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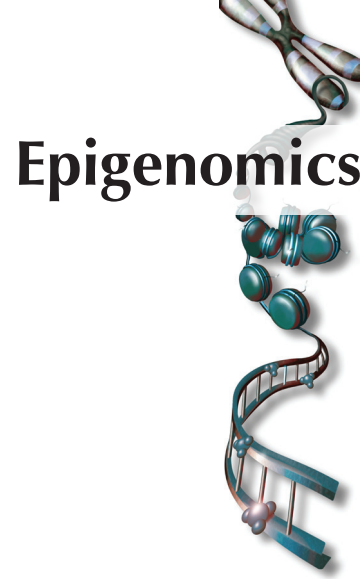

Neurobehavior related to epigenetic differences in preterm infants

Preterm birth is associated with medical problems affecting the neuroendocrine system, altering cortisol levels resulting in negative effects on newborn neurobehavior. Newborn neurobehavior is regulated by DNA methylation of *NR3C1* and *HSD11B2.* **Aim**: Determine if methylation of *HSD11B2* and *NR3C1* is associated with neurobehavioral profiles in preterm infants. **Patients & methods:** Neurobehavior was measured before discharge from the hospital in 67 preterm infants. Cheek swabs were collected for DNA extraction. **Results:** Infants with the high-risk neurobehavioral profile showed more methylation than infants with the low-risk neurobehavioral profile at CpG3 for *NR3C1* and less methylation of CpG3 for *HSD11B2*. Infants with these profiles were more likely to have increased methylation of *NR3C1* and decreased methylation of *HSD11B2* at these CpG sites. **Conclusion:** Preterm birth is associated with epigenetic differences in genes that regulate cortisol levels related to high-risk neurobehavioral profiles.

Keywords: cortisol • DNA methylation • epigenetics • high-risk infants • NICU Network Neurobehavioral Scale • prematurity • programming

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Preterm birth in the USA is a significant public health problem with a prevalence rate of approximately 10% (450,000 infants) per year [1]. Although, advances in perinatal and neonatal care have improved survival rates for preterm infants, this increase in survival is accompanied by a parallel increase in the risk for developmental impairment. Approximately 28–50% of infants born less than 32 weeks gestational age in the 1990s developed neuromotor, sensory, cognitive, language and/or behavioral impairments that require extensive healthcare, educational and psychosocial community resources through adulthood [2].

Developmental outcome in preterm infants

Neurodevelopmental impairment is a critically important outcome of preterm birth. Rates of cognitive impairment alone at 24 months in preterm infants, especially those born less than 32 weeks gestational age, range from 23 to 47% [2–4]. Behavior problems are also associated with lower cognitive scores [5]. Although improvements in cognitive scores have been seen by 8 years, preterm infants continued to score -0.5 to -1 standard deviations (SD) below their full-term peers. As many as 50–70% have impairments in executive functioning, visual motor skills, verbal memory, visual processing and/or adaptive tasks [6]. Even in children without specific cognitive deficits, those with chronic lung disease (CLD) and/or severe intraventricular hemorrhage (IVH) [7] are at increased risk of poor school achievement (24–41%), receipt of special education services (25–62%), repetition of grades (15–34%) and a 60–75% lower rate of graduation from high school. Cognitive impairments persist and remain significant in the areas of visual-perceptual tasks, reading and math achievement. In a meta-anaysis, preterm children were 0.48–0.76 SD behind term peers in reading, math, spelling and showed poor academic achievement [8].

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Significant motor impairments in preterm infants include cerebral palsy (CP) and generally impaired gross motor function [6]. At school age, 10–20% have neurological 'soft signs' that do not indicate localized brain injury, but reflect disorders of speech, balance, tone, gait and fine or visual-motor skills that are also associated with low IQ, learning disabilities, attention deficits and behavioral disorders at 6–11 years of age [9]. Mild visual and hearing impairments affect 9–25% of these infants, moderate-to-severe sensory impairments affect 13%, and at 11–14 years, 27–32% show impaired functional outcomes involved in daily living and social participation [10].

Behavioral and psychological disorders are evident in toddlers and children (8–12 year olds) born preterm including social withdrawal and/or anxiety or depression, attention deficits [11–14] and increased risk for Autism Spectrum Disorders [15,16]. When these infants reach adolescence, they have more difficulty establishing social relationships, and continue to experience social withdrawal and anxiety disorders at 20 years of age [13].

Medical problems & the neuroendocrine system

Although the range of morbidity in preterm infants is due, in part, to the immaturity of their organ systems, preterm birth is often accompanied by severe medical problems, such as bronchopulmonary dysplasia, retinopathy of prematurity, infection and structural brain abnormalities [17–19]. These complications are inversely proportional to gestational age at birth and are often the result of hemodynamic instability, cardiovascular dysfunction and hypotension and are correlated with increased mortality and morbidity [20]. Such problems can also affect the neuroendocrine system and disrupt the hypothalamic pituitary adrenocortical (HPA) axis, specifically; affected brain regions include elements of the limbic system that stimulate the release of corticotropin-releasing hormone (CRH). These regions include the hypothalamus, amygdala and hippocampus. CRH activates the pituitary gland to release adrenocorticotropic hormone (ACTH) into the bloodstream, which in turn stimulates cells in the adrenal glands to produce cortisol which is also released into the bloodstream [21,22]. The association of illnesses with HPA function and dysregulation has been investigated intensively in preterm infants [23]. These illnesses affect the secretion of CRH [20,24] altering cortisol levels with consequent negative effects on newborn neurobehavior and later development including behavioral and cognitive disorders.

Cortisol levels and the effects of cortisol are regulated, in part, by the cross talk of the sensitive feedback loop between the hormone products of the hypothalamus, the pituitary and the adrenal gland. There are complex mechanisms for the regulation of hormone and hormone receptor expression and processing. One example is the regulation of cortisol responsiveness by epigenetic differences in the expression of the glucocorticoid receptor (GR) gene *NR3C1* and 11β-hydroxysteroid dehydrogenase type 2 (*HSD11B2*). *NR3C1* encodes GR, a nuclear receptor that binds cortisol and facilitates cortisol's transcriptional activity including regulation of *HSD11B2*. *HSD11B2* converts cortisol to its inert form of cortisone, thus regulating levels of the active hormone in circulation. Regulation of the expression of these genes has been, in part, reported to occur through epigenetic mechanisms, particularly through DNA methylation of the promoter regions of these genes. In rodents, maternal care is related to DNA methylation, increased expression of the hippocampal *NR3C1* promoter region and HPA responses to stress including lower levels of corticosterone [25]. Human infants born to mothers with depression during pregnancy show increased DNA methylation of *NR3C1* in the comparable promoter region in blood and increased salivary cortisol levels at 3 months of age [26].

