OPEN

# Cord Blood *PRF1* Methylation Patterns and Risk of Lower Respiratory Tract Infections in Infants

## *Findings From the Ulm Birth Cohort*

*Magdeldin Elgizouli, MBBS, MSc, Chad Logan, MPH, Alexandra Nieters, PhD, MPH, Hermann Brenner, MD, MPH, and Dietrich Rothenbacher, MD, MPH*

**Abstract:** Lower respiratory tract infections (LRTIs) are a major cause of morbidity in children. DNA methylation provides a mechanism for transmitting environmental effects on the genome, but its potential role in LRTIs is not well studied. We investigated the methylation pattern of an enhancer region of the immune effector gene perforin-1 (*PRF1*), which encodes a cytolytic molecule of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, in cord blood DNA of children recruited in a German birth cohort in association with LRTIs in the first year of life.

Pyrosequencing was used to determine the methylation levels of target cytosine–phosphate–guanines (CpGs) in a 2-stage case–control design. Cases were identified as children who developed ≥2 episodes of physician-recorded LRTIs during the first year of life and controls as children who had none. Discovery (n = 87) and replication (n = 90) sets were arranged in trios of 1 case and 2 controls matched for sex and season of birth.

Logistic regression analysis revealed higher levels of methylation at a CpG that corresponds to a signal transducer and activator of transcription 5 (STAT5) responsive enhancer in the discovery (odds ratio [OR] per 1% methylation difference 1.24, 95% confidence interval [CI] 1.03–1.50) and replication (OR per 1% methylation difference 1.25, 95% CI 1.04–1.50) sets. Adjustment for having siblings <5 years old in the discovery and replication sets produced ORs of 1.19 (95% CI 0.98–1.45) and 1.25 (95% CI 1.04–1.50), respectively. Adjustment for gestational age in the replication set had no influence on the results. Methylation levels at adjacent CpGs varied with maternal age, smoking, education, and having siblings <5 years old.

Our data support an association between cord blood *PRF1* enhancer methylation patterns and subsequent risk of LRTIs in infants. Methylation levels at specific CpGs of the *PRF1* enhancer varied according to maternal and family environmental factors suggesting a role for DNA methylation in mediating environmental influences on gene function.

(*Medicine* 94(1):e332)

**Abbreviations**: BMI = body mass index, CI = confidence interval, CpG = cytosine-phosphate-guanine, CTL = cytotoxic T lymphocyte, EDTA = ethylenediaminetetraacetic acid, IL = interleukin, JAK = Janus kinase, LRTI = lower respiratory tract infection, MSR = methylation-sensitive region, NK cell = natural killer cell, OR = odds ratio, PCR = polymerase chain reaction, *PRF1* = perforin-1, RSV = respiratory syncytial virus, STAT = signal transducer and activator of transcription, TSS = transcription start site.

## INTRODUCTION

Lower respiratory tract infections (LRTIs), comprising pneumonia and bronchiolitis, constitute a major cause of morbidity and mortality in children. In industrialized countries, the incidence of hospital admissions for severe LRTIs was about 20 episodes per 1000 children per year in infants in 2010 compared with around 52 episodes per 1000 children per year in developing countries in the same age group.[1] Respiratory syncytial virus (RSV) is by far the most common causative pathogen accounting for an estimated 22% of acute LRTIs in children <5 years of age. The greatest proportion of RSV-associated disease occurs in the first year of life. Infants suffer 2 to 3 times the incidence observed in children <5 years old.[2]

Natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) are crucial for the elimination of cells infected by viruses. The 2 cell types, representing the innate and adaptive immune systems, respectively, employ the secretory granule-dependent pathway to initiate the apoptosis of virus-infected cells. Perforin-1 (*PRF1*) is a unique component of this cytotoxic pathway; it facilitates the delivery of granzymes to target cells through the formation of pores in the cell membrane.[3] Two enhancer sites, located 15 and 1 kb upstream of the *PRF1* locus, regulate the transcription of the gene under the control of signal transducer and activator of transcription 5 (STAT5) factors driven by interleukin 2 (IL-2) stimulation.[4] The proximal enhancer is likely under the primary control of STAT5. Intact IL-2Rβ/STAT5 signaling leads to *PRF1* expression in NK cells and CTLs and is a requirement for the differentiation of effector CTLs and the maintenance of memory CTLs in response to viral infection.[5]

DNA methylation of a sequence of cytosine–guanine dinucleotides (CpGs) within the proximal enhancer identified as a methylation-sensitive region (MSR) suppresses *PRF1*

Editor: Jing Liu.
Received: October 2, 2014; revised: November 10, 2014; accepted: November 12, 2014.
From the Center for Chronic Immunodeficiency (CCI) (ME, AN), University Medical Center Freiburg, Freiburg; Institute of Epidemiology and Medical Biometry (CL, DR), Ulm University, Ulm; and Division of Clinical Epidemiology and Aging Research (HB, DR), German Cancer Research Center, Heidelberg, Germany.
Correspondence: Alexandra Nieters, Center for Chronic Immunodeficiency, Molecular Epidemiology, University Medical Center Freiburg, Engesser Strasse 4, D-79108 Freiburg, Germany (e-mail: alexandra.nieters@uniklinik-freiburg.de).
The study was supported by grants of the German Research Council (BR 1704/3-1, BR 1704/3). It was supported by the German Federal Ministry of Education and Research (BMBF 01EO1303). ME was funded by a scholarship of the German Academic Exchange Service (DAAD A/10/90132).
The authors have no conflicts of interest to disclose.

