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Neonatal stress exposure and DNA methylation of stress-related and neurodevelopmentally relevant genes: An exploratory study

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ABSTRACT

Background: Stress exposure during Neonatal Intensive Care Unit (NICU) stay may have long-lasting effects on neurodevelopmental outcomes in extremely preterm infants. Altered DNA methylation of stress-related and neurodevelopmentally relevant genes may be an underlying mechanism.

Aims: This exploratory study aimed to investigate the association between neonatal stress exposure and DNA methylation in these genes at two different time points: early during the NICU stay (7–14 days after birth) and later, at discharge from the NICU.

Subjects: We included 45 extremely preterm infants in this prospective cohort study, gestational age 24–30 weeks.

Outcome measures: We collected fecal samples at days 7–14 (n = 44) and discharge (n = 28) and determined DNA methylation status in predefined regions of *NR3C1, SLC6A4, HSD11B2, OPRM1, SLC7A5, SLC1A2, IGF2, NNAT, BDNF* and *GABRA6* using pyrosequencing. Because of low DNA concentrations in some fecal samples, we could do so in 25–50 % of collected samples. We prospectively quantified daily neonatal stress exposure using the Neonatal Infant Stressor Scale (NISS) and explored associations between cumulative NISS scores and average DNA methylation status.

Results: Rates of methylation of most genes were not statistically different between day 7–14 and discharge, except for *OPRM1*. We found moderately high and mostly negative correlation coefficients upon discharge with the cumulative NISS for the *NR3C1*, *SLC6A4*, *SLC1A2*, *IGF2*, *BDNF* and *OPRM1* genes, albeit not statistically significant.

Conclusions: Findings suggest that expression of stress-related and neurodevelopmentally relevant genes may be differently regulated following higher neonatal stress exposure. Larger studies should challenge the findings of this study and ideally test the effects on gene expression.

1. Introduction

Survival rates of infants after preterm birth are rising, especially for extremely to very preterm infants born after a gestational age of 24 to 30 weeks [1]. After birth, these preterm infants are admitted to the Neonatal Intensive Care Unit (NICU). The NICU environment is highly different from the intrauterine environment and exposes these infants to various stressors, including pain-related procedures, medical procedures, noise and light, and parental separation [2–5]. Quantifying postnatal stress is increasingly receiving attention, and one measure that is gradually more implemented is the Neonatal Infant Stressor Scale (NISS). Using this scale, our group has identified that particularly during the first week after birth, neonatal stress exposure is high in preterm infants [2]. Neonatal stress may lead to adverse neurodevelopment over

Abbreviation: NISS, Neonatal Infant Stressor Scale; NICU, Neonatal Intensive Care Unit; HPA, hypothalamic-pituitary-adrenal; STRONG, Stress and Outcomes in NICU Graduates.

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the life course, in the cognitive, psychomotor, emotional, and behavioral domains [6].

Provenzi and colleagues have proposed a model of preterm behavioral epigenetics to understand the mechanisms through which neonatal stress may lead to adverse neurodevelopment [7]. This model assumes that epigenetic modifications induced by various environmental conditions contribute to neurodevelopmental and behavioral outcomes in preterm infants [7]. Epigenetic modifications refer to the ways in which heritable traits are associated with chemical modifications of DNA or the structural and regulatory proteins bound to DNA, without a change in nucleotide sequence. DNA methylation, by far the most studied epigenetic modification, is the binding of a methyl-group to a specific CpG dinucleotide often in the regulatory sequence of a gene. This binding may lead to altered transcriptional activity and gene functioning. Studies have reported that DNA methylation is susceptible to stressful environmental conditions, specifically in the genes involved in the hypothalamic-pituitary-adrenal (HPA)-axis and serotonergic system [8]. Relatively little attention in epigenetic research has been awarded to the NICU environment, even though the first year of life is believed to be rather determining for DNA methylation rates in brain tissues [9].

In this developing field of research, the two most often studied genes encode for the glucocorticoid receptor (*NR3C1*) and the serotonin transporter (*SLC6A4*). For *NR3C1*, increased methylation rates were reported for preterm infants between birth and a few days after birth, while these rates remained stable in fullterm infants during the same postnatal period [10]. This increase was associated with more neonatal complications [10]. Additionally, at discharge from the NICU, methylation rates were relatively lower in infants with higher stress exposure during NICU stay [11]. Cumulative stress exposure to the neuroendocrine system may lead to a less responsive or underactive HPA-axis [11]. For *SLC6A4*, studies report that preterm infants exposed to high levels of pain-related NICU stress are reported to have increased methylation at several CpG sites from birth to NICU discharge [12]. Several other stress-related and neurodevelopmentally relevant genes may also be affected by neonatal stress. Some of these have been implicated, but scarcely studied in the setting of neonatal stress exposure [7]. These include genes encoding for HPA-axis functioning, pain processing and neurodevelopmental processes, i.e., *HSD11B2, OPRM1, SLC7A5, SLC1A2, IGF2, NNAT, BDNF* and *GABRA6*.