Measuring newborn neurobehavior

We have shown that DNA methylation of *HSD11B2* and *NR3C1* in the placenta is related to newborn neurobehavior [27–29] using the Neonatal Intensive Care Unit Network Neurobehavioral Scale (NNNS) [30]. The NNNS is a comprehensive assessment of both neurologic integrity and behavioral functioning, including signs of stress. It assesses the full range of infant neurobehavioral performance (orientation to auditory and visual stimuli); infant stress (color changes, tremors and startles), neurologic functioning (reflexes, tone); some features of gestational age; self-soothing capacities; states and their organization. The neurobehavioral, reflex and stress items on the NNNS are reduced to summary scores (Table 1; attention, handling, self-regulation, arousal, excitability, lethargy, hypertonicity, hypotonicity, nonoptimal reflexes, asymmetric reflexes, quality of movement and stress/abstinence) with alpha coefficients ranging from 0.87 to 0.90 [31]. Methods including latent profile [32,33] and latent modeling have been developed to group infants into mutually exclusive categories or profiles based on the summary scores, with each profile showing a different pattern of performance over the course of the exam [34–36]. Collapsing the multidimensional NNNS exam into coherently integrated, statistically validated profiles facilitates their use in statistical comparison of behavioral outcome to biological

Table 1. NICU Network Neurobehavioral Scale summary scores.

NNNS summary scales	Description
Attention	Ability to localize and track objects, faces and voices
Handling	Handling strategies used during attention
Self-regulation	Organize behavior in response to stimulation
Arousal	Level of arousal during the examination
Excitability	High levels of motor, state and physiologic reactivity
Lethargy	Low levels of motor, state and physiologic reactivity
Hypertonicity	Hypertonic responses in arms, legs, trunk or tone
Hypotonicity	Hypotonic response in arms, legs, trunk or tone
Nonoptimal reflexes	Number of poor reflex scores
Asymmetric reflexes	Number of asymmetric reflex scores
Quality of movement	Smoothness, maturity, lack of startles, tremors
Stress/abstinence	Number of stress signs observed

NNNS: Neonatal Intensive Care Unit Network Neurobehavioral Scale.

variables. The profiles have been used to identify both normal and abnormal groups of infants and predict long-term outcome [32]. The NNNS can be used as early as 34 weeks' gestational age, depending on the medical status of the infant, through approximately 1-month post-term (44 weeks gestational age). It can be used with low-risk as well as extremely high-risk infants and is sensitive to a wide variety of medical conditions, prenatal exposures and demographic variables. In studies of preterm infants, the NNNS has been related to quality of care in the NICU, [37] medical problems [38] and white and gray matter abnormalities [39]. Especially important, the NNNS and NNNS-based profiles predict cognitive and motor scores at 18 months in preterm infants [40] and the development of CP and motor impairment [41]. Preterm infants and infants with prenatal cocaine exposure with an abnormal NNNS profile had an abnormal cranial ultrasound at 1 month, chronic neurological abnormalities and brain-related illness or CP by age 3, impaired cognitive and motor scores at 1 and 2 years, behavior problems at age 3, concept problems in school readiness at age 4 and low IQ at 4.5 years [32]. It is also both interesting and supportive of our analysis that in a low-risk sample, NNNS profiles predicted motor development and behavior problems at age 3 [33].

DNA methylation & newborn neurobehavior

In previous work with full-term infants, our group has studied relations between DNA methylation of placental *HSD11B2* and the *NR3C1* promoter and NNNS scores at birth. We found that DNA methylation of placental *HSD11B2* was related to quality of movement [27]. DNA methylation of the placental *NR3C1* promoter was also related to quality of movement as well as to attention (ability to maintain alert states and track visual and auditory stimuli) [28]. DNA methylation of both placental *NR3C1* and *HSD11B2* in mothers with depression or anxiety during pregnancy was related to self-regulation (able to modulate responsivity to stimulation), hypotonia (low muscle tone) and lethargy (low level of arousal) [29]. DNA methylation of somatic *HSD11B2* and *NR3C1* has not been studied in preterm infants. Our studies suggest that epigenetic mechanisms could play a role in the regulation of cortisol levels in preterm infants [27–29]. Specifically, DNA methylation of *HSD11B2* and *NR3C1* has the potential to disrupt HPA activity and affect the neurobehavior of these infants.

The purpose of the current study was to examine DNA methylation of somatic *HSD11B2* and *NR3C1* in preterm infants. In addition, we wanted to determine if DNA methylation of these genes was associated with different profiles on the NNNS in this preterm population. The rationale for this study is based on the impact of medical problems on the preterm infant's neuroendocrine system, the accompanying stress that could be involved and the role of *HSD11B2* and *NR3C1* in regulating cortisol. In addition, DNA placental methylation of these genes is associated with neurobehavior in term infants and we wanted to determine if there were similar DNA somatic methylation effects in preterm infants. As described above, it has been well documented that preterm infants are at high risk for the later development of cognitive, motor and social impairment as well as behavior problem and mental health disorders. Given that profiles on the NNNS predict long-term outcome in preterm infants, this study is an opportunity to identify potential epigenetic pathways in these infants that could alter early neu-

robehavior that has been related to later developmental outcome.

