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RESEARCH ARTICLE

Epigenetic investigation into *NR3C1* exon 1F and *HSD11B2*: associations with neurodevelopment and oral feeding skills in preterm infants

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ABSTRACT

Aims: To examine the relationship between DNA methylation of *NR3C1* exon 1F and *HSD11B2* promoters and neurodevelopment, and oral feeding skills in preterm infants.
Design: A longitudinal prospective cohort study was conducted.
Methods: Data from 61 preterm infants who were born between 26 to 34 weeks gestational age without major comorbidities were analyzed. DNA methylation was evaluated from buccal samples. Neurodevelopment and oral feeding skills were evaluated using the Neurobehavioral Assessment of the Preterm Infants and the Early Feeding Skills Assessment, respectively.
Results: Increased methylation at specific cytosine-guanine (CpG) dinucleotide sites within *NR3C1* exon 1F and *HSD11B2* promoters was associated with suboptimal neurodevelopmental and oral feeding outcomes, potentially reflecting heightened sensitivity to early life stress in the NICU. Conversely, certain methylation changes appear to be adaptive, promoting consistent suck-breathe coordination, or optimal behavioral and cardiorespiratory stability during oral feeding.
Conclusion: The study highlights the complex relationship between DNA methylation of *NR3C1* exon 1F and *HSD11B2* promoters and neurodevelopment, and oral feeding skills in preterm infants.
Implications for the profession and/or patient care: Findings emphasize the need for continued research into epigenetic mechanisms underlying neonatal adaptation and stress regulation, with potential implications for targeted epigenetic interventions to support optimal neurodevelopment and oral feeding skills.

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Preterm infants; epigenetics; *NR3C1*; *HSD11B2*; oral feeding skills; neurodevelopment; early life stress; NICU

1. Introduction

Preterm birth, defined as birth before 37 completed weeks of gestation, is a leading cause of infant morbidity and mortality worldwide, often requiring prolonged neonatal intensive care unit (NICU) hospitalization [1]. The NICU, designed to support the fragile health of preterm infants, paradoxically introduces early life stress through maternal separation, painful procedures, inappropriate handling and sensory stimuli, and sleep fragmentation [2,3]. Excessive and prolonged exposure to early life stress such as those in the NICU may lead to adverse DNA methylation patterns [4]. Neurodevelopment and oral feeding skills are two significant milestones for preterm infants. Yet, there is limited understanding regarding the relationship between DNA methylation, neurodevelopment, and oral feeding skills in preterm infants.

Established research in the field of epigenetics demonstrates that behavioral and environmental factors can influence gene expression through epigenetic modifications, primarily via three mechanisms: DNA methylation, histone modification, and microRNA regulation [5]. Our research program focuses on DNA methylation and particularly methylation leading to 5-Methylcytosine. DNA methylation is an enzymatic process by which methyl residues are added to standard cytosine base, and such methylation can affect gene expression without altering the DNA sequence. Increased methylation typically suppresses gene expression, whereas decreased methylation enhances it. Early life stress in the NICU exposes the preterm infant to behavioral and environmental factors, increasing risk for adverse DNA methylation patterns [4].

Although epigenetic research specific to preterm infants remains limited, emerging evidence indicates that early life stress during critical developmental periods contributes to differential methylation of stress-regulated genes [6]. Two key glucocorticoid-related genes involved in stress regulation are the glucocorticoid receptor (*NR3C1*) gene and the 11β-hydroxysteroid dehydrogenase type 2 (*HSD11B2*) gene [7,8]. Methylation of the *NR3C1* exon 1F promoter is associated with decreased expression of the glucocorticoid receptor, impairing the negative feedback regulation of the hypothalamic-pituitary-adrenal (HPA) axis, thus dysregulates cortisol release. Methylation of the *HSD11B2* promoter is associated with

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Article highlights

- The study addresses the critical knowledge gap regarding how epigenetic modifications (DNA methylation) of *NR3C1* exon 1F and *HSD11B2* promoters. The findings may represent key mechanisms underlying the relationships among early life stress, neurodevelopment, and oral feeding skills in preterm infants.
- Increased methylation at specific CpG sites within *NR3C1* exon 1F and *HSD11B2* promoters was associated with suboptimal neurodevelopment and oral feeding outcomes, potentially reflecting heightened sensitivity to early life stress in the NICU. Conversely, certain methylation changes can be theorized to be adaptive, promoting consistent suck-breath coordination, or optimal behavioral and cardiorespiratory stability during oral feeding.
- Understanding the dynamic nature of DNA methylation in response to NICU early life stress and its relationships with two critical developmental milestones, neurodevelopment and oral feeding skills, will pave the way for targeted epigenetic-informed clinical strategies to enhance health and developmental trajectories of preterm infants in the NICU.

decreased expression of the gene, reducing the levels of the enzyme that inactivates cortisol and protects tissues from overexposure to active glucocorticoids (cortisol stress hormone). Examining epigenetic modifications of these two glucocorticoid-related genes is particularly relevant to preterm infants who are at risk for a dysregulated stress response due to immature physiological systems [4,9].

Research demonstrates that stress-related epigenetic modifications, such as methylation of the *NR3C1* exon 1F and *HSD11B2* promoters, may adversely affect neurodevelopment in preterm infants across multiple domains, including cognitive, motor, and emotional regulation [6–10]. Altered methylation of these glucocorticoid-related genes is associated with increased cortisol exposure, which can disrupt neurodevelopment, particularly in brain regions responsible for cognitive, memory, and emotional regulation [11,12].

Beyond neurodevelopment, oral feeding skills – a critical milestone for preterm infants – is particularly vulnerable to stress dysregulation. Oral feeding requires the complex, coordinated integration of sucking, swallowing, and breathing while maintaining physiological stability, alertness, and engagement during feeding [13], all of which depends on neurodevelopmental integrity [14,15]. Emerging research suggests that early life stress may impair oral feeding skills through alterations in autonomic function and dysregulation of the HPA axis [3,4]. Preterm infants in the NICU frequently experience oral feeding difficulties, leading to prolonged hospitalizations [16]. These feeding challenges can persist beyond the NICU, negatively impacting growth, development, and parent-infant bonding [15,17]. There remains a critical need to elucidate the biological mechanisms through which early life stress influences the neurodevelopmental integrity that supports oral feeding competency.