Until date, only a few studies consider exposure to postnatal stress as an environmental factor explaining altered DNA methylation. Identifying target genes affected by postnatal stress and thereby possibly a mechanism for adverse neurodevelopment may increase our understanding of pathways leading to adverse neurodevelopment and may ultimately lead to early interventions improving outcomes. The main aim of this exploratory pilot study therefore was to investigate the association between neonatal stress exposure and DNA methylation in stress-related and neurodevelopmentally relevant genes at two different timepoints: early during the NICU stay (7–14 days after birth) and later, at discharge from the NICU. We chose these two timepoints to account for the highest burden of stress in the first 7 days after birth [2], but also to encompass the full NICU stay. Classically, DNA methylation is determined in blood samples. Here, we measured methylation of DNA derived from stool samples, which avoids the ethical problems associated with blood sampling in infants. To compare to existing literature, we also aimed to assess differences in DNA methylation between these two timepoints. We hypothesized that cumulative stress exposure would be associated with differential DNA methylation of the included target genes.

2. Methods

2.1. Setting and population

We included infants participating in the *Stress and Outcomes in NICU Graduates* (STRONG) study. These infants were all born between September 2019 and December 2020. All infants born were consecutively included in our study if they were born before 30 weeks' gestation and/or with a birth weight below 1000 g. They were all included in the first week after birth. Because our center is a tertiary referral center for preterm birth in the North of the Netherlands, we have a representative sample of the NICU population born before 30 weeks' gestation and/or 1000 g of birth weight, including those with medical morbidities and complications, and those with a relatively uncomplicated NICU stay. Infants were excluded when the parents did not understand Dutch. Both parents provided written informed consent. The STRONG study was approved by the Institutional Review Board of the University Medical Center Groningen (METc 2019/128).

2.2. Quantification of neonatal stress

Neonatal stress exposure was assessed using the Neonatal Infant Stressor Scale (NISS) as developed by Newham and colleagues [5]. This validated score includes acute and chronic items. The score incorporates skin-breaking procedures but also other items that may not necessarily be painful but could be considered as stressful. Examples include nappy changes, flushing of intravenous lines or receiving tube feeding. Individual items are weighted to be a little stressful (2 points) to extremely stressful (5 points). The weighing is based on expert opinions. Weighted items result in a daily NISS score, and over multiple days cumulative daily scores reflect cumulative stress exposure. We used the cumulative daily scores of the first seven days to reflect stress exposure early during the NICU stay (first week after birth). To quantify cumulative stress exposure for the full NICU stay, we used cumulative daily NISS scores between birth and discharge from the NICU.

2.3. Fecal samples

Fecal samples were collected at days 7–14 after birth and upon discharge for the same group of participating children. These samples are an ideal source for human DNA because of its non-invasive nature of collection. The human DNA in these samples is mainly originating from intestinal epithelium cells, which most likely renew every four to five days. Stool samples were collected using collection tubes with DNA stabilizer (Invitek Molecular, Berlin, Germany), stored at room temperature or 4 °C short-term and long term at −80 °C. This method was deemed feasible in a previous study from our laboratory [18].

2.4. Selection of genes and primer design

We selected relevant genes based on their involvement in either the stress system (*NR3C1, SLC6A4, HSD11B2* and *OPRM1*) or because of their implied neurodevelopmental role (*SLC7A5, SLC1A2, IGF2, NNAT, BDNF* and *GABRA6*) [7]. The glucocorticoid receptor, encoded by *NR3C1*, is an important regulator in the HPA-axis functioning. The serotonin transporter, encoded by *SLC6A4*, signals the pathway of emotion in reward in the brain and is believed to interact with the HPA-axis. *HSD11B2* encodes for Hydroxysteroid 11-Beta Dehydrogenase 2, which converts cortisone to cortisol, thereby regulating cortisol levels. Lester and colleagues report that in the case of higher neonatal stress exposure, this gene was hypomethylated [13]. *OPRM1* encodes for the mu opioid receptor, regulating pain, emotion and reward in the cerebral cortex neurons. This gene seems of interest because of a possible link with regulating pain, even though in a small study exposure to pain-related stress was not associated with alterations in DNA methylation levels [14]. *SLC7A5* is a regulator of the cell cycle and thyroid hormone uptake in the cerebral cortex, implicated in neurodevelopment [15]. Another member of the solute carrier family, *SLC1A2,* clears excitatory glutamate from the extracellular space in synapses in the central nervous system and is associated with psychiatric disorders and neurodegeneration [15]. *IGF2* is mainly known for its function as a regulator of growth. This gene is of interest because early growth may impact

neurodevelopment [16]. *IGF2* is also expressed during fetal development and present in the epithelial lining of the prefrontal cortex, thereby possibly regulating neurodevelopment as well. Dynamic changes in the first year of life have been reported in preterm infants, even though these were not studied in relation to stress exposure [17]. *NNAT* is a regulator of ion channels during brain development encoding for a protein that is involved in forming and maintaining the structure of nervous cells. *BDNF* promotes survival, growth, differentiation, and maintenance of neurons. Finally, *GABRA6* encodes for the inhibitory GABA neurotransmitter, part of the limbic system that is crucial in the stress-response. The genomic target regions that we chose (Table 1) were based on this existing literature, to incorporate the regions analyzed by others. All primers for the target regions were designed using the Pyromark Assay Design software (Qiagen). We preferably selected CpG-rich areas in the promotor part of the gene because of their putative regulatory function on transcriptional activity.

