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Postnatal Epigenetic Modification of Glucocorticoid Receptor Gene in Preterm Infants

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Complete List of Authors:	Kantake, Masato; Juntendo University Urayasu Hospital, Perinatal Medical Center Yoshitake, Hiroshi; Juntendo University Graduate School of Medicine, Institute for Environmental & Gender-specific Medicine Araki, Yoshihiko; Juntendo University Graduate School of Medicine, Institute for Environmental & Gender-specific Medicine Ishikawa, Hitoshi; Yamagata Saisei Hospital, Department of Health Information Management Shimizu, Toshiaki; Juntendo University Graduate School of Medicine, Department of Pediatrics
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1 ABSTRACT

2 3 Introduction

4 Early life experiences influence the physiological and mental health of an individual
5 through epigenetic modification of DNA. However, little is known about the epigenetic
6 regulation of genes in infants with adverse birth outcomes, including low birth weight
7 and preterm birth. To examine the environmental effects on cytosine methylation of
8 preterm infant's DNA, we compared epigenetic differences in the glucocorticoid
9 receptor (GR) gene between healthy term and preterm infants.

10 11 Methods

12 A cohort of 40 (20 term and 20 preterm) infants was recruited on the day of birth.
13 Peripheral blood was obtained from each infant at birth and on postnatal day 4. We
14 calculated the methylation rates in the 1-F promoter region of the GR gene using the
15 Mquant method.

16 17 Results

18 The methylation rate increased significantly between postnatal days 0 and 4 in preterm
19 infants but remained stable in term infants. Thus, the methylation rate was significantly
20 higher in preterm than in term infants at postnatal day 4. Several perinatal parameters
21 were significantly correlated with this change in the methylation rate. **Logistic
22 regression analysis revealed that methylation rates at postnatal day 4 predicted the
23 occurrence of later complications that required glucocorticoid administration during the
24 neonatal period. No gene polymorphism was detected within the GR promoter region
25 analyzed.**

26 27 Conclusion

28 **Although further large-scale studies are needed to detect the environmental factors that
29 explain the difference in epigenetic modification among infants after birth, our data
30 show that the postnatal environment influences epigenetic programming of GR
31 expression through methylation of the GR gene promoter in premature infants, which
32 may result in relative glucocorticoid insufficiency during the postnatal period.**

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1 Introduction

2 Many human and animal studies have documented the impact of early life experiences
3 on the neurobiological regulation of stress responsiveness, mood, and anxiety disorders
4 [1–4]. Maternal care, familial functioning, and childhood adversity all contribute to
5 neurobiological regulation through epigenetic modification of DNA, which is thought to
6 be highly stable across a lifespan [5–7]. Hippocampal glucocorticoid receptors (GRs)
7 play a central role in modulating hypothalamus pituitary adrenal (HPA) axis activity [8–
8 10]. Exon 1-F of the human GR gene is similar to rat exons 1–7, which is related to a
9 maternal effect on cytosine methylation and expression [11]. Several human studies
10 have reported a relationship between early life experience and epigenetic modification
11 of the GR 1-F promoter region [11–16]. These studies have shown that adverse early
12 life experiences, such as maternal depression in the third trimester, disruption of
13 adequate nurturing, and history of childhood abuse, are associated with increased
14 methylation of the GR promoter in leukocytes [12–15], the hippocampus [11], and the
15 placenta [16].

16 The stress of birth exceeds that of any other critical life event. Indeed, exposure to
17 preterm labor and postnatal environmental stress, including the requisite acute care and
18 prolonged physical separation under neonatal intensive care, may be the most adverse

1 environments during early life. Preterm birth is associated with significant long-term
2 neurodevelopmental impairments [17-21].

3 Increased methylation of the GR promoter in leukocytes may lead to down regulation of
4 cell surface GR expression that, in turn, results in glucocorticoid receptor resistance
5 (GCR). GCR refers to a decrease in the sensitivity of immune cells to glucocorticoid
6 hormones that normally terminate the inflammatory response [22-24]. Evidence for
7 GCR in response to prolonged stress has been found in humans, such as families of
8 patients with cancer or those reporting high levels of loneliness [25-27]. On the other
9 hand, relative adrenal insufficiency occurs when the HPA axis produces insufficient
10 cortisol relative to the degree of illness or stress. Several studies have reported a
11 relative adrenal insufficiency in infants who develop chronic lung disease (CLD)
12 [28,29] and cardiovascular instability [30-32] including late onset circulatory collapse
13 (LCC). LCC is thought to be caused by late-onset adrenal insufficiency. An
14 endocrinological study noted that even though relative glucocorticoid insufficiency in
15 preterm infants is an underlying factor contributing to LCC, cortisol concentrations did
16 not differ between the LCC and control infant groups [33,34]. Therefore, another
17 factor(s), including GCR, may be the cause of LCC.

18 To test the hypothesis that an increase in cytosine methylation within the GR promoter

1 during the perinatal period in preterm infants causes physiological impairment in later
2 life, we first compared epigenetic differences in the leukocyte GR promoter 1-F region
3 (containing 33 CpG sites ranging from -3479 bp to -3259 bp upstream of the ATG start
4 site) between healthy term and preterm infants on day 0 of birth and postnatal day 4.
5 Furthermore, we examined the relationship between the postnatal change in the
6 methylation rates and perinatal parameters and whether the methylation status on
7 postnatal day 4 caused complications in the neonatal period, such as CLD and LCC,
8 which are thought to be caused by relative adrenal insufficiency.

9 10 **Methods**

11 12 *Prospective cohort study*

13 Our study was approved by the Juntendo University Urayasu Hospital Research Ethics
14 Board, and the parents provided informed consent. We recruited a cohort of 40
15 consecutive infants (20 term and 20 preterm) on the day of birth between April and
16 October 2012 at our hospital. Eligible term infants were born within 37–41 weeks of
17 gestation and received routine postnatal care as follows: day 0, skin to skin contact for
18 30 min from 30 min after birth; day 1, nursing four times (vaginal delivery (VD)) or one

1 time (Caesarean section (C/S)); day 2, rooming with the baby (VD), nursing one time
2 (C/S); day 3, rooming with the baby (VD), nursing six times (C/S); day 4, discharge
3 (VD), rooming with the baby (C/S); day 5, rooming with the baby (C/S); day 6,
4 discharge (C/S)

5 An infant was defined as preterm if born before week 37 of gestation and admitted to
6 the neonatal intensive care unit (NICU) as premature. Patients born before 37 weeks of
7 gestation and who did not require admission to the NICU were excluded. All preterm
8 infants in our study were exposed to maternal separation for at least 4 days after birth.

9 Small for gestational age (SGA) was defined as weight below the 10th percentile for
10 gestational age. The criteria for CLD were the need for additional oxygen after 28 days
11 of age [35]. The criteria for LCC were (1) sudden systemic hypotension (mean arterial
12 pressure < 35 mm Hg sustained for at least 3 h); (2) oliguria (< 1 ml kg⁻¹ h⁻¹ during an
13 8-h interval); (3) electrolyte abnormality (hyponatremia < 130 mEqL⁻¹, hyperkalemia >
14 5.5 mEqL⁻¹); and (4) excess body weight gain per day (15 gkg⁻¹ per day or 1.5% per
15 day). When more than two of these symptoms were detected 7 or more days postnatally,
16 and after all other common conditions causing systemic hypotension and oliguria, such
17 as hypovolemia, dehydration, symptomatic patent ductus arteriosus, and sepsis were
18 ruled out, corticosteroid treatment was started. LCC was diagnosed once the infant

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6 1 responded to corticosteroid therapy. Daily changes in body weight, mean arterial
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9 2 pressure, serum sodium and potassium concentrations, and urine output before and at
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12 3 symptom onset were also assessed to help the attending physician make the diagnosis of
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14 4 LCC [34].
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22 *Methylation analysis*

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24 7 Peripheral blood was obtained from the umbilical cord (day 0) or peripheral vein (day
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26 8 4) from each infant and stored at -80°C until use. Blood was sampled at day 4 in term
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29 9 infants in conjunction with a metabolic screening test recommended for all infants born
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32 10 in Japan. According to the manufacture's instruction, genomic DNA was extracted from
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35 11 2 μL of frozen peripheral blood and bisulfite treated using an EZ DNA Methylation
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38 12 Direct Kit (Zymo Research Corp., Irvine, CA, USA); 1 μL of the resulting 10 μL
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41 13 bisulfite-treated genomic DNA solution was then subjected to polymerase chain
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44 14 reaction (PCR) to amplify the GR promoter 1-F region, as described previously [11].
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47 15 The Mquant method described by Leakey et al. was used to calculate the GR promoter
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50 16 methylation rate [36] (see Supplemental Methods).
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18 *Statistical analysis*

1 We performed Wilcoxon's signed-rank test to evaluate the longitudinal difference
2 between days 0 and 4 after birth in preterm and term group infants. We performed the
3 Mann-Whitney *U*-test to compare the methylation rates in the preterm and term groups
4 at each CpG site. We performed the chi-square tests (for gestational age, birth weight,
5 and Apgar score (at 1 and 5 minutes)), and Mann-Whitney *U*-tests (for intrauterine
6 growth retardation, Caesarean section delivery, respiratory distress, mechanical
7 ventilation, intracranial hemorrhage, bacterial infection, and antenatal steroid
8 administration) to compare the characteristics of each group of infants as shown in
9 Table 1. Stepwise multiple regression analysis was performed to investigate the
10 relationships of the increased ratio of methylation rates as a dependent variable, with the
11 perinatal parameters in preterm infants as independent variables. To perform this
12 stepwise multiple regression analysis, several nominal variables were converted to
13 categorical variables by grouping values into two categories. The adjusted coefficient
14 of determination (R^2) is the fraction of information of the dependent variable that is
15 explained by the independent variables. Logistic regression analysis was performed to
16 investigate the relationships between the methylation rate at 4 days as a dependent
17 variable with later complications in the neonatal period as independent variables. The
18 goodness of fit of the logistic regression models was assessed using Pearson residuals.

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6 1 The pseudo-R² is the fraction of information of the dependent variable that is explained
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9 2 by the independent variables [37]. We used the likelihood ratio test to test the hypothesis
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12 3 regarding which dependent variable is significantly better among the four CpGs. A *p*-
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15 4 value < 0.05 (two-tailed) was considered significant. All statistical analyses were
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18 5 carried out using the StatView R 5.0J for Windows (SAS Institute, Cary, NC, USA)
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21 6 software package.
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8 **Results**

9 10 *Participant characteristics*

11 Thirty-nine of the 40 participants completed the analysis. Bisulfite-treated genomic
12 DNA from one infant from the preterm group could not be amplified by PCR for
13 unknown reasons. The characteristics of the infants are shown in Table 1. Ten of 19
14 preterm infants received antenatal steroids. The preterm infants had higher rates of
15 intrauterine growth retardation than that of infants delivered by Caesarean section.
16 They had lower Apgar scores at 1 min and 5 min compared with those of term infants.
17 Preterm infants displayed higher rates of respiratory distress and required mechanical
18 ventilation more frequently during the postnatal period than did term infants. None of

1 the infants experienced a bacterial infection or intracranial hemorrhage through
2 postnatal day 4. No gene polymorphism was detected within the GR promoter region
3 analyzed.

4 *Longitudinal changes in methylation status*

5 We examined 33 CpG sites in the GR 1-F promoter. In preterm infants, methylation
6 rates significantly increased between birth and postnatal day 4 at 11 CpG sites (1, $p =$
7 0.022; 2, $p = 0.044$; 8, $p = 0.00029$; 9, $p = 0.049$; 10, $p = 0.044$; 14, $p = 0.0013$; 16, $p =$
8 0.0022; 25, $p = 0.036$; 26, $p = 0.049$; 28, $p = 0.00097$; 29, $p = 0.016$) and significantly
9 decreased at one CpG site (4, $p = 0.030$). In contrast, methylation rates between birth
10 and postnatal day 4 were stable in term infants (Figure 1).

