

## OBSTETRICS

# DNA methylation at imprint regulatory regions in preterm birth and infection

Ying Liu, MPH; Cathrine Hoyo, PhD; Susan Murphy, PhD; Zhiqing Huang, MD, PhD; Francine Overcash, MPH; Jennifer Thompson, MD; Haywood Brown, MD; Amy P. Murtha, MD



**OBJECTIVE:** To aid in understanding long-term health consequences of intrauterine infections in preterm birth, we evaluated DNA methylation at 9 differentially methylated regions that regulate imprinted genes by type of preterm birth (spontaneous preterm labor, preterm premature rupture of membranes, or medically indicated [fetal growth restriction and preeclampsia]) and infection status (chorioamnionitis or funisitis).

**STUDY DESIGN:** Data on type of preterm birth and infection status were abstracted from medical records and standardized pathology reports in 73 preterm infants enrolled in the Newborn Epigenetics Study, a prospective cohort study of mother-infant dyads in Durham, NC. Cord blood was collected at birth, and infant DNA methylation levels at the *H19*, *IGF2*, *MEG3*, *MEST*, *SGCE/PEG10*, *PEG3*, *NNAT*, and *PLAGL1* differentially methylated regions were measured using bisulfite pyrosequencing. One-way analyses of variance and logistic regression models were used to compare DNA methylation levels by type of preterm birth and infection status.

**RESULTS:** DNA methylation levels did not differ at any of the regions ( $P > .20$ ) between infants born via spontaneous preterm labor (average  $n = 29$ ), preterm premature rupture of membranes (average  $n = 17$ ), or medically indicated preterm birth (average  $n = 40$ ). Levels were significantly increased at *PLAGL1* in infants with chorioamnionitis ( $n = 10$ , 64.4%) compared with infants without chorioamnionitis ( $n = 63$ , 57.9%),  $P < .01$ . DNA methylation levels were also increased at *PLAGL1* for infants with funisitis ( $n = 7$ , 63.3%) compared with infants without funisitis ( $n = 66$ , 58.3%),  $P < .05$ .

**CONCLUSION:** Dysregulation of *PLAGL1* has been associated with abnormal development and cancer. Early-life exposures, including infection/inflammation, may affect epigenetic changes that increase susceptibility to later chronic disease.

**Key words:** chorioamnionitis, epigenetic, funisitis, imprinting, preterm birth

Cite this article as: Liu Y, Hoyo C, Murphy S, et al. DNA methylation at imprint regulatory regions in preterm birth and infection. *Am J Obstet Gynecol* 2013;208:x-ex-x-ex.

In the US, about 12% of all deliveries result in preterm births (PTB), defined as births occurring at less than 37 weeks' gestation.<sup>1</sup> PTB and low birth-

weight (LBW) are associated with not only significant perinatal and childhood morbidity but also long-term morbidity and increased risk of chronic diseases in adulthood.<sup>2-4</sup> In addition, PTB is a heterogeneous process that includes 3 different obstetric phenotypes: medically indicated PTB, preterm premature rupture of membranes (PPROM), and spontaneous preterm labor (PTL).<sup>5</sup> Although there are multiple risk factors for PTB, including infections such as chorioamnionitis and funisitis, mechanisms linking infection, PTB, and perinatal and long-term morbidity, which could be used for risk stratification, are still unclear.

Epigenetics has been postulated as the mechanism linking the early developmental environment to adult disease.<sup>6,7</sup> Studies of survivors of the Dutch and Chinese famines have shown that those exposed to famine during the periconceptional period had increased risk of obesity and chronic diseases in adulthood as well as persistent epigenetic

changes at multiple imprinted genes.<sup>8-13</sup>

In support of the role of epigenetics in preterm birth, recent studies have also found associations between DNA methylation levels of repetitive sequences or long interspersed nucleotide elements (LINE-1) and gestational age/PTB.<sup>14</sup> In addition, increased homocysteine and global DNA methylation levels were seen in the placenta of women with preeclampsia compared with normotensive women.<sup>15</sup> Moreover, a recent study found hypermethylation of multiple imprinted genes associated with spontaneous abortions and stillbirths, suggesting methylation alterations may play a role in pregnancy loss.<sup>16</sup>

The early origins of adult disease hypothesis postulates that the environment encountered in early life modifies risk of adult chronic disease, and this may include risk for poor birth outcomes. Mechanisms for these effects are still unclear, but are strongly suspected to include epigenetic modifications.<sup>17,18</sup> We sought to examine DNA methylation at

From the Department of Obstetrics and Gynecology, Duke University School of Medicine, Durham, NC.

Received Nov. 7, 2012; revised Jan. 14, 2013; accepted Feb. 4, 2013.

Financial support was provided by grant numbers R01ES016772 and R21ES014947 (National Institute of Environmental Health Sciences) and R01DK085173 (National Institute of Diabetes and Digestive and Kidney Diseases), National Institutes of Health.