Current evidence suggests that epigenetic modifications of *NR3C1* exon 1F and *HSD11B2* promoters may represent key mechanisms underlying the relationships among early life stress, neurodevelopment, and oral feeding skills in preterm infants. However, these relationships remain insufficiently understood. This study aimed to: (1) examine the relationship between DNA methylation of *NR3C1* exon 1F and *HSD11B2* promoters and neurodevelopment, and (2) examine the relationship between the *NR3C1* exon 1F and *HSD11B2* promoters and oral feeding skills. By understanding these relationships, this study may inform future development of targeted interventions to attenuate adverse DNA methylation patterns and improve clinical outcomes for preterm infants in the NICU.

2. Materials and methods

2.1. Design

A longitudinal prospective cohort study was conducted. Confirmatory analysis was conducted for hypothesis testing. Ethical approval was provided by the Institutional Review Board of the Loyola University Chicago. Written informed consents were obtained from the parents/guardians. The full study protocol has been previously published [18].

2.2. Study setting and sampling

This study was conducted at a level III open-bay NICU located in the Midwestern region of the United States. All infants received the study site's standard of care (e.g., clustered nursing care and developmental care). The clinical team decided when to initiate and advance oral feeding. Seventy infants were enrolled through convenience sampling. After withdrawing nine infants due to custody issues, or the development of conditions outlined in the exclusion criteria, the final sample size was sixty-one infants.

2.2.1. Inclusion criteria

Infants who were born between 26 to 34 weeks gestational age (GA) were included.

2.2.2. Exclusion criteria

Infants who had major cardiac defects requiring surgery, gastrointestinal defects, necrotizing enterocolitis, intraventricular hemorrhage (grade III or IV), periventricular leukomalacia, chromosomal abnormalities, glucocorticoid therapy during NICU hospitalization, unresolved sepsis, unresolved pneumonia, or remaining on ventilation by 34 weeks postmenstrual age (PMA) were excluded.

3. Measures and instruments

Infant Characteristics were obtained from the electronic medical record. We collected the following data: race, ethnicity, sex, GA, birthweight, 1- and 5-minute Apgar score, delivery type (vaginal/C-section), small for gestational age (yes/no), intra-uterine growth restriction (yes/no), PMA at first oral feeding, full oral feeding, and discharge, duration of transition from first to full oral feeding, length of hospitalization, and medical conditions via the Neonatal Medical Index Classification (NMI) [19]. The NMI summarizes infants' medical conditions with classifications ranging from 1 for infants born weighing ≥1000 grams and without major complications to 5

for infants born weighing <1000 grams and with very serious complications.

3.1. *DNA methylation of* NR3C1 *exon 1F and* HSD11B2 *promoters*

DNA was collected from buccal samples at 34 weeks PMA and during the week of discharge. Buccal samples were collected before feeding to avoid milk contamination. Buccal samples were collected using the DNA/RNA Shield Collection Tube with Swab (Zymo Research, Inc., Irvine, CA). All samples were stored at −80°C until analysis. Genomic DNA was extracted from the samples using a Maxwell® RSC Buccal Swab DNA kit (Promega, Maddison, WI) implemented on an automated Maxwell RSC48 device. Methylation patterns of *NR3C1* exon 1F and *HSD11B2* promoters were assessed using deep amplicon sequencing from bisulfite-converted DNA using next-generation sequencing. Briefly, DNA was treated with bisulfite for methylation analysis using an EZ DNA Methylation-Lightning Kit (D5030, Zymo Research, Inc., Irvine, CA) according to the manufacturer's instructions. Cytosine-guanine (CpG) dinucleotide sites of interest were targeted using PCR amplification of the bisulfite-treated DNA, followed by sequencing of the amplicons on an Illumina MiSeq sequencer. Preparation of converted DNA for high-throughput amplicon sequencing was performed in two PCR stages. Primer sets targeted the *NR3C1* exon 1F and *HSD11B2* promoters, with amplicons covering 33 and 31 CpG sites, respectively. Primers targeting the *NR3C1* exon 1F promoter (GYGGTTTTAGATTTATTYGGGAGTT/TCCCTAAAACCTCCCCAAAAA) were previously described [20]. Primers targeting the *HSD11B2* promoter (GGAAGTGGGGTTGTGYGTTTTTAGGTTTAAGTT/GGTGGT GYGATTAGTAAAGGGTAT) were previously described [7]. All primers were synthesized with linkers (i.e., CS1 and CS2 linkers for forward and reverse primers, respectively to facilitate incorporation of sample-specific barcodes and Illumina sequencing adapters through a second PCR reaction. Bisulfite conversion controls (0% methylated human DNA and 100% methylated human DNA; Zymo Human Methylated & Non-methylated DNA Set, D5014, Zymo Research, Inc., Irvine, CA) were included with each sequencing run. Raw read pairs generated from the software package PEAR to merge overlapping read pairs, and only the merged reads were used for downstream analyses. Trimming was performed using Cutadapt to remove low-quality bases and adapter sequences. FastQC was used to assess the quality of raw and merged reads. The trimmed, merged reads were then aligned to the human reference genome (hg38) in a bisulfite-conversion-aware manner using Bismark. Methylation status per read was obtained with bismark_methylation_extractor tool. Percent methylation levels, plus counts of methylated and unmethylated reads, were then summarized per CpG site.

Neurodevelopment was evaluated using the Neurobehavioral Assessment of the Preterm Infants (NAPI) at 34 weeks PMA [21]. The evaluator conducted the NAPI in-person at the bedside prior to feeding. The evaluator was trained by the PI. The evaluator and PI routinely conducted the NAPI together and discussed any finding inconsistency until 100% agreement was achieved. The NAPI consists of 73 items within 7 clusters: scarf sign, motor development and vigor, popliteal angle, alertness and orientation, irritability, quality of cry, and asleep. The NAPI provides summary scores for each cluster. Higher scores in scarf sign, motor development and vigor, popliteal angle, alertness and orientation, and quality of cry are considered optimal neurodevelopment. Higher scores in irritability and asleep are considered suboptimal neurodevelopment. Construct validity, face validity, and sensitivity of the NAPI were described using an index of medical complications and general movements [22]. Inter-rater reliability coefficient was established with Cronbach $\alpha = 0.67$ to 0.97 [21].