11 *Cross-sectional differences in methylation status*

12 Methylation rates in preterm infants at birth were lower at three CpG sites (1; $p = 0.033$,
13 5; $p = 0.0024$, 8; $p = 0.0001$) and higher at one site (4; $p = 0.040$) compared with those
14 in term infants. Methylation rates on postnatal day 4 were significantly higher in
15 preterm infants compared with term infants at seven CpG sites (15, $p = 0.00061$; 16, $p =$
16 0.0083; 21, $p = 0.043$; 25, $p = 0.0098$; 26, $p = 0.0050$; 27, $p = 0.00040$; 28, $p = 0.038$).

1 Methylation rates in preterm infants were not lower than those of term infants at any
2 CpG site (Figure 2).

3 Figure 3 shows the changes in methylation status in each group between birth and
4 postnatal day 4 at CpG sites 16, 25, 26, and 28. Methylation rates at these sites
5 significantly increased only in preterm infants, resulting in a significantly higher
6 methylation rate in preterm than in term infants on postnatal day 4.

7 8 *Relationship between methylation rates at birth and perinatal parameters*

9 We analyzed the relationship between the methylation rates at birth at CpG sites 1, 4, 5,
10 and 8 and prenatal parameters such as gestational age, birth weight, SGA, antenatal
11 steroid administration, and mode of delivery. We found no significant correlation among
12 them.

13 14 *Relationship between the increase in the methylation rate ratio and perinatal 15 parameters*

16 To examine the relationship between the increased methylation rate ratio and perinatal
17 parameters, we chose 12 CpG sites (1, 2, 4, 8, 9, 10, 14, 16, 25, 26, 28, and 29) in which
18 the methylation rates were significantly increased (with the exception of CpG4), and

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6 1 calculated the ratio as the methylation rate at day 4 divided by that at day 0. Then we
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9 2 investigated the relationship between the change in ratio at each CpG site and the
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12 3 perinatal parameters of gestational age, birth weight, Apgar score, mode of delivery,
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15 4 SGA, respiratory distress syndrome (RDS), mechanical ventilation, antenatal steroid
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18 5 administration, and admission to the NICU. We found associations between these
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21 6 parameters and an increase in the methylation rate ratio at CpG4, CpG8, CpG10,
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24 7 CpG25, and CpG29. The change in methylation ratio at each CpG site was associated
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27 8 with following perinatal parameters in the parenthesis: CpG4 (gestational age and Apgar
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30 9 score at 1 min [regression coefficient = 0.029 and -0.085, respectively; overall p -value =
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33 10 0.004; adjusted- R^2 = 0.223]); CpG8 (SGA, Caesarean section, Apgar score at 5 min, and
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36 11 NICU admission [regression coefficient = 0.43, -0.31, 0.16, and 0.79, respectively;
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39 12 overall p -value < 0.0001; adjusted- R^2 = 0.573]); CpG10 (NICU admission [regression
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42 13 coefficient = 0.29; overall p -value = 0.037; adjusted- R^2 = 0.089]); CpG25 (SGA
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45 14 [regression coefficient = 0.86, overall p -value = 0.009, adjusted- R^2 = 0.15]); and CpG29
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48 15 (SGA [regression coefficient = 0.096, overall p -value = 0.039, adjusted- R^2 = 0.086]),
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18 *Relationship between methylation rate at postnatal day 4 and complications during the*

1 *neonatal period*

2 We chose four CpG sites (16, 25, 26, and 28) shown in Figure 3 to investigate the
3 relationship between methylation status on postnatal day 4 and complications during the
4 perinatal period. Then, we analyzed the relationship between the methylation rate at
5 these CpG sites on postnatal day 4 and subsequent complications. **There were four**
6 **preterm infants who had some complications and needed glucocorticoid administration**
7 **during the neonatal period. (CLD; n=3, LCC; n=3) Except for these four cases, no**
8 **infant received glucocorticoid therapy postnatally.**

9 The logistic regression models were statistically fit (Pearson residuals, $\chi^2 = 19.284$, $p =$
10 0.9799 , pseudo- $R^2 = 0.429$). CpG16 had a significantly better explanatory factor among
11 four CpGs (likelihood ratio test $\chi^2 = 3.889$, $p = 0.0486$).

13 **Discussion**

14 Our study is the first longitudinal analysis of GR promoter methylation in humans
15 during the perinatal period, the stage that is thought to be the most susceptible to
16 epigenetic regulation. The present study demonstrated that postnatal exposure to an
17 adverse environment in preterm infants influences epigenetic programming of the
18 glucocorticoid response via GR promoter methylation during the neonatal period.

1 We found that perinatal parameters such as gestational age, intrauterine growth, Apgar
2 score, and mode of delivery were predictors for changes in methylation rates at CpG
3 sites in the GR promoter as well as NICU admission.

4 It should be noted that gestational age was positively associated with an increase in the
5 methylation ratio at CpG4. Exposure to preterm labor may be an adverse environment
6 in early life. Therefore, the methylation rate ratio at CpG4 should be negatively
7 associated with gestational age. CpG4 was the only site at which the methylation rates
8 in preterm infants were significantly higher than those in term infants at birth.

9 Moreover, CpG4 was the only site at which the methylation rate was lower on postnatal
10 day 4 than on day 0. Taken together, the change in methylation at CpG4 seemed to be
11 regulated inversely, compared with other CpG sites. The mechanisms underlying DNA
12 methylation and demethylation probably develop due to unfavorable experiences during
13 early life; however, recent studies that have investigated other transcripts of the GR
14 gene have proposed a more complex child maltreatment-induced DNA methylation
15 model than previously described for exon 1-F [38]. In the present study, CpG4 may be a
16 transcriptionally silent methylation site that mediated aberrant epigenetic regulation, as
17 proposed by Lutz et al [38]. Low Apgar score, which is thought to be an unfavorable
18 experience during the perinatal period, is positively associated with the CpG4

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6 1 methylation ratio increase and negatively associated with that of CpG8. Thus, low
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9 2 Apgar score may have a negative effect on methylation in most CpG sites in the GR 1-F
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12 3 promoter. One possible explanation is that low Apgar score indicates an adverse
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15 4 condition of cardiopulmonary function, which, in turn, inactivates the enzymatic
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18 5 cascade for DNA methylation. Stable oxygen and energy supplements may be necessary
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21 6 to open the window for epigenetic programming by DNA methylation. Caesarean
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24 7 section delivery was negatively associated with an increase in the methylation ratio at
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27 8 CpG8. This obstetrical intervention may partially reduce stress-induced epigenetic
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30 9 programming of the GR promoter. SGA was positively associated with an increase in
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33 10 methylation rate ratio at CpG8, 25, and 29. The intrauterine environment, like the
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36 11 postnatal environment, can affect fetal epigenetic modification of DNA. SGA infants
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39 12 have been thought to demonstrate epigenomic dysregulation, which mediates the long-
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42 13 term consequences of intrauterine growth restriction at birth [39,40]. The present study
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45 14 showed that, in the GR gene, SGA might influence the infant's susceptibility to the
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48 15 postnatal environment rather than epigenomic dysregulation at birth. As we expected,
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51 16 NICU admission followed by maternal separation was positively associated with an
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54 17 increase in the methylation rate ratio at CpG8 and 10. Taken together, we could
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57 18 approximately estimate the relationship between perinatal parameters and changes in
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1 methylation rates at CpG sites in the GR promoter. Further precise analysis should give
2 insights into the host-environment interaction.

3 **Four preterm infants needed glucocorticoid administration during the postnatal period**

4 **due to CLD or LCC.** The logistic regression models were statistically fit to predict the

5 development of these complications (CLD and LCC), which were thought to be partly

6 related to relative adrenal insufficiency. CpG16 had a significantly better explanatory

7 factor among the four CpGs in which methylation rates increased significantly only in

8 preterm infants resulting in a significantly higher methylation rate in preterm than in

9 term infants on postnatal day 4. CpG16 is a binding site for nerve growth factor

10 inducible protein A (NGFI-A), which has emerged as a central regulator of early

11 inflammatory and immune processes and potentiates GR 1-F promoter activity [11,41-

12 43].

13 Taken together, our results suggest that the high methylation rate observed in leukocyte

14 GR promoters may result in GR unresponsiveness to early inflammatory stimuli by

15 NGFI-A, followed by GCR and/or a failure to downregulate inflammatory responses by

16 glucocorticoids [44].

17 **One limitation of our study was the small sample size. Further studies with larger**

18 **sample sizes that include patients with chronic inflammation are needed.**

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2 Conclusions

3 We found marked changes in DNA methylation induced by the postnatal environment

4 during the 4 days immediately after birth. Moreover, the methylation status at postnatal

5 day 4 may predict the development of complications during the neonatal period, which

6 is thought to be induced by relative glucocorticoid insufficiency in preterm infants.

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	Preterm (n=19)	Term (n=20)	<i>p</i> value (preterm vs term)
Gestational age (weeks) (Mean ± SD)	30.8 ± 3.2	39.8 ± 1.3	<0.001
Birth weight (g) (Mean ± SD)	1431 ± 605	3076 ± 271	<0.001
Intrauterine growth retardation (n)	7	1	0.014
Caesarean section delivery (n)	15	3	<0.001
Apgar score at 1 min (Mean ± SD)	6.3 ± 2.6	9 ± 0	<0.001
Apgar score at 5 min (Mean ± SD)	8.4 ± 1.2	10 ± 0	<0.001
Respiratory distress (n)	10	0	<0.001
Mechanical ventilation (n)	10	0	<0.001
Intracranial hemorrhage (n)	0	0	1
Bacterial infection (n)	0	0	1
Antenatal steroid administration (n)	10	0	<0.001

Table 1; Participant characteristics

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6 **1 What's Known on This Subject:**
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9 2 Early life experiences influence the physiological and mental health of an individual
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11 3 through epigenetic modification of DNA, which is thought to be highly stable across the
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13 4 lifespan.
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18 **5 What This Study Adds:**
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21 6 Postnatal environment resets the epigenetic modification of glucocorticoid receptor
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23 7 gene promoter during four days after birth in preterm infants.
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29 **9 Acknowledgements**
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32 10 We thank Dr. Satoru Yamaguchi for sample collection.
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35 11 The English in this document has been checked by at least two professional editors,
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37 12 both native speakers of English. For a certificate, please see:

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46 **15 Competing interests**
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49 16 Financial Disclosure: The authors have no financial relationships relevant to this article
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51 17 to disclose.
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55 18 Conflict of Interest: The authors have no conflict of interest to disclose.
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8 **Contributors**

9 Drs. Kantake and Shimizu conceptualized and designed the study.
10 Dr. Kantake carried out the initial analyses and drafted the initial manuscript.
11 Dr. Araki reviewed and revised the manuscript.
12 Dr. Yoshitake designed the data collection instruments, and coordinated and supervised
13 data collection.
14 Dr. Ishikawa performed statistical analysis and revised the manuscript.
15 Drs. Kantake, Yoshitake, Ishikawa, Araki and Shimizu approved the final manuscript as
16 submitted.

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30 **Figure Legends**

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33 12 Figure 1. Longitudinal analysis of the methylation rate of the glucocorticoid receptor
34 13 (GR) gene promoter in the leukocyte calculated using the Mquant method.
35 14 The percentage of methylation at each CpG site (mean \pm SEM) in the GR promoter
36 15 isolated in peripheral blood collected from term and preterm infants at birth (dark gray
37 16 bar) and postnatal day 4 (light gray bar), respectively. Wilcoxon's signed-rank test was
38 17 performed to compare the methylation rate on day 0 and day 4 at each CpG site. A *p*-
39 18 value <0.05 (two tailed) was considered statistically significant.
40 19 Closed star symbol, significant increase in methylation rate; Open star symbol,
41 20 significant decrease in methylation rate. No significant changes in methylation rate
42 21 between postnatal days 0 and 4 were observed in term infants.
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49 23 Figure 2. Cross-sectional analysis of the methylation rates of the glucocorticoid receptor
50 24 (GR) gene promoter in leukocytes determined using the Mquant method
51 25 Percentage of methylation at each CpG site (mean \pm SEM) in the GR promoter isolated
52 26 from peripheral blood collected at birth and on postnatal day 4 in term (dark gray bar)
53 27 and preterm (light gray bar) infants, respectively. Mann-Whitney *U*-tests were
54 28 performed to compare the methylation rates in preterm and term infants at each CpG
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1 site. A p -value <0.05 (two tailed) was considered statistically significant.

