount of DNA methylation in healthy infants with that in preterm infants at risk of NEC may help predict the risk of developing NEC.

**Tweetable abstract:** DNA samples from infants show higher DNA methylation of the marker gene *CTDSPL2* prior to necrotizing enterocolitis #NEC onset.

First draft submitted: 6 April 2023; Accepted for publication: 25 May 2023; Published online: 13 June 2023

**Keywords:** *CTDSPL2* • DNA methylation • epigenetics • necrotizing enterocolitis • preterm • *SCP4* • Toll-like receptor 4

Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in preterm infants, characterized by inflammation of the intestine. It primarily affects preterm neonates, with the peak incidence occurring at around 31 weeks of postconceptional age [1]. The condition can progress rapidly, with subtle symptoms escalating to a critical state that can ultimately lead to death [2]. Despite advances in medical care, the mortality rate of infants with NEC has remained stagnant over the past three decades, with approximately 30% of cases resulting in death [3]. The most important risk factor for developing NEC is prematurity, infants with the lowest gestational age at birth have the highest risk of developing NEC [4]. Additional risk factors include alterations in intestinal microbiota colonization, hypoxic-ischemic injury, antibiotic use and formula feeding [5–8]. However, not all infants with a similar risk profile develop NEC, leaving the question of why some develop NEC and others do not [9]. Considering the unchanged survival rate, incomplete risk profile and devastating effect of this disease on patients and their families [10], there is an urgent need for a better understanding of its pathophysiology, as well as an early detection method.

Alterations in DNA methylation of CpGs, especially in the promoter region of a gene, affect gene expression [11]. The fetal and neonatal periods are critical to establish a stable DNA methylation pattern, and numerous CpGs with differential methylation have been identified in preterm born infants [12]. These changes may influence the microbiome and immune processes, ultimately affecting susceptibility to NEC development [13,14]. In addition, changes in the microbiome may lead to changes in DNA methylation [15]. DNA methylation may also serve to improve diagnostic accuracy of a disease, and a panel of DNA methylation changes has been used to improve early lung and colorectal cancer detection [16,17]. Recently, the current authors identified an altered methylome signature, including changes in *TLR4* methylation, prior to NEC, corresponding to an altered immunological function predisposing infants to developing NEC [18]. *TLR4* plays a critical role in NEC development, as the starting point of a heightened inflammatory response after activation by lipopolysaccharide (LPS) on the bacterial cell wall [19,20]. A study of preterm-born pigs affected by NEC underlines the variable and dynamic nature of DNA methylation in the period after birth, which was strongly influenced by exposing the immature intestine to formula feeding [21]. In infants with surgically treated NEC, other investigators have observed global hypermethylation in both colon and intestinal tissue after surgical resection, resulting in changes in gene expression [22]. They observed a similar pattern in intestinal cells derived from stool samples of infants with NEC, after NEC onset [23].

In the current study, stool samples obtained prior to NEC onset were used to determine the DNA methylation pattern of four prominent genes reported to be commonly hypermethylated in surgical NEC specimens. This offers a unique opportunity to detect whether these changes in methylation are already present prior to NEC onset and may play a role in NEC susceptibility rather than an effect of NEC itself. In addition, noninvasive measurement of DNA methylation using stool samples could be used as an early detection method for NEC.

## Methods

Infants from a previously described case-control cohort were included [18]. All included infants were born after a gestation of less than 30 weeks. 24 infants in this cohort developed NEC, Bell's stage 2 or higher. 45 infants in the cohort were included as controls and matched to the cases based on gestational age, birth weight and sex. For this study, residual stool samples that were collected daily from birth onward were used. These samples were obtained from a completed multicenter observational cohort study on fecal volatile organic compounds. For all infants included in the study, written informed consent was obtained from all parents. The study was approved by the Institutional Review Board of the University Medical Center Groningen and use of these residual stool samples was permitted under the Dutch Medical Research with Human Subjects Law.

