CLINICAL RESEARCH ARTICLE

DNA methylation in former extremely low birth weight newborns: association with cardiovascular and endocrine function

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BACKGROUND: There is increased risk of cardiovascular, metabolic, and hypertensive disorders in later life in the preterm population. We studied school-age children who had been born extremely premature who had undergone endocrine, cardiovascular, and anthropometric evaluations.
METHODS: School age measurements of salivary cortisol, adrenal androgens, blood pressure, and anthropometric markers were correlated with DNA methylation of 11-betahydroxysteroid dehydrogenase type 2 (11BHSD2), leptin, and the LINE1 repetitive DNA element.
RESULTS: We observed a modest correlation between log AUC for salivary cortisol and methylation of leptin in preterm infants and a negative correlation between methylation of region 1 of the glucocorticoid receptor (GR in term-born infants. There was an association between LINE1 methylation and cortisol response to awakening and a negative correlation between LINE1 and systolic blood pressure at 6–7 years. Methylation of the GR promoter region showed a positive association with systolic blood pressure at 6–7 years of age.
CONCLUSIONS: These results show that extremely preterm birth, followed by complex patterns of endocrine, cardiovascular, and metabolic exposures during early postnatal life, is associated with lasting changes in DNA methylation patterns in genes involved in hypothalamic pituitary adrenal axis function, adrenal hormonal regulation, and cardiometabolic risk.

IMPACT:

- Preterm infants have significant environmental and physiological exposures during early life that may have lasting impact on later function.
- Alterations in hypothalamic pituitary adrenal axis (HPA) function have been associated with these exposures.
- We examined the associated changes in DNA methylation of important genes involved in HPA function, metabolism, and global DNA methylation.
- The changes we saw in DNA methylation may help to explain associated cardiovascular, metabolic, and growth disturbance in these children in later life.

INTRODUCTION

The concept of developmental programming underscores the widely accepted view that environmental exposures during "critical windows" can have lasting effects on later health across the life course.[1–3] The variety of exposures that induce programming effects include environmental toxicants, drugs, hormones, physiological and/or emotional stress, alterations in nutritional status, and alterations in metabolic status.[4] There are many "critical windows", but few are better demonstrated than the periods of intrauterine life and early postnatal development. The concept of developmental programming first arose from classical epidemiological studies of David Barker who identified increased risk of adult cardiovascular, metabolic, and hypertensive disorders in patients who had experienced intrauterine growth restriction.[2]

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Exposures during critical periods are important to infants born prematurely in whom the later stages of important intrauterine development take place postnatally. The "fetal origins" or Developmental Origins of Health and Disease (DOHaD) is now well accepted and is based on a series of studies which demonstrated that measures of birth size are associated with long-term chronic disease risk.[1–3] The impact is greatest when the exposure occurs early in pregnancy.[5] The majority of studies have focused on cardiovascular disease and metabolic syndromes. Numerous investigators and investigations have shown that antenatal environmental factors, including diet, xenobiotic exposures, stress, and lifestyle factors, can alter fetal growth and, through programming, result in permanent biological and physiologic changes in the offspring.[4–11]

Research in fetal programming and many other disciplines is now focusing on the paradigm that altered gene regulation is the result of epigenetic changes. We and others have reported on the effects of exposures on DNA methylation during this period of developmental plasticity.[6–10] This critical role of epigenetic regulation, the mitotically and meiotically heritable control of gene expression not related to DNA sequence, during development is becoming increasingly appreciated. The most amenable epigenetic alteration to study, particularly in humans, and thus the most thoroughly examined, is DNA methylation. The methylation occurs as the addition of a methyl group to the 5′-carbon of the cytosine in CpG dinucleotides, and in the context of multiple methylated cytosines typically within a gene regulatory region. It is often associated with transcriptional silencing of the downstream gene. The presence of the methyl group alone in this context is not sufficient for transcriptional silencing, but instead recruits component proteins related to gene repression. This is often associated with creation and maintenance of a silenced chromatin conformation. The presence of the methylation changes is indicative of these alterations. Our recent studies have examined the effects of an adverse intrauterine environment on the placental epigenome at both the candidate gene and genome-wide level.[6–10] We have evaluated the effect of the intrauterine environment on genome-wide placental DNA methylation and its association with birth weight,[6] the association of methylation of repetitive elements in placental genomic DNA with fetal growth,[7] the effects of the intrauterine environment on methylation of repetitive element DNA and changes in placental gene expression,[7] the effects of maternal depression and SSRI use on placental gene expression,[8] and the effects of maternal smoking and birth weight on placental microRNA expression.[9,10] These changes are part of the emerging paradigm that the intrauterine and early postnatal environment do not exert just passive effects but play an active, mediating role on health and developmental outcome across the lifespan.[11]

Infants who are born extremely preterm experience a high rate of growth failure as they approach term gestation.[12,13] In addition, they are exposed to higher cortisol concentrations after birth than fetuses at an equivalent gestation.[14–16] Growth failure and exposure to elevated concentrations of cortisol and adrenal androgens may predispose these infants to adverse outcomes later in life, similar to term-born low birthweight individuals. Available data suggest that the preterm population is at increased risk for some of these adverse outcomes including elevated blood pressure and insulin resistance. The Neuroimaging and Neurodevelopmental Outcome (NEURO) secondary study of the Neonatal Research Network Surfactant Positive Airway Pressure and Pulse Oximetry Randomized Trial (SUPPORT) afforded us a unique opportunity to evaluate the relationship between changes in DNA methylation in candidate genes in buccal epithelial cells and comparison to adrenal and cardiovascular function and their relationship to perinatal factors.[17] We studied school-age children who had been born extremely premature.[17]

