

# Erythrocyte folate concentrations, CpG methylation at genomically imprinted domains, and birth weight in a multiethnic newborn cohort

Cathrine Hoyo<sup>1,2,\*</sup>, Anne Kjersti Daltveit<sup>3</sup>, Edwin Iversen<sup>4</sup>, Sara E Benjamin-Neelon<sup>5</sup>, Bernard Fuemmeler<sup>6</sup>, Joellen Schildkraut<sup>7</sup>, Amy P Murtha<sup>8</sup>, Francine Overcash<sup>2</sup>, Adriana C Vidal<sup>2</sup>, Frances Wang<sup>7</sup>, Zhiqing Huang<sup>9</sup>, Joanne Kurtzberg<sup>10</sup>, Victoria Seewaldt<sup>11</sup>, Michele Forman<sup>12</sup>, Randy L Jirtle<sup>13</sup>, and Susan K Murphy<sup>9</sup>

<sup>1</sup>Department of Biological Sciences; North Carolina State University; Raleigh, NC USA; <sup>2</sup>Department of Obstetrics and Gynecology; Division of Clinical Epidemiology; School of Medicine; Duke University; Durham, NC USA; <sup>3</sup>Department of Global Public Health and Primary Care; University of Bergen; Bergen, Norway; <sup>4</sup>Department of Statistics; Duke University; Durham, NC USA; <sup>5</sup>Department of Community and Family Medicine; Duke University School of Medicine and Duke Global Health Institute; Durham, NC USA; <sup>6</sup>Department of Community and Family Medicine; Duke University; Durham, NC USA; <sup>7</sup>Department of Community and Family Medicine; Duke Cancer Institute; Duke University; Durham, NC USA; <sup>8</sup>Department of Obstetrics and Gynecology; Division of Maternal-Fetal Medicine; School of Medicine; Duke University; Durham, NC USA; <sup>9</sup>Department of Obstetrics and Gynecology; Division of Gynecologic Oncology; School of Medicine; Duke University; Durham, NC USA; <sup>10</sup>Duke University Department of Pediatrics; Duke Cancer Institute; School of Medicine; Duke University; Durham, NC USA; <sup>11</sup>Department of Medicine; Division of Oncology; School of Medicine; Duke University; Durham, NC USA; <sup>12</sup>Department of Nutritional Sciences; University of Texas; Austin, TX USA; <sup>13</sup>Department of Oncology; McArdle Laboratory for Cancer Research; University of Wisconsin-Madison; Madison, WI USA

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Epigenetic mechanisms are proposed to link maternal concentrations of methyl group donor nutrients with the risk of low birth weight. However, empirical data are lacking. We have examined the association between maternal folate and birth weight and assessed the mediating role of DNA methylation at nine differentially methylated regions (DMRs) of genomically imprinted genes in these associations. Compared with newborns of women with folate levels in the lowest quartile, birth weight was higher in newborns of mothers in the second ( $\beta = 143.2$ ,  $se = 63.2$ ,  $P = 0.02$ ), third ( $\beta = 117.3$ ,  $se = 64.0$ ,  $P = 0.07$ ), and fourth ( $\beta = 133.9$ ,  $se = 65.2$ ,  $P = 0.04$ ) quartiles, consistent with a threshold effect. This pattern of association did not vary by race/ethnicity but was more apparent in newborns of non-obese women. DNA methylation at the *PLAGL1*, *SGCE*, *DLK1/MEG3* and *IGF2/H19* DMRs was associated with maternal folate levels and also birth weight, suggestive of threshold effects. *MEG3* DMR methylation mediated the association between maternal folate levels and birth weight ( $P = 0.06$ ). While the small sample size and partial scope of examined DMRs limit our conclusions, our data suggest that, with respect to birth weight, no additional benefits may be derived from increased maternal folate concentrations, especially in non-obese women. These data also support epigenetic plasticity as a key mechanistic response to folate availability during early fetal development.

## Introduction

Folate is a water-soluble vitamin that decreases the risk of neural tube defects when taken periconceptionally. This has led to recommendations that women at risk of pregnancy supplement their daily diet with 400  $\mu\text{g}$  of folic acid (FA), a synthetic form of folate.<sup>1–6</sup> Because approximately half of US pregnancies are unplanned, FA fortification of milled grain was instituted in the late 1990s. A commensurate increase in erythrocyte folate concentrations was reported in women of reproductive age within two years.<sup>7</sup> What is described as “supra-physiological” levels of circulating folate<sup>8–10</sup> has been reported in a quarter of the US population since FA fortification began.<sup>7</sup> Approximately 10% of pregnant women report taking supplements containing FA in doses exceeding 1000  $\mu\text{g}$  per day, the maximum tolerable limit

recommended by the Institute of Medicine.<sup>11</sup> The long-term effects of high folate levels are unknown and remain an active topic of investigation.