Patients & methods

The sample included 67 preterm infants born 23–35 weeks gestational age (birthweight, 480–1495 g) enrolled between 2 weeks after birth and 2 weeks before hospital discharge from the Neonatal Intensive Care Unit (NICU) at Women & Infants Hospital of Rhode Island. Exclusion criteria included; non-English speaking or <18–year-old mother, infant congenital anomalies or transfer to another hospital. The hospital Institutional Review Board approved the study, and written informed consent was obtained from all participants. The NNNS was administered to the infants 3–4 days before discharge from the NICU by certified examiners. Cheek swabs to collect buccal cells for DNA extraction were collected following administration of the NNNS. Demographic and medical data were abstracted from the medical records. Demographic characteristics included infant gender, race, ethnicity, maternal education and partner status. Medical variables included birthweight, gestational age, length of stay, weight at discharge, gestational age at discharge, rate of weight gain, head circumference at birth, gestational age at full enteral feeding, necrotizing enterocolitis, intraventricular hemorrhage, periventricular leukomalacia, retinopathy of prematurity, sepsis, bronchopulmonary dysplasia and the Neonatal Therapeutic Intervention Scoring System (NTISS). The NTISS is a measure of therapeutic intensity and provides an index of neonatal illness severity and resource utilization throughout the length of stay in the NICU. The NTISS scores were used to assess illness severity and medical interventions [42].

Statistical analysis

One-Way analysis of variance and Pearson correlation was used for continuous variables, χ^2 and odds ratios (OR) for dichotomous variables. The calculation of the NNNS profiles was based on a Gaussian-distributed recursively partitioned mixture model RPMM, [34–36] and was used to cluster infants into discrete profiles from the NNNS summary scores. RPMM is a hierarchical, model-based clustering algorithm that estimates the number of underlying classes (e.g., *K*) and the posterior probability of class membership for each subject across the *K* estimated classes. This methodology is similar to finite mixture models, but differs from this approach in its computational efficiency and its ability to estimate the number of classes without the need for sequential mixture model fitting. RPMM was used to fit the 67 infants. Based on the RPMM solution fit to these data, an empirical Bayesian procedure was used to predict profile membership for these infants. The profile analysis was conducted using the R ([43]; RPMM package). The Benjamini and Hochberg method (FDR q<0.10) was used to determine the percent of findings that could be a false discovery [44].

DNA methylation analysis

Genomic DNA was extracted from oral swab samples of each infant collected using Oragene Discover for assisted collection using the prepIT kit (DNA Genotek, (Kanata, ON, Canada), and subjected to bisulfite modification using the EZ DNA methylation Kit (Zymo Research, CA, USA). The DNA methylation status for both the *NR3C1* exon 1F and *HSD11B2* promoter regions was assessed using quantitative bisulfite pyrosequencing as previously described [27,28]. Amplification of the bisulfite treated DNA was accomplished with primers for *NR3C1* (Forward-5′-TTTTTT TTT TGA AGT TTT TTT A-3′, Reverse-5′-biotin-CCC CCA ACT CCC CAA AAA-3)and for *HSD11B2* (Forward-5-GGA-AGTGGGGTTGTGYGTTTTTAGGTTTAAG TT-3′,Reverse-5′-biotin-ATACCCTTTACTAAT CRCACCACC-3′). Sequencing primers were for *NR3C1* (5′-GAG TGG GTT TGG AGT-3′) and for HSD11B2(5′-GGGGTAGAGATTTTAAGAA-3′). Sequencing primers were for *NR3C1* (5′-GAG TGG GTT TGG AGT-3′) and for *HSD11B2* (5′-GGGG-TAGAGATTTTAAGAA -3′). We used sequencing primers to interrogate the first 4 of the 13 *NR3C1* CpG sites in the 1F region (representing the following genomic coordinates: GRCh37/hg19 Chr 14: 142783501, 142783503, 142783513, 142783519), as this region has been shown to relate to regulation of the neuroendocrine system including the HPA axis and stress reactivity in rodent [25] and human studies, [26] and the four *HSD11B2* CpG sites (representing the following genomic coordinates: GRCh37/hg19 Chr 1667464389, 67464395, 67464399 and 67464412). Percent of DNA methylation at each CpG site (1–4 for *NR3C1* and 1–4 for *HSD11B2*) was quantified using the PyroMark MD instrument and the PyroMark Q-CpG software, version 1.0.11. Bisulfite conversion controls were included with each sequencing read. In order for the sample's DNA methylation percent to be called, the bisulfite conversion rate must be greater than 93%. For all samples examined, the conversion rate was greater than 95%. All samples were sequenced in triplicates from the same bisulfite-converted DNA template, and if the repeats differed by greater than 10% the sample was repeated. DNA methylation data are analyzed as a continuous measure based on percent of methylation at each CpG site.

Results

High-risk & low-risk neurobehavioral profiles

The RPMM analysis yielded two profiles (Figure 1) from the NNNS summary scores shown on the X axis. This was then converted to z (standard) scores shown on the Y axis. There were 38 infants (56.7%) characterized by positive neurobehavior (low-risk group) and 29 infants (43.3%) characterized by negative, problematic neurobehavior (high-risk group). The high-risk profile is virtually identical to the profile in the Liu *et al.* study ('profile 5') [32]. In the latter study, profile 5 predicted developmental outcome through 4 years of age in a sample of high-risk infants. On the specific summary scores, infants with the low-risk profile showed better attention that required less handling (Table 2). They had better self-regulation, they were less aroused, less excitable, had a better quality of movement and few signs of stress. Infants with the high-risk profile showed poor attention that required substantial handling. They had poor self-regulation, were highly aroused and excitable, showed poor quality of movement and substantial signs of stress. There were fewer females with the high-risk profile than in infants with the low-risk profile and more mothers of infants with the high-risk profile had less than a high school education than mothers of infants in the low-risk profile (Table 3). There were no differences in medical characteristics between infants in the two profile groups, including gestational age at birth (Table 4).