METHODS
This report describes the ancillary study of DNA methylation at follow-up at 6–7 years of age of a large cohort of children who had been born extremely preterm (24–27 weeks GA), and enrolled at birth into the Surfactant Positive Airway Pressure and Pulse Oximetry (SUPPORT) randomized controlled trial[18] conducted by the Neonatal Research Network (NRN) of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD). The results at school age of the comparison of salivary cortisol and adrenal androgens with blood pressure and anthropometric markers of cardiometabolic risk have recently been reported.[17] Cortisol and dehydroepiandrosterone (DHEA) correlated inversely with early postnatal growth and directly with systolic BP in extremely preterm-born children, suggesting perinatal programming. We measured the association of salivary cortisol and adrenal responsiveness with DNA methylation patterns of the GR and 11-betahydroxysteroid dehydrogenase type 2 (11BHSD2), important genes affecting corticosteroid regulation, leptin, a regulator of adiposity, and methylation of the LINE1 repetitive DNA element, as a marker of genome-wide methylation.

Population
Fifteen NRN sites participated. All institutional review boards approved the study, and children were enrolled after parental consent. Preterm-born children were eligible if they were enrolled in the SUPPORT NEURO study (ClinicalTrials.gov ID NCT00233324); perinatal data were obtained from the original study database. The school-age visit occurred between 6 years 4 months and 7 years 2 months of age. As a reference group, 40 term-born children were recruited from five NRN sites. Eligible children for the reference group were, by parental report:[1] singletons, 5th–95th percentile birth weight, and 37°–41[6] weeks gestation;[2] 6 years 4 months to <7 years old;[3] 5th–95th percentile for height and weight;[4] never hospitalized >2 days;[5] without known medical problems;[6] never identified as eligible for special education, speech or physical therapy;[7] without a sibling with autism or mental retardation; and[8] living with their birth parent(s).

Procedures
Detailed methods have been described in the previous report.[17] Briefly, blood pressure (BP), anthropometric measures, including weight, height, head, and waist circumference, and triceps, subscapular, and abdominal skinfolds were obtained at clinic visits as previously described. Salivary cortisol was measured four times during the study visit: baseline (at beginning of visit), halfway through, after a break, and at the end. Additionally, samples were collected at home on morning awakening, 30 min later, and in the evening.

Samples were frozen at −20 °C, then shipped overnight to RTI International (Research Triangle Park, NC). All samples for hormone analysis were sent to the Institute for Interdisciplinary Salivary Bioscience Research, where they remained at −80 °C until assay. Samples were thawed, centrifuged, and assayed in duplicate using commercially available immunoassays designed for use with saliva without modification to the manufacturer's protocol (Salimetrics LLC, Carlsbad, CA). Saliva from each patient was also collected and shipped to Providence, RI for analysis of DNA methylation.

DNA methylation
Quantitative DNA methylation was measured as previously described for the exon 1F region of GR,[19] the promoter region of 11BHSD2,[20] the promoter region of leptin,[21] and the LINE1 repetitive element[22] (Fig. 1). Briefly, genomic DNA was extracted from pooled saliva samples using QIAmp DNA Mini Kits (Qiagen, Inc.) following the manufacturer's protocols and purified DNA was used for quantitative methylation analysis by pyrosequencing (Qiagen Inc.). DNA samples (1 µg) were bisulfite-modified using
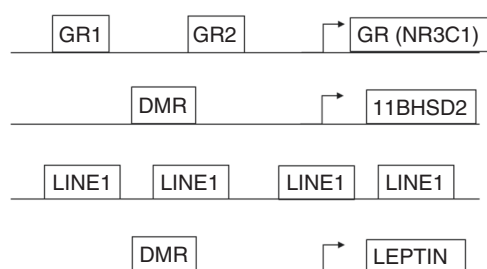
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Fig. 1 Schematic of genomic regions evaluated. DNA methylated regions (DMRs) were assayed in exon 1F region of GR,[19] the promoter region of 11BHSD2,[20] the promoter region of leptin,[21] and the LINE1 repetitive element.[22]

the EZ DNA methylation Kit (Zymo Research, CA) following the manufacturer's protocol. Pyrosequencing was performed on PCR product amplified from bisulfite-modified DNA as previously described using the assays defined above. Dilution series of fully methylated referent DNA into fully unmethylated referent DNA were run for each assay to assess the performance characteristics of the assay. As quality assurance for pyrosequencing, 10% of samples were assayed in duplicate. Laboratory personnel were blinded to patient identification.

Data analysis
Data were collected on the child at baseline (birth weight, gestational age, sex, race, antenatal steroids, necrotizing enterocolitis, bronchopulmonary dysplasia, days of ventilation, severe intracranial hemorrhage grade III or IV or cystic periventricular leukomalacia, late onset sepsis, severe retinopathy, postnatal steroids, length at 36 weeks, weight at 36 weeks) and follow-up at 6–7 years of age (test age, height, weight, body mass index, skinfolds [triceps, abdominal, subscapular], blood pressure [systolic, diastolic]). Maternal characteristics (highest education attainment, diabetes, hypertension) were captured as well.