Recent animal data demonstrate that high periconceptional and prenatal FA intake result in growth retardation that is partly rescued by maternal methylenetetrahydrofolate reductase (Mthfr) deficiency,<sup>12</sup> an enzyme central to folate metabolism.<sup>13</sup> In humans, evidence of associations between maternal folate concentrations and fetal growth is conflicting, as high levels have been linked to both low and high birth weight,<sup>14–24</sup> while other studies found no associations.<sup>25–31</sup> Extremes of the birth weight distribution have been associated with a wide range of childhood and adult-onset chronic diseases and conditions including obesity, impaired glucose tolerance, type 2 diabetes, coronary artery disease, congestive heart failure and some cancers.<sup>32–36</sup> Periconceptional and

\*Correspondence to: Cathrine Hoyo; Email: choyo@ncsu.edu

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**Table 1.** Characteristics of 496 study participants by maternal race/ethnicity

| Characteristic of study participants         | Non-Hispanic Black<br>(n = 189)<br>#(%) | Non-Hispanic White<br>(n = 133)<br>#(%) | Hispanic<br>(n = 154)<br>#(%) | Others<br>(n = 20)<br>#(%) | P values  |
|--|---|---|-------------------------------|----------------------------|-----------|
| <b>Maternal age</b>                          |   |   |                               |                            |           |
| 18–20 y                                      | 33 (17.5)                               | 3 (2.3)                                 | 16 (10.4)                     | 2 (10.0)                   | < 0.0001  |
| 21–25 y                                      | 73 (38.6)                               | 19 (14.3)                               | 46 (29.9)                     | 3 (15.0)                   |           |
| 25–30 y                                      | 42 (22.2)                               | 45 (33.8)                               | 42 (27.3)                     | 6 (30.0)                   |           |
| 31–35 y                                      | 21 (11.1)                               | 54 (40.6)                               | 35 (22.7)                     | 5 (25.0)                   |           |
| 36+years                                     | 20 (10.6)                               | 12 (9.0)                                | 15 (9.7)                      | 4 (20.0)                   |           |
| <b>Maternal Marital Status</b>               |   |   |                               |                            |           |
| Not partnered (never married/ divorced)      | 106 (56.1)                              | 12 (9.0)                                | 37 (24.0)                     | 4 (20.0)                   | < 0.0001* |
| Living with partner (cohabit/married)        | 71 (37.6)                               | 120 (90.2)                              | 114 (74.0)                    | 16 (80.0)                  |           |
| Other  | 7 (3.7)                                 | 1 (0.8)                                 | 3 (2.0)                       | 0 (0.0)                    |           |
| Missing                                      | 5 (2.6)                                 | 0 (0.0)                                 | 0 (0.0)                       | 0 (0.0)                    |           |
| <b>Maternal Education</b>                    |   |   |                               |                            |           |
| < High School                                | 5 (2.7)                                 | 3 (2.3)                                 | 62 (40.3)                     | 2 (10.0)                   | < 0.0001  |
| High School - < college                      | 99 (52.7)                               | 19 (14.3)                               | 75 (48.7)                     | 7 (35.0)                   |           |
| College graduate                             | 69 (36.7)                               | 55 (41.4)                               | 10 (6.5)                      | 4 (20.0)                   |           |
| Graduate/professional school                 | 15 (7.9)                                | 55 (41.4)                               | 7 (4.5)                       | 7 (35.0)                   |           |
| Missing                                      | 1 (0.5)                                 | 1 (0.8)                                 | 0 (0.0)                       | 0 (0.0)                    |           |
| <b>Parity</b>                                |   |   |                               |                            |           |
| None   | 50 (26.5)                               | 43 (32.3)                               | 33 (21.4)                     | 6 (30.0)                   | 0.02      |
| One  | 44 (23.2)                               | 44 (33.1)                               | 32 (20.8)                     | 6 (30.0)                   |           |
| Two  | 34 (18.0)                               | 25 (18.8)                               | 36 (23.4)                     | 3 (15.0)                   |           |
| Three or more                                | 61 (32.3)                               | 21 (15.8)                               | 53 (34.4)                     | 5 (25.0)                   |           |
| <b>Maternal BMI pre-pregnancy</b>            |   |   |                               |                            |           |
| < 25   | 60 (31.6)                               | 75 (56.4)                               | 37 (24.0)                     | 12 (60.0)                  | < 0.0001  |
| 25- < 30                                     | 50 (26.5)                               | 26 (19.6)                               | 62 (40.3)                     | 6 (30.0)                   |           |
| 30- < 35                                     | 33 (17.5)                               | 22 (16.5)                               | 21 (13.6)                     | 1 (5.0)                    |           |
| 35- < 40                                     | 21 (11.1)                               | 4 (3.0)                                 | 10 (6.5)                      | 0 (0.00)                   |           |
| 40+  | 19 (10.1)                               | 4 (3.0)                                 | 5 (3.3)                       | 0 (0.0)                    |           |
| Missing                                      | 6 (3.2)                                 | 2 (1.5%)                                | 19 (12.3)                     | 1 (5.0)                    |           |
| <b>Mean BMI (sd)</b>                         | 29.4 (8.2)                              | 25.2 (5.8)                              | 27.5 (5.5)                    | 23.4 (3.2)                 | < 0.0001  |
| <b>Maternal cigarette smoking</b>            |   |   |                               |                            |           |
| Ever smoked                                  | 16 (8.5)                                | 29 (68.2)                               | 8 (5.2)                       | 4 (20.0)                   | < 0.0001  |
| Quit sometime during pregnancy               | 48 (25.4)                               | 18 (13.5)                               | 6 (3.9)                       | 2 (10.0)                   |           |
| Never  | 125 (66.1)                              | 85 (63.9)                               | 139 (90.2)                    | 14 (70.0)                  |           |
| Missing                                      | 0 (0.0)                                 | 1 (0.8)                                 | 1 (0.7)                       | 0 (0.0)                    |           |
| <b>Gestational age a delivery</b>            |   |   |                               |                            |           |
| > 37 wk                                      | 157 (83.1)                              | 116 (87.2)                              | 135 (87.7)                    | 16 (80.0)                  | 0.5       |
| < = 37weeks                                  | 32 (16.9)                               | 17 (12.8)                               | 19 (12.3)                     | 4 (20.0)                   |           |
| <b>Sex of offspring</b>                      |   |   |                               |                            |           |
| Male   | 93 (49.2)                               | 75 (56.4)                               | 72 (46.8)                     | 9 (45.0)                   | 0.4       |
| Female                                       | 96 (50.8)                               | 58 (43.6)                               | 82 (53.2)                     | 11 (55.0)                  |           |
| <b>Prenatal physical activity</b>            |   |   |                               |                            |           |
| No   | 130 (68.8)                              | 119 (89.5)                              | 114 (74.0)                    | 17 (85.0)                  | 0.0002    |
| Yes  | 59 (31.2)                               | 14 (10.5)                               | 40 (26.0)                     | 3 (15.0)                   |           |
| <b>Gestational/Type II diabetes mellitus</b> |   |   |                               |                            |           |
| Yes  | 14 (7.4)                                | 5 (3.8)                                 | 6 (4.5)                       | 0                          | 0.001*    |
| No   | 174 (92.1)                              | 128 (96.2)                              | 146 (95.5)                    | 20 (100)                   |           |
| Missing                                      | 1 (0.5)                                 | 0                                       | 2 (1.3)                       | 0                          |           |
| <b>Birth weight (mean, sd)</b>               | 3164.0 (535.1)                          | 3452.9 (548.7)                          | 3342.1 (498.2)                | 3126.1 (512.2)             | < 0.0001  |
| <b>Maternal folate levels (µg/L) in all</b>  | 194.6 (83.2)                            | 247.6 (66.6)                            | 222.3 (64.1)                  | 204.4 (83.6)               | < 0.0001  |
| 1st trimester blood draw (n = 326)           | 191.3 (79.9)                            | 249.3 (66.1)                            | 215.5 (61.2)                  | 214.7 (55.1)               | < 0.0001  |
| 2nd trimester blood draw (n = 160)           | 198.2 (92.9)                            | 243.8 (69.5)                            | 228.9 (69.4)                  | 175.5 (119.7)              | 0.02      |
| 3rd trimester blood draw (n = 6)             | 208.3 (57.4)                            | 188.0 (NA)                              | 229.0 (35.2)                  | 283.5 (NA)                 | 0.4       |