Human DNA was isolated from stool samples and collected as close as possible to the onset of NEC. The median collection time was one day prior to NEC onset. Most of the human DNA found in stool samples is derived from intestinal epithelial cells that renew every few days and shed into the stool. In infants, the main cell types shed into the stool are enterocytes, goblet cells, Paneth cells and enteroendocrine cells, while only a small proportion of lymphoid cells are present [24]. Sample selection and DNA isolation were performed as previously reported using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) [18]. After DNA isolation, samples were treated with bisulfite using the EZ-96 DNA methylation Gold Kit (Zymo Research, CA, USA), following the protocol provided by the supplier. Genes were selected for analysis from a previously published dataset that included differentially methylated CpG sites in NEC colon. In that study, DNA methylation was calculated from colonic

**Table 1. DNA methylation of *CTDSPL2*, *HERC1*, *NXPE3* and *PTGDR*.**

Gene		NEC		Control		p-value
		N	% methylation	N	% methylation	
<i>CTDSPL2</i>	CpG 1 <sup>†</sup>	14	50.97 (20.50–70.09)	20	17.02 (12.20–57.06)	<b>0.047</b>
<i>HERC1</i>	CpG 1	9	100.0 (69.02–100.0)	8	98.53 (62.20–100.0)	0.815
	CpG 2 <sup>†</sup>	9	95.72 (50.57–100.0)	8	81.26 (24.55–100.0)	0.743
<i>NXPE3</i>	CpG 1 <sup>†</sup>	10	81.26 (34.48–83.8)	12	58.38 (35.82–81.78)	0.582
<i>PTGDR</i>	CpG 1 <sup>†</sup>	14	76.19 (51.03–92.31)	27	78.49 (39.78–95.88)	0.495
	CpG 2	14	77.35 (62.68–90.94)	27	66.94 (43.63–95.00)	0.242

Data are presented as median and interquartile range.  
<sup>†</sup>CpG positions identified as statistically significant by Good *et al.* [22].  
 NEC: Necrotizing enterocolitis.