2 Closed star symbol, methylation rate significantly higher in preterm compared to term
3 infants; Open star symbol, methylation rate significantly lower in preterm compared to
4 term infants.

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6 Figure 3. Change in methylation status between birth and postnatal day 4 at CpG sites
7 16, 25, 26, and 28. Methylation rates at these sites were significantly greater in preterm
8 than in term infants resulting in a significantly higher methylation rate ratio in the
9 preterm infants on postnatal day 4. The day 0 and day 4 methylation rate ratios were
10 compared using Wilcoxon's signed-rank test. The Mann–Whitney U -test was performed
11 to compare the methylation rates in preterm infants with those in term infants.

12 ns, not significant.

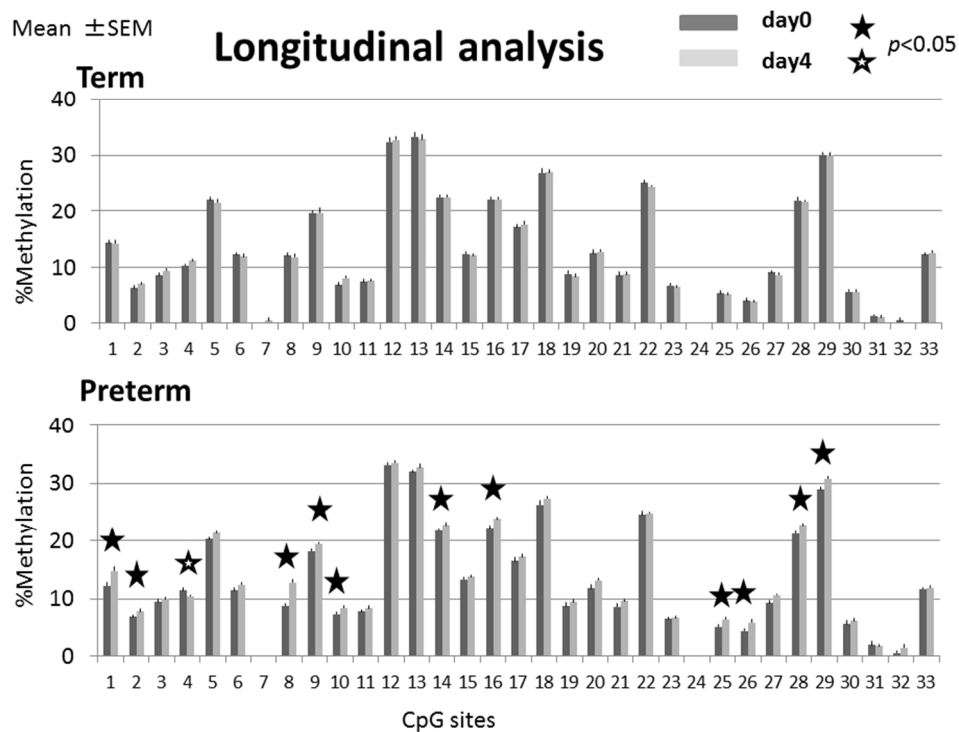


Figure 1. Longitudinal analysis of the methylation rate of the glucocorticoid receptor (GR) gene promoter in the leukocyte calculated using the Mquant method.

The percentage of methylation at each CpG site (mean \pm SEM) in the GR promoter isolated in peripheral blood collected from term and preterm infants at birth (dark gray bar) and postnatal day 4 (light gray bar), respectively. Wilcoxon's signed-rank test was performed to compare the methylation rate on day 0 and day 4 at each CpG site. A p-value < 0.05 (two tailed) was considered statistically significant.

Closed star symbol, significant increase in methylation rate; Open star symbol, significant decrease in methylation rate. No significant changes in methylation rate between postnatal days 0 and 4 were observed in term infants.

254x190mm (96 x 96 DPI)

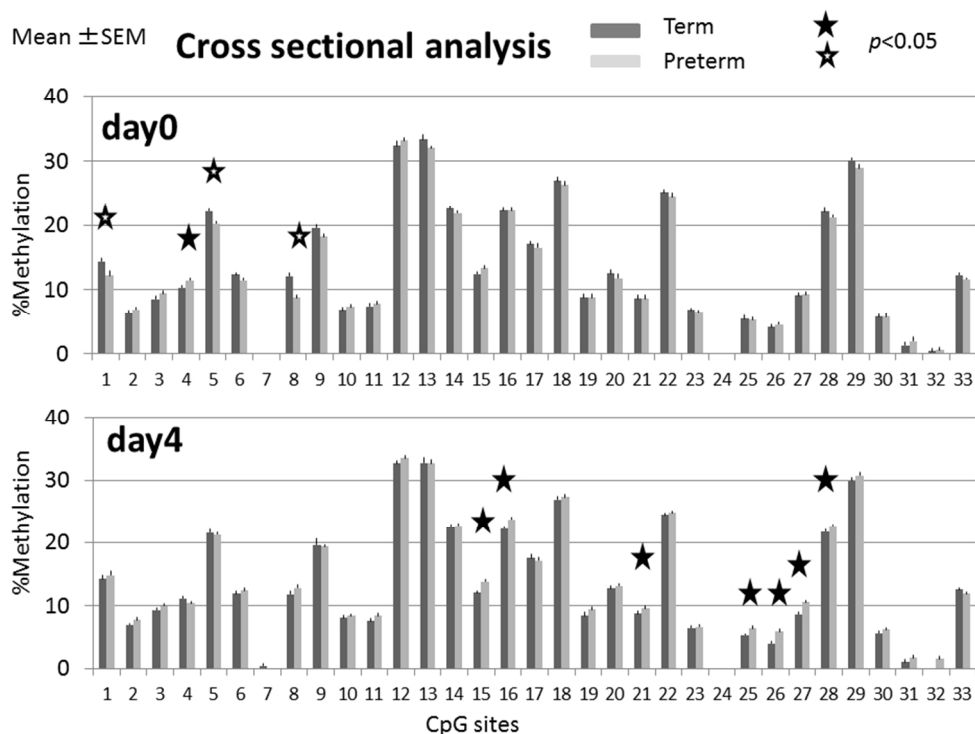


Figure 2. Cross-sectional analysis of the methylation rates of the glucocorticoid receptor (GR) gene promoter in leukocytes determined using the Mquant method

Percentage of methylation at each CpG site (mean \pm SEM) in the GR promoter isolated from peripheral blood collected at birth and on postnatal day 4 in term (dark gray bar) and preterm (light gray bar) infants, respectively. Mann-Whitney U-tests were performed to compare the methylation rates in preterm and term infants at each CpG site. A p-value < 0.05 (two tailed) was considered statistically significant.

Closed star symbol, methylation rate significantly higher in preterm compared to term infants; Open star symbol, methylation rate significantly lower in preterm compared to term infants.

254x190mm (96 x 96 DPI)

only

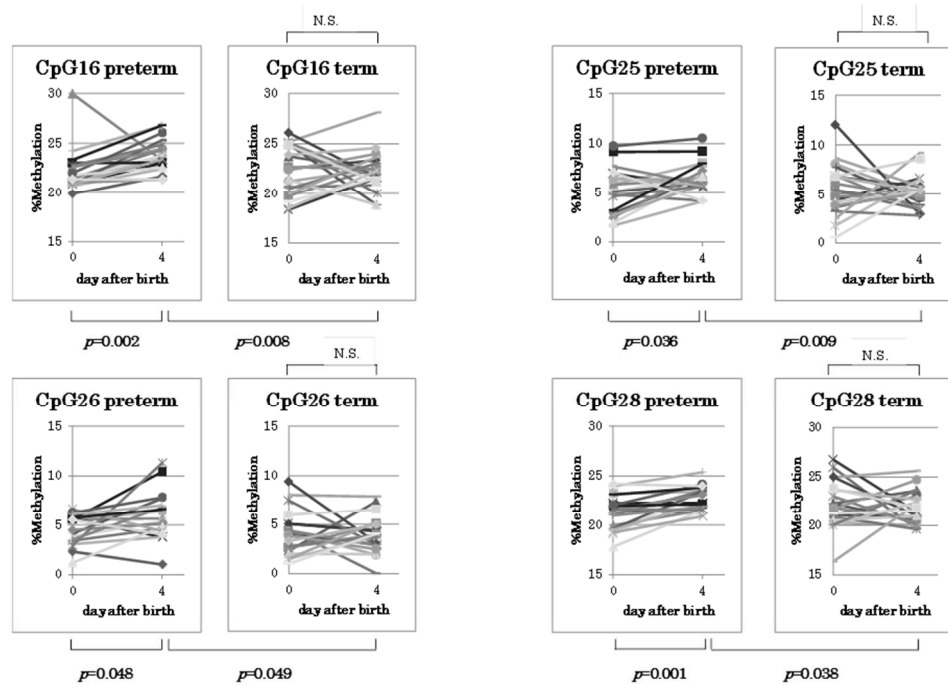


Figure 3. Change in methylation status between birth and postnatal day 4 at CpG sites 16, 25, 26, and 28. Methylation rates at these sites were significantly greater in preterm than in term infants resulting in a significantly higher methylation rate ratio in the preterm infants on postnatal day 4. The day 0 and day 4 methylation rate ratios were compared using Wilcoxon's signed-rank test. The Mann-Whitney U-test was performed to compare the methylation rates in preterm infants with those in term infants. ns, not significant.

254x190mm (96 x 96 DPI)

Supplemental methods

Primers for PCR were directed against the GR 1-F promoter using the following sequences: sense, 5'- GTG GTG GGG GAT TTG -3'; antisense, 5'- ACC TAA TCT CTC TAA AAC -3'. PCR was performed using a TaKaRa EpiTaq HS DNA polymerase kit (TAKARA BIO Inc., Shiga, Japan). Each 20 μ L PCR reaction included 1 U Taq polymerase, 0.25 mM dNTP mix, 1.25 mM $MgCl_2$, and 1 μ M each primer. The thermocycler protocol consisted of the following procedure: initial denaturation (5 min, 95°C); 35 cycles of denaturation (1 min, 95°C), annealing (2 min 30 s, 50°C), and extension (1 min, 72°C); and then a final extension (5 min, 72°C) with subsequent cooling to 20°C. The resulting product was separated by 2% agarose gel electrophoresis, and the corresponding 436 bp band was excised and purified using a Min Elute Gel Extraction Kit (QIAGEN N.V. Venlo, Netherlands). The purified product was amplified by nested PCR, using the following primers: sense, 5'- TTT TTG AAG TTT TTT TAG AGG G- 3'; antisense, 5'- AAT TTC TCC AAT TTC TTT TCT C- 3'. The PCR conditions were identical to the initial PCR procedure, with the exception that the annealing temperature was 54.2°C and 3 ng initial PCR product was used as template DNA. Then the product was purified using the same procedure as that used for the initial PCR (the corresponding band was 311 bp), and 20 ng of the resulting product was

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6 subjected to direct sequencing (Operon Biotechnology Co., Ltd. Tokyo, Japan) using
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9 antisense 'nested' primer. The ABL files from sequencing were processed using BioEdit
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12 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