\*P values based on Fisher's exact test. White pregnant mothers are older, more likely to be married, have a higher educational attainment, have a lower pre-pregnancy BMI > 30 or diabetes . NA-no variability.

**Table 2.** Regression coefficients for the association between maternal folate and offspring birth weight

| Characteristic                    | Erythrocyte folate concentrations<br>Regression coefficient (SE) P value |
|-----------------------------------|--|
| Intercept                         | 3044.68 (179.08)   |
| Maternal folate concentrations    | Referents  |
| 1st Quartile                      |  |
| 2nd Quartile                      | 143.18 (63.24) 0.024   |
| 3rd Quartile                      | 117.32 (63.99) 0.067   |
| 4th Quartile                      | 133.88 (65.20) 0.041   |
| <b>Male sex</b>                   | 140.82 (43.55) 0.001   |
| <b>Gestational age &lt; 37 wk</b> | -620.70 (61.84) 0.000  |
| <b>Race/ethnicity</b>             |  |
| Non-Hispanic Black                | -243.63 (60.96) 0.000  |
| Hispanic                          | -121.74 (62.43) 0.052  |
| Other                             | -213.85 (117.48) 0.069   |
| Non-Hispanic White                | Referents  |
| <b>Pre-pregnancy BMI</b>          | 11.88 (3.40) 0.001   |

After adjusting for maternal race/ethnicity, offspring sex, cigarette smoking, gestational age at delivery, gestational age at blood draw, physical activity, maternal pre-pregnancy body mass index (BMI) and delivery route, we note a relationship between maternal folate concentrations and birth weight consistent with a threshold effect.

perinatal exposure to FA supplements has also been linked to wheezing in mice<sup>37</sup> and humans,<sup>38</sup> as well as childhood asthma.<sup>39</sup> While these early studies support the developmental origins of adult disease susceptibility hypothesis,<sup>40,41</sup> low and high birth weight are used as an approximation for a wide range of adverse prenatal exposures, for which targeted interventions and monitoring will be difficult without mechanistic insights.