Oral Feeding Skills were evaluated using the Early Feeding Skills Assessment (EFS) [23]. The EFS was conducted weekly after the first oral feeding. To ensure consistency in the feeding method, the PI or Speech Therapist fed each infant following a standard protocol (e.g., 20–30 minutes, side-lying position, swaddling, and pacing as needed). The evaluator conducted the EFS in-person at the bedside. The evaluator was trained by the PI. The evaluator and PI routinely conducted the EFS together and discussed any finding inconsistency until 100% agreement was achieved. The EFS has 22 items within 5 subscales: respiratory regulation, oral-motor functioning, swallowing coordination, engagement, and physiologic stability. The EFS provides a summary score for each subscale and a total summary score for all 5 subscales. Higher summary scores indicate that higher oral feeding competency is observed. Inter-rater reliability and construct validity were established with Cronbach $\alpha = 0.81$ and correlations between EFS scores and GA, and PMA.

3.2. *Data collection*

Figure 1. After obtaining the written informed consent from the mother, the infant was enrolled in the study. Infant characteristics were collected from the electronic medical record. At 34 weeks PMA, the buccal sample was collected and the NAPI assessment was conducted. The first EFS assessment was conducted after the infant orally consumed ≥10% of their prescribed feedings for at least two consecutive days. The EFS assessment was then conducted once a week. During the week of discharge, the buccal sample was collected and the last EFS assessment was conducted.

3.3. *Data analysis*

Statistical analyses were performed using R statistical software.

Summary statistics were computed to describe clinical covariates, i.e., infant characteristics, NAPI, and EFS scores. NAPI and EFS scores were rated at bedside and testing for intra- and inter-rater reliability was not performed. The number of EFS assessments varied among infants depending on their oral feeding progression. The scores from the first and last EFS assessments were utilized. To identify confounding clinical covariates, simple bivariate analyses of the clinical covariates were performed using either Kendall Tau (τ), Kruskal-Wallis, or Chi-squared tests.

Differential analysis of the normalized methylation data, i.e., fraction of methylated reads, was performed using limma in R. The normalized methylation data, at two timepoints, i.e., 34 weeks PMA and discharge, were modeled as a function of the clinical covariates, i.e., NAPI and EFS scores, while controlling for clinical covariates, i.e., sex, race, GA, 1-minute Apgar

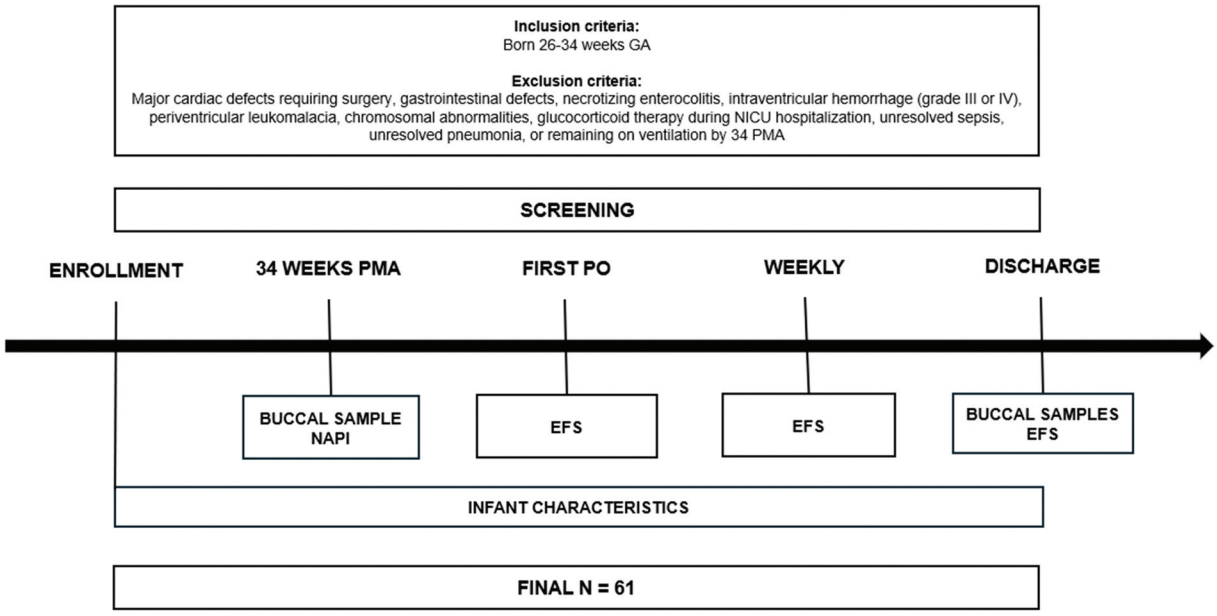

Figure 1. Study flow diagram illustrating screening, inclusion and exclusion, enrollment, and data collection at each study time point.

score, and NMI. The fitted coefficients of the model were then tested using empirical Bayes moderated t-statistic, i.e., ebayes function. In all cases, the resulting *p* values were adjusted for multiple testing using the false discovery rate (FDR) correction of Benjamini and Hochberg, yielding *q* values. *q* value ≤0.05 was considered statistically significant.

We used the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline to draft this manuscript, and the STROBE reporting checklist when editing, included in Supplementary Table S1 [24].

4. Results

4.1. Infant characteristics

The final sample size was $N = 61$. Descriptive statistics for infant characteristics are shown in Table 1. Briefly, the sample consisted of 22.95% Black or African American and 67.21% White, 36.07% Hispanic or Latino, and 54.10% female. Infants were born at a mean GA of 31.39 weeks with a mean birthweight of 1.71 kg. The majority of infants were classified in categories I, II, and III of the NMI, indicating minimal to moderate complications.