Descriptive statistics were calculated for child and maternal characteristics. Frequencies and percentages were reported for categorical variables with differences between preterm and term-born children tested for by chi-square and Fisher's exact test. Means, standard deviations, medians, and interquartile percentiles were reported for continuous variables with difference in means tested using the Kruskal–Wallis test.

DNA methylation was analyzed as described above at 3–8 positions within each gene region. Factor analysis was conducted to determine whether methylation variables could be characterized via a single factor. Results of our factor analyses showed that we could use one factor for each gene (those with low load bearings showed similar results to the one factor), so subsequent analyses were run using raw means.

Correlation between cortisol and DHEA levels with individual DNA methylation genes was tested using Spearman's correlation. Each single factor gene was then tested as mediating factors between outcomes (log AUC, logDHEA) and weight Z-score at 36 weeks, length Z-score at 36 weeks, weight velocity from birth to 36 weeks, and systolic BP at 6–7 years via mediation analyses using PROCESS macro. Multiple comparisons were not accounted for. All analyses were conducted in SAS 9.4.

RESULTS

Patient characteristics are shown in Table 1. The mean birth weight of the preterm-born infants was 880 g and the term reference patients weighed 3412 g. Their mean gestational ages were 26.4 vs 39.3 weeks, respectively. While sex did not differ between the two groups, there was a slightly greater proportion of white children in the term reference group. As expected, prenatal complications, including exposure to antenatal steroids, and postnatal morbidities experienced during the neonatal period were greater in the preterm infants. Maternal characteristics are also shown. The educational attainment of mothers of the preterm-born infants was significantly less than that of the term infants. The rate of hypertensive disorders of pregnancy was slightly higher in the mothers who delivered preterm. As we were striving for an age-matched reference group, the age at follow-up testing was similar between the preterm and the term control infants. However, their anthropometric measurements did differ modestly. Preterm infants were shorter, of lower weight, lower Z score, and a higher waist to height ratio than the term infants, suggesting some incipient changes in adiposity.

We compared DNA methylation across multiple CpG sites in the glucocorticoid receptor, 11BHSD2, leptin, and LINE1 (Fig. 1). The average DNA methylation values for each genomic region are shown in Table 2. The overall levels of DNA methylation were similar to overall methylation data previously reported by ourselves and others (Table 2). The overall levels of DNA methylation were similar to overall methylation data previously reported by ourselves and others (Table 2).[7,8,19–21] As shown in Table 2, there was a high level of methylation of the global methylation marker of LINE1, which was ~76%. Using factor analysis, it was determined that the methylation variables can be characterized as a single factor. There were some cases where one or two positions seemed to have a lower loading than other positions. Those positions got lower weights when computing the score. By t-test the only significant difference was between the group means for GR1.

The univariate correlations between outcomes and single genes in preterm and term-born children are shown in Table 3. The correlations between log AUC for salivary cortisol and single genes showed a modest correlation with methylation of leptin in preterm infants. In term-born infants the only significant univariate correlation seen was a modest negative correlation between methylation of region 1 of the glucocorticoid receptor and log AUC for salivary cortisol. There were no significant correlations between first morning cortisol and methylation of any of the marker genes. Similarly, there were no significant correlations between methylation of any gene region and awakening cortisol in preterm infants. There were no significant univariate correlations between measurable logDHEA and methylation in the promoters of any of the single genes. Only preterm infants were exposed to antenatal steroids. We tested the difference in median methylation values between those who were and were not exposed to antenatal steroids. There was a modest difference in methylation between exposures groups among 11BHSD2 and GR1, Table 4.

In order to analyze the effect of DNA methylation on anthropometrics and cardiovascular findings, we used mediation analyses to compare the methylation of gene regions as mediating factors between cortisol AUC, DHEA, and weight Z-scores, blood pressure at 36 weeks and 6–7 years. The results for cortisol AUC, for initial morning cortisol, for mediation on awakening cortisol response and for mediating effects of DNA methylation on DHEA at the beginning of the clinic visit in Table 5. As can be seen, there were no associations between regions of DNA methylation as mediating factors for cortisol AUC and cortisol at first awakening. As seen in Table 5, there was an unadjusted mediation effect of LINE1 methylation between cortisol response to awakening and systolic blood pressure. Likewise, LINE1 methylation had a negative mediation effect between systolic blood pressure and DHEA at 6–7 years, even after adjusting for confounders. Methylation of the glucocorticoid receptor promoter region showed a significant unadjusted mediation effect between DHEA and systolic blood pressure at 6–7 years of age.

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Table 1. Characteristics of patients (values mean ± SD, median [25th–75th percentile], or n (%))