DNA methylation of *NR3C1* & *HSD11B2* is related to neurobehavioral profiles

The percent of DNA methylation at each of the CpG sites in the *NR3C1* and *HSD11B2* genes were compared between infants in the low- and high-risk profile groups. For *NR3C1* (Figure 2) CPG sites 1 and 2, infants with the high-risk profile demonstrated less percent of methylation than infants with the low-risk, although these differences were not statistically significant. CpGs 3 and 4, though, demonstrated more methylation among infants with the high-risk profile than infants with the low-risk profile and reached statistical significance at CpG3. In fact, at CpG3, the methylation among infants with the high-risk profile was double that of the low-risk profile (high-risk M = 0.555; SD = 0.542; low-risk M = 0.264; SD = 0.412; p = 0.015, FDRq = 0.06). The opposite effect was observed for *HSD11B2* (Figure 3),

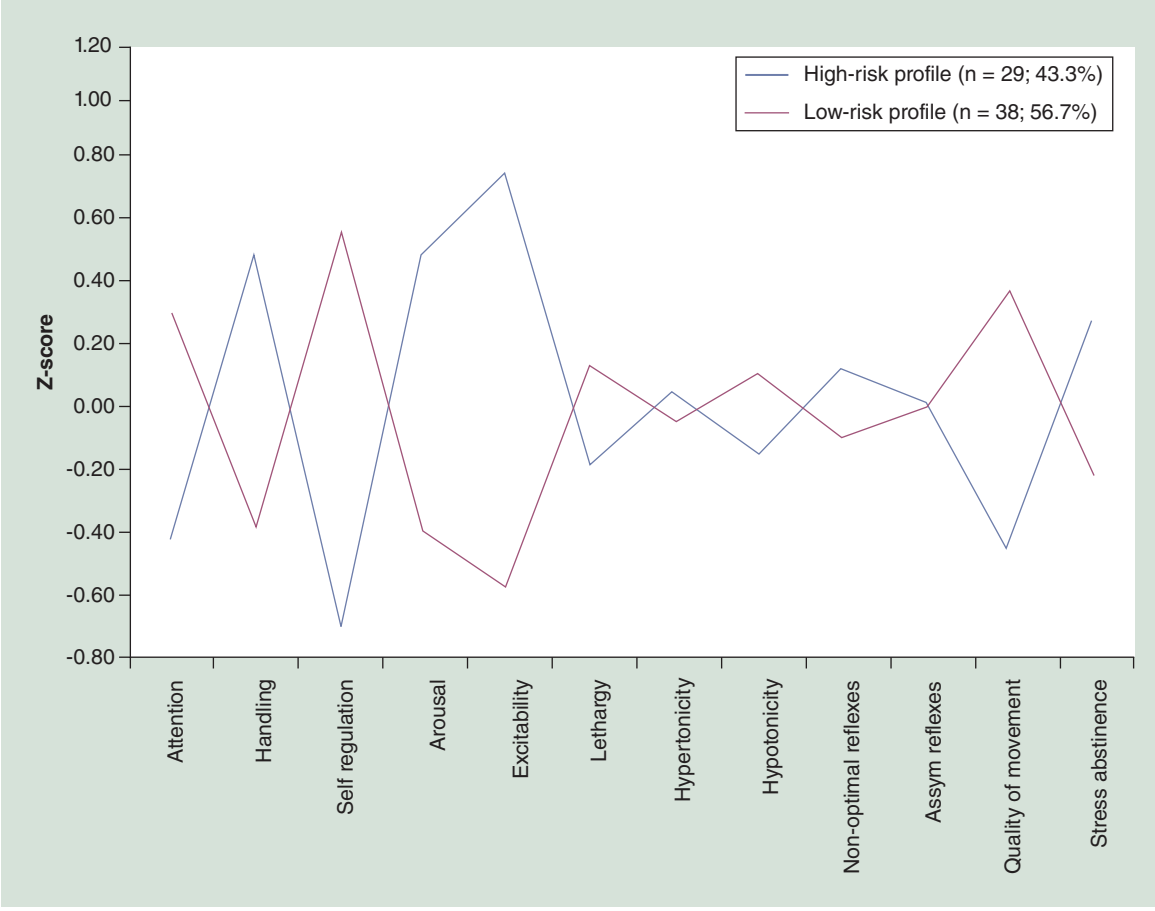

Figure 1. Neonatal Intensive Care Unit Network Neurobehavioral Scale profiles for high- and low-risk infants.

Table 2. NICU Network Neurobehavioral Scale summary scale scores by high- and low-risk profiles.

NNNS summary scales	High-risk profile (n = 29, 43.3%), mean (SD)	Low-risk profile (n = 38, 56.7%), mean (SD)	p-value
Attention	4.13 (0.83)	4.99 (1.29)	0.006
Handling	0.46 (0.20)	0.26 (0.21)	<0.001
Self-regulation	4.88 (0.58)	5.86 (0.59)	<0.001
Arousal	3.84 (0.72)	3.30 (0.39)	<0.01
Excitability	4.07 (1.89)	1.21 (1.34)	<0.001
Lethargy	4.90 (1.78)	5.50 (1.89)	0.188
Hypertonicity	0.10 (0.31)	0.08 (0.27)	0.733
Hypotonicity	0.21 (0.41)	0.34 (0.58)	0.292
Nonoptimal reflexes	5.93 (2.28)	5.47 (1.91)	0.376
Asymmetrical reflexes	1.03 (0.78)	1.03 (0.89)	0.969
Quality of movement	4.21 (0.58)	4.68 (0.48)	0.001
Stress/abstinence	0.15 (0.04)	0.13 (0.05)	0.044

NNNS: Neonatal Intensive Care Unit Network Neurobehavioral Scale; SD: Standard deviation.

where the percent methylation at CPG3 was higher for infants with the low-risk profile (M = 0.938; SD = 0.435) than infants with the high-risk profile (M = 0.723; SD = 0.254; p = 0.021; FDRq = 0.08).

Looking specifically at these two loci and using a median split of the percent of DNA methylation showed that infants with the high-risk profile were more likely to be above the median (more methylated group) than infants with the low-risk profile at CpG3 (OR: 4.27; CI = 1.53–12.01). For *HSD11B2,* infants with the high-risk profile were more likely to be below the median (less methylated group) than infants with the low-risk profile at CpG3 (OR: 3.05; CI = 1.10–8.44). Pairwise correlations between DNA methylation of *NR3C1* CpG1–4 and *HSD11B2* were all negative, and statistically significant at CpG4 (r = -0.324; p = 0.008).

Discussion

We studied DNA methylation of the promoter region of *HSD11B2* and *NR3C1* as this region relates to regu-

Table 3. Maternal & neonatal characteristics by high- and low-risk profiles.