4.2. Neurodevelopment

Each of the 61 infants had one NAPI assessment at 34 weeks PMA. Descriptive statistics for NAPI scores are shown in Table 2.

4.3. Oral feeding skills

All infants had at least one EFS assessment. The number of EFS assessments varied (range = 1–10) among infants due to their individual oral feeding progression and NICU discharge. A total of 196 weekly EFS assessments were completed for all infants. EFS scores from 56 infants who had both first and last EFS assessments were included. Descriptive statistics for EFS scores are shown in Table 3.

4.4. DNA methylation of NR3C1 exon 1F and HSD11B2 promoters

Methylation data were obtained for 33 CpG sites within the *NR3C1* exon 1F promoter and 31 CpG sites within the *HSD11B2* promoter. For clarity in reporting, the CpG sites were sequentially numbered from 1 to 33 for *NR3C1* and 1 to 31 for *HSD11B2*. Table 4 provides the CpG site numbering, genomic coordinates (hg38), and relative position from the transcription start site (TSS) for each targeted gene. Figure 2 illustrates the box plot summary graph of the DNA methylation. Supplementary Table S2 includes summary statistics for each CpG site as well as the aligned counts, methylated counts and fraction of methylated counts (percent methylation). The mean methylation at individual CpG sites of *NR3C1* exon 1F at 34 weeks PMA and discharge ranged from 0.09% to 2.58% and 0.10% to 1.08%, respectively. The mean methylation at individual CpG sites of *HSD11B2* promoters at 34 weeks PMA and discharge ranged from 0.14% to 3.50% and 0.14% to 3.62%, respectively.

Model 1a: Analysis of DNA Methylation of *NR3C1* Exon 1F Promoter at 34 Weeks PMA, Controlling for Clinical Covariates (Table 5).

NAPI: No significant correlations were found between methylation at individual CpG sites and NAPI scores.

First EFS Assessment: No significant correlations were found between methylation at individual CpG sites and EFS scores.

Last EFS Assessment: Higher methylation at CpG 31 was associated with lower physiologic stability scores ($\beta = -0.11$, $p = 1.26E\text{-}10$, $q = 4.15E\text{-}09$).

Table 1. Infant characteristics ($N = 61$).

Infant characteristics	Frequency	%
Race		
Asian	1	1.64
Black or African American	14	22.95
White	41	67.21
More than One Race	2	3.28
Unknown	3	4.92
Ethnicity		
Not Hispanic or Latino	36	59.02
Hispanic or Latino	22	36.07
Unknown	3	4.92
Sex		
Male	28	45.90
Female	33	54.10
Delivery Type		
Vaginal	24	39.34
C-section	37	60.66
NMI		
I	12	19.67
II	29	47.54
III	15	24.59
IV	4	6.56
V	1	1.64
SGA		
Yes	6	9.84
No	55	90.16
IUGR		
Yes	9	14.75
No	52	85.25

Infant Characteristics	Mean	SD	Median	IQR	Min	Max
GA at birth (weeks)	31.39	2.62	32.00	5.00	26.00	34.00
Birthweight (kg)	1.71	0.60	1.57	0.78	0.75	3.37
1-minute Apgar score	6.54	2.04	7.00	2.00	1.00	9.00
5-minute Apgar score	8.02	1.13	8.00	1.00	3.00	9.00
PMA at first PO (weeks)	33.74	1.51	34.00	0.86	25.57	36.86
PMA at full PO (weeks)	36.09	1.63	36.00	2.14	33.00	40.86
PMA at discharge (weeks)	37.06	1.59	37.00	1.86	34.43	41.14
Duration of transition from first to full PO (days)	15.48	10.76	13.00	12.00	3.00	48.00
Length of hospitalization (days)	39.38	24.01	34.00	35.00	8.00	105.00

Demographic and clinical characteristics of infants included in the study.
Abbreviations: NMI, Neonatal Medical Index Classification; SGA, small for gestational age; IUGR, intrauterine growth restriction; GA, gestational age; PMA, postmenstrual age; PO, oral feeding; SD, standard deviation; IQR, interquartile range.

Table 2. Neurodevelopment ($N = 61$).

NAPI cluster	Mean	SD	Median	IQR	Min	Max
Scarf sign	57.93	20.34	66.70	33.40	33.30	100.00
Motor development & vigor	42.41	14.48	41.67	22.39	13.57	74.04
Popliteal angle	37.15	32.27	33.30	66.70	0.00	100.00
Alertness and orientation	51.64	25.53	62.68	46.62	2.78	88.93
Irritability	32.52	21.90	33.35	20.27	0.00	82.15
Quality of cry	38.30	34.91	50.00	50.00	0.00	100.00
Asleep	11.02	11.32	7.14	19.05	0.00	33.33

Neurodevelopmental outcomes of infants included in the study.
Abbreviations: NAPI, the Neurobehavioral Assessment of the Preterm Infants; SD, standard deviation; IQR, interquartile range.

Model 2a: Analysis of DNA Methylation of *NR3C1* Exon 1F Promoter at Discharge, Controlling for Clinical Covariates (Table 5).

NAPI: No significant correlations were found between methylation at individual CpG sites and NAPI scores.

First EFS Assessment: Higher methylation at CpG 10 (β = −2.69E-04, $p = 3.23$E-03, q = 0.05) and CpG 13 (β = −2.07E-01, $p = 4.79$E-04, q = 0.02) was associated with poorer swallowing coordination scores.

Last EFS Assessment: Higher methylation at CpG 3 was associated with lower oral-motor function scores (β = −3.26E-04, $p = 6.77$E-04, q = 0.01) and lower total oral feeding scores (β = −1.38E-04, $p = 1.02$E-04, q = 3.37E-03). Higher methylation at CpG 18 was also associated with poorer oral-motor function scores (β = −2.63E-03, $p = 3.10$E-04, q = 0.01) and lower total oral feeding scores (β = −8.30E-04, $p = 2.98$E-03, q = 0.05).

Model 1b: Analysis of DNA Methylation of *HSD11B2* Promoter at 34 Weeks PMA, Controlling for Clinical Covariates (Table 6).