Neonatal characteristics	Preterm born N = 194	Term-born N = 38	P value
Birth weight (BW, g)	881 ± 189	3412 ± 473	<0.01
BW < 10th %ile (n, %)	10 (5)	3 (8)	0.45
Gestational age (GA, weeks)	26.4 ± 1.00	39.3 ± 0.97	<0.01
BW for GA Z score	0.12 ± 1.00	0.14 ± 0.88	0.48
Male sex (%)	101 (52)	19 (50)	0.86
Race (% white)	120 (62)	31 (82)	0.02
Received breast milk within first 28 days	172 (89)	N/A	N/A
Antenatal steroids (%)	182 (94)	0 (0)	
Any/complete course	156 (80)	0 (0)	N/A
Neonatal complications			
Necrotizing enterocolitis	12 (6)	0 (0)	
Bronchopulmonary dysplasia	59 (30)	0 (0)	
Days of ventilation	6 (1,22)	0 (0)	N/A
Severe ICH or cPVL[a]	12 (6)	0 (0)	
Late onset sepsis	52 (26)	0 (0)	
Severe retinopathy	20 (11)	0 (0)	
Postnatal steroids (%)	7 (4)	0 (0)	N/A
Length at 36 weeks (cm)	42.82 ± 2.79		
Length <10th %ile (n, %)	112 (71)	N/A	N/A
Z-score	−1.85 ± 1.11		
Weight at 36 weeks	2155 ± 363		
Wt < 10th %ile (n, %)	99 (59)	N/A	N/A
Z score	−1.52 ± 0.90		
Maternal characteristics			
Maternal education			
Did not complete high school	48 (25)	0 (0)	<0.01
Completed high school	61 (32)	2 (5)	
Some post-secondary	43 (23)	1 (3)	
Completed college	28 (15)	18 (49)	
Any graduate education	10 (5)	16 (43)	
Marital status	83 (43)	N/A	N/A
Diabetes	9 (5)	N/A	N/A
Hypertension	47 (24)	3 (9)	0.05
Characteristics at follow-up			
Child test age (years)	6.86 ± 0.40	6.71 ± 0.19	0.50
Height (cm)	119.33 ± 7.15	122.17 ± 5.04	0.03
Z score	−0.14 ± 1.27	0.48 ± 0.89	<0.01
Weight (kg)	22.86 ± 4.72	23.23 ± 3.46	0.48
Z score	−0.14 ± 1.21	0.18 ± 0.85	0.07
BMI	16.02 ± 2.49	15.51 ± 1.50	0.30
Waist/height ratio	0.48 ± 0.06	0.46 ± 0.03	0.02
Skinfolds (mm)			
Triceps	9.72 ± 4.47	8.65 ± 2.58	0.58
Abdominal	9.02 ± 6.24	7.46 ± 3.96	0.33
Subscapular	6.67 ± 3.98	5.95 ± 2.40	0.50
Blood pressure (mmHg)			
Systolic	101.63 ± 8.98	97.32 ± 10.51	0.15
Diastolic	63.31 ± 8.27	62.64 ± 7.73	0.94

[a]Severe ICH intracranial hemorrhage grade III or IV, cPVL cystic periventricular leukomalacia.

Table 2. Overall mean methylation of each gene: glucocorticoid receptor promoter region 1 (GR1) and region 2 (GR2), 11-beta hydroxysteroid dehydrogenase Type 2 (11-BHSD2), LINE1, and LEPTIN.

Gene region	Preterm born		Term born	
	Mean (SD)	Range (min−max)	Mean (SD)	Range (min−max)
11-BHSD2	4.58 (1.85)	1.00–15.66	4.52 (1.45)	2.50–7.92
GR1	4.25 (2.13)	0–10.06	3.29 (2.38)	0–9.58
GR2	4.79 (2.41)	0.56–18.93	4.30 (2.21)	1.28–12.08
LINE1	76.01 (5.29)	62.13–88.34	74.92 (5.30)	65.97–87.33
LEPTIN	21.21 (42.41)	8.29–594.47	18.01 (3.69)	3.81–22.48

DISCUSSION

We observed modest, univariate correlations between our primary outcomes of salivary cortisol and DHEA, anthropometrics and cardiovascular effects and the methylation of genes involved in HPA regulation, adiposity, and global methylation. These findings can be best understood when compared to prior studies of the effects of age, tissue, and exposures.[6,7,17,23–25] We have shown there is significant variation in tissue-specific DNA methylation in an extensive survey of non-pathologic human tissues from 10 anatomic sites as well as age and exposure-related differences.[23] Likewise, we have shown that intrauterine growth restriction is associated with distinct alterations in DNA methylation in human placenta that is dependent on intrauterine growth.[6] An altered intrauterine environment has been associated with changes in methylation of the promoter region of several of the genes we investigated here. We have seen a significant association between the placental glucocorticoid receptor promoter and early neurodevelopmental outcomes.[24] We have shown that infants whose mothers experienced the greatest levels of socioeconomic adversity during pregnancy had lowest extent of placental 11-beta HSD methylation.[25] We also showed that placental LINE1 methylation was positively correlated with birth weight and differentially affected by exposure to tobacco smoke and alcohol.[7]

Because of these prior results and because of our findings in the primary study of these children,[17] we anticipated the greatest changes in methylation of genes involved in cortisol regulation in the present study. We previously demonstrated decreased methylation in the glucocorticoid receptor promoter among very low birthweight infants with highest risks and the greatest clustering of neonatal morbidities from whom we examined DNA methylation from buccal epithelial cells prior to discharge from the Newborn Intensive Care Unit.[26] Among the subjects in this study, the preterm-born children evaluated at school age showed inverse correlations of cortisol and DHEA with weight and length, Z scores at 36 weeks post-menstrual age and positive correlations with systolic blood pressure. While cortisol was similar between preterm and term children at the school-age study visit, the preterm children showed a blunted morning cortisol. Nonetheless, we found only modest, univariate associations, with no significant correlations between first morning cortisol and any of the marker genes and only a modest negative correlation between methylation of the glucocorticoid receptor and log AUC for salivary cortisol. Epigenetic modifications of some of these genes have previously been studied in the postnatal period. Kantake et al.[27] demonstrated modest alterations in GR methylation between postnatal day 0 and 4 in preterm infants and modest variations in several CPG sites when comparing term and preterm infants. There were no relationships between GR promoter methylation and perinatal risk factors such as gestational age, birth weight, growth status, antenatal steroid exposure, and mode of delivery. Oberlander et al.[28] showed that increased methylation of the glucocorticoid receptor in umbilical cord blood was

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Table 3. Correlations between outcomes and single genes by preterm- and term-born children.