The authors report no conflicts of interest.

Presented orally at the 33rd annual meeting of the Society for Maternal-Fetal Medicine, San Francisco, CA, Feb. 11-16, 2013.

The racing flag logo above indicates that this article was rushed to press for the benefit of the scientific community.

Reprints not available from the authors.

0002-9378/\$36.00

© 2013 Mosby, Inc. All rights reserved.

<http://dx.doi.org/10.1016/j.ajog.2013.02.006>

differentially methylated regulatory regions associated with multiple imprinted genes. These genes function as critical growth effectors and regulators of development.<sup>8,13,19</sup> Our study was designed to evaluate these differentially methylated regions (DMRs) in relation to type of preterm birth and infection status.

## MATERIALS AND METHODS

### Study participants

Study participants included all singleton infants born prematurely (<37 weeks) to the cohort of women enrolled in the Newborn Epigenetics Study (NEST); a prospective study of mother/infant dyads aimed at investigating the effects of in utero exposures on epigenetic profiles and phenotypes in children. The target population and methods for participant identification and enrollment have been described in detail previously.<sup>20,21</sup> Briefly, between 2007 and 2011, pregnant women were recruited during their first or second trimester visits to prenatal clinics serving Duke Hospital or Durham Regional Hospital, the only 2 obstetric facilities in Durham County. Eligibility criteria were age 18 years and older, intention to use Duke Hospital or Durham Regional Hospital for delivery, and English and Spanish speaking. Subjects were excluded if they planned to give up custody of their children or had HIV because of the limited research on the interaction of HIV infection with potential DNA methylation alterations in the offspring. Of the 181 singleton preterm births in this cohort, infection, complete parturition, and methylation data were available in a subset of 73 mother-infant pairs. These mother-infant pairs are similar to the larger group of 181 with respect to maternal age ( $P = .3284$ ), race ( $P = .638$ ), maternal body mass index (BMI) ( $P = .8156$ ), type of preterm birth ( $P = .106$ ), and proportion of births with chorioamnionitis ( $P = .620$ ) and funisitis ( $P = .619$ ). The study protocol was approved by the Duke University Institutional Review Board.

### Data collection

Demographic information was collected from mothers at enrollment. Gestational

age at enrollment ranged from 5-36 weeks (mean, 17.5 weeks; standard deviation [SD], 9.0). Gestational age at enrollment was slightly higher in the group of 73 mother-infant pairs (mean, 20.8 weeks; SD, 9.1) as compared with the larger group of 181 (mean, 17.5 weeks; SD, 9.0),  $P = .01$ . Pregnant women self-reported birthdate (maternal age was computed by subtracting delivery date from birth date), current health status, parity, marital status, and usual weight and height at last menstrual period (LMP) for BMI. Because of changes in some questions in more recent demographic surveys, this study only characterizes women as “black” or “white”, and the remaining women (ones who chose “Asian,” “Native-American,” “Hispanic,” or “Other”) are considered “other.” Women who chose “white” or “caucasian” were classified as “white.” Women were classified as “black” if they identified themselves as “black/African-American” or if they identified as “biracial/other” and their mother was “black/African-American.” Smoking status was harmonized between questionnaires to 3 categories, none, smoking during pregnancy, and quitting during pregnancy, as previously described.<sup>22</sup> Education level was harmonized to less than high school, high school or GED, college, and graduate school.

After delivery of the infant, trained personnel abstracted parturition data from medical records including gestational age at birth, infant sex, delivery mode, and birthweight. PTB was defined as gestational age <37 weeks, and LBW was defined as birthweight <2500 g.<sup>23</sup> Trained abstractors examined the medical records of all preterm infants to determine the type of PTB. Medically indicated PTB was defined as emergent delivery because of a medical complication, intrauterine growth restriction (IUGR), or preeclampsia (defined for this study as medical order for magnesium and 24-hour urine protein level >300 mg).<sup>24</sup> PPROM was defined by a clinical diagnosis (listed on problem list) or presence of 2 of 3 of the following positive tests (pool, fern, or nitrazine). All other PTBs were defined as spontaneous. When the medical record was unclear, an obstetrician (A.P.M.) determined the

phenotype based on clinical expertise. Standard pathologic reports from the placenta of preterm infants were used to assess infection status. Chorioamnionitis was defined as a pathologic diagnosis of Stage II or III chorioamnionitis.<sup>25</sup> Funisitis was also a standardized pathologic diagnosis.<sup>25</sup> All data were collected and deidentified in STATA 12.0 (StataCorp, College Station, TX).