Epigenetic shifts in response to restricted or excessive prenatal nutrition have been hypothesized to mediate the associations between low birth weight and adult disease susceptibility, and could serve as intermediate endpoints for early monitoring<sup>42-44</sup>; however, empirical human data remain limited. In viable yellow agouti (*A<sup>y</sup>*) mice, supplementing pregnant dams with one carbon cycle nutrients, including FA, resulted in shifts in the coat color distribution in the offspring, from yellow to brown.<sup>45</sup> The shift to brown coat color was directly attributed to increased DNA methylation at a cryptic promoter in an intracisternal A particle (IAP) upstream of the *A<sup>y</sup>* locus, which, when unmethylated, drives ectopic *Agouti* expression. In humans, differences in DNA methylation of the differentially methylated regions (DMRs) regulating the imprinted expression of *insulin like growth factor-2* (*IGF2*), were reported in individuals exposed to FA during the periconceptional<sup>46</sup> and prenatal periods.<sup>47,48</sup> However, historical reports of self-reported FA intake may be fraught with recall concerns and do not account for individual variation in diet and metabolism. Higher *IGF2* and lower *PEG3* DMR methylation levels were recently reported in DNA from umbilical cord leukocytes of infants born to women with higher prenatal erythrocyte folate.<sup>49</sup> Herein, we expand on the repertoire of imprinted genes

for which we examined DNA methylation (*IGF2/H19*, *DLK1/MEG3*, *PEG1/MEST*, *PEG3*, *PEG10/SGCE*, *PLAGL1*, and *NNAT*) in relation to maternal folate levels in early pregnancy, and assess the extent to which DNA methylation changes at regulatory regions of these genes may influence birth weight.

## Results

### Study participants

Characteristics of pregnant women and their offspring previously shown to influence birth weight and/or DNA methylation are presented for each race/ethnicity in Table 1. Non-Hispanic Blacks comprised 38%, Hispanics 31%, Non-Hispanic Whites 27%, and other ethnicities including Asians and Native Americans 4% of the study population. Advanced maternal age (36+ years) was reported by 11% of pregnant women, comparable among all ethnic groups, although Non-Hispanic Blacks (56%) and Hispanics (40%) were more likely to be younger (< 25 y) at enrollment than Non-Hispanic Whites (17%) ( $P < 0.0001$ ). Mean maternal BMI was 28 Kg/m<sup>2</sup>, which was comparable in Hispanics and Non-Hispanic Blacks, but significantly lower in Non-Hispanic Whites ( $P < 0.0001$ ). Birth weight was lowest among infants born to Non-Hispanic Black women and highest in Non-Hispanic White women, despite similar proportions of infants born  $\leq 37$  wk gestation (16.9% of Non-Hispanic Blacks, vs. 12.8% Non-Hispanic Whites and 12.3% of Hispanics,  $P = 0.5$ ). Lower socio-economic correlates, including living without a partner and lower educational attainment, were also more frequent among Hispanics and Non-Hispanic Blacks ( $P < 0.0001$ ). Parity was higher in ethnic minorities, compared with Non-Hispanic White women, with approximately one-sixth of Non-Hispanic Black and Hispanic women reporting parity of three or more compared with one-fifth of Non-Hispanic Whites ( $P = 0.04$ ). Gestational age at enrollment was 12.7 wk (sd = 4.8 wk) and was comparable among ethnic groups as were gestational age at delivery, delivery mode (vaginal vs. caesarean section) and sex distribution of infants ( $P > 0.05$ ). Average maternal erythrocyte folate concentrations were 217.8 ng/ml (interquartile range 167–278) and it was highest in Non-Hispanic White women, moderate in Hispanics and lowest in Non-Hispanic Black women. We accounted for these differences in multivariable models and by restriction.

### Associations between maternal folate concentrations and birth weight

The average birth weight (3295 g) was higher in males (3350 g) than females (3237 g) and higher in infants born to Hispanics and Non-Hispanic Whites (3342 g and 3453 g, respectively) than Non-Hispanic Blacks (3164 g). After adjusting for sex, ethnicity/race, gestational age at delivery, delivery mode, cigarette smoking, prenatal physical activity and maternal pre-pregnancy BMI, we observed a relationship between maternal folate levels and birth weight consistent with a threshold effect (Table 2). Compared with infants born to women with folate levels in the lowest quartile, birth weight increased among newborns of women with folate levels in the second quartile ( $\beta_{2nd\ quartile} = 143.18$ , se = 63.24,

$P = 0.02$ ); however, the magnitude of this relationship did not significantly change when folate levels in the third and fourth were examined ( $\beta_{3rd\ quartile} = 117.32$ ,  $se = 63.99$  and  $\beta_{4th\ quartile} = 133.88$ ,  $se = 65.2$ ). Despite large differences in folate levels and birth weight among ethnic groups, this pattern of association did not vary significantly by race/ethnicity. Repeating these analyses stratified by maternal obesity (BMI > 30 vs. BMI  $\leq$  30) further adjusted for race/ethnicity revealed a pattern of association consistent with a threshold effect only in the subgroup of infants born to women with BMI  $\leq$  30 kg/m<sup>2</sup>, whereas a non-statistically significant U-shaped relationship was observed between maternal folate levels and birth weight when pre-pregnancy BMI was > 30 kg/m<sup>2</sup> (Fig. 1). However, none of the cross-product terms for maternal folate quartiles and continuous BMI in statistical models for the association between maternal folate and birth weight were statistically significant ( $P = 0.19$ ). Repeating these analyses in those with gestational age  $\geq$  37 wk and those with folate measured from blood obtained at gestational age < 13 wk, did not alter our findings, suggesting our results were unlikely to be influenced by preterm birth or gestational age at blood draw.