Gene	First morning cortisol value				Awakening cortisol response				log AUC				Measurable logDHEA			
	Preterm born		Term-born		Preterm born		Term-born		Preterm born		Term-born		Preterm born		Term-born	
	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value	ρ	P value
11-BHSD2	0.043	0.672	−0.062	0.760	0.155	0.374	0.063	0.846	−0.067	0.364	−0.251	0.735	−0.121	0.134	−0.072	0.762
GR1	−0.158	0.412		0.442			−0.119		−0.160	0.243	−0.097		0.069	0.496	0.098	0.098
GR2	0.360	0.018		0.812	−0.417		0.010	0.010	0.070	0.070			0.261	0.636	−0.019	
LINE1	−0.045	0.093		0.825	0.259	0.259	−0.035	0.371	0.914	0.914	0.068	0.091	0.043	0.565		
LEPTIN	−0.054	0.910		0.545	−0.183			0.770	0.011	0.881	0.043	−0.231		0.384	0.069	−0.119
	0.176	0.564		0.327	−0.100			0.050	0.114	0.622	0.128	−0.117		0.190		
	−0.133	0.443		0.544	0.036	0.036		0.583		0.198	0.533			0.562	0.261	
	0.238	0.130		0.457	0.093	0.155										

Table 4. Differences in DNA methylation values between those who were and were not exposed to antenatal steroids.

	Exposed to antenatal steroids, median (IQR)	Not exposed to antenatal steroids, median (IQR)	P value
BHSD2	4.34 (3.47, 5.60)	3.60 (3.01, 4.24)	0.018
GR1	4.22 (2.67, 5.75)	6.16 (4.90, 7.17)	0.016
GR2	4.66 (3.00, 5.90)	5.30 (4.83, 7.04)	0.071
Line 1	75.43 (72.30, 78.66)	75.48 (71.97, 81.96)	0.985
Leptin	18.28 (15.96, 20.58)	16.64 (13.71, 19.52)	0.234

associated with prenatal exposure to maternal third trimester depression and anxiety. Increased methylation of this promoter was associated with increased salivary cortisol stress responses at 3 months of age, as well. Methylation of the global marker LINE1 in the latter study did not vary between exposed and non-exposed infants or other perinatal factors.

11BHSD2 is an important enzyme in glucocorticoid metabolism and regulation. Oxidation of circulating cortisol to the inactive metabolite cortisone prevents binding to the glucocorticoid receptor and downstream biological effects. This would also be associated with alterations in feedback control of cortisol secretion in regulation of the HPA. We found little association between methylation of the 11BHSD2 promoter and first morning cortisol, log AUC of cortisol, and other parameters. This is in contrast to our studies showing alteration of 11BHSD2 promoter methylation with exposure to socioeconomic adversity during pregnancy.[25] We have also examined tissue-specific leptin promoter methylation in association with perinatal factors.[21] We showed cord blood leptin promoter methylation was higher in small for gestational age infants, lower in infants born to obese mothers, and correlated with maternal blood leptin, suggesting that leptin epigenetic control is influenced by perinatal factors. In subsequent studies we showed that increased placental leptin promoter methylation was associated with adverse neurobehavioral profiles which were restricted to male infants.[21] In the present study we found a modest positive correlation between leptin promoter methylation and log AUC in preterm infants but not in term infants. There was a similarly borderline correlation between awakening cortisol and leptin promoter methylation; again, only in the preterm infants.

LINE1 methylation is often used as a marker of global DNA methylation. LINE1 is actually a widely dispersed retrotransposon which is typically silenced by DNA methylation.[29] Interestingly, studies of early life exposures in developing animals showed significant alterations in LINE1 methylation in response to natural variations in maternal care. Mice that experienced low levels of maternal care had low levels of methylation across the LINE1 promoter.[29] This was associated with an increase in retro-transposon activity and LINE1 copy number in a region-specific fashion. The authors concluded that early life experiences resulted in somatic alterations in the genome due to L1 retrotransposon. LINE1 methylation in cord blood has shown a weak negative association with birth weight and a weak positive association with a need for neonatal treatment.[30] LINE1 methylation in peripheral blood has previously been associated with risk of cardiovascular and metabolic disorders. In a Samoan family study of overweight and diabetes, men were shown to have significantly higher LINE1 methylation than women and lower LINE1 methylation with higher levels of fasting LDL.[31] LINE1 methylation is also dynamic across the life span. During aging and the acquisition of a cellular senescence, LINE1 retrotransposable elements become transcriptionally derepressed, demethylated, and are involved in the senescence associated secretory phenotype[32] We found evidence of weak positive mediation of LINE1 on cortisol response to awakening and systolic blood pressure at 6–7 years of life. By