ISSN: 0025-7974
DOI: 10.1097/MD.0000000000000332

promoter function while demethylation is associated with increased gene expression.[6] It is hypothesized that epigenetic modifications mediate intrinsic developmental and external environmental effects on the genome and influence disease susceptibility.[7] A growing body of evidence supports the notion that maternal factors including age, body mass index (BMI),[8] diet,[9] and smoking[10,11] influence DNA methylation patterns in the neonate.

The aim of this study was to investigate cord blood methylation levels at the *PRF1* MSR of infants diagnosed with 2 or more episodes of LRTIs during the first year of life compared with those at the *PRF1* MSR of infants without such episodes after adjustment for potential confounding factors associated with the maternal and neonatal environment.

## METHODS

### Study Population

This study was performed on samples drawn from the Ulm Birth Cohort detailed here.[12] In brief, mothers who delivered at the Obstetrics and Gynaecology Department of the Ulm University Hospital in Germany between November 2000 and November 2001 and could speak German, Russian, or Turkish were recruited into the study. Exclusion criteria were: mothers who gave birth at less than 32 weeks gestation, had a child with birth weight less than 2500 g, or whose neonates required intensive care. Of 1593 mothers eligible for participation 1066 (1090 neonates) volunteered to take part. Mothers were interviewed at baseline by trained personnel during their hospital stay using standardized questionnaires. Cord blood samples were collected at birth in ethylenediaminetetraacetic acid (EDTA) tubes. Samples were immediately processed to obtain buffy coats and material aliquoted and stored since then at −80°C. Follow-up data were obtained from parents and children's physicians via mailed questionnaires at 6 weeks, 6 months, and 1 year postenrolment. The Ulm Birth Cohort study was approved by the University of Ulm Ethics Board and the Physicians' Boards of Baden-Württemberg and Bavaria. Written informed consent was obtained from all participants for data collection and analysis of biological samples. DNA extraction and methylation assays for this study were performed in 2012.

Two case–control subsets (discovery and replication sets) composed of trios of 1 case and 2 healthy controls (target n = 90) were selected from the Ulm Birth Cohort. A case was defined as an infant who developed 2 or more episodes of physician-recorded LRTIs during the first year of life, and a healthy control as an infant who had no physician-recorded LRTIs during the same period. The incidence of LRTIs in the first year of life was established by questionnaires completed by the infants' treating physicians. The definition of LRTIs in this cohort included the diagnoses of pneumonia, bronchitis, pertussis, tracheobronchitis, croup, and bronchiolitis (yes/no categories and if yes, how many episodes). The final discovery set comprised 87 samples, 29 cases and 58 respective controls, while the replication set comprised 90 samples, 30 cases and 60 respective controls. Cases and controls were matched according to the child's sex and season of birth (categorized as winter/spring vs summer/autumn). Matching addressed sex differences in methylation patterns[13] and the seasonal variation in prevalence of RSV[14] and vitamin D status.[15]

### Target Loci

Cytosine methylation levels in 6 CpGs in the MSR of *PRF1*, located 929 to 577 bp upstream of the transcription start site (TSS),[6] and 3 adjoining CpGs were determined by pyrosequencing using 4 assays adapted from Narasimhan et al[16] and identified further here as assays C, D, E, and F covering 3, 1, 2, and 3 CpGs, respectively. Figure 1 illustrates the *PRF1* enhancer region examined, the CpG loci assayed, and putative transcription factor binding sites.

### DNA Extraction and Bisulfite Conversion

Genomic DNA was manually extracted from cord blood buffy coat samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Quantity and quality of purified DNA were determined with an ND-1000 spectrophotometer. DNA was stored at −20°C before use. In preparation for pyrosequencing, 500 ng of DNA underwent bisulfite conversion using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. Nonmethylated cytosines in this procedure are converted to uracil, which is replaced in subsequent amplification by thymine, while methylated cytosines are preserved as such.

### Bisulfite Pyrosequencing

Design of the pyrosequencing assays was carried out using the PyroMark Assay Design software version 2.0.1.15 (Qiagen). All primers were obtained from Apara Biosciences GmbH (Denzlingen, Germany). The quantitative performance of each pyrosequencing assay was assessed by measuring methylation standards of known proportions of unmethylated (whole genome amplified) and fully methylated (*Sss-I* treated) genomic DNA and optimized by means of an annealing temperature

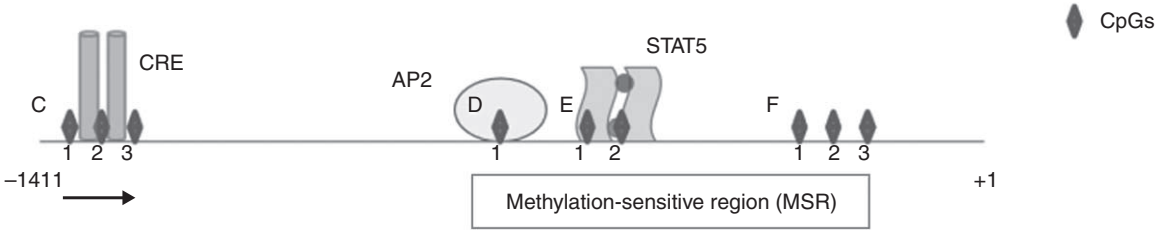


**FIGURE 1.** The perforin gene proximal enhancer, beginning −1411 bp upstream of the transcription start site with locations of assayed cytosine–guanine dinucleotides and transcription factor binding sites. Letters refer to separate pyrosequencing assays (C, D, E, F) with individual cytosine–guanine dinucleotides marked by diamond shapes and numbered (adapted from Narasimhan et al[16]). The arrow refers to direction of sequence. AP2 = activating protein 2, CpGs = cytosine-phosphate-guanines, CRE = cAMP responsive element, STAT5 = signal transducer and activator of transcription 5.