Characteristic	High-risk profile (n = 29, 43.3%)	Low-risk profile (n = 38, 56.7%)	p-value
Birth weight (g), M (SD)	1082 (257)	1017 (291)	0.346
Gestational age at birth (wks), M (SD)	28.4 (2.6)	28.3 (3.0)	0.879
Infant sex, female, n (%)	11 (37.9)	25 (65.8)	0.023
Infant race, n (%):			0.435
– White	21 (72.4)	23 (60.5)	–
– African–American	2 (6.9)	3 (7.9)	–
– Asian	0	3 (7.9)	–
– Multiracial	6 (20.7)	9 (23.7)	–
Low SES, n (%)	4 (14.8)	4 (10.8)	0.712
Maternal ethnicity, n (%):			
– Hispanic	3 (10.3)	6 (15.8)	0.721
Maternal educational status, n (%):			
– Less than high school	14 (50.0)	10 (26.3)	0.048
Maternal partner status, n (%):			
– No partner	4 (13.8)	5 (13.2)	0.940

M: Mean; SD: Standard deviation; SES: Socioeconomic status; wk: Week.

Table 4. Infant medical outcomes by high- and low-risk profiles.

Medical outcome	High-risk profile (n = 29, 43.3%)	Low-risk profile (n = 38, 56.7%)	p-value
Length of stay (d), M (SD)	76 (32)	81 (39)	0.584
Weight at discharge (g), M (SD)	3010 (666)	3010 (787)	1.00
Postmenstrual age at discharge (wk), M (SD)	39.21 (2.46)	39.80 (3.21)	0.412
Rate of weight gain (g/d), M (SD)	25.27 (3.01)	24.61 (3.75)	0.441
Head circumference (cm), M (SD)	25.84 (2.67)	25.23 (2.83)	0.375
GA at full enteral feeding (wk), M (SD)	31.10 (2.09)	30.93 (2.39)	0.755
Necrotizing enterocolitis (present), n (%)	6 (20.7)	4 (10.5)	0.247
Intraventricular hemorrhage (grade 3/4), n (%)	1 (3.4)	1 (2.6)	0.846
Periventricular leukomalacia (present), n (%)	1 (3.4)	1 (2.6)	0.846
Retinopathy of prematurity (stage 3, 4, 5), n (%)	1 (3.6)	3 (8.1)	0.451
Sepsis, n (%)	3 (10.3)	6 (15.8)	0.517
Bronchopulmonary dysplasia, n (%)	14 (48.3)	17 (44.7)	0.773
NTISS total score, M (SD)	26.79 (7.30)	26.76 (8.74)	0.988

d: Day; GA: Gestational age; M: Mean; NTISS: Neonatal therapeutic intervention scoring system; SD: Standard deviation; wk: Week.

lation of the neuroendocrine system including the HPA axis and stress reactivity. Our findings demonstrate differences in DNA methylation of the *HSD11B2* and *NR3C1* promoter at CpG3 between preterm infants with a high-risk neurobehavioral profile derived from summary scores on the NNNS compared with preterm infants with a low-risk neurobehavioral profile. Specifically, infants with the high-risk profile exhibited a greater extent of DNA methylation of *NR3C1* at CpG3 than infants with the low-risk profile whereas for *HSD11B2* infants with the high-risk profile exhibited less methylation than infants with the low-risk infant's profile. The false discovery rates of these findings were low, suggesting it was likely that these findings represent true discoveries, but like all findings from initial studies, we suggest replication in an independent cohort. We then conducted median split analysis at these two sites only. We found that, compared with infants with the low-risk profile, the likelihood of increased DNA methylation was higher for infants with the high-risk profile for *NR3C1* at CpG3 but lower for *HSD11B2* at CpG3. Thus, the median split analysis supported the mean comparison findings.

NR3C1 & *HSD11B2* methylation may affect transcription factor binding

The findings for *NR3C1* CpG3 and, to a lesser extent, the trend at CpG4, are consistent with findings from both rodent [25] and human work [26] as these CpG sites represent regions of the *NR3C1 1F* promoter that binds the nerve growth factor inducible protein transcription factor (NGF1-A) that is important for brain development including differentiation and neuronal plasticity [45]. In rodents, decreased methylation of the NGFI-A consensus binding site *NR3C1* promoter results in increased expression of exon 1_7 *NR3C1* hippocampal transcripts increasing negative HPA feedback and affecting stress regulation [25]. Human suicide victims also demonstrated increased methylation at NGF1-A consensus sites in the *NR3C1* 1F region in postmortem hippocampal samples, and *in vitro* studies demonstrated that methylation at these sites can inhibit expression in human cell as well [46]. Most human studies, though, cannot examine hippocampal tissue, but prior studies using peripheral samples demonstrated that DNA methylation of *NR3C1* at CpG3 in the newborn is related to maternal depression during pregnancy and more importantly, to the a phenotype of cortisol stress reactivity in the same infants at 3 months of age [26]. Our findings here best align with this prior study of newborns. We have also found that newborn infants of mothers with depression during pregnancy showed more methylation of placental *NR3C1* at CpG2 and these infants also showed poorer scores on the NNNS [29]. The absolute differences in the extent of methylation we observe are much smaller in peripheral samples than those observed in more selected hippocampal tissue, likely reflecting the

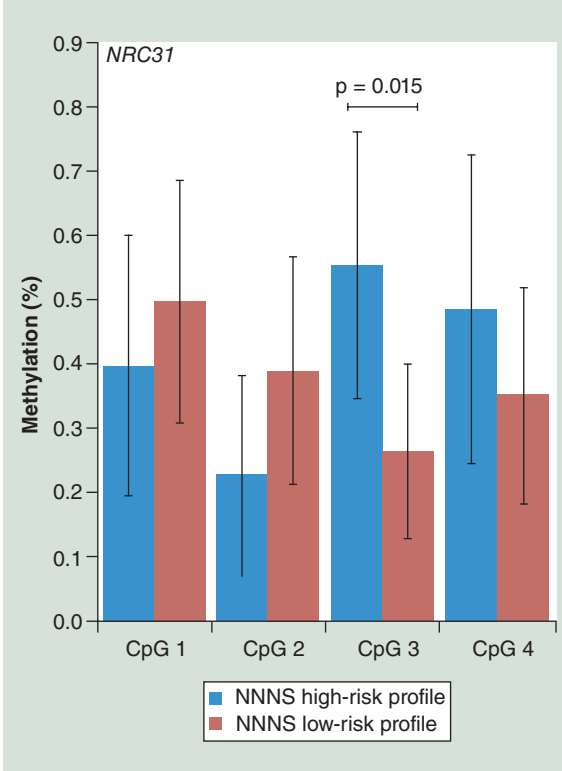

Figure 2. Percentage methylation of *NR3C1* by CpG site.
NNNS: Neonatal Intensive Care Unit Network Neurobehavioral Scale.

cellular heterogeneity of our samples. Thus, while it is unlikely that all cells are demonstrating differences in methylation within the sample, some proportion appear to be, and it would be among those cells where we would suggest the increased methylation observed in this region among high-risk infants in our study could affect *NR3C1* expression and later HPA-mediated stress reactivity. Likely, these cells represent those that are glucocorticoid responsive.