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15 To calculate the GR promoter methylation rate, the Mquant method described by
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18 Leakey et al. was used. First, the mean T height (T bar) from an equal number of each
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21 side surrounding the target CpG site was determined in conventional four-dye-trace
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24 direct sequencing electrophoregrams of this PCR product. We used 10 Ts (5 Ts from
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27 each side) around the CpG site. Ts used to calculate the T bar should be at least 10 times
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30 the height of their secondary base (C, G, or A). Second, the height of the T at the target
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33 CpG site was subtracted from T bar to yield delta T. Third, the level of methylation on
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36 the site was calculated as a ratio ($\Delta T/T$ bar). The calculations were performed
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39 using Excel 2013 (Microsoft, Redmond, WA, USA).
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Postnatal epigenetic modification of glucocorticoid receptor gene in preterm infants: a prospective cohort study

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5 **Postnatal epigenetic modification of glucocorticoid receptor gene in preterm**
6 **infants: a prospective cohort study**
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10 Masato Kantake¹, MD, Hiroshi Yoshitake², MD, Hitoshi Ishikawa³, MD,
11 Yoshihiko Araki², MD, and Toshiaki Shimizu⁴, MD
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14 Affiliations: ¹Perinatal Medical Center, Juntendo University Urayasu Hospital; ²Institute
15 for Environmental & Gender-specific Medicine, Juntendo University Graduate School
16 of Medicine; ³Department of Health Information Management, Yamagata Saisei
17 Hospital; ⁴Department of Pediatrics, Juntendo University Graduate School of Medicine
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21 Address correspondence to: Masato Kantake, Perinatal Medical Center, Juntendo
22 University Urayasu Hospital, 2-1-1 Tomioka, Urayasu, Chiba 279-0021, Japan
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24 Tel: 81-47-353-3111 Fax:81-47-353-3138
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26 E-mail: kantake@juntendo-urayasu.jp
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29 Keywords: DNA methylation, glucocorticoid receptor, promoter 1-F, postnatal
30 environment, glucocorticoid receptor resistance
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ABSTRACT

Objective: To examine the environmental effects on cytosine methylation of preterm infant's DNA, because early life experiences are considered to influence the physiological and mental health of an individual through epigenetic modification of DNA.

Design: A prospective cohort study, comparison epigenetic differences in the glucocorticoid receptor (GR) gene between healthy term and preterm infants.

Setting: Neonatal Intensive Care Unit in a Japanese University Hospital

Participants: A cohort of 40 (20 term and 20 preterm) infants was recruited on the day of birth, and peripheral blood was obtained from each infant at birth and on postnatal day 4.

Main outcome measures: The methylation rates in the 1-F promoter region of the GR gene using the Mquant method.

Results: The methylation rate increased significantly between postnatal days 0 and 4 in preterm infants but remained stable in term infants. Thus, the methylation rate was significantly higher in preterm than in term infants at postnatal day 4. Several perinatal parameters were significantly correlated with this change in the methylation rate. Logistic regression analysis revealed that methylation rates at postnatal day 4 predicted the occurrence of later complications that required glucocorticoid administration during the neonatal period. No gene polymorphism was detected within the GR promoter region analyzed.

Conclusions: Although further large-scale studies are needed to detect the environmental factors that explain the difference in epigenetic modification among infants after birth, our data show that the postnatal environment influences epigenetic programming of GR expression through methylation of the GR gene promoter in premature infants, which may result in relative glucocorticoid insufficiency during the postnatal period.

Strengths and limitations of the study

- Postnatal environment resets the epigenetic modification of GR gene promoter during four days after birth in preterm infants.
- This epigenetic modification may result in relative glucocorticoid insufficiency during the postnatal period.
- Large-scale studies will be expected to clarify the environmental factors that explain the difference in epigenetic modification among infants after birth.

Introduction

Many human and animal studies have documented the impact of early life experiences on the neurobiological regulation of stress responsiveness, mood, and anxiety disorders [1–4]. Maternal care, familial functioning, and childhood adversity all contribute to neurobiological regulation through epigenetic modification of DNA, which is thought to be highly stable across a lifespan [5–7]. Hippocampal glucocorticoid receptors (GRs) play a central role in modulating hypothalamus pituitary adrenal (HPA) axis activity [8–10]. Exon 1-F of the human GR gene is similar to rat exons 1–7, which is related to a maternal effect on cytosine methylation and expression [11]. Several human studies have reported a relationship between early life experience and epigenetic modification of the GR 1-F promoter region [11–16]. These studies have shown that adverse early life experiences, such as maternal depression in the third trimester, disruption of adequate nurturing, and history of childhood abuse, are associated with increased methylation of the GR promoter in leukocytes [12–15], the hippocampus [11], and the placenta [16].

The stress of birth exceeds that of any other critical life event. Indeed, exposure to preterm labor and postnatal environmental stress, including the requisite acute care and prolonged physical separation under neonatal intensive care, may be the most adverse

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6 environments during early life. Preterm birth is associated with significant long-term
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9 neurodevelopmental impairments [17-21].

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11 Increased methylation of the GR promoter in leukocytes may lead to down regulation of
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13 cell surface GR expression that, in turn, results in glucocorticoid receptor resistance
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15 (GCR). GCR refers to a decrease in the sensitivity of immune cells to glucocorticoid
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17 hormones that normally terminate the inflammatory response [22-24]. Evidence for
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19 GCR in response to prolonged stress has been found in humans, such as families of
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21 patients with cancer or those reporting high levels of loneliness [25-27]. On the other
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23 hand, relative adrenal insufficiency occurs when the HPA axis produces insufficient
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25 cortisol relative to the degree of illness or stress. Several studies have reported a
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27 relative adrenal insufficiency in infants who develop chronic lung disease (CLD)
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29 [28,29] and cardiovascular instability [30-32] including late onset circulatory collapse
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31 (LCC). LCC is thought to be caused by late-onset adrenal insufficiency. An
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33 endocrinological study noted that even though relative glucocorticoid insufficiency in
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35 preterm infants is an underlying factor contributing to LCC, cortisol concentrations did
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37 not differ between the LCC and control infant groups [33,34]. Therefore, another
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39 factor(s), including GCR, may be the cause of LCC.

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55 To test the hypothesis that an increase in cytosine methylation within the GR promoter
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6 during the perinatal period in preterm infants causes physiological impairment in later
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9 life, we first compared epigenetic differences in the leukocyte GR promoter 1-F region
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11 (containing 33 CpG sites ranging from -3479 bp to -3259 bp upstream of the ATG start
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13 site) between healthy term and preterm infants on day 0 of birth and postnatal day 4.
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16 Furthermore, we examined the relationship between the postnatal change in the
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18 methylation rates and perinatal parameters and whether the methylation status on
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20 postnatal day 4 caused complications in the neonatal period, such as CLD and LCC,
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26 which are thought to be caused by relative adrenal insufficiency.
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Methods

Prospective cohort study

Our study was approved by the Juntendo University Urayasu Hospital Research Ethics Board, and the parents provided informed consent. We recruited a cohort of 40 consecutive infants (20 term and 20 preterm) on the day of birth between April and October 2012 at our hospital. Eligible term infants were born within 37–41 weeks of gestation and received routine postnatal care as follows: day 0, skin to skin contact for 30 min from 30 min after birth; day 1, nursing four times (vaginal delivery (VD)) or one time (Caesarean section (C/S)); day 2, rooming with the baby (VD), nursing one time (C/S); day 3, rooming with the baby (VD), nursing six times (C/S); day 4, discharge (VD), rooming with the baby (C/S); day 5, rooming with the baby (C/S); day 6, discharge (C/S)

An infant was defined as preterm if born before week 37 of gestation and admitted to the neonatal intensive care unit (NICU) as premature. Patients born before 37 weeks of gestation and who did not require admission to the NICU were excluded. All preterm infants in our study were exposed to maternal separation for at least 4 days after birth. Small for gestational age (SGA) was defined as weight below the 10th percentile for

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6 gestational age. The criteria for CLD were the need for additional oxygen after 28 days
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8 of age [35]. The criteria for LCC were (1) sudden systemic hypotension (mean arterial
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10 pressure < 35 mm Hg sustained for at least 3 h); (2) oliguria (< 1 ml kg⁻¹ h⁻¹ during an
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12 8-h interval); (3) electrolyte abnormality (hyponatremia < 130 mEqL⁻¹, hyperkalemia >
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14 5.5 mEqL⁻¹); and (4) excess body weight gain per day (15 gkg⁻¹ per day or 1.5% per
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16 day). When more than two of these symptoms were detected 7 or more days postnatally,
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18 and after all other common conditions causing systemic hypotension and oliguria, such
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20 as hypovolemia, dehydration, symptomatic patent ductus arteriosus, and sepsis were
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22 ruled out, corticosteroid treatment was started. LCC was diagnosed once the infant
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24 responded to corticosteroid therapy. Daily changes in body weight, mean arterial
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26 pressure, serum sodium and potassium concentrations, and urine output before and at
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28 symptom onset were also assessed to help the attending physician make the diagnosis of
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30 LCC [34].
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47 *Methylation analysis*

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49 Peripheral blood was obtained from the umbilical cord (day 0) or peripheral vein (day
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51 4) from each infant and stored at -80°C until use. Blood was sampled at day 4 in term
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53 infants in conjunction with a metabolic screening test recommended for all infants born
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6 in Japan. According to the manufacture's instruction, genomic DNA was extracted from
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9 2 μL of frozen peripheral blood and bisulfite treated using an EZ DNA Methylation
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11 Direct Kit (Zymo Research Corp., Irvine, CA, USA); 1 μL of the resulting 10 μL
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13 bisulfite-treated genomic DNA solution was then subjected to polymerase chain
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15 reaction (PCR) to amplify the GR promoter 1-F region, as described previously [11].
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18 The Mquant method described by Leakey et al. was used to calculate the GR promoter
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20 methylation rate [36] (see Supplemental Methods).
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29 *Statistical analysis*

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32 We performed Wilcoxon's signed-rank test to evaluate the longitudinal difference
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34 between days 0 and 4 after birth in preterm and term group infants. We performed the
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36 Mann-Whitney *U*-test to compare the methylation rates in the preterm and term groups
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38 at each CpG site. We performed the chi-square tests (for gestational age, birth weight,
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40 and Apgar score (at 1 and 5 minutes)), and Mann-Whitney *U*-tests (for intrauterine
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42 growth retardation, Caesarean section delivery, respiratory distress, mechanical
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44 ventilation, intracranial hemorrhage, bacterial infection, and antenatal steroid
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46 administration) to compare the characteristics of each group of infants as shown in
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6 relationships of the increased ratio of methylation rates as a dependent variable, with the
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8 perinatal parameters in preterm infants as independent variables. To perform this
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10 stepwise multiple regression analysis, several nominal variables were converted to
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12 categorical variables by grouping values into two categories. The adjusted coefficient
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14 of determination (R^2) is the fraction of information of the dependent variable that is
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16 explained by the independent variables. Logistic regression analysis was performed to
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18 investigate the relationships between the methylation rate at 4 days as a dependent
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20 variable with later complications in the neonatal period as independent variables. The
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22 goodness of fit of the logistic regression models was assessed using Pearson residuals.
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24 The pseudo- R^2 is the fraction of information of the dependent variable that is explained
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26 by the independent variables [37]. We used the likelihood ratio test to test the hypothesis
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28 regarding which dependent variable is significantly better among the four CpGs. A p -
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30 value < 0.05 (two-tailed) was considered significant. All statistical analyses were
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32 carried out using the StatView R 5.0J for Windows (SAS Institute, Cary, NC, USA)
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34 software package.
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Results

Participant characteristics

Thirty-nine of the 40 participants completed the analysis. Bisulfite-treated genomic DNA from one infant from the preterm group could not be amplified by PCR for unknown reasons. The characteristics of the infants are shown in Table 1. Ten of 19 preterm infants received antenatal steroids. The preterm infants had higher rates of intrauterine growth retardation than that of infants delivered by Caesarean section. They had lower Apgar scores at 1 min and 5 min compared with those of term infants. Preterm infants displayed higher rates of respiratory distress and required mechanical ventilation more frequently during the postnatal period than did term infants. None of the infants experienced a bacterial infection or intracranial hemorrhage through postnatal day 4. No gene polymorphism was detected within the GR promoter region analyzed.