gradient. Each assay included non-CpG cytosines as internal controls to verify efficient bisulfite conversion. The target fragments within the candidate genes were amplified by polymerase chain reaction (PCR) using the primers detailed in Supplemental Digital Content (Table S1, http://links.lww.com/MD/A119). A total of 5 pmol each of the forward and reverse primers, one being biotinylated, was employed to amplify the fragments of interest from an input amount of 0.5 μL bisulfite-converted DNA, 20 ng assuming 80% recovery from bisulfite conversion, in a final reaction volume of 12.5 μL using the PyroMark PCR Kit (Qiagen). PCR conditions were as follows: 95°C for 10 minutes; 50 cycles of 94°C for 30 seconds, annealing for 30 seconds, 72°C for 30 seconds; and a final extension step at 72°C for 10 minutes.

PCR products were rendered single stranded according to established protocol.[17] A total of 3 pmol of the sequencing primers in Supplemental Digital Content (Table S1, http://links.lww.com/MD/A119) was used in each case to carry out pyrosequencing of the amplified fragments on the PyroMark Q96 MD apparatus (Qiagen). The percentage methylation at each CpG was calculated using the PyroMark CpG software version 1.0.11 build 14 (Qiagen). Matched cases and controls were assayed in the same batch. All samples were assayed in duplicates and the mean value of the 2 measurements calculated and used for statistical analyses.

## Statistical Analysis

Univariate analyses were conducted on each candidate gene locus to assess methylation results for normality and identify potential outliers. Methylation measurements greater than 3 standard deviations from the mean were considered as potential outliers. In order to identify potential confounders that were not accounted for by matching, Kruskal–Wallis tests were used to determine if associations existed between both individual demographic or a priori–determined LRTI risk factors and methylation levels. Potential confounders considered included gestational age (<37 or ≥37 weeks), delivery mode (vaginal or cesarean section), birth weight (continuous), mother's country of birth (Germany or other), maternal age (in years), maternal education (primary school or greater than or equal to secondary education), maternal smoking during the child's first year (yes or no), breast-feeding duration (categorized as <3, >3 to ≤6, >6 to ≤9, and >9 months), and whether or not the child had a sibling <5 years of age (yes or no). Sibling status was associated with both the primary exposure and outcome in the discovery set and entered into the model for adjustment. Bivariate and adjusted conditional logistic regression models were used to calculate the odds ratio (OR) per 1% difference in methylation, 95% confidence interval (CI), and *P* for the association with case status at each candidate gene locus. In order to determine the effect of potential outliers on the results, a second bivariate analysis was performed restricted to trios (case and 2 matched controls) containing no outlier values. All statistical analyses were performed using SAS 9.3 for Windows (SAS Institute, Cary, NC).

# RESULTS

## Population Characteristics

Demographic characteristics of the discovery set (n = 87) are detailed in Table 1 according to case–control status. The discovery set comprised slightly more male children (58.6%) and most were born between November and April (62.1%). The majority were delivered vaginally (83.9%) to mothers of a median age of 32.8 years with 11 or more years of formal education (73.3%). None of the factors analyzed differed significantly between cases and controls in the discovery set apart from having siblings younger than 5 years of age. Sixty-two percent of cases had a sibling <5 years old compared with only 31% of controls ($P = 0.01$).

The majority of newborns in the replication set (n = 90) were also male (56.7%) and born between November and April (56.7%). Further characteristics of the replication set are presented in Table 2. Most were delivered vaginally (82.2%) to mothers at a median age of 31.5 years with an intermediate education or above (71.6%). There was no association between case status and having a sibling <5 years old in the replication set. 56.7% of cases had a sibling <5 years old compared with 41.7% of controls. However, cases had a higher proportion of children with a gestation period <37 weeks compared with controls ($P = 0.04$).

## Differential Methylation of the *PRF1* Enhancer MSR

The methylation levels observed in cases and controls of the discovery set across the CpGs assayed in the MSR of the *PRF1* enhancer are plotted in Figure 2 as means with 95% CIs. Assessment of normality revealed an outlier control sample in the discovery set. The matched case–control trio containing the outlier sample was excluded from the final analysis. When analyzed by case–control status methylation levels at the first CpG of the E fragment (E1) were higher in cases. Cases showed a mean methylation level of 82.5% (95% CI 81.3–83.7) at the *PRF1* E1 locus compared with 80.3% (95% CI 79.1–81.5) in controls ($P = 0.02$). Bivariate conditional logistic regression demonstrated an OR of 1.24 for each 1% increase in methylation in association with case status in an unadjusted model (95% CI 1.03–1.50, $P = 0.02$).

Analysis of methylation levels against demographic variables and exposures revealed that children with a sibling <5 years of age had higher levels of methylation at the loci C2 and E1 in cord blood compared with children who had none ($P = 0.01$). Neonates of mothers reporting less than 11 years of formal education had higher levels of methylation at the loci F1 and F3 compared with children whose mothers enjoyed a greater education ($P = 0.05$ and 0.02).