### DNA methylation analysis

Infant cord blood specimens were collected at birth. Samples were collected in EDTA-containing vacutainer tubes and centrifuged using standard protocols to allow for collection of plasma and buffy coat, with buffy coat used for DNA extraction (Qiagen, Valencia, CA). Specimens were stored at  $-80^{\circ}\text{C}$  until time of analysis. DNA was extracted using Puregene reagents according to the manufacturer's protocol (Qiagen) and quantity and quality assessed using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Infant genomic DNAs (800 ng) were modified by treatment with sodium bisulfite using the Zymo EZ DNA Methylation kit (Zymo Research, Irvine, CA). Bisulfite treatment of denatured DNA converts all unmethylated cytosines to uracils, leaving methylated cytosines unchanged, allowing for quantitative measurement of cytosine methylation status. Pyrosequencing was performed using a Pyromark Q96 MD pyrosequencer (Qiagen). The bisulfite pyrosequencing assays were used to quantitatively measure the level of methylation at CpG sites contained within 9 imprinted DMRs. DMRs analyzed were the paternally methylated *IGF2* DMR, *H19* DMR, *MEG3-IG* DMR (located intergenic to *DLK1* and *MEG3*) and *MEG3* DMR (promoter), and the maternally methylated *PEG3* DMR, *MEST* DMR, *SGCE/PEG10* DMR, *NNAT* DMR, and *PLAGL1* DMR. For all DMRs except *PLAGL1*, pyrosequencing assay design, genomic coordinates, assay conditions and assay validation are described in detail elsewhere.<sup>26, 27</sup> Assays were designed to query established imprinted gene DMRs using the Pyromark Assay Design Software (Qiagen). For amplification of *PLAGL1* from bisulfite

modified DNAs, polymerase chain reaction (PCR) primers were forward, 5'-GTA GGG TAG GTG TTT GGG TGT T-3' and reverse, 5'-[biotin]CRA CAA AAA CAC ACC CTC CTC-3'. PCR was performed as previously described<sup>26</sup> using the following conditions: 95°C for 15 minutes, 55 cycles of 94°C for 30 seconds, 68°C for 30 seconds, and 72°C for 30 seconds, and a final 10-minute extension at 72°C. Pyrosequencing was performed using primer 5'-TGA GGT GTT TGG GTG TT-3' to analyze sequence at genomic coordinates 144,329,179 to 144,329,209 on chromosome 6 (UCSC Genome Browser, Feb. 2009 release, GRCh37/hg19). PCR conditions were optimized to produce a single, robust amplification product. Defined mixtures of fully methylated and unmethylated control DNAs were used to show a linear increase in detection of methylation values as the level of input DNA methylation increased (Pearson  $r > 0.98$  for all DMRs). Once optimal conditions were defined, each DMR was analyzed using the same amount of input DNA from each specimen (40 ng, assuming complete recovery after bisulfite modification). Percent methylation for each CpG cytosine was determined using Pyro Q-CpG Software (Qiagen).

### Statistical analysis

Fisher exact tests were used to determine associations between type of PTB and infection status (chorioamnionitis and funisitis). To examine the role of epigenetics, we then assessed each DMR for normality using the Kolmogorov-Smirnov test. We found that with the exception of *SGCE/PEG10* ( $P < .01$ ), all other infant DMRs were normally distributed ( $P > .05$ ). Confirmatory factor analysis for individual maternal and infant CpGs revealed Cronbach's alphas for all DMRs were  $> .74$ , suggesting mean methylation levels for each DMR could be used in models. One-way analyses of variance (ANOVA) were used to compare infant DNA methylation differences at the 9 DMRs by type of PTB (medically indicated, PPROM, or spontaneous PTL).  $t$  tests were used to compare infant DNA methylation differences at DMRs by infection status (chorioamnionitis or fu-

TABLE 1

### Summary of characteristics of preterm mother-infant dyads in NEST

Variable	n (%)
<b>Age, y</b>	
<20	6 (3)
20-24	51 (28)
25-29	33 (18)
30-34	48 (27)
35-39	36 (20)
>40	7 (4)
<b>Race</b>	
White	47 (28)
Black	93 (54)
Other	31 (18)
<b>BMI</b>	
<18.5	9 (6)
18.5 to <25	41 (29)
25 to <30	41 (29)
30 to <35	30 (21)
35 to <40	13 (10)
>40	7 (5)
<b>Smoking</b>	
None	109 (63)
Smoking	35 (20)
Quit	30 (17)
<b>Delivery mode</b>	
Vaginal	91 (51)
Cesarean section	90 (49)
<b>Type of PTB</b>	
Medically indicated	67 (37)
PPROM	43 (24)
Spontaneous	71 (39)
<b>Chorioamnionitis</b>	
Chorioamnionitis	22 (16)
None	113 (84)
<b>Gestational age at birth</b>	
Range	24-36 wks
Mean	33.9 wks
SD	2.9 wks

(continued)