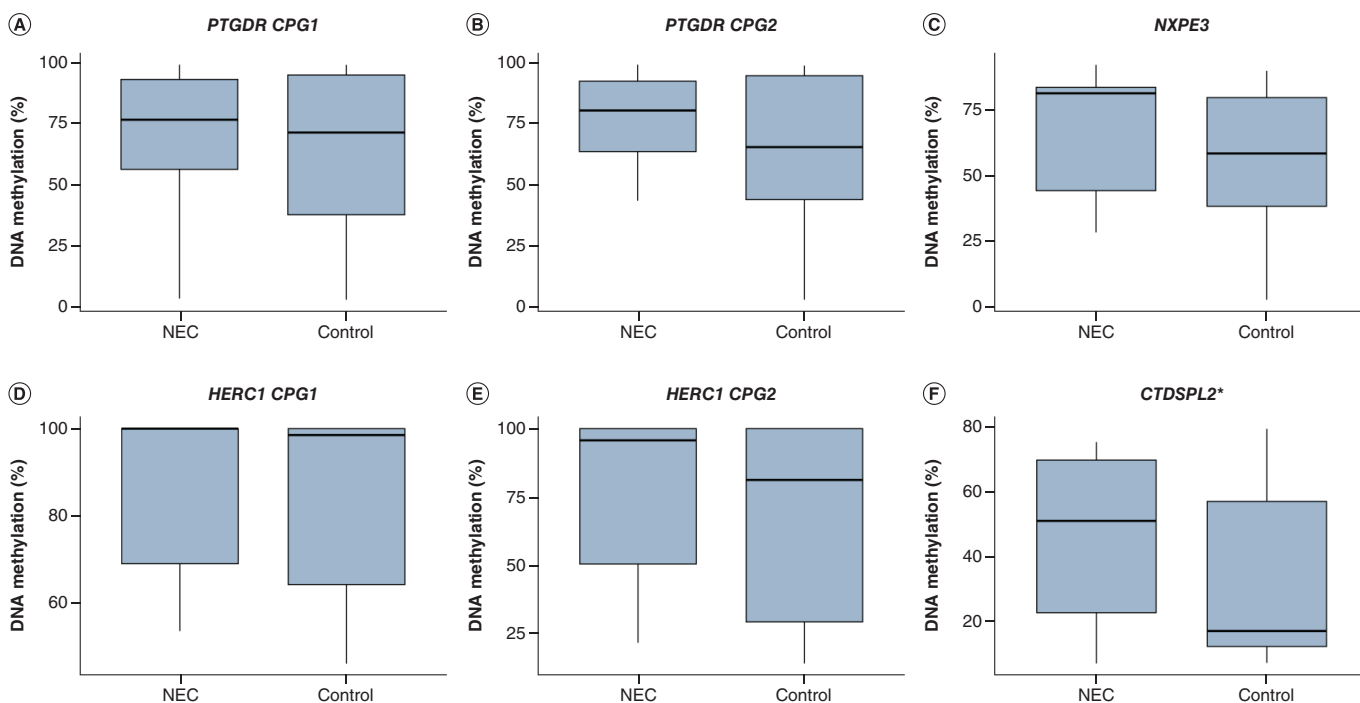
tissue obtained during surgical treatment of NEC [22]. All differentially methylated single CpG sites identified in the colon by Good *et al.* (in their Supplemental Table 4A) were ranked based on q-values [22]. Genes were excluded if they were not expressed in colon tissue, were nonprotein-coding or if SNPs were present at the CpG site. The location of the identified CpG position within the gene (e.g., inside or outside the promoter region) was not considered. This resulted in the selection of four genes with a highly significant differentially methylated CpG site in surgically resected colon tissue from NEC patients: *PTGDR*, *HERC1*, *NXPE3* and *CTDSPL2*. Primers for these genes were designed to include the significant CpG position reported [22] using Pyromark Assay Design software (Qiagen, Hilden, Germany). For *HERC1* and *PTGDR*, two additional CpG positions were included that were in close proximity to the originally reported CpG positions. The *CTDSPL2* CpG position of interest is located on chromosome 15, starting at nucleotide 44429117. For *HERC1*, the CpG position of interest was located on chromosome 15, starting at nucleotide 63678136. The CpG position of interest for *NXPE3* was located on chromosome 3, starting at nucleotide 101804213. For *PTGDR*, the CpG position of interest started at nucleotide 52272903 on chromosome 14.

In total, methylation of six CpG positions was analyzed for these four genes (Table 1). DNA amplification was done using 12.5 µl of Qiagen HotStar Taq Master Mix, 1 µl of 10 µM forward and reverse primer, 5 µl of bisulfite converted DNA and 6.5 µl purified water. Cycling conditions on a T100 Thermal Cycler (Bio-Rad, CA, USA) were as follows: 95°C for 15 min, 50 cycles of 94°C for 30 s, 54°C (for *PTGDR* and *NXPE3* this step was performed at 52°C) for 30 s, 72°C for 30 s, followed by a final step of 72°C for 7 min and storage at 4°C. Due to the generally low yield of human DNA from stool samples, the PCR protocol was optimized by increasing the number of cycles to 50 to enhance DNA amplification. Methylation was measured by pyrosequencing using the Pyromark Q24 (Qiagen, Hilden, Germany), following the provided manufacturer's protocol. Data was analyzed using PyroMark Q24 Advanced Software (Qiagen). Samples that failed quality control or displayed low peak height were excluded from the data analysis. For *HERC1*, because of the low yield after PCR, only samples with a visible band on gel electrophoresis were analyzed using pyrosequencing. Samples that showed low peak height during pyrosequencing due to low DNA content were analyzed separately but were not included in the current data analysis.