Children born to smoking mothers as established through measurements of cord blood cotinine showed elevated methylation levels at the locus F3 ($P = 0.05$). Cord blood vitamin D levels were inversely associated with methylation levels at the loci F1 and F2 ($P = 0.05$ and 0.03). Interestingly, a significant inverse correlation between maternal age and methylation levels was observed at the CpGs C1, C2, and F2 (Spearman coefficients −0.23, −0.23, −0.30, respectively; all $P \leq 0.05$).

Results of the association analysis between DNA methylation levels at individual CpGs of the *PRF1* enhancer MSR and the variables explored in the discovery set are presented in Supplemental Digital Content (Table S2, http://links.lww.com/MD/A119). Having a sibling aged <5 years was associated with both primary exposure and outcome and therefore considered for adjustment. Differential methylation at *PRF1* E1 did not reach statistical significance after adjustment for sibling status (OR 1.19, 95% CI 0.98–1.45, $P = 0.07$).

To ensure the validity of the findings, DNA methylation levels of the 2 CpGs of the E fragment in the *PRF1* enhancer

**TABLE 1.** Demographic and Clinical Characteristics of the Ulm Birth Cohort Lower Respiratory Tract Infection Discovery Set According to Case–Control Status*

| Characteristic | Cases | Controls | Total | *P* |
|---|---|---|---|---|
| Neonatal | | | | |
| Number of neonates | 29 | 58 | 87 | |
| Gender | | | | |
| Male | 17 (58.6%) | 34 (58.6%) | 51 (58.6%) | |
| Female | 12 (41.4%) | 24 (41.4%) | 36 (41.4%) | |
| Birth season | | | | |
| November–April | 18 (62.1%) | 36 (62.1%) | 54 (62.1%) | |
| May–October | 11 (37.9%) | 22 (37.9%) | 33 (37.9%) | |
| Delivery mode | | | | 0.54 |
| Vaginal birth | 23 (79.3%) | 50 (86.2%) | 73 (83.9%) | |
| Cesarean section | 6 (20.7%) | 8 (13.8%) | 14 (16.1%) | |
| Gestation period | | | | 0.67 |
| ≥37 wk | 28 (96.6%) | 54 (93.1%) | 82 (94.3%) | |
| <37 wk | 1 (3.4%) | 4 (6.9%) | 5 (5.7%) | |
| Birth weight, kg | 3.4 (3.2, 3.7) | 3.4 (3.1, 3.8) | 3.4 (3.1, 3.8) | 0.83 |
| Breast-feeding duration, mo | | | | 0.78 |
| <3 | 7 (24.1%) | 14 (24.6%) | 21 (24.4%) | |
| >3 to ≤6 | 6 (20.7%) | 15 (26.3%) | 21 (24.4%) | |
| >6 to ≤9 | 8 (27.6%) | 10 (17.5%) | 18 (20.9%) | |
| >9 | 8 (27.6%) | 18 (31.6%) | 26 (30.2%) | |
| Cord blood vitamin D, nmol/L | 24.5 (16.1, 45.2) | 22.3 (15.6, 37.1) | 22.6 (15.7, 38.4) | 0.60 |
| Sibling <5 y old | | | | 0.01 |
| No | 11 (37.9%) | 40 (69%) | 51 (58.6%) | |
| Yes | 18 (62.1%) | 18 (31%) | 36 (41.4%) | |
| Maternal | | | | |
| Age, y | 33.0 (3.0, 35.5) | 32.3 (28.8, 34.9) | 32.8 (28.8, 35.0) | 0.60 |
| Country of birth | | | | 1.00 |
| Germany | 23 (79.3%) | 46 (79.3%) | 69 (79.3%) | |
| Outside Germany | 6 (20.7%) | 12 (20.7%) | 18 (20.7%) | |
| Maternal allergy | | | | 1.00 |
| No self-reported atopic condition | 22 (75.9%) | 44 (75.9%) | 66 (75.9%) | |
| Self-reported atopic condition | 7 (24.1%) | 14 (24.1%) | 21 (24.1%) | |
| Maternal education | | | | 0.12 |
| No school certificate or primary | 11 (37.9%) | 12 (21.1%) | 23 (26.7%) | |
| Secondary education and above | 18 (62.1%) | 45 (78.9%) | 63 (73.3%) | |
| Smoking (cord blood cotinine) | | | | 0.15 |
| No (<10 ng/mL) | 28 (96.6%) | 48 (84.2%) | 76 (88.4%) | |
| Yes (>10 ng/mL) | 1 (3.4%) | 9 (15.8%) | 10 (11.6%) | |
| Maternal BMI | | | | 0.41 |
| <18.5 (underweight) | 2 (6.9%) | 1 (1.8%) | 3 (3.5%) | |
| ≥18.5 to <25 (normal) | 19 (65.5%) | 39 (68.4%) | 58 (67.4%) | |
| ≥25 to <30 (overweight) | 5 (17.2%) | 14 (24.6%) | 19 (22.1%) | |
| >30 (obese) | 3 (10.3%) | 3 (5.3%) | 6 (7.0%) | |

BMI = body mass index.
* Categorical data reported as n (%), continuous data as median (25th, 75th percentiles). *P* for categorical variables calculated by Fisher exact test, continuous variables by Kruskal–Wallis test.

were assayed in a replication set comprising 30 matched trios, 30 cases and 60 controls, drawn from the same Ulm Birth Cohort. Assessment of normality revealed 2 outlier controls who were subsequently excluded together with matching samples from the final analysis. Mean cord blood DNA methylation at *PRF1* E1 was 82.6% (95% CI 81.5–83.8) in cases compared with 79.5% (95% CI 77.7–81.3) in controls ($P = 0.02$).