TABLE 1

### Summary of characteristics of preterm mother-infant dyads in NEST (continued)

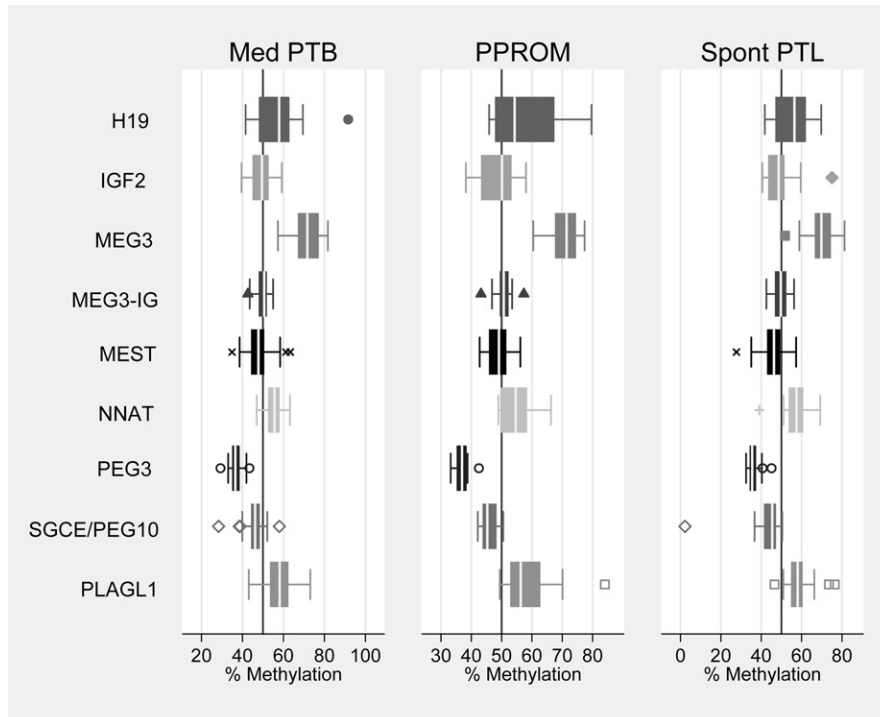
Variable	n (%)
<b>Marital status</b>	
Never married	54 (31)
Married	65 (38)
Widowed	0 (0)
Living with partner	38 (22)
Divorced/separated	13 (8)
Other	1 (1)
<b>Parity</b>	
Nulliparous	54 (32)
Multiparous	117 (68)
<b>Health</b>	
Excellent	17 (10)
Very good	49 (29)
Good	76 (44)
Fair	26 (15)
Poor	3 (2)
<b>Education</b>	
Less than high school	14 (8)
High school/GED	58 (34)
Some college	82 (48)
Graduate school	17 (10)
<b>Baby sex</b>	
Male	102 (56)
Female	79 (44)
<b>Birthweight</b>	
Range	580-3765 g
Mean birthweight	2346 g
SD	677 g
<b>Funisitis</b>	
Funisitis	16 (12)
None	119 (88)

BMI, body mass index; GED, general equivalency degree; NEST, newborn epigenetics study; PPROM, preterm premature rupture of membranes; PTB, preterm birth; SD, standard deviation.

Liu. DNA methylation in preterm birth and infection. *Am J Obstet Gynecol* 2013.

nisitis). Wilcoxon-rank sum tests were used to confirm differences found by  $t$  testing in all DMRs that were not normally distributed. For DMRs that differed by type of PTB or infection status,

**FIGURE 1**  
**Distribution of DMRs by type of PTB**



Median and interquartile range of infant DNA methylation levels at nine DMRs by type of PTB. There are no significant differences between mean methylation levels at any of the 9 DMRs by type of PTB,  $P > .20$ . Reference line represents 50% methylation.

DMR, differentially methylated regions; PTB, preterm birth.

Liu. DNA methylation in preterm birth and infection. *Am J Obstet Gynecol* 2013.

logistic regression models were fit to examine associations between PTB or infection status and DNA methylation at DMRS. Models were initially fit with all variables considered clinically relevant including maternal age at delivery, maternal race, health status, parity, marital status, maternal BMI, smoking, education level, delivery mode, infant sex, infant birthweight, and gestational age at delivery. A backward stepwise approach was used to refine the model, and log likelihood tests for individual covariates were used to create the final parsimonious model.

## RESULTS

### Demographic characteristics

Table 1 describes the characteristics of the 181 preterm mother-infant dyads. Maternal age ranged from 18–49, with mean age of 30 years and SD of 6.6 years. Thirty-eight percent of women were married, 31% were never married, and

the rest were either living with a partner, divorced/separated or chose “other.” Fifty-four percent of the mothers were black, 28% were white, and 18% were grouped as other, which included women choosing a race/ethnicity of Hispanic, Asian, Native American, or other. The majority of the women were multiparous (68%). BMI ranged from 16–67 with a mean BMI of 28, SD of 7.7. The majority of the women endorsed good, very good, or excellent health with 17% endorsing fair or poor health. 20% of women smoked during pregnancy, whereas 17% reported quitting. The majority (>50%) of women had some college education or more. Fifty-one percent of the births were delivered vaginally, and 49% were delivered via cesarean section. Fifty-six percent of the infants delivered were male, and 44% were female. Of the PTBs, 37% were medically indicated, 24% were a result of PPROM, and 39% were spontaneous.