For *HSD11B2, in silico* analyses suggest that CpG3 may be part of a sequence region representing binding sites for the E2F1 transcription factor, which is involved in cell proliferation, cell cycle regulation, DNA synthesis and apoptosis. This CpG site is also adjacent to a GR-α-binding region, consistent with GR's role in the control of *HSD11B2* expression through a negative feedback loop. This could suggest that methylation in this region affects *HSD11B2* expression by altering the binding of E2F1 or GR. The role of these transcription factors and the potential disruption by methylation is consistent with the impact of illnesses associated with prematurity on the neuroendocrine system and disruption of the HPA axis. Moreover, control of *HSD11B2* and *NR3C1* are critical to the recovery and development of preterm infants. Disruption or enhancement of the transcription factors that elicit their expression could play an important role in establishing infants who are at risk or resilient following preterm birth. Although our current findings are not conclusive on this issue, they do suggest that further work to resolve the effects of methylation on the activity of these pathways is of interest.

NNNS profiles could reflect cortisol regulation & may have diagnostic value

It is both reasonable and noteworthy that *HSD11B2* and *NR3C1* have different methylation patterns related to newborn neurobehavior as these genes work in concert to regulate cortisol and it is well documented that cortisol affects infant behavior [21]. This is supported by our findings of a statistically significant negative correlation between *HSD11B2* and *NR3C1* at CpG3 as increased methylation of *NR3C1,* indicative of decreased expression would be expected to be related to decreased methylation of *HSD11B2.* These correlated patterns may represent the known feedback loop between GR and *HSD11B2,* and could suggest that in these infants, there may be compensatory reductions in methylation at *HSD11B2* in response to reduced *NR3C1* expression. The fact that this regulatory process is also reflected in the NNNS profiles speaks to the sensitivity of neurobehavior as a marker of physiological processes. In our previous work [34,36] we used placental DNA and found relations between DNA methylation of *HSD11B2* and *NR3C1* and NNNS profiles in healthy term infants. Here we used buccal cells and found an association between promoter methylation of these genes and NNNS profiles in preterm infants. This observation supports the utility of accessible samples such as saliva to assess these epigenetic marks. The generalizability and robustness of the relationship is suggested by these findings showing that relations have been observed in different populations with different underlying risks.

The profiles of neurobehavior based on the NNNS in these studies are very similar to previous observations. Both the low-risk and high-risk profiles in the present study are virtually identical to profiles that we have reported using different statistical methods [32,35] and in different populations. These two profiles represent extremes of positive and negative neurobehavior. The low-risk profile is that of the 'optimal' baby; attentive, alert, easy to handle, calm, not over-reactive, with fluid movements and minimal stress. The high-risk profile is quite the opposite; nonattentive, difficult to handle, very reactive, easily upset, jittery movements, stiff muscle tone and highly stressed. These profiles have now been observed in several populations including normal healthy infants, [34,36] low-risk infants, [33] term and preterm infants with prenatal cocaine exposure [32] and preterm infants in the present study. In

addition, these profiles have been used with infants at birth through 5 weeks of age [32,33] here in preterm infants at NICU discharge and with placental DNA methylation of the same two genes [34,36] and now with somatic DNA methylation. In two of these studies, the profiles predicted 3.5 [33] and 4.5-year developmental outcomes [32]. This could suggest that the profiles capture 'true' characteristics or developmental typologies of infants that may not be evident in individual summary scores. It is also possible that some infants with the high-risk profile in the current study will have some form of developmental impairment in the long-term. If this hypothesis is confirmed, the high-risk profile could have diagnostic value, and be used to identify those infants in need of early intervention to potentially impact future deficits in health and behavioral outcomes.

The role of developmental programming

Our findings could suggest the possibility of a compensatory regulatory system involving *HSD11B2* and *NR3C1* (Figure 4A & B) to re-establish a balance in cortisol activity in infants demonstrating the high-risk profile. As described above, the reciprocity between *HSD11B2* and *NR3C1* could function to decrease methylation of the high-risk profile in *HSD11B2* as this could result in reduced cortisol levels, representing a compensatory mechanism as a result of developmental programming.

Developmental programming or the resetting of physiological parameters due to external events, in this case prematurity, can endure into adulthood. Epigenetic regulation has been implicated as a key mechanism underlying developmental programming [47]. Modification of gene expression as a result of epigenetic differences could alter regulatory systems such as the regulation of cortisol through changes in DNA methylation of *NR3C1* and *HSD11B2* as seen in our study. We suggest that this regulatory system is differentially affected in infants with the high-risk profile versus infants with the low-risk profile. It is possible that developmental programming was impeded in infants with the high-risk profile interfering with compensatory mechanisms that would reduce cortisol levels with continued disruption of the HPA axis which then becomes manifested as the abnormal high-risk profile.

Impaired developmental programming could be due to the effects of methylation of *NR3C1* on transcription factors that bind to this region. Specifically, NGF1-A is important for differentiation and for neuronal plasticity and neuronal plasticity is necessary for developmental programming. Decreased activity of this transcription factor could reduce neuronal plasticity and impede developmental programming. It is possible that decreased expression of GR would result in reduced binding potential for cortisol and reduction in its transcriptional activity, which is critical for appropriate neurodevelopmental programming. A compensatory decrease in methylation within the *HSD11B2* region could allow for increased expression of this cortisol regulator to then inactivate the high levels of circulating cortisol resulting from the lack of GRs related to its increased methylation. Impaired developmental programming could also reduce the activity of the E2F1 transcription factor that binds to the CpG3 region of *HSD11B2* thereby affecting cell proliferation, cell cycle regulation, DNA synthesis and apoptosis and through these potentially global effects could be responsible for the high-risk profile. The potential permanency of this epigenetic developmental programming could explain why many infants with the high-risk profile develop later impairment. Thus, epigenetic effects, especially those related to the HPA system may be one mechanism involved in the long-term outcome of preterm and other high-risk infants.