NAPI: Higher methylation at CpG 7 (β = 8.07E-04, $p = 1.16$E-04, q = 2.66E-03) and CpG 8 (β = 8.70E-04, $p = 1.77$E-04, q = 2.66E-03) was associated with higher asleep scores.

Table 3. Oral feeding skills ($N = 56$).

	First EFS Assessment (n = 56)					
EFS Subscale	Mean	SD	Median	IQR	Min	Max
Respiratory regulation	10.70	2.37	10.00	4.00	6.00	15.00
Oral-motor functioning	8.91	1.92	9.00	2.25	5.00	12.00
Swallowing coordination	11.61	0.68	12.00	1.00	9.00	12.00
Engagement	4.11	1.40	4.00	1.25	2.00	6.00
Physiologic stability	11.23	1.08	12.00	1.00	7.00	12.00
Total	46.55	4.47	47.00	8.00	35.00	54.00
	Last EFS Assessment ($n = 56$)					
EFS Subscale	Mean	SD	Median	IQR	Min	Max
Respiratory regulation	12.96	1.74	13.00	3.00	9.00	15.00
Oral-motor functioning	10.50	1.49	11.00	2.00	6.00	12.00
Swallowing coordination	11.41	1.06	12.00	1.00	8.00	12.00
Engagement	4.77	1.29	5.00	2.00	2.00	6.00
Physiologic stability	11.64	0.82	12.00	0.25	7.00	12.00
Total	51.29	3.74	52.00	4.00	38.00	57.00

Oral feeding skills at the first and last Early Feeding Skills Assessment of infants included in the study.
Abbreviations: EFS, Early Feeding Skills Assessment; SD, standard deviation; IQR, interquartile range.

Table 4. CpG site coordinates[a].

NR3C1 exon 1F promoter[b]			*HSD11B2* promoter[c]		
CpG	Genomic coordinate	TSS relative coordinate	CpG	Genomic coordinate	TSS relative coordinate
1	143,404,098	−412	1[d]	67,430,428	−693
2	143,404,113	−427	2	67,430,465	−656
3	143,404,120	−434	3	67,430,486	−635
4	143,404,123	−437	4	67,430,492	−629
5	143,404,137	−451	5	67,430,496	−625
6	143,404,147	−461	6	67,430,509	−612
7	143,404,151	−465	7	67,430,541	−580
8	143,404,165	−479	8	67,430,543	−578
9	143,404,170	−484	9	67,430,562	−559
10	143,404,177	−491	10	67,430,564	−557
11	143,404,179	−493	11	67,430,580	−541
12	143,404,190	−504	12	67,430,594	−527
13	143,404,201	−515	13	67,430,600	−521
14	143,404,203	−517	14	67,430,606	−515
15	143,404,206	−520	15	67,430,616	−505
16	143,404,209	−523	16	67,430,619	−502
17	143,404,212	−526	17	67,430,621	−500
18	143,404,215	−529	18	67,430,627	−494
19	143,404,220	−534	19	67,430,631	−490
20	143,404,227	−541	20	67,430,633	−488
21	143,404,244	−558	21	67,430,637	−484
22	143,404,256	−570	22	67,430,643	−478
23	143,404,266	−580	23	67,430,649	−472
24	143,404,272	−586	24	67,430,655	−466
25	143,404,278	−592	25	67,430,662	−459
26	143,404,283	−597	26	67,430,673	−448
27	143,404,288	−602	27	67,430,685	−436
28	143,404,292	−606	28	67,430,687	−434
29	143,404,294	−608	29	67,430,707	−414
30	143,404,298	−612	30	67,430,711	−410
31	143,404,304	−618	31	67,430,713	−408
32	143,404,308	−622			
33	143,404,318	−632			

Genomic coordinates of CpG sites within the promoter regions of *NR3C1* exon 1F and *HSD11B2*.
Abbreviations: TSS, transcription start site.
[a]Based on human reference genome (hg38).
[b]Located on chromosome 5, compliment strand, TSS is located at 143,403,686 on the complement strand.
[c]Located on chromosome 16, TSS is located at 67,431,121.
[d]Located within the primer annealing site, thus excluded from analysis.

First EFS Assessment: No significant correlations were found between methylation at individual CpG sites and EFS scores.

Last EFS Assessment: Higher methylation at CpG 11 was correlated with lower oral-motor function scores (β = −0.02, p = 1.24E-03, q = 0.04).

Model 2b: Analysis of DNA Methylation of *HSD11B2* Promoter at Discharge, Controlling for Clinical Covariates (Table 6).

NAPI: Higher methylation at CpG 8 was associated with higher asleep scores (β = 6.39E-04, p = 1.50E-05, q = 4.51E-04).