Birthweight ranged from 580–3765 g with a mean birthweight of 2346 g (SD, 677 g). Gestational age at birth ranged from 24–36 weeks with a mean of 33.9 weeks (SD, 2.9 weeks). Sixteen percent of the PTB infants had chorioamnionitis, and 12% had funisitis. Of the 22 cases of chorioamnionitis and 16 cases of funisitis, 13 cases overlapped and had pathologic features of both.

### Types of PTB and infection

When examining type of preterm birth by infection status, more cases of chorioamnionitis occurred in preterm births that were the result of PPROM ( $n = 14$ ) or spontaneous PTL ( $n = 8$ ) when compared with medically indicated PTB ( $n = 0$ ), Fisher exact  $P$  value  $< .001$ , which was expected based on previous epidemiologic studies.<sup>5</sup> A similar phenomenon was observed in those infants with funisitis with more cases in preterm births that occurred as a result of PPROM ( $n = 8$ ) or spontaneous PTL ( $n = 7$ ) when compared with medically indicated PTB ( $n = 1$ ), Fisher exact  $P$  value = .003. In addition, chorioamnionitis and funisitis were highly correlated (correlation coefficient, 0.6449;  $P < .001$ ).

### Type of PTB, infection, and DNA methylation at imprint regulatory regions

When examining the role of epigenetics in our subset of 73 preterm infants with completed methylation data, infant DNA methylation levels did not differ by type of PTB (medically indicated, PPROM, and spontaneous PTL) at any of the 9 DMRs examined,  $P > .20$  (Figure 1). We found that infants born with pathologic evidence of chorioamnionitis had higher methylation levels at the DMRs regulating the imprinted genes *PLAGL1* and *PEG3* (Table 2,  $P < .05$ ). We also found that infants born with pathologic evidence of funisitis had higher methylation levels at the DMR regulating the imprinted gene *PLAGL1* (Table 2,  $P < .05$ ). After Bonferroni correction for multiple testing, differences at *PLAGL1* by chorioamnionitis persisted ( $P = .0026$ ). In logistic regression models (Table 3), this association per-



**TABLE 2**  
**Infant DNA methylation by infection status**

DMR	n	Mean % Methylation (SE)	n	Mean % Methylation (SE)	t test P value	Rank sum P value
Chorioamnionitis		None	Chorioamnionitis			
H19	43	54.6 (1.25)	6	54.1 (2.68)	.8882	.9999
IGF2	38	49.4 (1.07)	5	48.9 (3.05)	.8824	.8945
MEG3-IG	58	49.6 (0.40)	10	50.6 (1.19)	.3829	.4358
MEG3	58	71.0 (0.81)	9	70.1 (1.84)	.6858	.6994
MEST	59	47.7 (0.68)	9	44.9 (2.35)	.1670	.4311
PEG3	62	36.2 (0.27)	10	38.1 (0.49)	.0081	.0050
NNAT	58	55.5 (0.54)	6	58.2 (2.20)	.1282	.1971
SGCE/PEG10	61	45.6 (0.54)	10	42.9 (4.57)	.2239	.4670
PLAGL1 <sup>a</sup>	63	57.9 (0.70)	10	64.4 (2.87)	.0026	.0218
Funisitis		None	Funisitis			
H19	46	54.6 (1.19)	3	53.3 (4.16)	.7824	.8348
IGF2	41	49.1 (1.02)	2	53.5 (4.59)	.3685	.2732
MEG3-IG	61	49.8 (0.39)	7	49.4 (1.57)	.7398	.3800
MEG3	61	70.6 (0.78)	6	73.0 (1.98)	.3606	.3564
MEST	61	47.7 (0.66)	7	43.8 (2.82)	.0717	.2459
PEG3	65	36.3 (0.27)	7	37.6 (0.63)	.1105	.0837
NNAT	60	55.7 (0.54)	4	55.8 (2.73)	.9573	.5982
SGCE/PEG10	64	45.6 (0.52)	7	41.4 (6.58)	.1065	.4234
PLAGL1	66	58.3 (0.78)	7	63.3 (2.48)	.0517	.0492

DMR, differentially methylated region; SE, standard error.

<sup>a</sup> Bonferroni correction for multiple testing,  $P < .005$ .