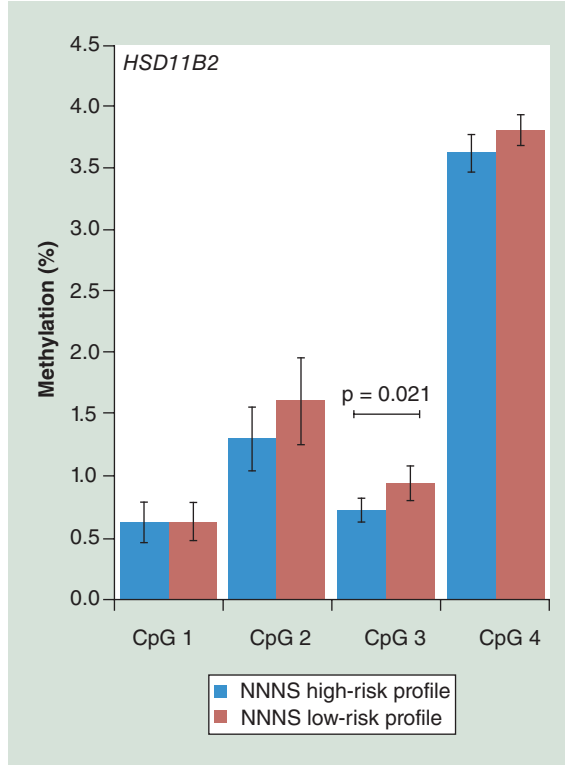

Figure 3. Percentage methylation of *HSD11B2* by CpG site.
NNNS: Neonatal Intensive Care Unit Network Neurobehavioral Scale.

Stress & allostatic load

We also know that stress can impact the neuroendocrine system. Thus, in addition to the specific effects of medical problems on the infant's neuroendocrine

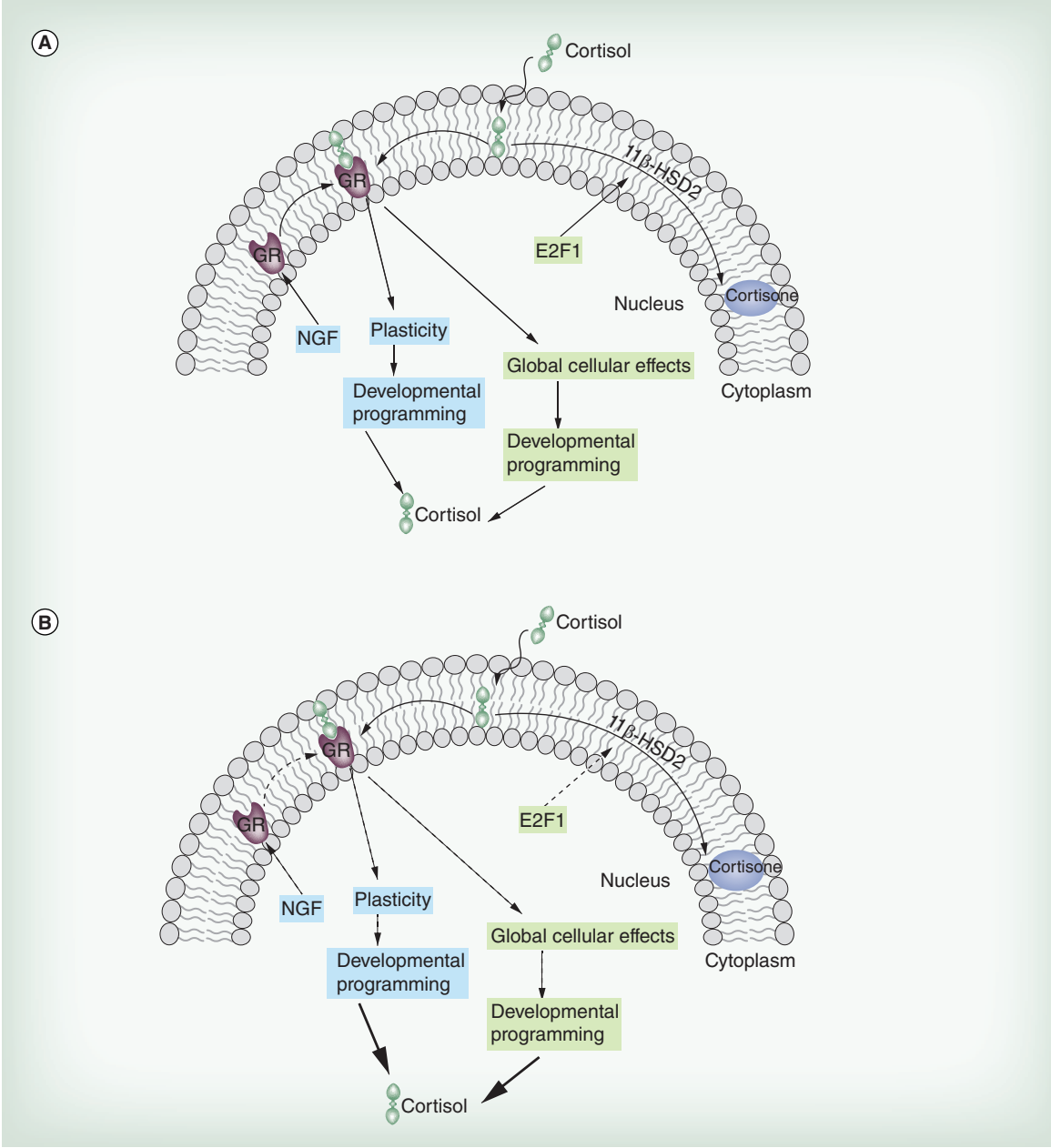

Figure 4. Proposed pathways leading to low-risk and high-risk and neurobehavioral profiles based on Neonatal Intensive Care Unit Network Neurobehavioral Scale summary scores. The solid arrows in infants with the low-risk profile **(A)** indicate normal developmental processes and appropriate levels of cortisol. The dashed lines in infants with the high-risk profile **(B)** indicate disrupted processes including transcription factors and thick arrows for increased levels of cortisol.