Bivariate conditional logistic regression confirmed the association between higher methylation and case status at the locus *PRF1* E1 in the unadjusted model (OR 1.25, 95% CI 1.04–1.50, $P = 0.02$) and after adjustment for sibling status (OR 1.21, 95% CI 1.00–1.46, $P = 0.05$). Adjustment for gestational age (instead of siblings <5 years of age) resulted in an OR of 1.25 (95% CI 1.04–1.50, $P = 0.02$), equal to the unadjusted model.

Table 3 summarizes the results of the logistic regression analysis of the cord blood DNA methylation levels at *PRF1* E1 according to case status in both LRTI discovery and replication sets of the Ulm Birth Cohort.

**TABLE 2.** Demographic and Clinical Characteristics of the Ulm Birth Cohort Lower Respiratory Tract Infection Replication Set According to Case–Control Status*

| Characteristic | Cases | Controls | Total | *P* Value |
|---|---|---|---|---|
| Neonatal | | | | |
| Number of neonates | 30 | 60 | 90 | |
| Gender | | | | |
| Male | 17 (56.7%) | 34 (56.7%) | 51 (56.7%) | |
| Female | 13 (43.3%) | 26 (43.3%) | 39 (43.3%) | |
| Birth season | | | | |
| November–April | 17 (56.7%) | 34 (56.7%) | 51 (56.7%) | |
| May–October | 13 (43.3%) | 26 (43.3%) | 39 (43.3%) | |
| Delivery mode | | | | 0.15 |
| Vaginal birth | 22 (73.3%) | 52 (86.7%) | 74 (82.2%) | |
| Cesarean section | 8 (26.7%) | 8 (13.3%) | 16 (17.8%) | |
| Gestation period | | | | 0.04 |
| ≥37 wk | 25 (83.3%) | 58 (96.7%) | 83 (92.2%) | |
| <37 wk | 5 (16.7%) | 2 (3.3%) | 7 (7.8%) | |
| Birth weight, kg | 3.4 (2.9, 3.9) | 3.4 (3.1, 3.9) | 3.4 (3.0, 3.9) | 0.92 |
| Breast-feeding duration, mo | | | | 0.07 |
| <3 | 13 (43.3%) | 10 (16.7%) | 23 (25.6%) | |
| >3 to ≤6 | 5 (16.7%) | 17 (28.3%) | 22 (24.4%) | |
| >6 to ≤9 | 5 (16.7%) | 14 (23.3%) | 19 (21.1%) | |
| >9 | 7 (23.3%) | 19 (31.7%) | 26 (28.9%) | |
| Cord blood vitamin D, nmol/L | 21.3 (15.3, 42.4) | 21.2 (16.4, 37.6) | 27.2 (16.4, 40.2) | 0.93 |
| Sibling <5 y old | | | | 0.19 |
| No | 13 (43.3%) | 35 (58.3%) | 48 (53.3%) | |
| Yes | 17 (56.7%) | 25 (41.7%) | 42 (46.7%) | |
| Maternal | | | | |
| Age, y | 32.0 (28.6, 36.9) | 32.0 (28.1, 35.0) | 31.5 (28.1, 35.1) | 0.46 |
| Country of birth (mother) | | | | 0.80 |
| Germany | 22 (73.3%) | 46 (76.7%) | 68 (75.6%) | |
| Outside Germany | 8 (26.7%) | 14 (23.3%) | 22 (24.4%) | |
| Maternal allergy | | | | 0.14 |
| No self-reported atopic condition | 18 (60.0%) | 46 (76.7%) | 64 (71.1%) | |
| Self-reported atopic condition | 12 (40.0%) | 14 (23.3%) | 26 (28.9%) | |
| Maternal education | | | | 0.32 |
| No school certificate or primary | 10 (35.7%) | 15 (25.0%) | 25 (28.4%) | |
| Secondary education and above | 18 (64.3%) | 45 (75.0%) | 63 (71.6%) | |
| Smoking (cord blood cotinine) | | | | 0.20 |
| No (<10 ng/mL) | 23 (79.3%) | 52 (89.7%) | 75 (86.2%) | |
| Yes (>10 ng/mL) | 6 (20.7%) | 6 (10.3%) | 12 (13.8%) | |
| Maternal BMI | | | | 0.31 |
| <18.5 (underweight) | 2 (6.7%) | 0 (0.0%) | 2 (2.2%) | |
| ≥18.5 to <25 (normal) | 19 (63.3%) | 41 (69.5%) | 60 (67.4%) | |
| ≥25 to <30 (overweight) | 6 (20.0%) | 12 (20.3%) | 18 (20.2%) | |
| >30 (obese) | 3 (10.0%) | 6 (10.2%) | 9 (10.1%) | |

BMI = body mass index.
* Categorical data reported as n (%), continuous data as median (25th, 75th percentiles). *P* for categorical variables calculated by Fisher exact test, continuous variables by Kruskal–Wallis test.