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Table 5. (A) Analysis of gene regions as mediating factors between cortisol AUC and weight Z-score at 36 weeks, length Z-score at 36 weeks, weight velocity from birth to 36 weeks, and systolic BP at 6–7 years, unadjusted and adjusted for center, starting time at clinic, sex, birthweight Z-score, maternal education, and marital status. (B) Analysis of gene regions as mediating factors between cortisol upon first awakening and weight Z-score at 36 weeks, length Z-score at 36 weeks, weight velocity from birth to 36 weeks, and systolic BP at 6–7 years, unadjusted and adjusted for center, time of awakening, sex, birthweight Z-score, maternal education, and marital status. (C) Analysis of gene regions as mediating factors between cortisol response to awakening and weight Z-score at 36 weeks, length Z-score at 36 weeks, weight velocity from birth to 36 weeks, and systolic BP at 6–7 years, unadjusted and adjusted for center, time of awakening, sex, birthweight Z-score, maternal education, and marital status. (D) Analysis of single factor genes as mediating factors between DHEA at beginning of visit and weight Z-score at 36 weeks, length Z-score at 36 weeks, weight velocity from birth to 36 weeks, and systolic BP at 6–7 years, unadjusted and adjusted for center, time of day, race, sex, birthweight Z-score, maternal education, and marital status.

Study samples	BHSD2 Unadjusted	BHSD2 Adjusted	GR1 Unadjusted	GR1 Adjusted	GR2 Unadjusted	GR2 Adjusted	Line 1 Unadjusted	Line 1 Adjusted	Leptin Unadjusted	Leptin Adjusted
(A)										
Weight Z-score at 36 weeks	−0.0001 (−0.0180, 0.0154)	0.0033 (−0.0131, 0.0338)	−0.0022 (−0.0321, 0.0068)	−0.0046 (−0.0371, 0.0123)	−0.0063 (−0.0358, 0.0047)	0.0030 (−0.0085, 0.0404)	−0.0033 (−0.0491, 0.0311)	0.0087 (−0.0157, 0.0470)	−0.0038 (−0.0155, 0.0203)	0.0002 (−0.0165, 0.0132)
Length Z-score at 36 weeks	0.0020 (−0.0078, 0.0245)	0.0030 (−0.0107, 0.0310)	−0.0034 (−0.0329, 0.0082)	0.0024 (−0.0048, 0.0246)	−0.0089 (−0.0442, 0.0036)	−0.0026 (−0.0253, 0.0097)	−0.0104 (−0.0495, 0.0157)	0.0016 (−0.0186, 0.0288)	−0.0017 (−0.0097, 0.0156)	0.0009 (−0.0094, 0.0142)
Weight velocity birth to 36 weeks	−0.0042 (−0.3718, 0.3266)	0.0202 (−0.2633, 0.5716)	−0.1340 (−1.0851, 0.1663)	−0.0036 (−0.4293, 0.2835)	−0.1661 (−1.0389, 0.1696)	−0.0074 (−0.4627, 0.4330)	−0.2306 (−1.4084, 0.5861)	0.0231 (−0.5770, 0.6854)	−0.0681 (−0.3544, 0.5858)	−0.0187 (−0.4031, 0.3157)
Systolic BP at 6–7 weeks	−0.0002 (−0.0030, 0.0009)	−0.0001 (−0.0017, 0.0013)	−0.0005 (−0.0037, 0.0015)	0.0004 (−0.0005, 0.0027)	−0.0004 (−0.0028, 0.0007)	−0.0003 (−0.0021, 0.0003)	−0.0019 (−0.0057, 0.0000001)	−0.0009 (−0.0034, 0.0001)	−0.0005 (−0.0018, 0.0008)	−0.0005 (−0.0021, 0.0004)
Control samples										
Systolic BP at 6–7 years	−0.0012 (−0.0145, 0.0068)	0.0037 (−0.0044, 0.0210)	−0.0039 (−0.0188, 0.0061)	0.0019 (−0.0078, 0.0144)	−0.0001 (−0.0055, 0.0044)	−0.0001 (−0.0110, 0.0076)	−0.0003 (−0.0067, 0.0024)	0.0007 (−0.0030, 0.0122)	0.0015 (−0.0026, 0.0245)	−0.0001 (−0.0085, 0.0060)
(B)										
Weight Z-score at 36 weeks	−0.0083 (−0.0605, 0.0117)	−0.0781 (−0.3478, 0.0583)	0.0123 (−0.0094, 0.0828)	−0.0189 (−0.2571, 0.0438)	−0.0099 (−0.0611, 0.0074)	−0.0001 (−0.1129, 0.0999)	−0.0024 (−0.0384, 0.0068)	−0.0258 (−0.3251, 0.0370)	0.0010 (−0.0187, 0.0108)	0.0051 (−0.0823, 0.1748)
Length Z-score at 36 weeks	−0.0179 (−0.0798, 0.0029)	−0.0138 (−0.1834, 0.0263)	0.0118 (−0.0082, 0.0724)	0.0032 (−0.0412, 0.1032)	−0.0048 (−0.0431, 0.0067)	0.0001 (−0.0787, 0.0732)	0.0023 (−0.0044, 0.0308)	0.0159 (−0.0537, 0.2331)	0.0006 (−0.0162, 0.0086)	−0.0020 (−0.1676, 0.0990)
Weight velocity birth to 36 weeks	0.0007 (−0.8468, 0.7039)	−1.5144 (−8.5520, 0.7137)	0.3732 (−0.2467, 2.1000)	0.5165 (−0.9422, 6.4305)	−0.0945 (−1.2433, 0.2236)	−0.0113 (−3.5232, 2.5005)	−0.0137 (−0.6657, 0.3092)	−1.1731 (−8.5984, 0.7935)	0.0138 (−0.3801, 0.3573)	−0.1209 (−4.6107, 2.1780)
Systolic BP at 6–7 years	−0.0017 (−0.0076, 0.0005)	−0.0011 (−0.0196, 0.0026)	0.0019 (−0.0009, 0.0096)	0.0041 (−0.0013, 0.0351)	−0.0007 (−0.0050, 0.0006)	0.0001 (−0.0046, 0.0077)	−0.0003 (−0.0039, 0.0012)	0.0024 (−0.0023, 0.0272)	0.0003 (−0.0015, 0.0017)	−0.0018 (−0.0260, 0.0036)
Control samples										
Systolic BP at 6–7 years	−0.0008 (−0.0206, 0.0025)	−0.0050 (−0.0494, 0.0208)	−0.0016 (−0.0181, 0.0033)	−0.0002 (−0.0994, 0.1558)	0.0007 (−0.0026, 0.0136)	0.0008 (−0.0161, 0.0610)	0.0000 (−0.0057, 0.0063)	0.0048 (−0.0354, 0.0598)	0.0019 (−0.0024, 0.0182)	−0.0209 (−0.0669, 0.0367)
(C)										
Weight Z-score at 36 weeks	−0.0095 (−0.1155, 0.0243)	−0.0006 (−0.1621, 0.1337)	0.0027 (−0.0333, 0.0694)	−0.0044 (−0.1506, 0.0575)	−0.0087 (−0.0975, 0.0289)	0.0081 (−0.0415, 0.2491)	−0.0398 (−0.1732, 0.0240)	−0.0346 (−0.2372, 0.0330)	−0.0135 (−0.1196, 0.0166)	0.0081 (−0.0400, 0.1485)