Liu. DNA methylation in preterm birth and infection. *Am J Obstet Gynecol* 2013.

sisted, and increasing levels of methylation at *PLAGL1* was associated with increased risk for chorioamnionitis, after adjusting for the significant covariates maternal BMI and gestational age at birth (odds ratio [OR], 1.22; 95% confidence interval [CI], 1.04–1.44;  $P = .015$ ). Decreasing gestational age at birth and increasing maternal BMI were also associated with increased risk of chorioamnionitis. Infant birthweight and gestational age at birth were highly correlated (correlation coefficient, 0.7661;  $P < .0001$ ). As a result, although both were associated with chorioamnionitis, only gestational age at birth was included in overall models. After adjusting for significant covariates maternal age and infant sex, increasing levels of methylation at *PLAGL1* was associated with increased risk for funisitis (OR, 1.17; 95% CI, 1.03–1.32;  $P = .016$ ). Increasing maternal age

and male gender were also associated with increased risk for funisitis. Gestational age at birth did not differ for funisitis ( $P = .380$ ), and it was not included in overall models. Figure 2 shows the distribution of DNA methylation levels at *PLAGL1* by infection status (chorioamnionitis or funisitis).

### COMMENT

We examined the role of DNA methylation at multiple imprint regulatory regions implicated in growth and development by type of PTB and infection status. Both chorioamnionitis and funisitis were more common in PPRM and spontaneous PTL as compared with medically indicated PTB. We found no differences in DNA methylation at any of the 9 DMRs examined by type of PTB. However, in preterm infants with patho-

logically defined chorioamnionitis or funisitis, DNA methylation may be increased at the *PLAGL1* DMR, an association that persisted after adjustment for significant covariates.

PTB has been associated with significant mortality and morbidity, both at birth and later in development. Children born preterm are at increased risk for respiratory distress and apnea, hypoglycemia and infant death.<sup>4</sup> Although long-term effects of PTB are still poorly understood, children and adolescents born preterm are at increased risk for behavioral and developmental disorders.<sup>4</sup> In addition, epidemiologic studies have seen associations between LBW and PTB and increased risk for obesity and cardiovascular disease in adulthood.<sup>6</sup> Environmental factors such as nutrition, inflammation, and toxic exposures may increase susceptibility for PTB or infec-

**TABLE 3**  
**Logistic regression model of infection and *PLAGL1* with covariates**

Variable	OR <sup>a</sup>	95% CI	P value
<b>Chorioamnionitis</b>			
<i>PLAGL1</i>	1.22	1.04–1.44	.015
Gestational age at birth	0.695	0.527–0.917	.010
Maternal BMI	1.06	0.980–1.14	.147
<b>Funisitis</b>			
<i>PLAGL1</i>	1.17	1.03–1.32	.016
Maternal age	1.09	0.97–1.23	.134
Sex (M->F)	0.11	0.009–1.35	.085

BMI, body mass index; CI, confidence interval; OR, odds ratio.

<sup>a</sup> OR mutually adjusted for all the covariates listed in each model.

Liu. DNA methylation in preterm birth and infection. *Am J Obstet Gynecol* 2013.

tion through epigenetic perturbations and subsequent alterations in early growth and development.<sup>28</sup> Chorioamnionitis has been associated with inflammation and changes in chemokine profiles and signaling.<sup>29</sup> These endocrine disruptors may alter appropriate maintenance of epigenetic profiles during pregnancy, resulting in changes that ad-

versely affect birth outcomes and may also be perpetuated through somatic cell division to increase susceptibility to disease in adulthood.<sup>30</sup>

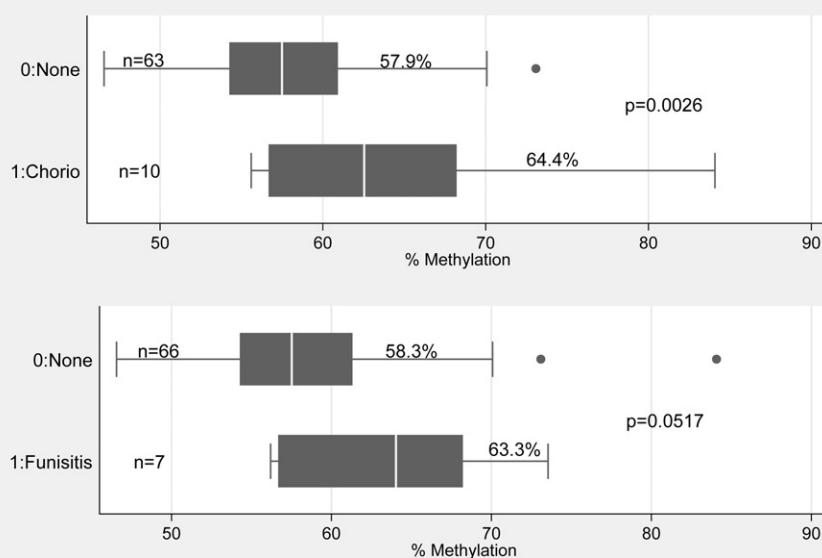
Our study found differences in DNA methylation at the *PLAGL1* DMR associated with chorioamnionitis and funisitis. *PLAGL1* is located at chromosome 6q24.2 and encodes a zinc-finger tran-