## DISCUSSION

In this study, we investigated cord blood DNA methylation in an enhancer region of *PRF1* in relation to frequency of physician-recorded LRTIs during the first year of life using a prospective case–control design of matched trios, each composed of 1 case and 2 controls, through 2 phases, a discovery and a replication phase. Our results support an association between higher DNA methylation at a CpG in the MSR of the *PRF1* proximal enhancer and increased risk of LRTIs during the first year of life. Matching for season of birth controlled for the seasonal variation in RSV prevalence and vitamin D status, both important determinants of risk.[18,19]

*PRF1* methylation was assayed in blood, a composite tissue of different cell types, each with a distinctive DNA methylation signature.[20] Accordingly, the DNA methylation alterations identified might reflect a shift in cellular composition between cases and controls[21] since measurements were performed on DNA derived from the entire leucocyte compartment of cord blood and not specific cell populations. This

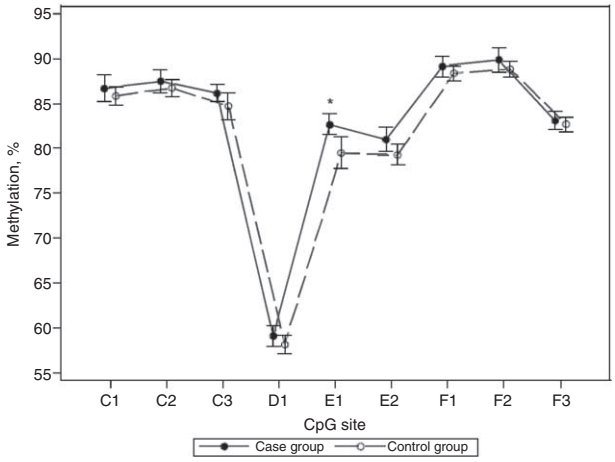


**FIGURE 2.** DNA methylation levels in 9 cytosine–guanine dinucleotides of the perforin gene proximal enhancer methylation-sensitive region in the lower respiratory tract infections discovery case–control set nested in the Ulm Birth Cohort (n = 87). Letters C, D, E, and F denote separate pyrosequencing assays and numbers the order of the cytosine–guanine dinucleotides examined. Methylation levels are plotted as means and 95% confidence intervals. $^{*}P = 0.02$. CpG = cytosine-phosphate-guanine.

limitation however does not compromise the relevance of the findings when considering methylation changes as markers of disease susceptibility. Liang and Cookson argued recently that the overall methylation variance in DNA from a heterogeneous tissue such as blood would mask rather than magnify associations in case the variance in a locus arises from a particular cell type,[22] in this study CTL and NK cells. Another limitation of the study is the lack of distinction between bacterial and viral causes of LRTIs in the cohort. Additionally, the biomaterial available did not allow for extraction of messenger RNA precluding an investigation of the correlation between methylation variation and expression levels of *PRF1* in the study population.

The variant CpG, identified here as E1, lies within a STAT5 responsive element in the proximal enhancer of the gene. Members of the STAT family play highly specific roles in innate and acquired immunity through their interaction with members of the *Janus* family of protein tyrosine kinases (Janus kinases [JAKs]) in what is referred to as the JAK/STAT signaling pathway.[23] The molecules STAT5a and STAT5b are encoded by closely linked genes on human chromosome 17, share above 90% homology in amino acid sequence,[24] and contribute to normal T-cell activation.[25] STAT5a and STAT5b are crucial for NK cell proliferation and cytolysis. Expression of *PRF1* in NK cells and CTLs is under the regulation of STAT5, which in turn is stimulated by IL-2Rβ signaling.[5]

STAT5b deficiency features a phenotype comprising severe postnatal growth retardation, growth hormone insensitivity, and characteristically an immunodeficiency disorder.[26] Immunologically, these patients have moderate lymphopenia with very low numbers of NK and T cells as well as functional defects of T cells, and present a clinical picture of severe pulmonary disease associated with viral infections, compounded by atopy and autoimmune diatheses from as early as the first 6 months of life.[27] Methylation of the *PRF1* MSR suppresses expression of the gene in healthy CD8+ T cells while demethylation of the locus is associated with increased gene expression.[6] Kaplan et al reported demethylation of the MSR as a contributing factor to *PRF1* overexpression in CD4+ T cells of patients with active systemic lupus erythematosus, cells that normally demonstrate no *PRF1* expression secondary to methylation of the MSR.[28]

Higher methylation levels at the variant CpG identified here, E1, were associated with having a sibling younger than 5 years and premature birth (less than 37 weeks gestation) in the discovery and replication sets, respectively. Both are established risk factors for RSV infection in infants[29,30] and were also associated with the LRTI outcome in this study. Statistical analysis was adjusted accordingly. Higher levels of methylation at adjacent CpGs of the MSR were noted in relation to additional risk factors of RSV disease in infants, namely, lower maternal education, maternal smoking,[29] and lower cord blood vitamin D levels.[19] These associations suggest a role for DNA methylation in mediating risk of infection, assuming that alterations in DNA methylation reflect the maternal environment as indicated by social and behavioral determinants such as education and smoking. Indeed, a growing body of literature points toward the impact of maternal factors on the epigenome of the newborn, for instance, BMI[8,30] and smoking,[10,31,32] commensurate with the developmental origins of health and disease hypothesis[33,34] whereby DNA methylation links adverse intrauterine events and exposures with disease later in life. A negative correlation was observed at 3 CpGs of the *PRF1* MSR with maternal age. Using a genome-wide approach, Adkins et al established a strong correlation between cord blood DNA methylation and maternal age. The correlations were predominantly negative, in all but 9 of 144 CpGs that achieved genome-wide significance, and involved disproportionately CpGs within CpG islands. The authors argued that the observed shift represented an accentuation of a normal pattern of decreased DNA methylation at promoter regions.[35]