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Table 5. continued

Study samples	BHSD2 Unadjusted	BHSD2 Adjusted	GR1 Unadjusted	GR1 Adjusted	GR2 Unadjusted	GR2 Adjusted	Line 1 Unadjusted	Line 1 Adjusted	Leptin Unadjusted	Leptin Adjusted
Length Z-score at 36 weeks	−0.0011 (−0.0824, 0.0258)	0.0078 (−0.0247, 0.1129)	0.0045 (−0.0155, 0.0718)	0.0000 (−0.0409, 0.0431)	−0.0069 (−0.1002, 0.0360)	0.0006 (−0.0617, 0.0769)	0.0112 (−0.0544, 0.1084)	0.0146 (−0.0449, 0.1648)	−0.0063 (−0.1185, 0.0307)	−0.0017 (−0.0876, 0.0606)
Weight velocity birth to 36 weeks	−0.2198 (−4.7687, 0.4344)	0.0376 (−3.0548, 2.7518)	0.3921 (−0.3614, 2.5624)	0.0025 (−2.3384, 1.8983)	−0.2724 (−3.1218, 0.9291)	0.0412 (−1.8232, 4.7203)	−1.2387 (−5.3047, 0.7691)	−1.7071 (−7.4638, 0.5332)	−0.4055 (−4.1433, 0.3703)	0.1196 (−1.4867, 3.2124)
Systolic BP at 6–7 years	−0.0002 (−0.0057, 0.0012)	0.0003 (−0.0029, 0.0076)	0.0018 (−0.0027, 0.0109)	0.0005 (−0.0039, 0.0121)	−0.0004 (−0.0058, 0.0044)	0.0002 (−0.0044, 0.0099)	0.0043 (0.0001, 0.0156)	0.0022 (−0.0020, 0.0197)	0.0013 (−0.0013, 0.0094)	−0.0012 (−0.0156, 0.0028)
Control samples										
Systolic BP at 6–7 years	−0.0005 (−0.0387, 0.0018)	−0.0085 (−0.0578, 0.0286)	−0.0017 (−0.0202, 0.0124)	−0.0141 (−0.2302, 0.1297)	0.0010 (−0.0030, 0.0139)	0.0013 (−0.0395, 0.0434)	0.0016 (−0.0040, 0.0136)	0.0068 (−0.0720, 0.0358)	−0.0002 (−0.0135, 0.0032)	−0.0300 (−0.0834, 0.0487)
(D)										
Weight Z-score at 36 weeks	0.0068 (−0.0075, 0.0410)	0.0105 (−0.0078, 0.0534)	0.0120 (−0.0120, 0.0665)	0.0044 (−0.0241, 0.0580)	0.0020 (−0.0135, 0.0318)	0.0017 (−0.0096, 0.0296)	−0.0086 (−0.0578, 0.0173)	0.0009 (−0.0294, 0.0375)	−0.0025 (−0.0111, 0.0342)	−0.0023 (−0.0222, 0.0223)
Length Z-score at 36 weeks	0.0067 (−0.0056, 0.0408)	0.0090 (−0.0069, 0.0512)	0.0132 (−0.0061, 0.0545)	0.0116 (−0.0073, 0.0549)	−0.0015 (−0.0319, 0.0156)	0.0003 (−0.0161, 0.0203)	−0.0062 (−0.0455, 0.0086)	−0.0008 (−0.0298, 0.0183)	−0.0014 (−0.0102, 0.0179)	−0.0009 (−0.0183, 0.0125)
Weight velocity birth to 36 weeks	0.0470 (−0.2197, 0.8107)	0.1162 (−0.2344, 1.1523)	0.6579 (−0.0922, 2.2971)	0.3844 (−0.3399, 2.0066)	0.0236 (−0.4261, 0.7595)	0.0021 (−0.5713, 0.5333)	−0.3525 (−1.7361, 0.2143)	−0.0644 (−1.1886, 0.6139)	−0.0406 (−0.3123, 0.6826)	−0.0407 (−0.6196, 0.6512)
Systolic BP at 6–7 years	−0.0001 (−0.0029, 0.0016)	−0.0001 (−0.0038, 0.0011)	0.0031 (0.0001, 0.0088)	0.0025 (−0.0001, 0.0085)	0.0003 (−0.0011, 0.0034)	0.0005 (−0.0006, 0.0041)	−0.0030 (−0.0079, −0.0004)	−0.0022 (−0.0071, −0.0000)	−0.0004 (−0.0018, 0.0017)	−0.0005 (−0.0025, 0.0013)
Control samples										
Systolic BP at 6–7 years	0.0005 (−0.0053, 0.0148)	0.0010 (−0.0049, 0.0201)	−0.0029 (−0.0158, 0.0046)	0.0021 (−0.0048, 0.0191)	0.0007 (−0.0042, 0.0130)	0.0016 (−0.0036, 0.0200)	−0.0000 (−0.0045, 0.0037)	−0.0014 (−0.0158, 0.0019)	−0.0015 (−0.0128, 0.0022)	0.0000 (−0.0065, 0.0087)