scription factor thought to be involved in tumor development and growth via IGF2 signaling.<sup>31</sup> Aberrant epigenetic marks at this site are also associated with transient neonatal diabetes mellitus (TNDM), a disorder of growth restriction and hyperglycemia.<sup>32</sup> Moreover, the *PLAGL1* gene product is thought to function as a major regulatory hub that coordinates the expression of a network of genes, including many that are imprinted such as *IGF2*, *H19*, and *MEST*.<sup>33</sup> The *IGF2/H19* domain is one of the best characterized imprinted regions, located at chromosome 11p15.5, and was originally associated with Beckwith-Wiedemann syndrome, a somatic overgrowth disorder associated with increased risk of Wilms tumor and hepatoblastoma.<sup>34</sup> Elevated levels of DNA methylation at *H19* have also been observed in assisted reproductive technology (ART-related) pregnancy loss as compared with spontaneously conceived pregnancy loss.<sup>35</sup>

We found changes at the *PLAGL1* DMR associated with infection, and these alterations may not only affect growth/development at birth but also be maintained throughout life to increase susceptibility to adult-onset disease, as postulated by the developmental origins of adult disease hypothesis. This has potential implications for early screening of infection during pregnancy and novel epigenetic-based therapies to modulate clinical sequelae of infection related PTB. It is also possible that these marks were established earlier in pregnancy and may have increased risk for infection during pregnancy; however, our study cannot differentiate between these 2 possibilities. Interestingly, we found no differences in DNA methylation at any of the 9 DMRs examined by type of PTB, and this may reflect the heterogeneity in causes and risk factors for PTB.

Our study is one of the first to examine epigenetic regions in regard to type of PTB and infection. We used rigorous definitions verified by obstetric experts for the various types of PTB and standardized pathologic reports to define infection status. We also investigated methylation at multiple regions thought to be important in growth and development. Interestingly, we found that male

**FIGURE 2**  
**Infant DNA methylation at *PLAGL1* by infection**



Median and interquartile range of infant DNA methylation levels at *PLAGL1* by chorioamnionitis and funisitis. Mean DNA methylation levels at *PLAGL1* are higher in infants who experienced chorioamnionitis (difference 6.5%,  $P = .0026$ ) and funisitis (difference 5.0%,  $P = .0517$ ) compared with infants who experienced no infection at birth.

Liu. DNA methylation in preterm birth and infection. *Am J Obstet Gynecol* 2013.

sex was associated with increased risk of funisitis as compared with female sex, which may relate to sex-dependent epigenetic findings reported previously.<sup>13,22</sup> Limitations include the small sample size, cross-sectional nature of the study, which prevents assessment of causation, and relatively small regions of the imprintome examined. Although multiple testing may be a concern, the finding of differences in methylation at *PLAGL1* by chorioamnionitis persisted after Bonferroni correction, and the difference by funisitis may be limited by small sample size. In addition, only cord blood methylation was examined in this study; however, prior studies have shown that DNA methylation at DMRs of these imprinted genes is consistent across tissues and cord blood fractions<sup>26</sup> including *PLAGL1*, for which methylation of cord blood polymorphonuclear cells (PMNs) is slightly lower than that in matched peripheral blood mononuclear cells (PBMCs) by 0.68% (n = 25; S.K.M., unpublished data). Future larger studies are required to verify these intriguing preliminary results and to further characterize these complex epigenetic networks and their interactions. ■

#### ACKNOWLEDGMENTS

We are especially grateful to the women and families involved in the Newborn Epigenetics Study and acknowledge the expert contributions of study coordinator Stacy Murray, research nurse Tammy Bishop, and laboratory technicians Carole Grenier, Erin Erginer, Cara Davis, and Allison Barratt.