Longitudinal changes in methylation status

We examined 33 CpG sites in the GR 1-F promoter. In preterm infants, methylation rates significantly increased between birth and postnatal day 4 at 11 CpG sites (1, $p =$

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6 0.022; 2, $p = 0.044$; 8, $p = 0.00029$; 9, $p = 0.049$; 10, $p = 0.044$; 14, $p = 0.0013$; 16, $p =$
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9 0.0022; 25, $p = 0.036$; 26, $p = 0.049$; 28, $p = 0.00097$; 29, $p = 0.016$) and significantly
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11 decreased at one CpG site (4, $p = 0.030$). In contrast, methylation rates between birth
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13 and postnatal day 4 were stable in term infants (Figure 1).
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20 *Cross-sectional differences in methylation status*

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23 Methylation rates in preterm infants at birth were lower at three CpG sites (1; $p = 0.033$,
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25 5; $p = 0.0024$, 8; $p = 0.0001$) and higher at one site (4; $p = 0.040$) compared with those
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27 in term infants. Methylation rates on postnatal day 4 were significantly higher in
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29 preterm infants compared with term infants at seven CpG sites (15, $p = 0.00061$; 16, $p =$
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31 0.0083; 21, $p = 0.043$; 25, $p = 0.0098$; 26, $p = 0.0050$; 27, $p = 0.00040$; 28, $p = 0.038$).
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38 Methylation rates in preterm infants were not lower than those of term infants at any
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40 CpG site (Figure 2).
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44 Figure 3 shows the changes in methylation status in each group between birth and
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46 postnatal day 4 at CpG sites 16, 25, 26, and 28. Methylation rates at these sites
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48 significantly increased only in preterm infants, resulting in a significantly higher
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50 methylation rate in preterm than in term infants on postnatal day 4.
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6 *Relationship between methylation rates at birth and perinatal parameters*
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9 We analyzed the relationship between the methylation rates at birth at CpG sites 1, 4, 5,
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11 and 8 and prenatal parameters such as gestational age, birth weight, SGA, antenatal
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13 steroid administration, and mode of delivery. We found no significant correlation among
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18 them.
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23 *Relationship between the increase in the methylation rate ratio and perinatal*
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27 *parameters*
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29 To examine the relationship between the increased methylation rate ratio and perinatal
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31 parameters, we chose 12 CpG sites (1, 2, 4, 8, 9, 10, 14, 16, 25, 26, 28, and 29) in which
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33 the methylation rates were significantly increased (with the exception of CpG4), and
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35 calculated the ratio as the methylation rate at day 4 divided by that at day 0. Then we
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37 investigated the relationship between the change in ratio at each CpG site and the
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39 perinatal parameters of gestational age, birth weight, Apgar score, mode of delivery,
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47 SGA, respiratory distress syndrome (RDS), mechanical ventilation, antenatal steroid
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49 administration, and admission to the NICU. We found associations between these
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51 parameters and an increase in the methylation rate ratio at CpG4, CpG8, CpG10,
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56 CpG25, and CpG29. The change in methylation ratio at each CpG site was associated
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6 with following perinatal parameters in the parenthesis: CpG4 (gestational age and Apgar
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8 score at 1 min [regression coefficient = 0.029 and -0.085, respectively; overall p -value =
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10 0.004; adjusted- R^2 = 0.223]); CpG8 (SGA, Caesarean section, Apgar score at 5 min, and
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12 NICU admission [regression coefficient = 0.43, -0.31, 0.16, and 0.79, respectively;
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14 overall p -value < 0.0001; adjusted- R^2 = 0.573]); CpG10 (NICU admission [regression
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16 coefficient = 0.29; overall p -value = 0.037; adjusted- R^2 = 0.089]); CpG25 (SGA
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18 [regression coefficient = 0.86, overall p -value = 0.009, adjusted- R^2 = 0.15]); and CpG29
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20 (SGA [regression coefficient = 0.096, overall p -value = 0.039, adjusted- R^2 = 0.086]),
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29 respectively.

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35 *Relationship between methylation rate at postnatal day 4 and complications during the*
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38 *neonatal period*

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40 We chose four CpG sites (16, 25, 26, and 28) shown in Figure 3 to investigate the
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42 relationship between methylation status on postnatal day 4 and complications during the
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44 perinatal period. Then, we analyzed the relationship between the methylation rate at
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46 these CpG sites on postnatal day 4 and subsequent complications. There were four
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48 preterm infants who had some complications and needed glucocorticoid administration
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50 during the neonatal period. (CLD; $n=3$, LCC; $n=3$) Except for these four cases, no
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6 infant received glucocorticoid therapy postnatally.
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9 The logistic regression models were statistically fit (Pearson residuals, $\chi^2 = 19.284$, $p =$
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12 0.9799, pseudo- $R^2 = 0.429$). CpG16 had a significantly better explanatory factor among
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15 four CpGs (likelihood ratio test $\chi^2 = 3.889$, $p = 0.0486$).
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Discussion

Our study is the first longitudinal analysis of GR promoter methylation in humans during the perinatal period, the stage that is thought to be the most susceptible to epigenetic regulation. The present study demonstrated that postnatal exposure to an adverse environment in preterm infants influences epigenetic programming of the glucocorticoid response via GR promoter methylation during the neonatal period.

We found that perinatal parameters such as gestational age, intrauterine growth, Apgar score, and mode of delivery were predictors for changes in methylation rates at CpG sites in the GR promoter as well as NICU admission.

It should be noted that gestational age was positively associated with an increase in the methylation ratio at CpG4. Exposure to preterm labor may be an adverse environment in early life. Therefore, the methylation rate ratio at CpG4 should be negatively associated with gestational age. CpG4 was the only site at which the methylation rates in preterm infants were significantly higher than those in term infants at birth. Moreover, CpG4 was the only site at which the methylation rate was lower on postnatal day 4 than on day 0. Taken together, the change in methylation at CpG4 seemed to be regulated inversely, compared with other CpG sites. The mechanisms underlying DNA methylation and demethylation probably develop due to unfavorable experiences during

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6 early life; however, recent studies that have investigated other transcripts of the GR
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9 gene have proposed a more complex child maltreatment-induced DNA methylation
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11 model than previously described for exon 1-F [38]. In the present study, CpG4 may be a
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13 transcriptionally silent methylation site that mediated aberrant epigenetic regulation, as
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15 proposed by Lutz et al [38]. Low Apgar score, which is thought to be an unfavorable
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17 experience during the perinatal period, is positively associated with the CpG4
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19 methylation ratio increase and negatively associated with that of CpG8. Thus, low
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21 Apgar score may have a negative effect on methylation in most CpG sites in the GR 1-F
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23 promoter. One possible explanation is that low Apgar score indicates an adverse
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25 condition of cardiopulmonary function, which, in turn, inactivates the enzymatic
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27 cascade for DNA methylation. Stable oxygen and energy supplements may be necessary
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29 to open the window for epigenetic programming by DNA methylation. Caesarean
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31 section delivery was negatively associated with an increase in the methylation ratio at
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33 CpG8. This obstetrical intervention may partially reduce stress-induced epigenetic
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35 programming of the GR promoter. SGA was positively associated with an increase in
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37 methylation rate ratio at CpG8, 25, and 29. The intrauterine environment, like the
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39 postnatal environment, can affect fetal epigenetic modification of DNA. SGA infants
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41 have been thought to demonstrate epigenomic dysregulation, which mediates the long-
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6 term consequences of intrauterine growth restriction at birth [39,40]. The present study
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9 showed that, in the GR gene, SGA might influence the infant's susceptibility to the
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12 postnatal environment rather than epigenomic dysregulation at birth. As we expected,
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15 NICU admission followed by maternal separation was positively associated with an
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18 increase in the methylation rate ratio at CpG8 and 10. Taken together, we could
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21 approximately estimate the relationship between perinatal parameters and changes in
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24 methylation rates at CpG sites in the GR promoter. Further precise analysis should give
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27 insights into the host-environment interaction.

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29 Four preterm infants needed glucocorticoid administration during the postnatal period
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32 due to CLD or LCC. The logistic regression models were statistically fit to predict the
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35 development of these complications (CLD and LCC), which were thought to be partly
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38 related to relative adrenal insufficiency. CpG16 had a significantly better explanatory
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41 factor among the four CpGs in which methylation rates increased significantly only in
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44 preterm infants resulting in a significantly higher methylation rate in preterm than in
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47 term infants on postnatal day 4. CpG16 is a binding site for nerve growth factor
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50 inducible protein A (NGFI-A), which has emerged as a central regulator of early
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53 inflammatory and immune processes and potentiates GR 1-F promoter activity [11,41-
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6 Taken together, our results suggest that the high methylation rate observed in leukocyte
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9 GR promoters may result in GR unresponsiveness to early inflammatory stimuli by
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12 NGFI-A, followed by GCR and/or a failure to downregulate inflammatory responses by
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15 glucocorticoids [44].

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18 One limitation of our study was the small sample size. Further studies with larger
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21 sample sizes that include patients with chronic inflammation are needed.
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Conclusions

We found marked changes in DNA methylation induced by the postnatal environment during the 4 days immediately after birth. Moreover, the methylation status at postnatal day 4 may predict the development of complications during the neonatal period, which is thought to be induced by relative glucocorticoid insufficiency in preterm infants.

Contributors

MK and TS conceptualized and designed the study. MK carried out the initial analyses.

HY designed the data collection instruments, and coordinated and supervised data collection. HI performed statistical analysis. MK and drafted the initial manuscript, and revised the manuscript.

MK, HY, HI, YA and TS approved the final manuscript as submitted.

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Competing interests

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Data sharing

No additional data are available.

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Figure Legends

Figure 1. Longitudinal analysis of the methylation rate of the glucocorticoid receptor (GR) gene promoter in the leukocyte calculated using the Mquant method.

The percentage of methylation at each CpG site (mean \pm SEM) in the GR promoter isolated in peripheral blood collected from term and preterm infants at birth (dark gray bar) and postnatal day 4 (light gray bar), respectively. Wilcoxon's signed-rank test was performed to compare the methylation rate on day 0 and day 4 at each CpG site. A *p*-value <0.05 (two tailed) was considered statistically significant.

Closed star symbol, significant increase in methylation rate; Open star symbol, significant decrease in methylation rate. No significant changes in methylation rate between postnatal days 0 and 4 were observed in term infants.

Figure 2. Cross-sectional analysis of the methylation rates of the glucocorticoid receptor (GR) gene promoter in leukocytes determined using the Mquant method

Percentage of methylation at each CpG site (mean \pm SEM) in the GR promoter isolated from peripheral blood collected at birth and on postnatal day 4 in term (dark gray bar) and preterm (light gray bar) infants, respectively. Mann-Whitney *U*-tests were performed to compare the methylation rates in preterm and term infants at each CpG site. A *p*-value <0.05 (two tailed) was considered statistically significant.

Closed star symbol, methylation rate significantly higher in preterm compared to term infants; Open star symbol, methylation rate significantly lower in preterm compared to term infants.

Figure 3. Change in methylation status between birth and postnatal day 4 at CpG sites 16, 25, 26, and 28. Methylation rates at these sites were significantly greater in preterm than in term infants resulting in a significantly higher methylation rate ratio in the preterm infants on postnatal day 4. The day 0 and day 4 methylation rate ratios were compared using Wilcoxon's signed-rank test. The Mann-Whitney *U*-test was performed to compare the methylation rates in preterm infants with those in term infants. ns, not significant.