To overcome the limitations of the current study future investigations should consider analysis of individual cell types when feasible or account for the heterogeneous cellular composition of blood based on knowledge of distinct cell-specific methylation patterns using recently developed methods.[36,37] Moreover, detailed knowledge of clinical and epidemiological covariates, including causative respiratory pathogens, disease severity, family history, immunization, and nutritional status, as well as other exposures emanating from a rural or an urban

**TABLE 3.** Conditional Logistic Regression of DNA Methylation Levels at the Cytosine–Guanine Dinucleotide E1 of the Perforin Gene Enhancer Methylation-Sensitive Region in the Lower Respiratory Tract Infection Discovery and Replication Sets of the Ulm Birth Cohort by Case Status: Adjusted and Unadjusted Models

| | OR (95% CI)* | *P* |
|---|---|---|
| Discovery | | |
| Unadjusted | 1.24 (1.03, 1.50) | 0.02 |
| Adjusted† | 1.19 (.98, 1.45) | 0.07 |
| Replication | | |
| Unadjusted | 1.25 (1.04, 1.50) | 0.02 |
| Adjusted† | 1.21 (1.00, 1.46) | 0.05 |

CI = confidence interval, OR = odds ratio.
* Odds ratios calculated per 1% difference in methylation level.
† Model adjusted for having a sibling <5 year old.

environment, is necessary to expand on confounding analysis and probe the multitude of potential effects on the epigenome.

*PRF1* overexpression in T lymphocyte subsets has been suggested as a contributing mechanism in the pathogenesis of asthma.[38] Likewise, increased expression of *PRF1* and granzyme B in invariant NK cells marking heightened cytotoxicity has been identified in allergic asthma patients.[39] Considering the evidence in support of an association between respiratory tract infections in infancy and childhood asthma[40–44] it is worth speculating whether alterations in the DNA methylation patterns of *PRF1* under the influence of maternal and environmental factors and in response to respiratory infections play a role in the genesis of asthma or atopic lung disease. Furthermore, appropriately powered studies could help investigate whether *PRF1* methylation could be utilized as a predictive marker of severe infant respiratory infections and their sequelae.

Here, we presented preliminary evidence supporting an association between alterations in DNA methylation at functional CpGs of the *PRF1* MSR and subsequent risk of a common infection in a longitudinal pediatric cohort. The study derives its strength from its prospective 2-stage design and the available covariates for analysis but has the drawbacks of a limited sample size, cellular heterogeneity of the sample, and potential confounding. However, the findings remain valid when considering the DNA methylome as a proxy for the environment and variant patterns as avenues for potential effects on the genome. To comprehensively capture these effects, longitudinal epigenome-wide DNA methylation studies investigating the impact of the maternal environment on the neonatal epigenome and infectious outcomes in the growing child are needed.