#### REFERENCES

- Martin JA, Hamilton BE, Sutton PD, et al. Births: final data for 2007. *Natl Vital Stat Rep* 2010;58:1-125.
- Iams JD, Romero R, Culhane JF, Goldenberg RL. Primary, secondary, and tertiary interventions to reduce the morbidity and mortality of preterm birth. *Lancet* 2008;371:164-75.
- McCormick MC. The contribution of low birth weight to infant mortality and childhood morbidity. *N Engl J Med* 1985;312:82-90.
- Saigal S, Doyle LW. An overview of mortality and sequelae of preterm birth from infancy to adulthood. *Lancet* 2008;371:261-9.
- Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet* 2008;371:75-84.
- Barker DJ. The origins of the developmental origins theory. *J Intern Med* 2007;261:412-7.
- Dolinoy DC, Weidman JR, Jirtle RL. Epigenetic gene regulation: linking early developmental environment to adult disease. *Reprod Toxicol* 2007;23:297-307.
- Heijmans BT, Tobi EW, Stein AD, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A* 2008;105:17046-9.
- Huang C, Li Z, Wang M, Martorell R. Early life exposure to the 1959-1961 Chinese famine has long-term health consequences. *J Nutr* 2010;140:1874-8.
- Lumey LH, Stein AD, Kahn HS, Romijn JA. Lipid profiles in middle-aged men and women after famine exposure during gestation: the Dutch Hunger Winter Families Study. *Am J Clin Nutr* 2009;89:1737-43.
- Ravelli GP, Stein ZA, Susser MW. Obesity in young men after famine exposure in utero and early infancy. *N Engl J Med* 1976;295:349-53.
- St Clair D, Xu M, Wang P, et al. Rates of adult schizophrenia following prenatal exposure to the Chinese famine of 1959-1961. *JAMA* 2005;294:557-62.
- Tobi EW, Lumey LH, Talens RP, et al. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. *Hum Mol Genet* 2009;18:4046-53.
- Burris HH, Rifas-Shiman SL, Baccarelli A, et al. Associations of LINE-1 DNA Methylation with Preterm Birth in a Prospective Cohort Study. *J Dev Orig Health Dis* 2012;3:173-81.
- Kulkarni A, Chavan-Gautam P, Mehendale S, Yadav H, Joshi S. Global DNA methylation patterns in placenta and its association with maternal hypertension in pre-eclampsia. *DNA Cell Biol* 2011;30:79-84.
- Pliushch G, Schneider E, Weise D, et al. Extreme methylation values of imprinted genes in human abortions and stillbirths. *Am J Pathol* 2010;176:1084-90.
- Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med* 2008;359:61-73.
- Menon R, Conneely KN, Smith AK. DNA methylation: an epigenetic risk factor in preterm birth. *Reprod Sci* 2012;19:6-13.
- Skaar DA, Li Y, Bernal AJ, Hoyo C, Murphy SK, Jirtle RL. The human imprintome: regulatory mechanisms, methods of ascertainment, and roles in disease susceptibility. *ILAR J* 2012;53:337-54.
- Liu Y, Murphy SK, Murtha AP, et al. Depression in pregnancy, infant birth weight and DNA methylation of imprint regulatory elements. *Epigenetics* 2012;7:735-46.
- Hoyo C, Murtha AP, Schildkraut JM, et al. Folic acid supplementation before and during pregnancy in the Newborn Epigenetics Study (NEST). *BMC Public Health* 2011;11:46.
- 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.
- Kramer MS. The epidemiology of adverse pregnancy outcomes: an overview. *J Nutr* 2003;133:1592S-6S.
- American College of Obstetricians and Gynecologists. ACOG practice bulletin no. 33. Diagnosis and management of preeclampsia and eclampsia. January 2002. *Int J Gynaecol Obstet* 2002;77:67-75.
- Redline RW, Faye-Petersen O, Heller D, Qureshi F, Savell V, Vogler C. Amniotic infection syndrome: nosology and reproducibility of placental reaction patterns. *Pediatr Dev Pathol* 2003;6:435-48.
- Murphy SK, Huang Z, Hoyo C. Differentially methylated regions of imprinted genes in prenatal, perinatal and postnatal human tissues. *PLoS One* 2012;7:e40924.
- Nye MD, Hoyo C, Huang Z, et al. Associations between methylation of paternally expressed gene 3 (PEG3), cervical intraepithelial neoplasia and invasive cervical cancer. *PLoS One* 2013;8:e56325.
- Waterland RA, Michels KB. Epigenetic epidemiology of the developmental origins hypothesis. *Annu Rev Nutr* 2007;27:363-88.
- Redline RW. Inflammatory response in acute chorioamnionitis. *Semin Fetal Neonatal Med* 2012;17:20-5.
- Robins JC, Marsit CJ, Padbury JF, Sharma SS. Endocrine disruptors, environmental oxygen, epigenetics and pregnancy. *Front Biosci (Elite Ed)* 2011;3:690-700.
- Van Dyck F, Declercq J, Braem CV, Van de Ven WJ. *PLAG1*, the prototype of the *PLAG* gene family: versatility in tumour development (review). *Int J Oncol* 2007;30:765-74.
- Temple IK, Mackay DJG, Docherty LE. Diabetes mellitus, 6q24-related transient neonatal. In: Pagon RA, Bird TD, Dolan CR, et al. eds. *GeneReviews*[Internet]. Seattle, WA: University of Washington, Seattle; 1993-2005 [updated 2012 Sep 27].
- Varrault A, Gueydan C, Delalbre A, et al. *Zac1* regulates an imprinted gene network critically involved in the control of embryonic growth. *Dev Cell* 2006;11:711-22.
- Engel JR, Smallwood A, Harper A, et al. Epigenotype-phenotype correlations in Beckwith-Wiedemann syndrome. *J Med Genet* 2000;37:921-6.
- Zechner U, Pliushch G, Schneider E, et al. Quantitative methylation analysis of developmentally important genes in human pregnancy losses after ART and spontaneous conception. *Mol Hum Reprod* 2010;16:704-13.