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comparison, we found a weak negative effect of LINE1 methylation as a mediating factor between DHEA and systolic blood pressure at the same ages. We observed modest alteration in DNA methylation of 11BDHSD2, GR1, and GR2 in infants exposed to antenatal steroids. There were many more infants exposed to antenatal steroids among the cases than not exposed. Epigenetic effects of exposure to antenatal dexamethasone have been observed.[33] The effects are gene and methylation region specific and include both increased and decreased levels of DNA methylation in genes involved in inflammation, development, and maintenance of methylation. Regulatory elements were identified in several different DNA methyltransferase genes. Likewise, increased methylation of the glucocorticoid receptor was seen in placenta, cord blood, and maternal blood and was associated with with enhanced performance on cognitive testing.[33]

These results show that extremely preterm birth followed by complex patterns of endocrine, cardiovascular, and metabolic exposures during early postnatal life is associated with lasting changes in DNA methylation patterns in genes involved in hypothalamic pituitary adrenal axis function, adrenal hormonal regulation, and global DNA methylation. The strongest effects were seen in association with the repetitive DNA element LINE1. As noted above, changes in methylation of the LINE1 repetitive element in the central nervous system are associated with responses to environmental exposures during critical periods of development. The strengths in this study include use of a large and very well phenotyped group of former extremely preterm infants. We observed no center-related differences. Given this was a multi-center study, the observations have increased generalizability. While this was a convenience sample the availability of such a cohort provides valuable information to other investigators interested in similar outcomes. There are limitations in this study including the lack of availability of blood samples for both endocrine and methylation measurements. We were only able to measure changes in DNA methylation in buccal endothelial cells which might reflect "somatic effects." DNA methylation is cell-type-specific and it is possible that the observed changes in DNA methylation are due to changes in cell populations or changes driven by genetic variation in *cis* and *trans* that may or may not affect gene expression.[34] Studies of DNA methylation in brain tissue or with immune function would add to understanding of the association of these patterns of methylation and long-term outcomes and disease risk. Blood samples might have allowed a more precise estimate of insulin sensitivity. Blood samples for comparisons of DNA methylation on circulating mononuclear immune cells might provide even greater insights into immune-endocrine regulation during this important developmental period.

AUTHOR CONTRIBUTIONS

J.P. made substantial contributions to conception and design of the study, acquisition, analysis, and interpretation of data. He supervised the conduct of the laboratory analyses of DNA methylation. He drafted the initial manuscript, revised it, and has given final approval of the version to be published. B.D. made substantial contributions to the formal analysis and interpretation of the data, writing and review and editing of the manuscript, and has given final approval of the version to be published. C.B. made substantial contributions to the formal analysis and interpretation of the data, writing and review and editing of the manuscript, and has given final approval of the version to be published. C.M. made substantial contributions to conception and design of the study, analysis, and interpretation of the DNA methylation data. He provided technical guidance and support for the laboratory analyses. He reviewed the manuscript and has given final approval of the version to be published. S.H. provided resources for the study, made substantial contributions to the investigation, reviewed and edited the manuscript, and has given final approval of the version to be published. B.V. made substantial contribution to the investigation, review, and editing of the manuscript, and has given final approval of the version to be published. J.L. made substantial contribution to the methodology and investigation, reviewed the manuscript, and has given final approval of the version to be published. J.N. made substantial contribution to the study methodology, provided supervision/oversight, reviewed and edited the manuscript, and has given final approval of the version to be published. D.G. made substantial contribution to the parent study methodology and data curation, provided supervision/oversight, participated in writing and editing of this manuscript, and has given final approval of the version to be published. A.P. provided substantial contribution to the study investigation, participated in writing and editing of the manuscript, and has given final approval of the version to be published. K.W. made substantial contributions to conception and design of the study, acquisition, analysis, and interpretation of data. She drafted the initial manuscript, revised it, and has given final approval of the version to be published.

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