2.5. Quantification of methylation and pyrosequencing

DNA isolation was performed using the PSP® Spin Stool DNA Kit (Invitek Molecular, Berlin, Germany). Quality and concentration of the isolated DNA was assessed using the NanoDrop®ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and on agarose gels. DNA was bisulfite converted, using the EZ DNA methylation Gold-Kit (Zymo Research, Irvine, CA), according to the supplier's protocol. In total, 500 ng of DNA sample was bisulfite converted for subsequent analysis. For PCR amplification, we used a master mix of 12.5 μL HotStarTaq DNA Polymerase (Thermo Fisher Scientific, Hilden, Germany), 10.5 μL sterile water, a 1 μL mix of forward and reverse primer and a 1 μL bisulfite template, according to the manufacturer's instructions. We included a negative control to check for contamination. Cycling conditions for PCR were: 95 °C for 15 min, 50 cycles of 94 °C for 30 s, 54–58 °C for 30 s, 72 °C for 30 s, followed by a final step of 72 °C for 7 min. Afterwards, a DNA ladder and 5 μL of each PCR product was loaded and run on 2 % agarose gel with ethidium bromide staining to visualize presence or absence of PCR products and contamination. Next, pyrosequencing was performed using the PyroMarkQ24 system (Qiagen, Hilden, Germany). Methylation levels were analyzed with associated software. Each of the CpG sites was quality control checked and the percentage of DNA methylation at individual CpG sites was calculated.

2.6. Statistical analyses

First, we described participant characteristics using descriptive statistics. Second, we visually inspected distribution of included variables using Q-Q plots and tested whether they were normally distributed using the Shapiro-Wilk test. Next, we performed our comparative analyses. We first addressed the differences between DNA methylation status early

Table 1
PCR forward and reverse primer sequences accompanied by sequencing primer and the sequence to analyze, and their genomic region.

Gene	Primers	Sequence to analyze	Genomic region
NR3C1	F:5′- AGTTTTAGAGTGGGTTTGGAG −3′ R:5′- Biotin- CCCCCAACTCCCCAAAAA-3′ S:5′-GAGTGGGTTTGGAGT −3’	YGYGGAGTTGGGYGGGGGYG GGAAGGAGGTAGYGAGAAAAGAAATTGGAGAAATT	Chromosome 5, NC_000005.10, 143404021-143404075
SLC6A4	F: 5’-GGGGAAGAAGGTTTGGAAAGA-3 R: 5’ - Biotin- AAAATCCCTCCCCTCCTA-3′ S: 5′-TTTGAGGAGAATAAATTTAATGTTT-3’	TTTYGYGGTYGYGGTTTYGY GTTTTYGTTGGATGGGGTTGYGT TYGTTAGGGAGGGGT	Chromosome 17, NC_000017.11, 30235826-30235883
HSD11B2	F: 5’-Biotin- AAATTTGTTTGGGATAGGGGTTGG-3′ R: 5’-CTAACCCCCACACACATACATTTAT-3′ S: 5’-ACAAAAACTCCAAAAACC-3’	RAAAACRATC RTCCTATTCC CCCRCCAACC CCTATCCCAA ACAAATTTT	Chromosome 16, NC_000016.10, 67430487-67430535
SLC7A5	F:5’- GTTTGGGGGTTGTATTAGTGG-3′ R:5′- Biotin- ACTTCCCAAAACCTATATTATTACTACTT-3′ S:5′- AGTGGGGTTGGTGAG-3’	TYGYGAATTG GGAYGAGGGA AGAATYGAAT YGAGGTTATT TAGTAATTTT TATTGT	Chromosome 16, NC_000016.10, 87870064-87870119
SLC1A2	F5’- GGAGGGTTATAGGAGAATTAAATAGA- 3′ R:5′- Biotin- AAAATTTACCCCAAATTACTCAACT-3′ S:5′- GTGTAAATTTTTTGAGTAGGT-3’	T TYGGGGATG GTGTTAGAGG AGTYGGAGTT TTAGTAGGAT YGATTGGTAG TTAAATGTTA GGYGTTGATA TTYGAAGGTT TGAGTTTTTT ATTATTTT	Chromosome 11, NC_000011.10, 35420616-35420713
IGF2	F:5’- GGGGGTTTTTGTATAGTATATGGGT −3′ R:5′- Biotin- ACTCCTATAAATATCCTATTCCCAAATAAC S:5’-TGGTTGTAGTTGTGG-3’	AATYGGAAGTGGTYGYGYGGYGGTAGTGTAGGTTTATATATTATAGTT [CpG4 = C/T snp ~50/50 %] CpG4 cannot be used because of the snp position.	Chromosome 11, NC_000011.10, 2002618 - 2002636
NNAT	F5’- TAGGTTAGGGATTGGGGAGAA-3′ R:5’- Biotin- AAATAATCCATCTACTCTTTCCATAC-3′ S:5′- TTAAAGTAAAATTTAAAAGTAAGT-3’	AYGGYGGAAT TTTTTGGAAG GGGGTTAAGA TGGAATTTAG GAGGYGGGGG TYGGTATGGA AAGAGTAGAT GGATTAT	Chromosome 20, NC_000020.11, 37521049-37521125
BDNF	F:5’- GTGAATGGGTTTAAGGTAGGTTTAAGA-3′ R:5′- Biotin- ACCCTCATAAACATATTTACAACATCT-3′ S:5′- GGTTTGATATTATTGGTTGATATTT-3’	TYGAATAYGT GATAGAAGAG TTGTTGGATG AGGATTAGAA AGTTYGGTTT AATGAAGAAA ATAATAAGG	Chromosome 11, NC_000011.10, 27658308-27658377
OPRM1	F:5′- TAGTTAGGATTGGTTTTTGTAAGAAATAG-3′ R:5′- Biotin- ATCCAAAAACACAAACTATCTCTCCC-3′ S:5′- GTAGGAGTTGTGGTAG-3’	YGGYGAAAGG AAGYGGTTGA GGYGTTTGGA ATTYGAAAAG TTTYGGTGTT TTTGGTTATT T YGTATAGYG GTGTTYGTTY GGTYGTTAGT ATTATGGATA GTAG	Chromosome 6, NC_000006.12, 154039452-154039555
GABRA6	F:5’-AGGAGAGTTTGAAGGGATAG-3′ R:5’- Biotin- CCTCACAAAACCCTACTAATTCC-3′ S:5′- TTGAAGGGATAGTGTATG-3’	GTYGGAGAGT AGTGATAATA ATYGAATAAG GTATTAAGGG AGATTAGA	Chromosome 5, NC_000005.10, 161685633-161685680