system, the number of medical problems probably results in cumulative stress and further exacerbation of effects on the neuroendocrine system. Since there is abundant evidence that the NICU environment itself is stressful, the construct of allostatic load may apply here. Allostatic load refers to chronic, cumulative stress that becomes biologically embedded through repeated activation of the HPA system. This 'wear and tear' on stress response systems that has long-term effects on adult cardiovascular, metabolic, nervous and immune systems, increases the likelihood of disease, [48–50] psychological and behavioral abnormalities [51–54] and psychopathology [55]. Although this construct is not typically used in the current context, it is important to note that the mean gestational age at birth of our sample was 28.26 weeks with a mean length of stay of 11.5 weeks. This represents 28% of their lifespan and can be considered 'chronic stress' for these infants.

This hypothesis is consistent with rodent work relating hippocampal transcripts to increased negative HPA feedback that affects stress regulation [25].

There is also a rich literature on prenatal stress although we cannot say with certainty what specific stressors, if any, were responsible for these infants' preterm birth. Poor health outcomes have been related to prenatal stress including low birth weight, preterm birth and intrauterine growth retardation [56,57]. Low birth weight as a proxy for the quality of the intrauterine environment has also been associated with greater HPA reactivity in both childhood and adolescence [58]. It is also interesting that infants exposed to stress *in utero* show high reactivity, activity and irritability [59–61] reminiscent of the high-risk profile in the current study. In addition, in our study, more mothers of infants with the high-risk profile had less than a high school education than mothers of infants with the low-risk profile. Having less than a high school education could be a proxy for sociodemographic factors indicative of a high stress environment and contributed to the development of the high-risk profile [36].

Importance of this study

This is the first study of somatic DNA methylation of *HSD11B2* and *NR3C1* in preterm infants and is important for several reasons. We have shown that DNA methylation of these two genes that play a critically important role in regulating cortisol levels seems to be related to distinct neurobehavioral profiles. Moreover, these profiles replicate previous work including the high-risk abnormal profile that predicts long-term developmental outcome. It is well known that preterm infants are at high risk for the later development of cognitive, motor and social impairment as well as behavior problem and mental health disorders. We add to this literature the possibility of epigenetic pathways that could be involved in the developmental outcome of these infants and raise the role of developmental programming involvement. These findings are also important because the methylation effects that we found were relatively small, yet they were related to not only newborn neurobehavior, but to neurobehavioral profiles that have been related to long-term developmental outcome in similar populations. Even small epigenetic effects may have 'big' consequences for later development. Thus, epigenetics may play an important role in our understanding of the consequences of prematurity.

DNA methylation of *NR3C1* in particular, has been studied in many populations and is related to newborn neurobehavior, [27–29] cortisol reactivity in infants, [26] environmental adversity including parental loss and child maltreatment, [62] childhood psychopathology and suicide [46]. There is an extensive literature on cortisol reactivity and the development of mental health disorders [63] and disturbances in HPA regulation have also been associated with affective and anxiety disorders [64–67]. We suggest that epigenetic differences that perturb the HPA axis could predispose infants to neurobehavioral profiles that interact with postnatal environmental factors leading to later mental health disorders [68].

Limitations

This study has limitations. This is an associational study and as such we cannot establish causal relations. Although specimens such as blood and buccal swabs are increasingly being used to study epigenetic processes in human populations, there is no direct evidence that these specimens indicate epigenetic mechanisms in the brain or that they directly represent the rodent work on which much of this is based [25]. It is possible that findings from different specimens indicate an epigenetic 'footprint;' that epigenetic processes were involved and that additional research is needed to elucidate the role of these 'footprints'. Due to sample collection constraints, we were not able to measure gene expression or cortisol levels. Thus, we still lack direct evidence that cortisol levels differed between the two groups. However, we did measure an epigenetic mechanism that regulates cortisol levels. We only studied two genes and it is unlikely that only two genes are involved in these processes. Coordinated alterations in many genes may be particularly important for our understanding of neurobehavioral development. DNA methylation at the genome-wide level would complement the study of candidate genes.

Conclusion

We have presented a unique study that measures epigenetic mechanisms that regulate cortisol levels and are potentially responsible for differences in cortisol levels related to neurobehavior in preterm infants. Thus, epigenetics may play an important role in our understanding of neurobehavior related to prematurity. The fact that the NNNS exam and the NNNS profiles have predictive validity suggests that our findings could have implications for the long-term developmental outcome of preterm infants. We suggest that preterm birth can result in epigenetic differences in genes that regulate the HPA system and that disruption of this system can lead to abnormal neonatal neurobehavior and later developmental impairment.

Future perspective

The study of epigenetic processes involved in human behavior is just beginning and holds great promise for the future. Epigenetics is the quintessential gene–environment interaction as it enables us to study

how environmental factors change gene expression at the cellular level. This enables us to understand the molecular underpinnings of both normal and abnormal behavior and development. This is not only a scientific 'sea change' but also opens the door to epigenetically based interventions to prevent disorders of behavior and development. For example, if increased cortisol levels are responsible for the high-risk profile described in this paper leads to adverse developmental outcomes, reducing cortisol levels could become a target for intervention.

Financial & competing interests disclosure

The authors have no 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. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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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.

Executive summary

Background

- Epigenetic differences related to cortisol levels has the potential to alter neurobehavior in preterm infants.

Results

- Neurobehavior in preterm infants at hospital discharge shows low-risk versus high-risk neurobehavioral profiles.
- Infants with the high-risk profile showed more DNA methylation than infants with the low-risk profile at CpG3 for *NR3C1* and less methylation of CpG3 for *HSD11B2*.

Discussion

- A compensatory regulatory system involving *HSD11B2* and *NR3C1* to re-establish a balance in cortisol activity in infants with the high-risk profile is involved.
- Perturbations in this system due to reduced activity of transcription factors that impedes developmental programming increases cortisol levels resulting in the high-risk neurobehavioral profile.

Conclusion

- Epigenetics may play an important role in our understanding of consequences of prematurity including long-term developmental outcome.

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