Statistical analyses were conducted using IBM SPSS Statistics for Windows, version 28.0 (IBM Corp., NY, USA). A parametric or nonparametric approach was used based on the examination of Q-Q plots. Continuous variables were analyzed using either the Mann-Whitney U test or Student's *t*-test. Correlations were assessed through visual inspection of scatterplots and further evaluated using Pearson's test or Spearman's Rho. p-values less than 0.05 (two-tailed) were considered statistically significant. Figures were constructed using R, version 4.2.3. (R Foundation for Statistical Computing, Vienna, Austria).

## Results

A large variation in DNA methylation across all samples was observed at all CpG positions (Figure 1). Only *CTDSPL2* showed a significant difference in DNA methylation prior to NEC onset, with a 51% median methylation in infants with NEC compared with 17% in controls ( $p = 0.047$ ; Table 1). The variation in methylation of *CTDSPL2* is also visualized in Figure 1F. *PTGDR* CpG position 1 (Figure 1A) showed a median methylation of 76.19% in infants with NEC compared with 78.49% in controls ( $p = 0.495$ ), and position 2 (Figure 1B) showed a median methylation of 77.35% versus 66.94% ( $p = 0.242$ ). *NXPE3* (Figure 1C) displayed a median methylation of 81.26% in infants with NEC compared with 58.38% in controls ( $p = 0.582$ ). For infants with NEC, the median



**Figure 1.** Data are presented as median and interquartile range. Boxes (A–F) show the DNA methylation of PTGDR, NXPE, HERC1 and CTDSPL2 respectively. \*Only CTDSPL2 showed a significant difference in DNA methylation prior to NEC onset, with a 51% median methylation in infants with NEC compared with 17% in controls;  $p = 0.047$ . NEC: Necrotizing enterocolitis.

**Table 2.** Absence of association between DNA methylation of CTDSPL2, HERC1, NXPE3 and PTGDR and clinical characteristics.

Gene		N	GA	BW	NEC severity	Postnatal age	Day to NEC †
CTDSPL2	CpG 1	24	0.263	0.042	-0.194	-0.293	-0.273
HERC1	CpG 1	17	0.108	-0.133	-0.133	0.051	-0.172
	CpG 2	17	0.051	-0.047	0.054	0.032	-0.374
NXPE3	CpG 1	22	-0.29	-0.103	-0.174	-0.096	0.301
PTGDR	CpG 1	41	0.050	0.106	0.044	0.010	-0.119
	CpG 2	41	-0.018	0.249	0.073	-0.008	0.048

NEC severity = Bell's stage 2A, 2B or 3, Day to NEC = Days counted between the day of stool sample collection and the day of NEC onset.

Spearman correlation coefficients (rho) presented, no significant correlations present.

† For day to NEC, only infants with NEC were included in correlation tests.

BW: Birth weight; GA: Gestational age; NEC: Necrotizing enterocolitis.

methylation of *HERC1* CpG position 1 (Figure 1D) was 100% versus 98.53% in controls ( $p = 0.815$ ), and for CpG position 2 (Figure 1E) this was 95.72% compared with 81.26% in controls ( $p = 0.743$ ). The DNA methylation of all tested genes did not correlate significantly with gestational age, birth weight, NEC severity or postnatal age at stool sample collection (Table 2). Due to the lower quality of human DNA isolated from stool samples, many of the samples did not pass the strict quality control checks after pyrosequencing, resulting in a lower number of included infants. The main clinical characteristics for infants included in the analysis for *CTDSPL2* are presented in Supplementary Table 1. After excluding missing samples for this gene, no significant differences were found in gestational age, birthweight or sex between the NEC and control groups.

To investigate whether the methylation of these genes was affected by NEC or a precursor of NEC, whether the collection date of the stool samples in relation to NEC onset was related to the percentage of DNA methylation was assessed. No correlation was found between the exact number of days before NEC onset and DNA methylation (Table 2).