#### Association between maternal folate concentrations and DNA methylation

We evaluated the extent to which epigenetic mechanisms may mediate the association between maternal folate levels and birth weight by examining associations between maternal folate and DNA methylation levels at each of the nine DMRs measured (Table 3). We also examined associations between DMR methylation and birth weight, overall and stratified by pre-pregnancy BMI (Table 4), prior to evaluating the subset of DMRs associated with both maternal folate levels and birth weight for potential mediation. Mean DNA methylation levels at DMRs regulating *MEG3* was 67%,  $se = 0.64$ . Mean DNA methylation levels at DMRs were also estimated for *MEG3-IG* (mean = 49.9%,  $se = 0.69$ ), *NNAT* (mean = 57%,  $se = 0.84$ ), *PEG10/SGCE* (mean = 45%,  $se = 0.37$ ), *PLAGL1* (mean = 50%,  $se = 0.46$ ), *MEST* (mean = 44%,  $se = 0.48$ ), *H19* (mean = 46%,  $se = 0.37$ ), *IGF2* (mean = 51.0,  $se = 0.49$ ), and *PEG3* (mean = 36%,  $se = 0.26$ ).

After adjusting for maternal race/ethnicity, cigarette smoking, delivery route, gestational age at blood draw, gestational age at delivery, physical activity and maternal pre-pregnancy obesity among all participants, we observed decreases in offspring DNA methylation levels with increasing maternal folate levels in at least one quartile, which were consistent with a threshold effect at the *MEG3*, *NNAT*, *PEG10/SGCE*, *MEG3-IG*, *PLAGL1*, *PEG3* and the *H19* DMRs. Linear increases in methylation levels with increasing maternal folate levels were noted at the *IGF2* and *PEG1/MEST* DMRs. These associations were statistically significant only for *MEG3* and *PLAGL1*, where the magnitude of associations increased with increasing quartile of maternal folate ( $\beta_{2nd\ quartile} = -1.16$ ,  $P = 0.041$ ,  $\beta_{3rd\ quartile} = -1.93$ ,  $P = 0.001$ ,  $\beta_{4th\ quartile} = -2.02$ ,  $P = 0.001$  for *MEG3* and  $\beta_{2nd\ quartile} = 0.20$ ,  $P = 0.607$ ,  $\beta_{3rd\ quartile} = -1.01$ ,  $P = 0.011$ ,  $\beta_{4th\ quartile} = -0.08$ ,  $P = 0.851$  for *PLAGL1*), and for the *PEG3* and *IGF2* DMRs, where the magnitude of associations weakened with increasing quartile ( $\beta_{2nd\ quartile} = 0.43$ ,  $P = 0.031$ ,  $\beta_{3rd\ quartile} = 0.22$ ,  $P = 0.294$ ,  $\beta_{4th\ quartile} = 0.06$ ,  $P = 0.602$  for *PEG3* and  $\beta_{2nd\ quartile} = -1.22$ ,  $P = 0.004$ ,  $\beta_{3rd\ quartile} =$

$-0.47$ ,  $P = 0.271$ ,  $\beta_{4th\ quartile} = -0.04$ ,  $P = 0.929$  for the *IGF2* DMR). Remarkably, repeating these analyses in infants born to women with pre-pregnancy BMI  $\leq$  30 and BMI > 30 revealed that, with the exception of the *NNAT* and *IGF2* DMRs, these patterns of association were stronger in newborns of women with pre-pregnancy BMI > 30 ( $P < 0.05$ ). Nonetheless, the cross-product terms for the interaction of maternal pre-pregnancy BMI and maternal folate were not statistically significant at any DMR ( $P > 0.59$ ), presumably because we were underpowered.

#### Association between DNA methylation and birth weight

Table 4 shows that five of the DMRs associated with maternal folate were also associated with birth weight. Higher birth weight was associated with higher DNA methylation levels at the *PEG10/SGCE* ( $\beta = 18.1$ ,  $P = 0.002$ ), *PLAGL1* ( $\beta = 12.3$ ,  $P = 0.001$ ), and *H19* ( $\beta = 20.2$ ,  $P = 0.003$ ) DMRs, and lower methylation levels at the *MEG3* ( $\beta = -10.9$ ,  $P = 0.008$ ) and *NNAT* ( $\beta = -7.6$ ,  $P = 0.062$ ) DMRs. To evaluate the potential for confounding by gestational age at blood draw (folate levels decrease with increasing gestational age) and preterm deliveries, we repeated these analyses among the 320 women with blood draws at gestational age < 120 d, and gestational age at birth > 37 wk and our findings remained essentially unaltered. Inclusion of DMRs associated with both maternal folate and birth weight into refined models attenuated these associations, suggesting that DNA methylation at the *MEG3*, *H19* and *PLAGL1* DMRs may mediate these associations.