	Preterm (n=19)	Term (n=20)	<i>p</i> value (preterm vs term)
Gestational age (weeks) (Mean ± SD)	30.8 ± 3.2	39.8 ± 1.3	<0.001
Birth weight (g) (Mean ± SD)	1431 ± 605	3076 ± 271	<0.001
Intrauterine growth retardation (n)	7	1	0.014
Caesarean section delivery (n)	15	3	<0.001
Apgar score at 1 min (Mean ± SD)	6.3 ± 2.6	9 ± 0	<0.001
Apgar score at 5 min (Mean ± SD)	8.4 ± 1.2	10 ± 0	<0.001
Respiratory distress (n)	10	0	<0.001
Mechanical ventilation (n)	10	0	<0.001
Intracranial hemorrhage (n)	0	0	1
Bacterial infection (n)	0	0	1
Antenatal steroid administration (n)	10	0	<0.001

Table1; Participant characteristics

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5 **Postnatal epigenetic modification of glucocorticoid receptor gene in preterm**
6 **infants: a prospective cohort study**
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10 Masato Kantake¹, MD, Hiroshi Yoshitake², MD, Hitoshi Ishikawa³, MD,
11 Yoshihiko Araki², MD, and Toshiaki Shimizu⁴, MD
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14 Affiliations: ¹Perinatal Medical Center, Juntendo University Urayasu Hospital; ²Institute
15 for Environmental & Gender-specific Medicine, Juntendo University Graduate School
16 of Medicine; ³Department of Health Information Management, Yamagata Saisei
17 Hospital; ⁴Department of Pediatrics, Juntendo University Graduate School of Medicine
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21 Address correspondence to: Masato Kantake, Perinatal Medical Center, Juntendo
22 University Urayasu Hospital, 2-1-1 Tomioka, Urayasu, Chiba 279-0021, Japan
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24 Tel: 81-47-353-3111 Fax:81-47-353-3138
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26 E-mail: kantake@juntendo-urayasu.jp
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29 Keywords: DNA methylation, glucocorticoid receptor, promoter 1-F, postnatal
30 environment, glucocorticoid receptor resistance
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ABSTRACT

Objective: To examine the environmental effects on cytosine methylation of preterm infant's DNA, because early life experiences are considered to influence the physiological and mental health of an individual through epigenetic modification of DNA.

Design: A prospective cohort study, comparison epigenetic differences in the glucocorticoid receptor (GR) gene between healthy term and preterm infants.

Setting: Neonatal Intensive Care Unit in a Japanese University Hospital

Participants: A cohort of 40 (20 term and 20 preterm) infants was recruited on the day of birth, and peripheral blood was obtained from each infant at birth and on postnatal day 4.

Main outcome measures: The methylation rates in the 1-F promoter region of the GR gene using the Mquant method.

Results: The methylation rate increased significantly between postnatal days 0 and 4 in preterm infants but remained stable in term infants. Thus, the methylation rate was significantly higher in preterm than in term infants at postnatal day 4. Several perinatal parameters were significantly correlated with this change in the methylation rate. Logistic regression analysis revealed that methylation rates at postnatal day 4 predicted the occurrence of later complications that required glucocorticoid administration during the neonatal period. No gene polymorphism was detected within the GR promoter region analyzed.

Conclusions: Although further large-scale studies are needed to detect the environmental factors that explain the difference in epigenetic modification among infants after birth, our data show that the postnatal environment influences epigenetic programming of GR expression through methylation of the GR gene promoter in premature infants, which may result in relative glucocorticoid insufficiency during the postnatal period.

Strengths and limitations of the study

- Postnatal environment resets the epigenetic modification of GR gene promoter during four days after birth in preterm infants.
- This epigenetic modification may result in relative glucocorticoid insufficiency during the postnatal period.
- Large-scale studies will be expected to clarify the environmental factors that explain the difference in epigenetic modification among infants after birth.

Introduction

Many human and animal studies have documented the impact of early life experiences on the neurobiological regulation of stress responsiveness, mood, and anxiety disorders [1–4]. Maternal care, familial functioning, and childhood adversity all contribute to neurobiological regulation through epigenetic modification of DNA, which is thought to be highly stable across a lifespan [5–7]. Hippocampal glucocorticoid receptors (GRs) play a central role in modulating hypothalamus pituitary adrenal (HPA) axis activity [8–10]. Exon 1-F of the human GR gene is similar to rat exons 1–7, which is related to a maternal effect on cytosine methylation and expression [11]. Several human studies have reported a relationship between early life experience and epigenetic modification of the GR 1-F promoter region [11–16]. These studies have shown that adverse early life experiences, such as maternal depression in the third trimester, disruption of adequate nurturing, and history of childhood abuse, are associated with increased methylation of the GR promoter in leukocytes [12–15], the hippocampus [11], and the placenta [16].

The stress of birth exceeds that of any other critical life event. Indeed, exposure to preterm labor and postnatal environmental stress, including the requisite acute care and prolonged physical separation under neonatal intensive care, may be the most adverse

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6 environments during early life. Preterm birth is associated with significant long-term
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9 neurodevelopmental impairments [17-21].

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11 Increased methylation of the GR promoter in leukocytes may lead to down regulation of
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13 cell surface GR expression that, in turn, results in glucocorticoid receptor resistance
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15 (GCR). GCR refers to a decrease in the sensitivity of immune cells to glucocorticoid
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17 hormones that normally terminate the inflammatory response [22-24]. Evidence for
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19 GCR in response to prolonged stress has been found in humans, such as families of
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21 patients with cancer or those reporting high levels of loneliness [25-27]. On the other
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23 hand, relative adrenal insufficiency occurs when the HPA axis produces insufficient
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25 cortisol relative to the degree of illness or stress. Several studies have reported a
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27 relative adrenal insufficiency in infants who develop chronic lung disease (CLD)
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29 [28,29] and cardiovascular instability [30-32] including late onset circulatory collapse
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31 (LCC). LCC is thought to be caused by late-onset adrenal insufficiency. An
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33 endocrinological study noted that even though relative glucocorticoid insufficiency in
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35 preterm infants is an underlying factor contributing to LCC, cortisol concentrations did
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37 not differ between the LCC and control infant groups [33,34]. Therefore, another
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39 factor(s), including GCR, may be the cause of LCC.
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55 To test the hypothesis that an increase in cytosine methylation within the GR promoter
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6 during the perinatal period in preterm infants causes physiological impairment in later
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9 life, we first compared epigenetic differences in the leukocyte GR promoter 1-F region
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11 (containing 33 CpG sites ranging from -3479 bp to -3259 bp upstream of the ATG start
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13 site) between healthy term and preterm infants on day 0 of birth and postnatal day 4.
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16 Furthermore, we examined the relationship between the postnatal change in the
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18 methylation rates and perinatal parameters and whether the methylation status on
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20 postnatal day 4 caused complications in the neonatal period, such as CLD and LCC,
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23 which are thought to be caused by relative adrenal insufficiency.
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Methods

Prospective cohort study

Our study was approved by the Juntendo University Urayasu Hospital Research Ethics Board, and the parents provided informed consent. We recruited a cohort of 40 consecutive infants (20 term and 20 preterm) on the day of birth between April and October 2012 at our hospital. Eligible term infants were born within 37–41 weeks of gestation and received routine postnatal care as follows: day 0, skin to skin contact for 30 min from 30 min after birth; day 1, nursing four times (vaginal delivery (VD)) or one time (Caesarean section (C/S)); day 2, rooming with the baby (VD), nursing one time (C/S); day 3, rooming with the baby (VD), nursing six times (C/S); day 4, discharge (VD), rooming with the baby (C/S); day 5, rooming with the baby (C/S); day 6, discharge (C/S)

An infant was defined as preterm if born before week 37 of gestation and admitted to the neonatal intensive care unit (NICU) as premature. Patients born before 37 weeks of gestation and who did not require admission to the NICU were excluded. All preterm infants in our study were exposed to maternal separation for at least 4 days after birth. Small for gestational age (SGA) was defined as weight below the 10th percentile for

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6 gestational age. The criteria for CLD were the need for additional oxygen after 28 days
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9 of age [35]. The criteria for LCC were (1) sudden systemic hypotension (mean arterial
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12 pressure < 35 mm Hg sustained for at least 3 h); (2) oliguria (< 1 ml kg⁻¹ h⁻¹ during an
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15 8-h interval); (3) electrolyte abnormality (hyponatremia < 130 mEqL⁻¹, hyperkalemia >
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18 5.5 mEqL⁻¹); and (4) excess body weight gain per day (15 gkg⁻¹ per day or 1.5% per
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21 day). When more than two of these symptoms were detected 7 or more days postnatally,
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24 and after all other common conditions causing systemic hypotension and oliguria, such
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27 as hypovolemia, dehydration, symptomatic patent ductus arteriosus, and sepsis were
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30 ruled out, corticosteroid treatment was started. LCC was diagnosed once the infant
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33 responded to corticosteroid therapy. Daily changes in body weight, mean arterial
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36 pressure, serum sodium and potassium concentrations, and urine output before and at
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39 symptom onset were also assessed to help the attending physician make the diagnosis of
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42 LCC [34].
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46 47 *Methylation analysis*

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50 Peripheral blood was obtained from the umbilical cord (day 0) or peripheral vein (day
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53 4) from each infant and stored at -80°C until use. Blood was sampled at day 4 in term
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56 infants in conjunction with a metabolic screening test recommended for all infants born
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6 in Japan. According to the manufacture's instruction, genomic DNA was extracted from
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9 2 μL of frozen peripheral blood and bisulfite treated using an EZ DNA Methylation
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11 Direct Kit (Zymo Research Corp., Irvine, CA, USA); 1 μL of the resulting 10 μL
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13 bisulfite-treated genomic DNA solution was then subjected to polymerase chain
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15 reaction (PCR) to amplify the GR promoter 1-F region, as described previously [11].
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18 The Mquant method described by Leakey et al. was used to calculate the GR promoter
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20 methylation rate [36] (see Supplemental Methods).
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29 *Statistical analysis*

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32 We performed Wilcoxon's signed-rank test to evaluate the longitudinal difference
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34 between days 0 and 4 after birth in preterm and term group infants. We performed the
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36 Mann–Whitney *U*-test to compare the methylation rates in the preterm and term groups
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38 at each CpG site. We performed the chi-square tests (for gestational age, birth weight,
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40 and Apgar score (at 1 and 5 minutes)), and Mann–Whitney *U*-tests (for intrauterine
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42 growth retardation, Caesarean section delivery, respiratory distress, mechanical
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44 ventilation, intracranial hemorrhage, bacterial infection, and antenatal steroid
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46 administration) to compare the characteristics of each group of infants as shown in
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6 relationships of the increased ratio of methylation rates as a dependent variable, with the
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8 perinatal parameters in preterm infants as independent variables. To perform this
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10 stepwise multiple regression analysis, several nominal variables were converted to
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12 categorical variables by grouping values into two categories. The adjusted coefficient
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14 of determination (R^2) is the fraction of information of the dependent variable that is
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16 explained by the independent variables. Logistic regression analysis was performed to
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18 investigate the relationships between the methylation rate at 4 days as a dependent
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20 variable with later complications in the neonatal period as independent variables. The
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22 goodness of fit of the logistic regression models was assessed using Pearson residuals.
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24 The pseudo- R^2 is the fraction of information of the dependent variable that is explained
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26 by the independent variables [37]. We used the likelihood ratio test to test the hypothesis
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28 regarding which dependent variable is significantly better among the four CpGs. A p -
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30 value < 0.05 (two-tailed) was considered significant. All statistical analyses were
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32 carried out using the StatView R 5.0J for Windows (SAS Institute, Cary, NC, USA)
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34 software package.
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Results

Participant characteristics

Thirty-nine of the 40 participants completed the analysis. Bisulfite-treated genomic DNA from one infant from the preterm group could not be amplified by PCR for unknown reasons. The characteristics of the infants are shown in Table 1. Ten of 19 preterm infants received antenatal steroids. The preterm infants had higher rates of intrauterine growth retardation than that of infants delivered by Caesarean section. They had lower Apgar scores at 1 min and 5 min compared with those of term infants. Preterm infants displayed higher rates of respiratory distress and required mechanical ventilation more frequently during the postnatal period than did term infants. None of the infants experienced a bacterial infection or intracranial hemorrhage through postnatal day 4. No gene polymorphism was detected within the GR promoter region analyzed.