## REFERENCES


1. Nair H, Simoes EA, Rudan I, et al. Global and regional burden of hospital admissions for severe acute lower respiratory infections in young children in 2010: a systematic analysis. *Lancet.* 2013;381:1380–1390.
2. Nair H, Nokes DJ, Gessner BD, et al. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. *Lancet.* 2010;375:1545–1555.
3. Voskoboinik I, Dunstone MA, Baran K, et al. Perforin: structure, function, and role in human immunopathology. *Immunol Rev.* 2010;235:35–54.
4. Zhang J, Scordi I, Smyth MJ, et al. Interleukin 2 receptor signaling regulates the perforin gene through signal transducer and activator of transcription (Stat)5 activation of two enhancers. *J Exp Med.* 1999;190:1297–1308.
5. Pipkin ME, Rao A, Lichtenheld MG. The transcriptional control of the perforin locus. *Immunol Rev.* 2010;235:55–72.
6. Lu Q, Wu A, Ray D, et al. DNA methylation and chromatin structure regulate T cell perforin gene expression. *J Immunol.* 2003;170:5124–5132.
7. Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. *Nat Rev Genet.* 2007;8:253–262.
8. Teh AL, Pan H, Chen L, et al. The effect of genotype and in utero environment on interindividual variation in neonate DNA methylomes. *Genome Res.* 2014;24:1064–1074.
9. Dominguez-Salas P, Moore SE, Baker MS, et al. Maternal nutrition at conception modulates DNA methylation of human metastable epialleles. *Nat Commun.* 2014;5:3746.
10. Joubert BR, Haberg SE, Nilsen RM, et al. 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. *Environ Health Perspect.* 2012;120:1425–1431.
11. Markunas CA, Xu Z, Harlid S, et al. Identification of DNA methylation changes in newborns related to maternal smoking during pregnancy. *Environ Health Perspect.* 2014;122:1147–1153.
12. Weyermann M, Adler G, Brenner H, et al. The mother as source of *Helicobacter pylori* infection. *Epidemiology.* 2006;17:332–334.
13. Liu J, Morgan M, Hutchison K, et al. A study of the influence of sex on genome wide methylation. *PLoS One.* 2010;5:e10028.
14. Terletskaia-Ladwig E, Enders G, Schalasta G, et al. Defining the timing of respiratory syncytial virus (RSV) outbreaks: an epidemiological study. *BMC Infect Dis.* 2005;5:20.
15. Holick MF. Vitamin D: a D-Lightful health perspective. *Nutr Rev.* 2008;66 (10 suppl 2):S182–S194.
16. Narasimhan S, Falkenberg VR, Khin MM, et al. Determination of quantitative and site-specific DNA methylation of perforin by pyrosequencing. *BMC Res Notes.* 2009;2:104.
17. Tost J, Gut IG. DNA methylation analysis by pyrosequencing. *Nat Protoc.* 2007;2:2265–2275.
18. Belderbos ME, Houben ML, Wilbrink B, et al. Cord blood vitamin D deficiency is associated with respiratory syncytial virus bronchiolitis. *Pediatrics.* 2011;127:e1513–e1520.
19. Luczynska A, Logan C, Nieters A, et al. Cord blood 25(OH)D levels and the subsequent risk of lower respiratory tract infections in early childhood: the Ulm birth cohort. *Eur J Epidemiol.* 2014;29:585–594.
20. Zilbauer M, Rayner TF, Clark C, et al. Genome-wide methylation analyses of primary human leukocyte subsets identifies functionally important cell-type-specific hypomethylated regions. *Blood.* 2013;122:e52–e60.
21. Reinius LE, Acevedo N, Joerink M, et al. Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility. *PLoS One.* 2012;7:e41361.
22. Liang L, Cookson WO. Grasping nettles: cellular heterogeneity and other confounders in epigenome-wide association studies. *Hum Mol Genet.* 2014;23:R83–R88.
23. Kisseleva T, Bhattacharya S, Braunstein J, et al. Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene.* 2002;285:1–24.
24. Lin JX, Mietz J, Modi WS, et al. Cloning of human Stat5B. Reconstitution of interleukin-2-induced Stat5A and Stat5B DNA binding activity in COS-7 cells. *J Biol Chem.* 1996;271:10738–10744.
25. Moriggl R, Sexl V, Piekorz R, et al. Stat5 activation is uniquely associated with cytokine signaling in peripheral T cells. *Immunity.* 1999;11:225–230.
26. Jenks JA, Seki S, Kanai T, et al. Differentiating the roles of STAT5B and STAT5A in human CD4+ T cells. *Clin Immunol.* 2013;148:227–236.
27. Nadeau K, Hwa V, Rosenfeld RG. STAT5b deficiency: an unsuspected cause of growth failure, immunodeficiency, and severe pulmonary disease. *J Pediatr.* 2011;158:701–708.
28. Kaplan MJ, Lu Q, Wu A, et al. Demethylation of promoter regulatory elements contributes to perforin overexpression in CD4+ lupus T cells. *J Immunol.* 2004;172:3652–3661.
29. Simoes EA. Environmental and demographic risk factors for respiratory syncytial virus lower respiratory tract disease. *J Pediatr.* 2003;143 (5 suppl):S118–S126.
30. Liu X, Chen Q, Tsai HJ, et al. Maternal preconception body mass index and offspring cord blood DNA methylation: exploration of early life origins of disease. *Environ Mol Mutagen.* 2014;55:223–230.

31. Murphy SK, Adigun A, Huang Z, et al. Gender-specific methylation differences in relation to prenatal exposure to cigarette smoke. *Gene.* 2012;494:36–43.

32. Breton CV, Byun HM, Wenten M, et al. Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. *Am J Respir Crit Care Med.* 2009;180:462–467.

33. Reynolds RM, Jacobsen GH, Drake AJ. What is the evidence in humans that DNA methylation changes link events in utero and later life disease? *Clin Endocrinol.* 2013;78:814–822.

34. Szyf M. The early life social environment and DNA methylation: DNA methylation mediating the long-term impact of social environments early in life. *Epigenetics.* 2011;6:971–978.

35. Adkins RM, Thomas F, Tylavsky FA, et al. Parental ages and levels of DNA methylation in the newborn are correlated. *BMC Med Genet.* 2011;12:47.

36. Houseman EA, Accomando WP, Koestler DC, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics.* 2012;13:86.

37. Liu Y, Aryee MJ, Padyukov L, et al. Epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis. *Nat Biotechnol.* 2013;31:142–147.

38. Arnold V, Balkow S, Staats R, et al. Increase in perforin-positive peripheral blood lymphocytes in extrinsic and intrinsic asthma. *Am J Respir Crit Care Med.* 2000;161:182–186.

39. Nguyen KD, Vanichsarn C, Nadeau KC. Increased cytotoxicity of CD4+ invariant NKT cells against CD4+CD25hiCD127lo/− regulatory T cells in allergic asthma. *Eur J Immunol.* 2008;38:2034–2045.

40. Nafstad P, Magnus P, Jaakkola JJ. Early respiratory infections and childhood asthma. *Pediatrics.* 2000;106:E38.

41. Klinnert MD, Nelson HS, Price MR, et al. Onset and persistence of childhood asthma: predictors from infancy. *Pediatrics.* 2001;108:E69.

42. Szabo SM, Gooch KL, Korol EE, et al. A population-based study of childhood respiratory morbidity after severe lower respiratory tract infections in early childhood. *J Pediatr.* 2014;165:123–128e123.

43. Sigurs N, Bjarnason R, Sigurbergsson F, et al. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *Am J Respir Crit Care Med.* 2000;161:1501–1507.

44. Garcia-Garcia ML, Calvo C, Casas I, et al. Human metapneumovirus bronchiolitis in infancy is an important risk factor for asthma at age 5. *Pediatr Pulmonol.* 2007;42:458–464.