Structural equation models using Mplus version 7.1 with bootstrapping were used to test for a significant mediated effect of *MEG3*, *PLAGL1*, and *H19* DMRs in the relationship between folate measured on a continuous scale and birth weight adjusting for sex, ethnicity/race, prenatal cigarette smoking, gestational age at delivery, delivery mode, cigarette smoking, prenatal physical activity and maternal pre-pregnancy BMI. We found that the indirect effect of DNA methylation at the *MEG3* DMR approached significance (estimate = 0.09,  $s.e. = 0.05$ ,  $P = 0.06$ ), while that of *PLAGL1* and *H19* were not statistically significant (estimate = -0.007,  $s.e. = 0.04$ ,  $P = 0.87$ ; estimate = -0.02,  $s.e. = 0.03$ ,  $P = 0.64$ , respectively).

## Discussion

We have examined maternal folate concentrations in early pregnancy, representing folate from all sources, in relation to birth weight in a multiethnic cohort, and the extent to which DNA methylation at multiple regulatory sequences of genomically imprinted genes mediates these associations, accounting for differences in maternal race/ethnicity, offspring sex, cigarette smoking, gestational age at blood draw or delivery, maternal pre-pregnancy BMI, physical activity and delivery route. We found associations consistent with a nonlinear relationship between maternal folate levels at median 12 wk gestation, and birth weight that was consistent with a threshold effect. Restricting these analyses by maternal obesity status revealed that this pattern of association was limited to newborns of women with BMI < 30. Birth weight is a robust, albeit non-specific indicator of

fetal wellbeing, and both low and high birth weight are consistently associated with a wide range of common adult onset diseases. We also found nonlinear relationships largely consistent with a threshold effect between maternal folate and DNA methylation levels of newborns of women with BMI  $\geq 30$ , at seven of nine DMRs examined; methylation levels in five of these DMRs were also linearly associated with birth weight and *MEG3* DMR methylation may mediate the relationship between maternal folate and birth weight.

#### Associations between maternal folate and birth weight

While clinical trials have demonstrated that folate deficiency increases risk of neural tube defects, evidence for excess folate availability, altering birth weight is inconsistent, regardless of whether folate is measured in plasma or erythrocytes, or is self-reported as diet rich in one carbon cycle nutrients. Our findings that moderate folate levels in early pregnancy are associated with higher birth weight regardless of race/ethnicity are consistent with previous studies,<sup>14-24,50</sup> although an equally large number of studies from Europe, Asia and the US<sup>25-31</sup> have not confirmed these associations. Reasons for discordant findings are unclear. Some of the discordant findings could be due to population differences in the prevalence of competing risk factors for low birth weight, including maternal obesity, nutritional status, cigarette and alcohol use and genetic susceptibility. In general, maternal obesity is higher and periconceptional FA use is lower in the present study.<sup>11,51</sup>

Discordant findings could also be due to differences in the gestational age at folate measurement; maternal folate concentrations measured in peripheral blood decrease with increasing gestational age, as folate demands increase during fetal growth and development.<sup>52</sup> The majority of studies with null findings collected blood specimens during the second or third trimester. In our analysis, restricted to 326 infant-mother pairs in whom maternal specimens were collected at gestational age < 12 wk, the magnitude of associations was somewhat higher than when all women were included. If the influence of folate supply on birth weight differs by gestational age, weaker associations in previous studies could be due, in part, to wide variability in gestational age in which folate measurements were made. Nonetheless, this multiethnic cohort reveals a pattern of association consistent with a threshold, interpretable as a lack of additional benefit to birth weight related to high folate levels beyond the second quartile; a pattern that may vary by maternal obesity status. Because up to 20% of American women enter their pregnancies at BMI > 30,<sup>53</sup> the seeming U-shaped relationship between maternal folate levels and birth weight among obese women requires urgent clarification in larger studies.

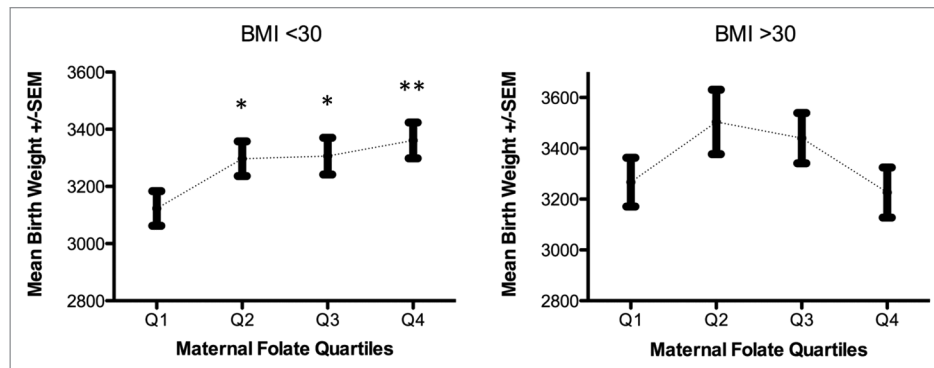
#### Associations between maternal folate levels, DMR methylation and birth weight