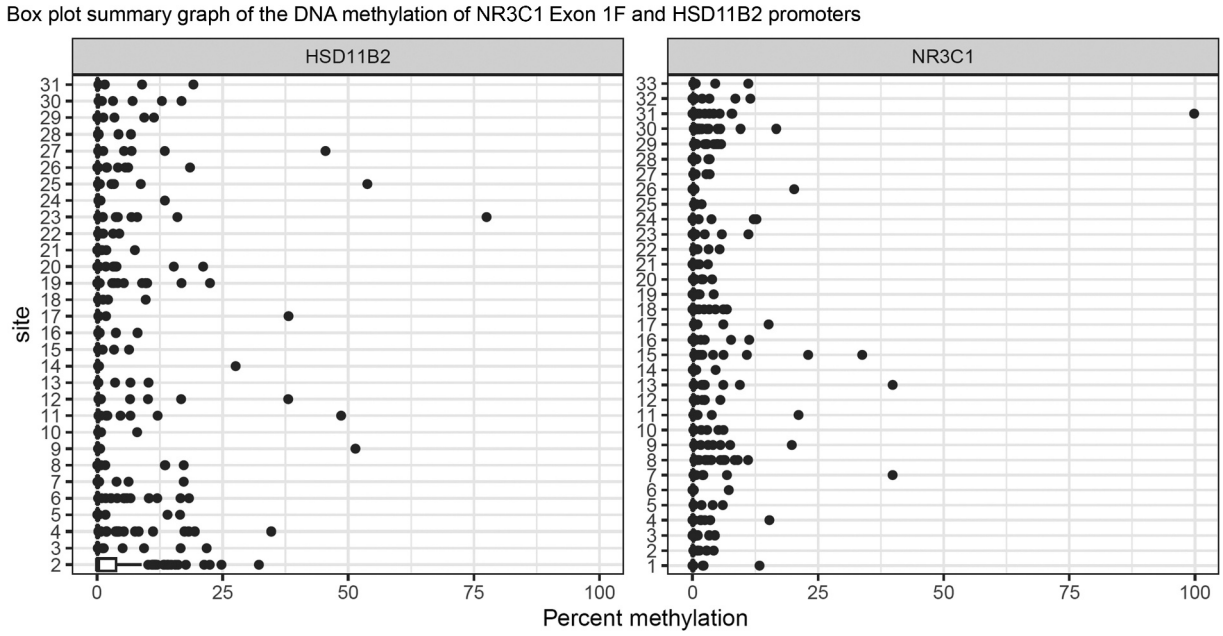

Figure 2. DNA methylation of *NR3C1* exon 1F and *HSD11B2* promoters.

Box plot summary graph of the DNA methylation of *NR3C1* Exon 1F and *HSD11B2* promoters.

Table 5. Study findings related to DNA methylation of *NR3C1 exon 1F* promoter.

NR3C1 exon 1F promoter	34 Weeks PMA	Discharge
NAPI	No significant correlations	No significant correlations
First EFS	No significant correlations	**CpG 10** • **Swallowing coordination** (β = −2.69E-04, q = 0.05) **CpG 13** • **Swallowing coordination** (β = −2.07E-01, q = 0.02)
Last EFS	**CpG 31** • **Physiologic stability** (β = −0.11, q = 4.15E-09)	**CpG 3** • **Oral-motor function** (β = −3.26E-04, q = 0.01) • **Total oral feeding** (β = −1.38E-04, q = 3.37E-03) **CpG 18** • **Oral-motor function** (β = −2.63E-03, q = 0.01) • **Total oral feeding** (β = −8.30E-04, q = 0.05)

Study findings related to DNA methylation of the *NR3C1* exon 1F promoter and associations with neurobehavioral assessment and early feeding skills in preterm infants at the first and last assessments.

Abbreviations: NAPI, the Neurobehavioral Assessment of the Preterm Infants; EFS, Early Feeding Skills Assessment; PMA, Postmenstrual age.

First EFS Assessment: Higher methylation at CpG 6 was associated with higher respiratory regulation scores (β = 3.66E-03, p = 9.24E-04, q = 0.03).

Last EFS Assessment: Higher methylation at CpG 16 was associated with lower swallowing coordination scores (β = −4.32E-03, p = 1.67E-03, q = 0.05).

5. Discussion

This study examined the relationship between DNA methylation of *NR3C1* exon 1F and *HSD11B2* promoters, neurodevelopment, and oral feeding skills in preterm infants. The findings revealed significant associations, suggesting that differential methylation of these glucocorticoid-related genes may influence neurodevelopment and oral feeding skills in preterm infants.

It was unexpected that no association was found between methylation at individual CpG sites of *NR3C1* exon 1F promoter and neurodevelopment. On the other hand, associations of individual CpG methylation and neurodevelopment were observed for the *HSD11B2* promoter. For example, the findings revealed higher methylation at specific CpG sites of *HSD11B2* promoter (i.e., 7 and 8) was associated with more time spent in sleep states during the neurobehavioral assessment. These findings add to the growing body of evidence regarding the potential role of epigenetic modifications in preterm neurodevelopment. Thus far, a study by Lester et al., found that high-risk preterm infants exhibited higher buccal cell methylation (same tissue type as our study) of *NR3C1*

Table 6. Study findings related to DNA methylation of *HSD11B2* promoter.

HSD11B2 promoter	34 weeks PMA	Discharge
NAPI	**CpG 7** • **Asleep** (β = 8.07E-04, q = 2.66E-03) **CpG 8** • **Asleep** (β = 8.70E-04, q = 2.66E-03)	**CpG 8** • **Asleep** (β = 6.39E-04, q = 4.51E-04)
First EFS	No significant correlations	**CpG 6** • **Respiratory regulation** (β = 3.66E-03, q = 0.03)
Last EFS	**CpG 11** • **Oral-motor function** (β = −0.02, q = 0.04)	**CpG 16** • **Swallowing coordination** (β = −4.32E-03, q = 0.05)

Study findings related to DNA methylation of the *HSD11B2* promoter and associations with neurobehavioral assessment and early oral feeding skills in preterm infants at the first and last assessments.
Abbreviations: NAPI, the Neurobehavioral Assessment of the Preterm Infants; EFS, Early Feeding Skills Assessment; PMA, Postmenstrual age.

exon 1F at CpG 3 and lower *HSD11B2* methylation at CpG 3 compared to low-risk preterm infants [7]. In that study, high-risk preterm infants displayed poorer attention and movement quality, increased stress signs, heightened arousal and excitability, and required more handling during attention assessments. We recognize that CpG methylation is likely dependent upon the tissue evaluated. Yet it is worth noting that others found that extremely preterm infants with atypical Motor Optimality Score-Revised (MOS-R) scores exhibited lower average placental methylation of *NR3C1* compared to near-optimal MOS-R scores at 3 months corrected age [8]. Lower placental methylation at CpG 1, 3, and 5 of *NR3C1* was also noted for infants with atypical MOS-R scores compared to near-optimal MOS-R scores at 3 months corrected age [8]. Prior research in full-term infants demonstrated similar patterns. For instance, decreased placental methylation of *NR3C1* was found to be associated with neurodevelopment in full-term infants, showing poorer regulatory behaviors in infancy [25,26]. In contrast, Sheinkopf and colleagues reported that higher mean methylation of *NR3C1* (from placental tissue) associated with poorer regulation, as reflected in full-term cry acoustics [27]. Lower placental methylation at CpG 2 of *NR3C1* exon 1F promoter and CpG 4 of *HSD11B2* promoter was linked to poor behavioral self-regulation, weak muscle tone, and lethargy [28]. Conversely, higher placental methylation at specific CpG sites of *NR3C1* exon 1F promoter (i.e., 6, 7, 8, and 10) [26] and *HSD11B2* promoter [29] was associated with poorer movement quality and attention. Differential methylation of *NR3C1* exon 1F and *HSD11B2* promoters (from placental tissue) was associated with varying levels of excitability and reflex asymmetry [30]. The variation in direction of the relationships, patterns of methylation and tissue-specific nature of methylation highlight the complexity of the field. Although evidence establishing causality remains to be determined, collectively the associations found in our study and that of others suggests epigenetic modifications of glucocorticoid-related genes may serve as an underlying mechanism contributing to neurodevelopmental outcomes and phenotypes.