Longitudinal changes in methylation status

We examined 33 CpG sites in the GR 1-F promoter. In preterm infants, methylation rates significantly increased between birth and postnatal day 4 at 11 CpG sites (1, $p =$

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6 0.022; 2, $p = 0.044$; 8, $p = 0.00029$; 9, $p = 0.049$; 10, $p = 0.044$; 14, $p = 0.0013$; 16, $p =$
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9 0.0022; 25, $p = 0.036$; 26, $p = 0.049$; 28, $p = 0.00097$; 29, $p = 0.016$) and significantly
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11 decreased at one CpG site (4, $p = 0.030$). In contrast, methylation rates between birth
12
13 and postnatal day 4 were stable in term infants (Figure 1).
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16 17 18 19 20 *Cross-sectional differences in methylation status* 21

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23 Methylation rates in preterm infants at birth were lower at three CpG sites (1; $p = 0.033$,
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25 5; $p = 0.0024$, 8; $p = 0.0001$) and higher at one site (4; $p = 0.040$) compared with those
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27 in term infants. Methylation rates on postnatal day 4 were significantly higher in
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29 preterm infants compared with term infants at seven CpG sites (15, $p = 0.00061$; 16, $p =$
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31 0.0083; 21, $p = 0.043$; 25, $p = 0.0098$; 26, $p = 0.0050$; 27, $p = 0.00040$; 28, $p = 0.038$).
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37 Methylation rates in preterm infants were not lower than those of term infants at any
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39 CpG site (Figure 2).
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43 Figure 3 shows the changes in methylation status in each group between birth and
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45 postnatal day 4 at CpG sites 16, 25, 26, and 28. Methylation rates at these sites
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47 significantly increased only in preterm infants, resulting in a significantly higher
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49 methylation rate in preterm than in term infants on postnatal day 4.
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6 *Relationship between methylation rates at birth and perinatal parameters*
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9 We analyzed the relationship between the methylation rates at birth at CpG sites 1, 4, 5,
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11 and 8 and prenatal parameters such as gestational age, birth weight, SGA, antenatal
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13 steroid administration, and mode of delivery. We found no significant correlation among
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18 them.
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23 *Relationship between the increase in the methylation rate ratio and perinatal*
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27 *parameters*
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29 To examine the relationship between the increased methylation rate ratio and perinatal
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31 parameters, we chose 12 CpG sites (1, 2, 4, 8, 9, 10, 14, 16, 25, 26, 28, and 29) in which
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33 the methylation rates were significantly increased (with the exception of CpG4), and
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35 calculated the ratio as the methylation rate at day 4 divided by that at day 0. Then we
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37 investigated the relationship between the change in ratio at each CpG site and the
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39 perinatal parameters of gestational age, birth weight, Apgar score, mode of delivery,
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48 SGA, respiratory distress syndrome (RDS), mechanical ventilation, antenatal steroid
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50 administration, and admission to the NICU. We found associations between these
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52 parameters and an increase in the methylation rate ratio at CpG4, CpG8, CpG10,
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60 CpG25, and CpG29. The change in methylation ratio at each CpG site was associated

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6 with following perinatal parameters in the parenthesis: CpG4 (gestational age and Apgar
7 score at 1 min [regression coefficient = 0.029 and -0.085, respectively; overall p -value =
8 0.004; adjusted- R^2 = 0.223]); CpG8 (SGA, Caesarean section, Apgar score at 5 min, and
9 NICU admission [regression coefficient = 0.43, -0.31, 0.16, and 0.79, respectively;
10 overall p -value < 0.0001; adjusted- R^2 = 0.573]); CpG10 (NICU admission [regression
11 coefficient = 0.29; overall p -value = 0.037; adjusted- R^2 = 0.089]); CpG25 (SGA
12 [regression coefficient = 0.86, overall p -value = 0.009, adjusted- R^2 = 0.15]); and CpG29
13 (SGA [regression coefficient = 0.096, overall p -value = 0.039, adjusted- R^2 = 0.086]),
14 respectively.
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35 *Relationship between methylation rate at postnatal day 4 and complications during the*
36 *neonatal period*
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40 We chose four CpG sites (16, 25, 26, and 28) shown in Figure 3 to investigate the
41 relationship between methylation status on postnatal day 4 and complications during the
42 perinatal period. Then, we analyzed the relationship between the methylation rate at
43 these CpG sites on postnatal day 4 and subsequent complications. There were four
44 preterm infants who had some complications and needed glucocorticoid administration
45 during the neonatal period. (CLD; $n=3$, LCC; $n=3$) Except for these four cases, no
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6 infant received glucocorticoid therapy postnatally.
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9 The logistic regression models were statistically fit (Pearson residuals, $\chi^2 = 19.284$, $p =$
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11 0.9799, pseudo- $R^2 = 0.429$). CpG16 had a significantly better explanatory factor among
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13 four CpGs (likelihood ratio test $\chi^2 = 3.889$, $p = 0.0486$).
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Discussion

Our study is the first longitudinal analysis of GR promoter methylation in humans during the perinatal period, the stage that is thought to be the most susceptible to epigenetic regulation. The present study demonstrated that postnatal exposure to an adverse environment in preterm infants influences epigenetic programming of the glucocorticoid response via GR promoter methylation during the neonatal period.

We found that perinatal parameters such as gestational age, intrauterine growth, Apgar score, and mode of delivery were predictors for changes in methylation rates at CpG sites in the GR promoter as well as NICU admission.

It should be noted that gestational age was positively associated with an increase in the methylation ratio at CpG4. Exposure to preterm labor may be an adverse environment in early life. Therefore, the methylation rate ratio at CpG4 should be negatively associated with gestational age. CpG4 was the only site at which the methylation rates in preterm infants were significantly higher than those in term infants at birth. Moreover, CpG4 was the only site at which the methylation rate was lower on postnatal day 4 than on day 0. Taken together, the change in methylation at CpG4 seemed to be regulated inversely, compared with other CpG sites. The mechanisms underlying DNA methylation and demethylation probably develop due to unfavorable experiences during

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6 early life; however, recent studies that have investigated other transcripts of the GR
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9 gene have proposed a more complex child maltreatment-induced DNA methylation
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11 model than previously described for exon 1-F [38]. In the present study, CpG4 may be a
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13 transcriptionally silent methylation site that mediated aberrant epigenetic regulation, as
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15 proposed by Lutz et al [38]. Low Apgar score, which is thought to be an unfavorable
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17 experience during the perinatal period, is positively associated with the CpG4
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19 methylation ratio increase and negatively associated with that of CpG8. Thus, low
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21 Apgar score may have a negative effect on methylation in most CpG sites in the GR 1-F
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23 promoter. One possible explanation is that low Apgar score indicates an adverse
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25 condition of cardiopulmonary function, which, in turn, inactivates the enzymatic
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27 cascade for DNA methylation. Stable oxygen and energy supplements may be necessary
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29 to open the window for epigenetic programming by DNA methylation. Caesarean
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31 section delivery was negatively associated with an increase in the methylation ratio at
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33 CpG8. This obstetrical intervention may partially reduce stress-induced epigenetic
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35 programming of the GR promoter. SGA was positively associated with an increase in
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37 methylation rate ratio at CpG8, 25, and 29. The intrauterine environment, like the
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39 postnatal environment, can affect fetal epigenetic modification of DNA. SGA infants
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41 have been thought to demonstrate epigenomic dysregulation, which mediates the long-
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6 term consequences of intrauterine growth restriction at birth [39,40]. The present study
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9 showed that, in the GR gene, SGA might influence the infant's susceptibility to the
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12 postnatal environment rather than epigenomic dysregulation at birth. As we expected,
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15 NICU admission followed by maternal separation was positively associated with an
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18 increase in the methylation rate ratio at CpG8 and 10. Taken together, we could
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21 approximately estimate the relationship between perinatal parameters and changes in
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24 methylation rates at CpG sites in the GR promoter. Further precise analysis should give
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27 insights into the host-environment interaction.

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29 Four preterm infants needed glucocorticoid administration during the postnatal period
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32 due to CLD or LCC. The logistic regression models were statistically fit to predict the
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35 development of these complications (CLD and LCC), which were thought to be partly
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38 related to relative adrenal insufficiency. CpG16 had a significantly better explanatory
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41 factor among the four CpGs in which methylation rates increased significantly only in
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44 preterm infants resulting in a significantly higher methylation rate in preterm than in
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47 term infants on postnatal day 4. CpG16 is a binding site for nerve growth factor
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50 inducible protein A (NGFI-A), which has emerged as a central regulator of early
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53 inflammatory and immune processes and potentiates GR 1-F promoter activity [11,41-
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6 Taken together, our results suggest that the high methylation rate observed in leukocyte
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9 GR promoters may result in GR unresponsiveness to early inflammatory stimuli by
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12 NGFI-A, followed by GCR and/or a failure to downregulate inflammatory responses by
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15 glucocorticoids [44].

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18 One limitation of our study was the small sample size. Further studies with larger
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21 sample sizes that include patients with chronic inflammation are needed.
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Conclusions

We found marked changes in DNA methylation induced by the postnatal environment during the 4 days immediately after birth. Moreover, the methylation status at postnatal day 4 may predict the development of complications during the neonatal period, which is thought to be induced by relative glucocorticoid insufficiency in preterm infants.

Contributors

MK and TS conceptualized and designed the study. MK carried out the initial analyses. HY designed the data collection instruments, and coordinated and supervised data collection. HI performed statistical analysis. MK and drafted the initial manuscript, and revised the manuscript. MK, HY, HI, YA and TS approved the final manuscript as submitted.

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Competing interests

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Data sharing

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No additional data are available.

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For peer review only

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Figure Legends

Figure 1. Longitudinal analysis of the methylation rate of the glucocorticoid receptor (GR) gene promoter in the leukocyte calculated using the Mquant method.

The percentage of methylation at each CpG site (mean \pm SEM) in the GR promoter isolated in peripheral blood collected from term and preterm infants at birth (dark gray bar) and postnatal day 4 (light gray bar), respectively. Wilcoxon's signed-rank test was performed to compare the methylation rate on day 0 and day 4 at each CpG site. A *p*-value <0.05 (two tailed) was considered statistically significant.

Closed star symbol, significant increase in methylation rate; Open star symbol, significant decrease in methylation rate. No significant changes in methylation rate between postnatal days 0 and 4 were observed in term infants.

Figure 2. Cross-sectional analysis of the methylation rates of the glucocorticoid receptor (GR) gene promoter in leukocytes determined using the Mquant method

Percentage of methylation at each CpG site (mean \pm SEM) in the GR promoter isolated from peripheral blood collected at birth and on postnatal day 4 in term (dark gray bar) and preterm (light gray bar) infants, respectively. Mann-Whitney *U*-tests were performed to compare the methylation rates in preterm and term infants at each CpG site. A *p*-value <0.05 (two tailed) was considered statistically significant.

Closed star symbol, methylation rate significantly higher in preterm compared to term infants; Open star symbol, methylation rate significantly lower in preterm compared to term infants.

Figure 3. Change in methylation status between birth and postnatal day 4 at CpG sites 16, 25, 26, and 28. Methylation rates at these sites were significantly greater in preterm than in term infants resulting in a significantly higher methylation rate ratio in the preterm infants on postnatal day 4. The day 0 and day 4 methylation rate ratios were compared using Wilcoxon's signed-rank test. The Mann-Whitney *U*-test was performed to compare the methylation rates in preterm infants with those in term infants. ns, not significant.