## Discussion

DNA methylation may play a role in predisposing infants to NEC. Of four previously identified hypermethylated genes in patients who already developed NEC [22], only *CTDSPL2* displayed significantly higher DNA methylation prior to NEC onset. At the same CpG position as previously identified by Good *et al.* (1487 nucleotides after the transcription start site), our findings demonstrated a methylation difference of 34 percentage points when comparing samples taken prior to NEC onset with those of healthy preterm age-matched controls. Good *et al.* demonstrated hypermethylation of this gene after NEC onset, with a methylation difference of 47 percentage points when comparing infants with surgical NEC to controls [4]. Their control group consisted of an unmatched group of infants who had either healed from NEC and underwent an operation for reanastomosis or infants with a spontaneous intestinal perforation or anorectal malformation. In a follow-up study using stool samples, general hypermethylation was reported, although *CTDSPL2* was not mentioned. In the latter study, a small number of infants were included and stool samples were collected after NEC onset [23].

*CTDSPL2*, also known as *SCP4*, has a role in regulating cell mitosis, proliferation and motility. *CTDSPL2* plays a role in pancreatic tumors and B-cell lymphoma because its overexpression promotes cell migration and immortalization [25,26]. Hypermethylation of *CTDSPL2* may result in reduced expression, leading to *CTDSPL2* depletion. This may restrict cell cycle progression and induce inflammation through the NF- $\kappa$ B pathway [26]. This pathway is also utilized by *TLR4* to induce an exaggerated inflammatory response, as observed in NEC [20,27]. Thus, *CTDSPL2* depletion may contribute to NEC onset. Only one other study has reported the methylation of *CTDSPL2*, in a cohort of obese asthmatic preadolescent children [28]. In this study, hypermethylation of *CTDSPL2* was reported in cases compared with controls, but gene expression was not measured.

The current results demonstrated that hypermethylation of a previously identified CpG site in *CTDSPL2* is already present in human DNA derived from stool samples prior to the onset of NEC. We did not find any differences in the other three genes defined based on published data [22]. Changes in DNA methylation in these genes may not yet be present prior to NEC or may be present only in severe surgical NEC cases. In our dataset, we could not confirm the latter because we did not find a correlation between DNA methylation and NEC severity. Alternatively, changes in DNA methylation may be more subtle prior to NEC and therefore not significantly different in this small cohort. Lastly, the studies by Good *et al.* [22,23] were specifically performed on epithelial cells, whereas our samples contain human DNA from various cell types. A limitation of the current study was the low quality of human DNA isolated from stool. Despite including a second or third sample to optimize DNA isolation from the included infants to reduce missing data, we still lost over 50% of the included infants for some genes after pyrosequencing. Other investigators report increasing amplification cycles as was done in this study as well, to increase DNA yield from stool samples for analysis [23]. These missing infants in the study are a direct result of the decision to adhere to robust quality control criteria, which excluded samples that displayed low peak height during pyrosequencing, to ensure the reliability and accuracy of our data. This decision led to a reduction in the number of valid samples analyzed and impacted the statistical power of this study. The samples with low peak heights, identified after pyrosequencing, were analyzed separately and showed similar results to the included samples of high quality, but were not included in this analysis (data available upon request).

Finally, we note that there is a large overlap in methylation levels at the *CTDSPL2* CpG site (Figure 1F), which makes it difficult to distinguish between NEC cases and controls based on DNA methylation alone. The *CTDSPL2* CpG site in this study is just one of many CpG sites that could potentially be used as a biomarker for NEC. Further research is needed to identify a potential panel of CpG sites that are specific to NEC and have less overlap in methylation levels between cases and controls. Especially, the promoter region of the gene would be of interest for these future investigations, as this region is heavily involved in regulating gene expression.