With respect to oral feeding skills, the findings demonstrated that infants with higher methylation at specific CpG sites of *NR3C1* exon 1F promoter (i.e., 3, 10, 13, 18, and 31) and *HSD11B2* promoter (i.e., 11 and 16) were associated with poorer oral-motor function, swallowing coordination, physiologic stability, and overall oral feeding competency during oral feeding. Based on the attributes of the items evaluated in the EFS [23], the associations support the notion that increased methylation at multiple CpG sites of *NR3C1* Exon 1F promoter may be linked to suboptimal oral feeding skills, particularly difficulties with mouth and jaw movements, suck strength, lip seal, swallowing difficulties, frequent behavioral stress cues or skin color changes, or difficulty maintaining stable oxygen saturation or heart rate during oral feeding. These findings extend previous research. For example, prior studies found that infants with higher methylation at specific CpG sites of *NR3C1* exon 1F promoter (i.e., 17 and 31) and *HSD11B2* promoter (i.e., 4 and 28) exhibited less competent oral feeding skills, including impaired respiratory regulation, oral-motor function, and engagement, difficulty maintaining physiologic stability, and longer transition to full oral feeding [3]. Aligning with these findings, current evidence suggests that increased methylation, as opposed to decreased methylation, in certain CpG sites of glucocorticoid-related genes may reflect a heightened stress response, leading to dysregulation of autonomic function and the HPA axis [31,32]. We theorized that aberrant stress regulation can elicit behavioral responses such as disorganized self-regulation, motor function, lethargy, hyper or hypoactive muscle tone and energy. Thus, these disorganized behaviors may impair coordination of sucking and swallowing reflexes [33]. Dysregulated stress responses can disrupt behavioral states, contributing to excessive sleepiness or agitation during oral feeding, making it harder to relax and achieve optimal suck-breath-swallow coordination [34]. Physiologic instability in response to stress leads to bradycardia, tachypnea, and oxygen desaturation, further compromising the infants' limited energy and fragile airways [35]. Such biological dysregulation may lead to poor suck-swallow-breath coordination, engagement, and difficulty maintaining physiologic stability. Ultimately, biological dysregulation can contribute to delays in developing the motor skills and coordination necessary for competent oral feeding.

While higher methylation at CpG 6 of *HSD11B2* promoter was associated with better respiratory regulation during oral feeding, this pattern of CpG methylation was not consistently observed across all CpG sites evaluated. Similarly, a prior pilot

study showed that higher methylation at CpG 25 of *NR3C1* exon 1F promoter and CpG 21 of *HSD11B2* promoter was linked to better swallowing coordination and respiratory regulation, respectively [3]. Perhaps, greater methylation in certain CpG sites of glucocorticoid-related genes may also have a potential role in supporting the development of specific skills, such as respiratory and swallowing, necessary for competent oral feeding.

DNA methylation is a dynamic biological process that is sensitive to environmental influences and shaped by complex interactions between genes and CpG sites. Also, Kantake et al. noted that DNA methylation at multiple CpG sites of *NR3C1* increased significantly between postnatal month 1 and 2 [36]. We speculate that age-related changes in DNA methylation along with environmental influences may account for the variability in findings across studies. As a result, identifying consistent methylation patterns that can serve as predictors of oral feeding skills remains challenging. It is likely that multiple CpG sites are involved in influencing the development of oral feeding skills, and their effects may depend on the specific interactions between genes and CpG sites at the time of sample collection. Despite these challenges, our findings to date offer valuable insights into the potential role of methylation in shaping oral feeding skills in preterm infants during their early days of life.

5.1. Strength and limitations of the work

This study has several notable strengths. The repeated-measures design provides data at two critical timepoints during NICU hospitalization, allowing for a more nuanced understanding of developmental changes and relationships over time. The use of the NAPI and EFS instruments to evaluate neurodevelopment and oral feeding skills strengthens the study, as both instruments demonstrate high reliability and validity, enhancing the robustness of the outcome data. In addition, the DNA methylation analysis protocol has been optimized and validated in a pilot study, ensuring accuracy and reliability of the molecular data. The study is further strengthened by the integration of biobehavioral and molecular outcomes, allowing for a more comprehensive evaluation of the interplay between early life stress, neurodevelopment, and oral feeding skills. The statistical analysis plan, grounded in confirmatory analyses to test predefined hypotheses, adds rigor and reliability to the interpretation of results. Finally, strict adherence to standardized data collection protocol ensured consistency across measures, enhancing both internal validity and reproducibility of the findings.

Despite the novel findings of this study, several limitations are acknowledged. The sample size is small and included infants who were born between 26 to 34 weeks GA, which limits the generalizability of the findings. While statistical adjustments were made for clinical covariates, unmeasured factors such as genetic predisposition and prenatal stress may have influenced the observed methylation patterns, neurodevelopment, and oral feeding skills. Given the large range in GA of the infants evaluated, it is possible that a variety of age-related parameters may influence our findings. For example, the associations found may be confounded by ongoing developmental changes in DNA methylation. In addition, the lack of methylation measures in a term infant reference group and/or control group, may hinder a comprehensive understanding of methylation in the target genes. Multiple studies and a systematic review found that methylation at numerous CpG sites of *NR3C1* exon 1F and *HSD11B2* were associated with reduced expression of these genes [37–39]. However, our analyses were limited to DNA methylation and did not include measures of mRNA or gene protein products. As a result, this limits our understanding of the interaction between DNA methylation, gene expression, and clinical outcomes. Although our study identified associations between DNA methylation of the targeted genes, neurodevelopment, and oral feeding skills, we did not identify whether the associations were linked to mRNA expression for these genes. Future studies incorporating functional analyses, such as gene expression profiling and measurement of gene protein products are necessary to validate these findings. While this study highlights potential epigenomic targets for early NICU interventions, it does not establish whether modifying DNA methylation can directly improve neurodevelopment and oral feeding skills. Thus, further research is warranted to determine the efficacy of targeted interventions in mitigating adverse epigenetic modifications and supporting optimal neurodevelopment and oral feeding skills.