	Preterm (n=19)	Term (n=20)	<i>p</i> value (preterm vs term)
Gestational age (weeks) (Mean ± SD)	30.8 ± 3.2	39.8 ± 1.3	<0.001
Birth weight (g) (Mean ± SD)	1431 ± 605	3076 ± 271	<0.001
Intrauterine growth retardation (n)	7	1	0.014
Caesarean section delivery (n)	15	3	<0.001
Apgar score at 1 min (Mean ± SD)	6.3 ± 2.6	9 ± 0	<0.001
Apgar score at 5 min (Mean ± SD)	8.4 ± 1.2	10 ± 0	<0.001
Respiratory distress (n)	10	0	<0.001
Mechanical ventilation (n)	10	0	<0.001
Intracranial hemorrhage (n)	0	0	1
Bacterial infection (n)	0	0	1
Antenatal steroid administration (n)	10	0	<0.001

Table1; Participant characteristics

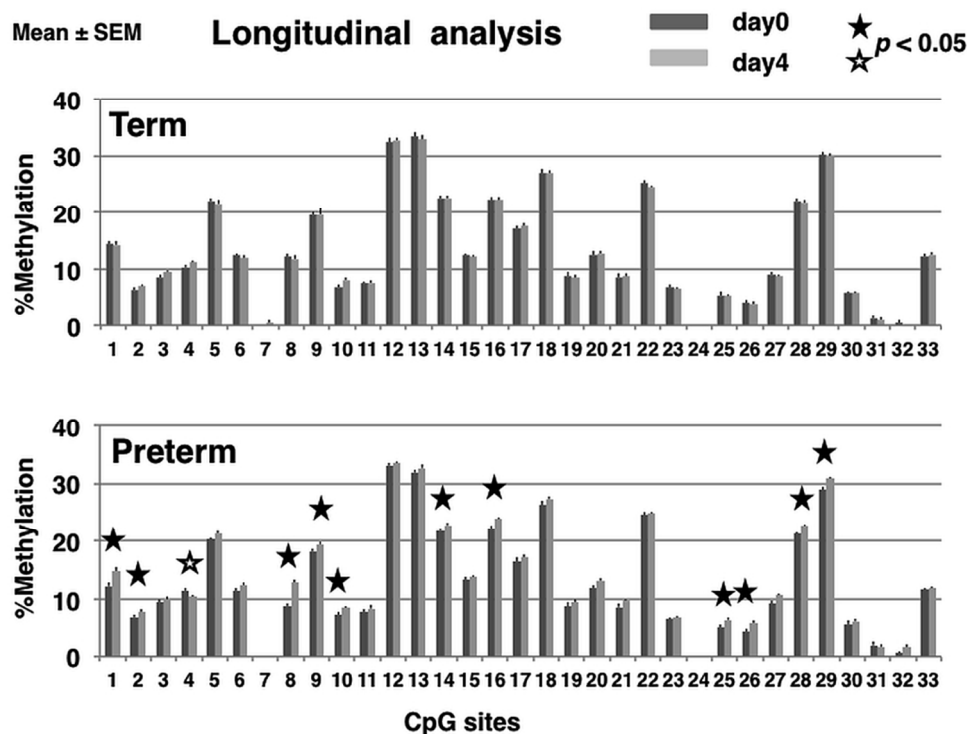


Figure 1. Longitudinal analysis of the methylation rate of the glucocorticoid receptor (GR) gene promoter in the leukocyte calculated using the Mquant method.

The percentage of methylation at each CpG site (mean \pm SEM) in the GR promoter isolated in peripheral blood collected from term and preterm infants at birth (dark gray bar) and postnatal day 4 (light gray bar), respectively. Wilcoxon's signed-rank test was performed to compare the methylation rate on day 0 and day 4 at each CpG site. A p-value < 0.05 (two tailed) was considered statistically significant.

Closed star symbol, significant increase in methylation rate; Open star symbol, significant decrease in methylation rate. No significant changes in methylation rate between postnatal days 0 and 4 were observed in term infants.

90x67mm (300 x 300 DPI)

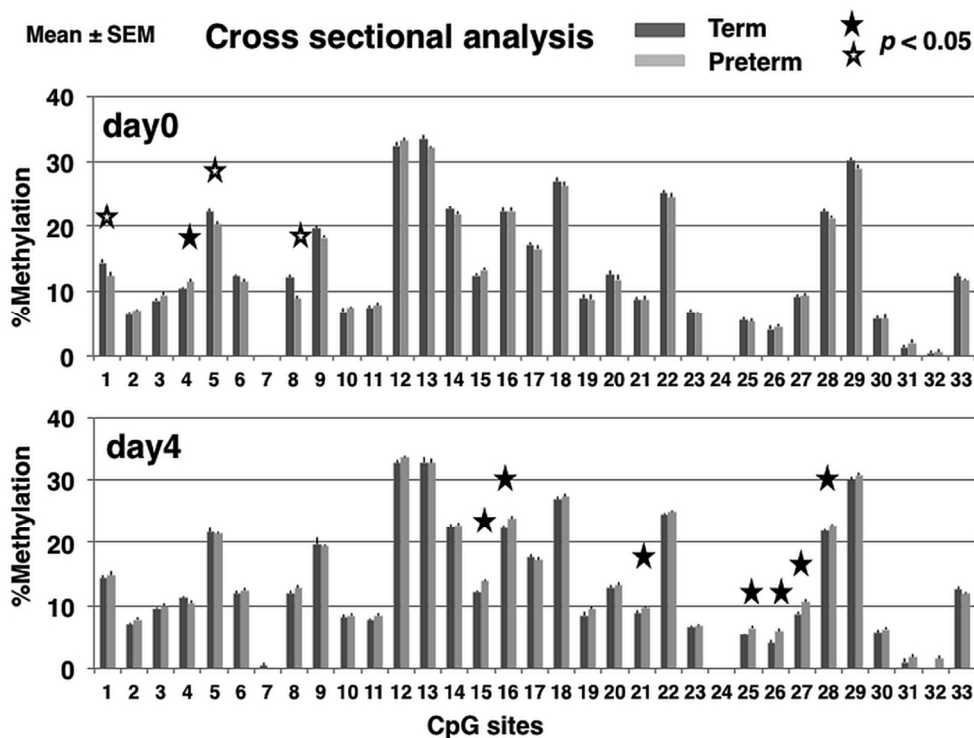


Figure 2. Cross-sectional analysis of the methylation rates of the glucocorticoid receptor (GR) gene promoter in leukocytes determined using the Mquant method

Percentage of methylation at each CpG site (mean ± SEM) in the GR promoter isolated from peripheral blood collected at birth and on postnatal day 4 in term (dark gray bar) and preterm (light gray bar) infants, respectively. Mann-Whitney U-tests were performed to compare the methylation rates in preterm and term infants at each CpG site. A p-value <0.05 (two tailed) was considered statistically significant.

Closed star symbol, methylation rate significantly higher in preterm compared to term infants; Open star symbol, methylation rate significantly lower in preterm compared to term infants.

90x67mm (300 x 300 DPI)

only

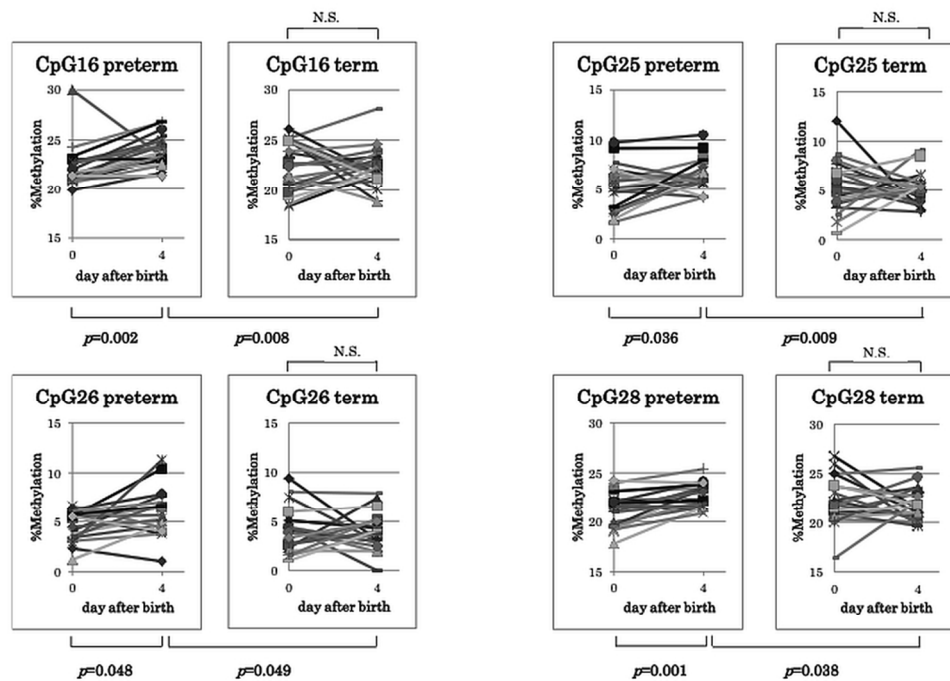


Figure 3. Change in methylation status between birth and postnatal day 4 at CpG sites 16, 25, 26, and 28. Methylation rates at these sites were significantly greater in preterm than in term infants resulting in a significantly higher methylation rate ratio in the preterm infants on postnatal day 4. The day 0 and day 4 methylation rate ratios were compared using Wilcoxon's signed-rank test. The Mann-Whitney U-test was performed to compare the methylation rates in preterm infants with those in term infants. ns, not significant.

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Supplemental methods

Primers for PCR were directed against the GR 1-F promoter using the following sequences: sense, 5'- GTG GTG GGG GAT TTG -3'; antisense, 5'- ACC TAA TCT CTC TAA AAC -3'. PCR was performed using a TaKaRa EpiTaq HS DNA polymerase kit (TAKARA BIO Inc., Shiga, Japan). Each 20 μ L PCR reaction included 1 U Taq polymerase, 0.25 mM dNTP mix, 1.25 mM MgCl₂, and 1 μ M each primer. The thermocycler protocol consisted of the following procedure: initial denaturation (5 min, 95°C); 35 cycles of denaturation (1 min, 95°C), annealing (2 min 30 s, 50°C), and extension (1 min, 72°C); and then a final extension (5 min, 72°C) with subsequent cooling to 20°C. The resulting product was separated by 2% agarose gel electrophoresis, and the corresponding 436 bp band was excised and purified using a Min Elute Gel Extraction Kit (QIAGEN N.V. Venlo, Netherlands). The purified product was amplified by nested PCR, using the following primers: sense, 5'- TTT TTG AAG TTT TTT TAG AGG G- 3'; antisense, 5'- AAT TTC TCC AAT TTC TTT TCT C- 3'. The PCR conditions were identical to the initial PCR procedure, with the exception that the annealing temperature was 54.2°C and 3 ng initial PCR product was used as template DNA. Then the product was purified using the same procedure as that used for the initial PCR (the corresponding band was 311 bp), and 20 ng of the resulting product was

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6 subjected to direct sequencing (Operon Biotechnology Co., Ltd. Tokyo, Japan) using
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9 antisense 'nested' primer. The ABL files from sequencing were processed using BioEdit
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12 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

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15 To calculate the GR promoter methylation rate, the Mquant method described by
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18 Leakey et al. was used. First, the mean T height (T bar) from an equal number of each
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21 side surrounding the target CpG site was determined in conventional four-dye-trace
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24 direct sequencing electrophoregrams of this PCR product. We used 10 Ts (5 Ts from
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27 each side) around the CpG site. Ts used to calculate the T bar should be at least 10 times
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30 the height of their secondary base (C, G, or A). Second, the height of the T at the target
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33 CpG site was subtracted from T bar to yield delta T. Third, the level of methylation on
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36 the site was calculated as a ratio ($\Delta T/T$ bar). The calculations were performed
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39 using Excel 2013 (Microsoft, Redmond, WA, USA).
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