F: forward primer, R: reversed primer, S: sequence primer.

during the NICU stay and upon discharge from the NICU. We calculated average percentages of DNA methylation in the ten target genes for 7–14 days after birth and at discharge from the NICU and used a student's *t*-test or a Mann-Whitney-*U* test where appropriate to test for differences in averages between these two moments. Because few infants had a valid measurement at both timepoints, we were unable to perform paired comparisons. Then, we graphically displayed differences between 7–14 days and discharge between individual CpG positions of each gene and tested differences using student's *t*-test or a Mann-Whitney-*U* test where appropriate. After that, we addressed the main aim of this study by performing correlation analyses calculating either Pearson or Spearman's correlation coefficients where appropriate. The first correlation analysis was performed between cumulative daily NISS scores for the first seven days and DNA methylation status early during the NICU stay (7–14 days after birth). The second correlation analysis was performed between cumulative daily NISS scores for the entire NICU stay and DNA methylation status upon discharge from the NICU. Throughout our analyses, we did not adjust for confounding or influencing factors, because of small sample sizes.

3. Results

3.1. Participant characteristics

In total, 93 infants were eligible for participation in the STRONG study. Of these, 48 were excluded because they declined to participate in research (n = 15), did not speak Dutch (n = 2), deceased before informed consent could be asked (n = 6) or because of logistical reasons including the COVID-19 research stop at our department (n = 25). The STRONG study therefore eventually included 45 infants. The median gestational age was 27 weeks, and the median birth weight was 1000 g. Almost half of the participating children were males. More clinical and cohort details are presented in the Table 2. In this cohort, cumulative NISS scores were median 559 and 1857 at days 7 and discharge, with interquartile ranges or 163 and 604 respectively. Scores were highest in the first 7 days after birth and declining of scores was dependent on gestational age.

Table 2
Participant characteristics.

	Descriptive (N = 45)
Gestational age (weeks)	27 (26–28)
Birth weight (grams)	1000 (790–1248)
Male sex	22 (48.9)
Multiple birth	9 (20.0)
Apgar 1 min	5.5 (3.0–7.0)
Apgar 5 min	7.0 (6.0–8.0)
NICU admission for surviving infants (days)	35 (24–49)
Deceased during NICU admission	2 (0.4)
Delivery via caesarean section	23 (51.1)
Antenatal steroids	38 (84.4)
Complete course	23 (51.1)
IVH grade ≥ grade 3	6 (13.3)
Mechanical ventilation	30 (66.7)
Days (n = 30)	7.0 (2.8–20.5)
NEC	4 (8.9)
Sepsis	16 (35.6)
Circulatory insufficiency	5 (11.1)
PDA	20 (44.4)

Data are presented as median (25th – 75th percentile) or N (%) where appropriate. NICU: neonatal intensive care unit, IVH: intraventricular haemorrhage based on serial cranial ultrasound measurements routinely performed every week NEC: necrotising enterocolitis, PDA: hemodynamically significant patent ductus arteriosus determined by cardiac ultrasound. Complete course of antenatal steroids was defined as birth >48 h after the first dose. Presence of intestinal pathologies was based on clinical and radiographic examinations. Sepsis was defined as clinical signs of infection, combined with a positive blood culture and requiring antibiotic treatment. Circulatory insufficiency was defined as requiring fluid therapy and/or treatment with inotropic agent such as dopamine or dobutamine.