The effect of methylation changes in *CTDSPL2* on the differences in gene expression remains unclear, since the studied CpG position of *CTDSPL2* is not present in its promoter region. No previous studies have evaluated the effect of changes in DNA methylation of *CTDSPL2* on gene expression in the intestine. Analysis of mRNA might be helpful, but these stool samples were not suitable for such analyses. Stool was collected at routine diaper changes to not disturb the infants and may have been in the diaper for several hours prior to transfer to the freezer. As mRNA is not likely to remain stable for this amount of time at room temperature, we were concerned about the reliability of any data obtained from such samples [29]. In future studies, stool frozen immediately would be suitable for mRNA analysis using recent techniques such as droplet digital PCR [30].

## Conclusion

Significant hypermethylation of *CTDSPL2* was found prior to NEC onset. Stool samples enabled a comparison between preterm-born infants with NEC and a matched control group without subsequent NEC, which is impossible when surgically resected material is used. In addition, the use of stool samples is the only noninvasive method currently available to measure DNA methylation in human DNA predominantly from epithelial cells of the intestine, of preterm-born infants prior to NEC onset. Future research is needed for a broader evaluation of *CTDSPL2* DNA methylation to evaluate its role in NEC development beyond a possible role as biomarker or risk predictor. This research should include longitudinally collected stool samples prior to NEC, combined with surgically obtained samples or freshly frozen stool samples, which would allow for expression studies.

### Summary points

- In infants with surgically treated necrotizing enterocolitis (NEC), investigators have observed global hypermethylation in both colon and intestinal tissue after surgical resection.
- This is the first study evaluating those changes in DNA methylation prior to the onset of NEC and including healthy controls.
- *CTDSPL2* displayed a higher DNA methylation of 51% compared with 17% in controls, prior to NEC onset ( $p = 0.047$ ).
- *PTGDR*, *HERC1* and *NXPE3* did not show significant differences in DNA methylation when comparing cases to controls, prior to NEC onset.
- DNA methylation of all tested genes did not correlate significantly with gestational age, birth weight, NEC severity, time to NEC onset or postnatal age at stool sample collection.
- The effects of these methylation changes on the differences in gene expression remain unclear, since the studied CpG position of *CTDSPL2* is not present in the promoter region.
- Noninvasive measurement of methylation using stool samples allows for comparison with healthy preterm controls and potential biomarker or risk predictor use.
- Future research is needed for a broader evaluation of *CTDSPL2* DNA methylation to evaluate its role in NEC development, or as a possible biomarker or risk predictor.

### Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: [www.futuremedicine.com/doi/suppl/10.2217/epi-2023-0119](http://www.futuremedicine.com/doi/suppl/10.2217/epi-2023-0119)

### Author contributions

D Klerk, T Plösch, J Hulscher, E Kooi and A Bos conceptualized and designed the study. D Klerk and R Verkaik-Schakel collected and analyzed the data. D Klerk drafted the initial manuscript. T Plösch, E Kooi and A Bos supervised the study. R Verkaik-Schakel, T Plösch, J Hulscher, E Kooi and A Bos critically reviewed and revised the manuscript. All authors approved the final manuscript as submitted.

### Acknowledgments

The authors would like to thank the nurses and medical staff of the NICU of the Beatrix Children's Hospital in Groningen, as well as the laboratory staff of the obstetrics laboratory for helping with data collection and for creating the opportunity to carry out this study.

### Financial & competing interests disclosure

A research grant was obtained for this work from the Dutch de Cock-Hadders Foundation. D Klerk was financially supported by the Junior Scientific Masterclass of the University of Groningen. The open access article processing charges were paid through the University of Groningen open access fund. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

### Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

### Data sharing statement

The datasets analyzed in the current study, including deidentified participant data, are available immediately after publication, upon reasonable request. Proposals may be emailed to the corresponding author and include a methodological and sound proposal. Data requesters must sign a data access agreement.

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