5.2. Recommendation for future research

DNA methylation is increasingly recognized as a key mechanism of adaptive plasticity, allowing organisms to modify gene expression in response to environmental and behavioral challenges. For preterm infants, methylation of glucocorticoid-related genes may play a crucial role in stress regulation, neurodevelopment, and oral feeding skills. Emerging evidence suggests that early life stress can lead to persistent epigenetic modifications of glucocorticoid-related genes, potentially contributing to long-term neurodevelopmental consequences and increased risk for chronic health conditions [40]. However, critical gaps remain in our understanding of the impact of changes in methylation in this population. While this study measured methylation at two significant NICU milestones, most existing research captures methylation at a single time point, limiting insights into its stability and evolution. Ideally, future studies should consider longitudinal designs to track methylation changes over a longer time span, providing insight as to the persistence and reversibility of these modifications. Such research will help clarify how early-life methylation patterns influence long-term developmental and health outcomes.

Furthermore, nutrition parameters and the disturbed trans-sulfuration pathway in infants who are fed parenterally may impact methyl donation and should be incorporated in future research [41] DNA methylation is only one epigenetic mechanism, hence assessment of other epigenetic markers (histone modifications and micro-RNA regulations) will provide a more comprehensive epigenetic profile to enhance understanding of the impact of environmental factors on

development. In addition, the integration of epigenomics with other omics approaches, such as genomics, transcriptomics, proteomics, and metabolomics could provide a more comprehensive view of molecular pathways underlying developmental phenotypes in preterm infants. The application of multi-omics approaches within the context of environmental and behavioral factors would enable a holistic approach [42].

While associations between methylation, neurodevelopment, and oral feeding skills begin to come to light, further research is warranted to design and test early interventions. Particularly, caregiving behaviors and environmental factors may foster more favorable epigenetic programming. Thus, it is crucial to investigate the extent to which interventions, such as enhanced parental engagement, developmental care, skin-to-skin care, human milk, breastfeeding, sensory experiences, targeted nutrition, and pharmacologic treatments, are effective in attenuating adverse methylation patterns and improving associated developmental outcomes. Research related to epigenetics in preterm infants is in its early stages. Advancing our understanding in this field will support the development and standardization of targeted epigenetic interventions to optimize growth and development for preterm infants in the NICU.

5.3. Implications for practice

Emerging evidence underscores the role of early life stress – reflected in DNA methylation of glucocorticoid genes – in shaping neurodevelopment and oral feeding skills. These insights have important clinical implications for the care of preterm infants. For example, methylation patterns may serve as early biomarkers for developmental risk stratification. By integrating epigenetic profile with clinical assessments, precision diagnostics could be developed to identify high-risk infants, thus enabling early and individualized interventions. Through incorporating epigenetic findings into neonatal care, clinicians can better recognize the multifaceted impact of early life stress on preterm infant growth and development. This understanding reinforces the need for targeted, evidence-based interventions to minimize stress and optimize clinical outcomes.

Although it is not yet confirmed whether existing evidence-based NICU interventions – such as pain management, parent-infant bonding, multisensory stimulation, and optimized nutrition – modify adverse methylation patterns, they have been shown to improve stress responses, enhance neurodevelopment, and support oral feeding competency. Clinicians are encouraged to integrate these interventions into neonatal care protocols to mitigate the effects of early life stress and promote optimal developmental outcomes.

Additionally, educating parents about the impact of early life stress and caregiving on infant epigenetics may encourage supportive caregiving practices, such as skin-to-skin care, breastfeeding, and active parental engagement. By fostering family-centered care and emphasizing the importance of early interactions, clinicians can support parents to positively contribute to their infant's health.

6. Conclusion

This study highlights the complex relationship between DNA methylation of *NR3C1* exon 1F and *HSD11B2* promoters and neurodevelopment, and oral feeding skills in preterm infants. Increased methylation at specific CpG sites within *NR3C1* exon 1F and *HSD11B2* promoters was associated with suboptimal neurodevelopmental and oral feeding outcomes, potentially reflecting heightened sensitivity to early life stress in the NICU. These findings emphasize the need for continued research into epigenetic mechanisms underlying neonatal adaptation and stress regulation, with potential implications for targeted epigenetic interventions to support optimal neurodevelopment and oral feeding skills. The long-term effects of early life epigenetic modifications are not yet fully understood, necessitating further research into their persistence and functional consequences into adulthood. Understanding the dynamic nature of DNA methylation in response to NICU early life stress and interventions could pave the way for targeted clinical strategies to enhance neonatal health and developmental trajectories.

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Author contributions

Thao Griffith: conceptualization; acquisition, analysis, interpretation of the data; writing – original draft, review, editing; and final approval of the version to be published.
George E. Chlipala: analysis, interpretation of the data; writing – review, editing, and visualization; and final approval of the version to be published.
Ashley Ford: acquisition, analysis, interpretation of the data; writing – review and editing; and final approval of the version to be published.
Stefan J. Green: conceptualization; acquisition, analysis, interpretation of the data; writing – review and editing; and final approval of the version to be published.
Rosemary White-Traut: conceptualization; writing – review and editing; and final approval of the version to be published.
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Linda Janusek: conceptualization; acquisition, analysis, interpretation of the data; writing – original draft, review, editing; and final approval of the version to be published.

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Data availability statement

Data will be shared in accordance with the NIH Data Sharing Policy. De-identified data may be made available upon reasonable request through Loyola University Chicago under a Data Use Agreement.

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