3.2. Average percentage of DNA methylation at days 7–14 and upon discharge

In Table 3 we present the averages of DNA methylation of the target genes at days 7–14 after birth and at discharge from the NICU. In total, 44 infants had a stool sample taken upon inclusion at 7–14 days after birth, and 28 had a sample taken upon discharge from the NICU. Because of low human DNA concentrations in the stool samples and the fragmentation of the DNA, only approximately 25 % to 50 % of the samples were appropriate for pyrosequencing. The number of children with a suitable sample for pyrosequencing varied per gene (Table 3).

Apart from *OPRM1*, the percentages of average methylation of the selected genes were not statistically significantly different between day 7–14 and discharge (Table 3). For *OPRM1* the averages at days 7–14 after birth and upon discharge were 16.0 ± 8.6 at days 7–14 after birth vs. 3.1 ± 1.5 at discharge ($p = 0.007$). In Fig. 1 and Fig. 2 we present the averages of DNA methylation at individual CpG positions for the ten target genes at days 7–14 after birth and upon discharge from the NICU. Most CpG sites were not significantly different between the two timepoints. We did, however, observe significant differences for all *OPRM1* individual CpG sites (*p*-values between 0.003 and 0.028). Except that, we found a 9.9 % difference in methylation at in the *SLC1A2* gene at CpG position 3 ($p < 0.001$), and in the *NR3C1* gene, we observed a difference of 2.4 % in methylation at CpG position 4, which show the trend of statistical significance ($p = 0.060$).

3.3. Correlations between neonatal stress and average DNA methylation

In Fig. 3, we present a heatmap of the correlation coefficients between the average percentage of DNA methylation in the target genes and the NISS scores at the two time points. Upon discharge, moderately high correlation coefficients were found for the *NR3C1*, *SLC6A4*, *SLC1A2*, *IGF2*, *BDNF* and *OPRM1* genes, however, none of these correlations were statistically significant. Even so, correlation coefficients were generally higher upon discharge compared with at 7–14 days. Although statistically not significant, most of the correlation coefficients were negative.

4. Discussion

We investigated the association between neonatal stress exposure and DNA methylation in stress-related and neurodevelopmentally relevant genes at 7–14 days after birth and upon discharge from the NICU. Unfortunately, adequate DNA isolation only succeeded in 25 to 50 % of

Table 3
Average percentage of DNA methylation in the 10 stress-related and/or neurodevelopmentally relevant genes at days 17–14 after birth and at discharge from the NICU.

Target genes	Day 7–14 after birth		Discharge		*p*-Value*
	N	Average %	N	Average %	
NRC31	22	0.9 (0.6–1.5)	6	1.0 ± 0.6	0.39
SLC6A4	22	2.3 (1.7–6.2)	11	3.6 (2.5–8.3)	0.29
HSD11B2	39	5.9 ± 2.3	22	6.5 ± 2.0	0.28
SLC7A5	33	84.6 ± 9.6	21	81.3 ± 14.5	0.32
SLC1A2	24	10.0 (3.5–16.1)	14	19.5 ± 15.2	0.14
IGF2	10	41.5 ± 24.9	4	16.0 ± 27.2	0.85
NNAT	29	65.1 ± 29.2	14	72.7 ± 20.0	0.41
BDNF	16	98.9 (94.2–99.4)	4	98.4 (98.1–99.6)	0.78
OPRM1	7	16.0 ± 8.6	8	3.1 ± 1.5	0.007
GABRA6	24	91.3 ± 10.1	19	86.0 ± 15.8	0.21

Data are presented as mean ± SD or median (25th – 75th percentile) where appropriate, **p*-value of the independent sample t-test or Mann Whitney U test where appropriate.