We also found inverse relationships between maternal folate levels and DNA methylation in all participants are apparent for at least one folate quartile for *MEG3*, *MEG3-IG*, *NNAT*, *PEG10/SGCE*, *PLAGL1*, *PEG3* and the *H19* DMRs, while positive associations were found in relation to the *IGF2* and *PEG1/MEST* DMRs. These findings are consistent with the central role of folate in the conversion of methionine to S-adenosylmethionine,

which transfers methyl groups to proteins and nucleic acids, including for DNA methylation. These findings also support that DNA methylation variation at regulatory sequences of imprinted genes may provide important epigenetic *readouts* for early prenatal nutrient adequacy and/or excess regardless of race/ethnicity. However, only nine of the more than 65 known imprinted genes are examined here, therefore a cautious interpretation of these findings is warranted, at least until more DMRs are examined in relation to folate.

DNA methylation at four DMRs was also associated with birth weight; higher birth weight was associated with higher methylation levels at the *H19*, *PEG10/SGCE* and *PLAGL1* DMRs and lower *MEG3* methylation levels. Although we found statistical evidence for mediation of the relationship between maternal folate levels and birth weight by the *MEG3* DMR, due to limited statistical power and the knowledge that imprinted genes are over-selected for growth effectors, we cannot exclude the possibility that DNA methylation at other DMRs including *H19* and *PLAGL1* DMRs may also mediate the association between maternal folate concentration and birth weight. The *IGF2/H19* domain, one of the best-characterized imprinted regions, is located in chromosome 11p15.5, and was originally associated with Beckwith-Wiedemann syndrome, an overgrowth disorder that increases the risk of Wilms tumor and hepatoblastoma.<sup>54</sup> Higher DNA methylation levels at the *IGF2/H19* imprinting center (IC) in a diploid cell results from increased methylation of the normally unmethylated maternally-derived allele, and can result in biallelic expression of *IGF2* by inhibiting binding the CTCF insulator protein.<sup>55</sup> CTCF normally binds in a methylation-sensitive manner to the maternal allele at this region, blocking interaction between downstream enhancers and *IGF2*.<sup>56,57</sup> The *H19* DMR analyzed here encompasses one of the six CTCF binding motifs within the IC region of *IGF2/H19*. Hypermethylation of the *H19* DMR has been linked to relaxation of imprint controls, increased transcription and translation of the potent IGF2 growth mitogen,<sup>58</sup> and higher birth weight.<sup>59</sup> Our findings are consistent with those of previous studies in which FA intake before or/and during pregnancy or folate levels were associated with DNA methylation differences of a similar magnitude at this imprinted domain<sup>46-49,60</sup> and early childhood obesity.<sup>61-63</sup>

Similar to the *IGF2/H19* imprinted domain where *IGF2* and *H19* are reciprocally imprinted, the *DLK1* and *MEG3* genes, located on chromosome 14q32.2, are also reciprocally imprinted.<sup>64</sup> Methylation at the *MEG3* DMR is established post-fertilization on the paternally derived allele, while methylation of the other DMRs is established in the gametes. While the gametic DMRs must resist demethylation during post-fertilization epigenetic reprogramming, the *MEG3* DMR is first established during this time. It remains unclear if this temporal difference in imprint mark establishment renders the *MEG3* DMR somehow differentially responsive to periconceptional folate levels. Nonetheless, aberrant methylation of the DMRs in the *DLK1/MEG3* domain leads to widespread disruption of imprinting and gene expression.<sup>65,66</sup> Higher methylation of the *MEG3* DMR has been associated with fetal distress, associated with famine *in utero*<sup>67</sup>; in



**Figure 1.** Maternal folate and birth weight by pre-pregnancy BMI < 30 and BMI  $\geq$  30Kg/m<sup>2</sup>. Adjusted for race/ethnicity, gestational age, cigarette smoking, gestational age at delivery, pre-pregnancy BMI and physical activity during pregnancy. \*P value < 0.01, \*\* p-value < 0.001

the present study, lower methylation levels at the *MEG3* DMR were associated with moderate folate levels. *PLAGL1* is located at chromosome 6q24.2, and encodes a developmentally regulated zinc-finger transcription factor thought to be involved in tumor development and growth via IGF2 signaling.<sup>29</sup> Differences in DNA methylation at the *PLAGL1* DMR have been associated with chorioamnionitis and funisitis<sup>68</sup> as well as lower birth weight.<sup>63</sup> Aberrant epigenetic marks at this site are also associated with transient neonatal diabetes mellitus (TNDM), a disorder of growth restriction and hyperglycemia.<sup>69,70</sup> The *PLAGL1* gene product is proposed to function as a major regulatory “hub” that coordinates the expression of a network of genes, including imprinted *IGF2*, *H19*, *DLK1*, *MEG3* and *MEST*.<sup>71</sup> Collectively, DNA methylation at networked DMRs are thought to have additive effects.<sup>72</sup>