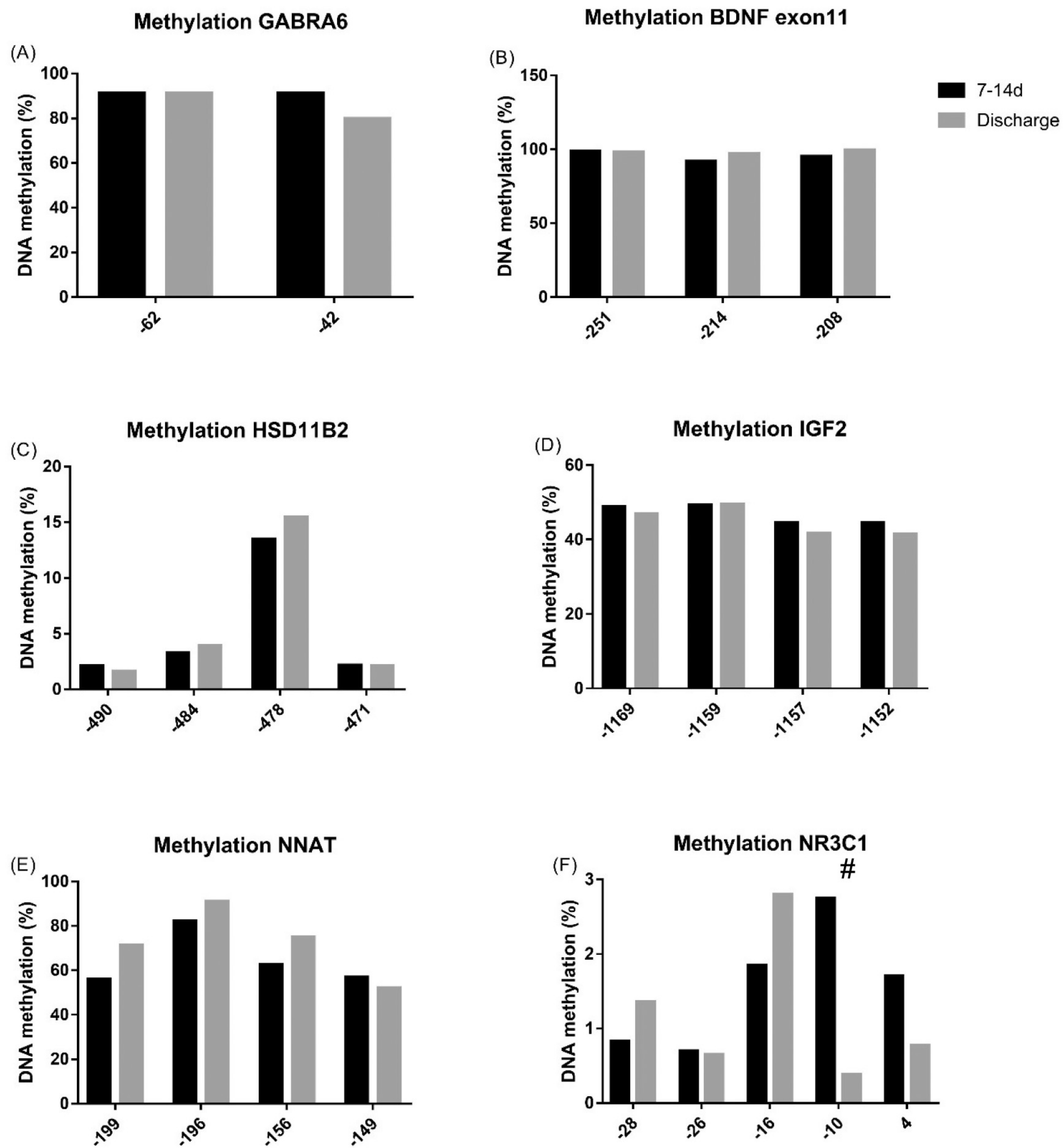

Fig. 1. DNA methylation per CpG position of (A) GABRA6, (B) BDNF exon11, (C) HSD11B2, (D) IGF2, (E) NNAT, (F) NR3C1. # $p = 0.06$.

samples, limiting the statistical power of our study. Even so, our findings from this exploratory study suggest that changes in DNA methylation may occur between those two time points in specific genes and that, particularly at discharge, cumulative neonatal stress exposure may play a role in altered DNA methylation.

Out of all ten genes investigated, the *OPRM1* gene showed importantly decreased average methylation percentages between days 7–14 and discharge from the NICU, as well as a probable association with neonatal stress exposure throughout NICU stay. We identified only one study in humans examining the effect of noxious stimuli on DNA methylation of this particular gene. In that study, no relationship was found [14]. Studies in mice report that the *OPRM1* pathway is involved in reactions to environmental stress, psychosocial stress, and analgesia as well, thereby making it plausible that this pathway is triggered in the case of environmental stress in preterm birth [27,28]. The mu-opioid receptor may also be involved in subsequent regulation of *BDNF* expression in the hippocampus, thereby predisposing for psychological disorders such as depression and anxiety after exposure to early stress [29]. Furthermore, although morphine activates the mu-opioid receptor and thus reduces pain, it does not fully counteract environmental stress, as witnessed by animal studies reporting that the developing brain is still affected by stress, despite morphine exposure [29]. In other areas of pain research, including fibromyalgia or other chronic pain conditions, the *OPRM1* gene was found to play an important role in pain perception and pain sensitivity [30]. In extremely preterm-born children, several studies have suggested increased sensitivity to pain later in life, up to and including adolescence, that seem to be associated with early exposure to pain and stress [19–26]. Our observation of altered DNA methylation in *OPRM1* implicates that higher neonatal stress exposure would result in hypomethylation and therefore gene upregulation and increased expression. When confirmed in larger studies, this finding could contribute to a mechanistic explanation for this altered sensitivity to pain as well as neurodevelopmental problems later in life in these children.

In the other genes, averages did not differ statistically. However, within two genes, two individual CpG positions showed importantly decreased DNA methylation between early NICU stay (7–14 days after birth) and upon discharge from the NICU, albeit statistically not

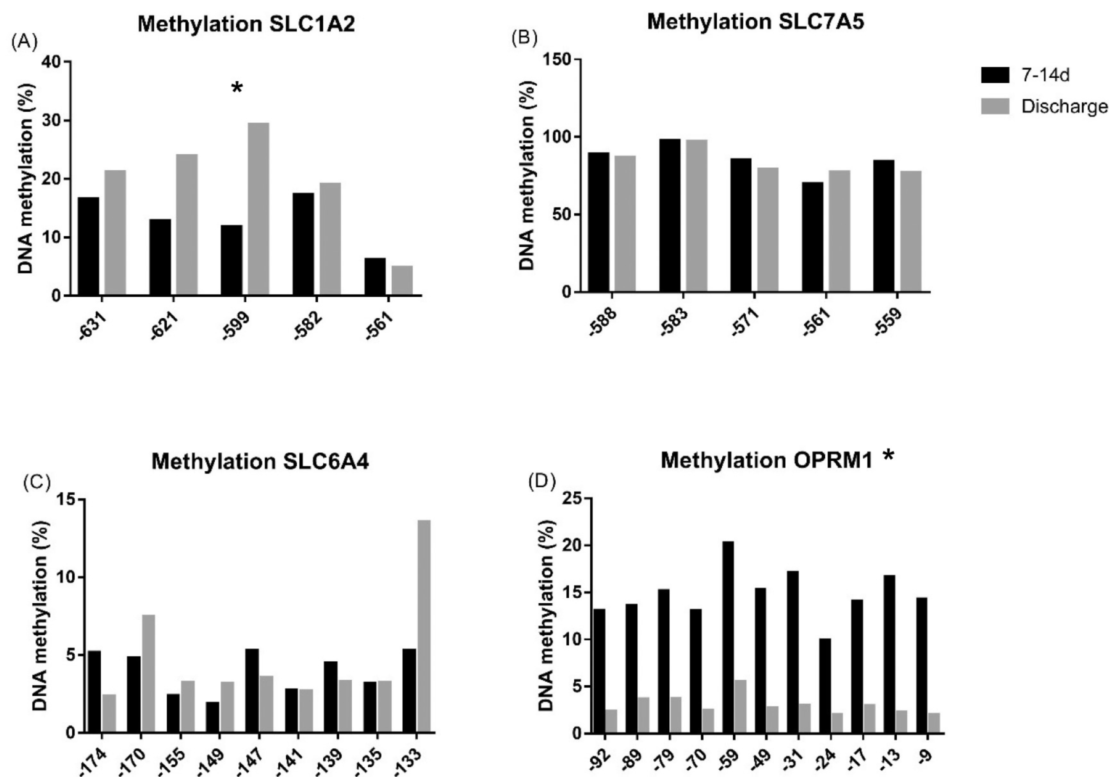

Fig. 2. DNA methylation per CpG position of (A) SLC1A2, (B) SLC7A5, (C) SLC6A4, (D) OPRM1. * $p < 0.05$. For OPRM1 * is for all CpG positions.

	Cumulative NISS score	
	7-14 days	NICU discharge
NRC31	0.07	0.59
SLC6A4	0.08	-0.38
HSD11B2	0.09	-0.1
SLC7A5	0	-0.05
SLC1A2	0.29	-0.31
IGF2	-0.19	0.8
NNAT	-0.08	-0.1
BDNF	-0.28	-0.4
OPRM1	-0.14	-0.43
GABRA6	0.21	-0.18

0.6	strong positive correlation
0.3	moderate positive correlation
0	no correlation
-0.3	moderate negative correlation
-0.6	strong negative correlation

Fig. 3. Heatmap of correlations between DNA methylation of target genes and Neonatal Infant Stressor Scale (NISS) scores at 7–14 days and NICU discharge.

significant in one instance. This concerns *NR3C1* CpG position 4. In a recent study, using the same assay in another cohort, we identified that infants with poorer neurodevelopmental outcomes at three months post-term had lower DNA methylation at this specific CpG site [31]. The individual CpG sites in this area are interesting, because this is where transcription factors bind that regulate the expression of the *NR3C1* gene, including nerve growth factor 1-a, that underlies healthy brain development [11]. Our finding of a potentially decreased methylation at this position upon discharge thus underscores the hypothesis that decreased DNA methylation may be caused by neonatal stress exposure and in turn may lead to poorer neurodevelopmental outcomes. The other position, in this instance statistically significant, is CpG position 3 of the *SLC1A2* gene. We did not find studies that investigated DNA methylation for this gene in preterm children either early or upon discharge from the NICU. However, *SLC1A2* is reported to be expressed in neurons in the brain during development [15]. It also is transcriptionally and post-transcriptionally regulated during the developmentally sensitive window for periventricular leukomalacia [32,33]. In preterm born children this takes place during NICU stay. Via this route, neonatal stress exposure may be a contributing factor to impaired neurodevelopmental outcomes. Consequently, these two genes, particularly these two specific CpG sites, should be included in future larger-scale studies, to better understand the influence of neonatal stress exposure on neurodevelopmental outcomes in preterm infants.

Apart from differences, we also investigated associations with neonatal stress. We found moderately high correlation coefficients between neonatal stress exposure and DNA methylation of *NR3C1, SLC6A4, SLC1A2, IGF2* and *BDNF* upon discharge from the NICU, although statistically not significant. For *NR3C1* and *SLC6A4* this is in line with previous research [10–12]. In that research, neonatal stress exposure was measured as the number of skin-breaking procedures while we used the NISS. The relative contribution of skin-breaking procedures in the NISS weighted scores is approximately 20 % [2]. With this small relative contribution, our findings indicate that exposure to other neonatal stressors than pain may contribute to decreased DNA methylation upon discharge as well. Expanding the definition of neonatal stress beyond skin-breaking procedures alone thus points towards the same direction of DNA methylation. For *SLC1A2, IGF2* and *BDNF,* we did not find studies examining the relationship between neonatal stress exposure and DNA methylation. The correlation coefficients we identified were mainly negative.

For two genes we observed positive correlation coefficients, again statistically not significant, being: *IGF2* and *NR3C1*. For *IGF2* we did not identify any studies to date to compare our findings with. The positive association may therefore reflect true directions, perhaps as a compensatory mechanism regarding brain repair but it may also be a chance finding due to low statistical power. For *NR3C1*, when confirmed in a larger sample, a positive correlation is a surprising finding, because a negative correlation between neonatal stress exposure and methylation of *NR3C1* had previously been reported [11]. However, the relationship between neonatal stress exposure and the functioning of the HPA-axis through cortisol also shows conflicting results. On the one hand, studies show that higher stress exposure leads to lower cortisol levels

[34,35], but on the other hand, Grunau and colleagues indicate that higher stress leads to higher cortisol levels [36], and Mello and colleagues even found no differences at all [37]. Future studies should therefore include both DNA methylation, gene expression as well as hormone levels to study the effects of level of neonatal stress exposure on the HPA-axis.

In this study, we also included several neurodevelopmentally relevant target genes that had not previously been studied in the context of neonatal stress. Some of these genes, particularly *IGF2*, *BDNF* and *SLC1A2*, showed moderately high correlation coefficients with neonatal stress Neonatal stress has been reported to have large effects on neurodevelopmental outcomes in preterm infants [6]. As previous studies have shown associations between altered DNA methylation of *NR3C1* and *SLC6A4* and neurodevelopmental outcomes [38–41], these other genes should also be incorporated in future studies examining such associations. Unravelling mechanisms through which adverse neurodevelopment may occur also calls for structural action to improve neonatal care.

4.1. Strengths and limitations

The major strength of this hypothesis-generating explorative study was its inclusion of a broader selection of target genes than in any other epigenetic neonatal stress-related studies to date. We used pyrosequencing methods to do so. While modern genome-wide assays would yield wider results, the method we used is the best applied in non-invasively collected fecal samples. We also acknowledge our limitations. First and foremost, the findings of this study should be interpreted with caution because of the small sample sizes for each individual gene. Smaller sample sizes were due to our inability to isolate enough human DNA from some fecal samples, even though the method that we used had been successful in fecal material in a study from our laboratory [18]. In that study, samples from multiple days were present, and samples with too little DNA could be replaced. This was not possible in the STRONG study, possibly explaining why our drop-out rate attributed to low DNA concentrations was high. These small sample sizes also limited our ability to perform the most suitable statistical tests, for example paired comparisons or corrections for multiple testing. As such, we were also unable to adjust for confounding or influencing factors, for example mode of delivery. Thus, future studies with larger sample sizes are needed to confirm the findings of this hypothesis generating study. A second limitation is that we were unable to investigate whether the DNA methylation alterations were associated with altered gene expression, because RNA degrades rapidly in stool samples due to its instability [18]. We did, however, purposely choose to use fecal samples as our tissue, because of the non-invasive nature compared with blood samples or saliva sampling in extremely preterm infants. Future studies also need to address whether changes in fecal DNA methylation correlate with those in other tissues. However, this is also unclear for the commonly used nucleated blood cell analysis.

4.2. Implications

With the results of this study, we contribute a first step into unravelling mechanisms underlying the association between neonatal stress exposure and adverse neurodevelopment. As a first exploration of several of these pathways, we propose that future studies focus on three pathways. First, the pathway of pain, i.e., through the *OPRM1*; second, via an altered set point of stress i.e., through HPA-axis related genes; and third, via direct effects on brain development, i.e., through neurodevelopmentally important genes. Future studies should also include how gene expression is influenced by differential DNA methylation patterns in neonatal stress. For clinical practice, we propose to continue all efforts towards minimizing pain and stress during NICU stay, preferably through non-pharmacological interventions such as kangaroo care, music therapy or massage therapy.

5. Conclusion

In conclusion, this exploratory study suggests that several stress-related and neurodevelopmentally relevant genes may be upregulated following higher neonatal stress exposure. Future studies should confirm our findings using larger cohorts and include mechanistical studies.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guidelines on human experimentation [Wet Medisch-wetenschappelijk onderzoek met mensen] and with the most recent version of the Helsinki Declaration of 2013. The study has been approved by the institutional medical ethical committee [Medisch Ethische Toetsingscommissie] of the University Medical Center Groningen [METc 2019/128].

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CRediT authorship contribution statement

Nienke H. van Dokkum conceptualized and designed the study, performed the statistical analyses, acquired funding, was responsible for project administration and wrote the original manuscript. Mian Bao performed the laboratory analyses and critically revised the manuscript. Rikst Nynke Verkaik-Schakel was responsible for data curation, performed the laboratory analyses and critically revised the manuscript. Sijmen A. Reijneveld was involved in conceptualization and methodology, and critically revised the manuscript. Arend F. Bos conceptualized and designed the study, supervised the statistical analyses and critically revised the manuscript. Marlou L.A. de Kroon conceptualized and designed the study, supervised the statistical analyses and critically revised the manuscript. Torsten Plosch conceptualized and designed the study, supervised the laboratory and statistical analyses and critically revised the manuscript.

Declaration of competing interest

The authors have no conflict of interest to disclose.

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