Our study strengths include the prospective design, the multiethnic composition of the cohort and examination of erythrocyte folate levels with the majority of specimens collected in early pregnancy, demonstrating that the steady-state folate concentrations during the periconceptional period have similar epigenetic and physiological effects regardless of race/ethnicity. We also consider measurement of erythrocyte folate, which accounts for folate derived from all sources, a distinct strength of this study which improves upon numerous prior studies in which maternal folate was estimated by FA supplementation. DNA methylation examined here was measured from unfractionated umbilical cord blood specimens containing multiple cell types. Thus, one concern is that findings here may be a function of the major cell types. However, methylation profiles at these DMRs in fractionated polymorphonuclear and mononuclear cells were comparable between these cell fractions, suggesting that DMR methylation profiles measured using unfractionated umbilical cord blood may not sizably differ by cell type.<sup>47,73</sup> Furthermore, DNA methylation in the regions examined was comparable in multiple tissues representing the three germ layers in human conceptual tissues.<sup>73</sup> Lastly, at the well-characterized *IGF2/H19* IC, inverse associations have been reported for DMR methylation and in utero FA exposure using DNA methylation measurements from umbilical cord endothelial cells,<sup>48</sup> placenta<sup>48</sup> and umbilical cord leukocytes.<sup>47-49</sup>

The main limitation of this study is the relatively small sample size for maternal pre-pregnancy obesity- and race/ethnic-specific analyses to disentangle the role of maternal obesity from that of race/ethnicity in the associations between maternal folate, DMR methylation and birth weight. In addition, although the goal was to determine the extent to which periconceptional folate availability altered DNA methylation levels, some maternal blood from which folate levels were measured, was collected after 120 d gestation, the average life span of a red blood cell. However, the majority of specimens (>95%) were collected prior to 120 d gestation and should have included red blood cells that were present during the periconceptional period, a potential window of vulnerability for epigenetic alterations due to the methylation reprogramming that occurs during this time. Moreover, our findings remained unaltered after repeating the analysis in the subgroup in which maternal folate was measured from maternal blood obtained in the first trimester. Another study limitation is that we examined only nine imprint regulatory sequences, a small fraction of the 65 imprinted genes that are currently known.<sup>74</sup> Moreover, epigenetic changes resulting from variable maternal folate concentrations in early pregnancy are likely not restricted to imprinted domains. Nonetheless, while these data clearly require replication in larger studies, they support the contention that at least for birth weight, excessively high folate concentrations may have no added benefit, and that epigenetic plasticity, measurable at some genomically imprinted DMRs, may mediate these relationships. Such plasticity could be developed as biomarkers to improve monitoring folate adequacy or inadequacy much earlier than is currently possible. These findings also raise questions about potential links between declining birth weight in the US<sup>63</sup> and high FA intake.

In summary, we found a relationship between maternal folate and birth weight consistent with a threshold effect, an association stronger in newborns of non-obese women. We also found DNA methylation at some imprinted loci may mediate these associations. Larger studies are required to confirm these findings and clarify the pattern of associations in children born to obese women.

**Table 3.** Regression coefficients and standard errors for associations between maternal folate levels in quartiles and DNA methylation fractions at 9 DMRs regulating imprinted genes

| DMR                          | All                 |              | BMI < 30            |              | BMI ≥ 30            |              |
|------------------------------|---------------------|--------------|---------------------|--------------|---------------------|--------------|
|                              | Mean, $\beta$ , SE  | P-value      | $\beta$ , SE        | P value      | $\beta$ , SE        | P value      |
| <b>MEG3 (mean, se)</b>       | 67.2% (0.64)        |              |                     |              |                     |              |
| Quartile 2                   | -1.16 (0.56)        | <b>0.041</b> | -0.77 (0.64)        | 0.237        | <b>-2.02 (1.17)</b> | <b>0.092</b> |
| Quartile 3                   | -1.93 (0.57)        | <b>0.001</b> | -1.47 (0.69)        | <b>0.037</b> | <b>-2.72 (1.05)</b> | <b>0.013</b> |
| Quartile 4                   | -2.02 (0.58)        | <b>0.001</b> | -1.68 (0.68)        | <b>0.017</b> | <b>-2.47 (1.16)</b> | <b>0.039</b> |
| <b>NNAT (mean, se)</b>       | 56.7% (0.84)        |              |                     |              |                     |              |
| Quartile 2                   | -0.83 (0.71)        | 0.241        | -0.85 (0.84)        | 0.314        | -1.02 (1.32)        | 0.441        |
| Quartile 3                   | <b>-1.34 (0.73)</b> | <b>0.068</b> | -1.90 (0.92)        | <b>0.039</b> | -0.21 (1.21)        | 0.866        |
| Quartile 4                   | <b>-1.15 (0.74)</b> | <b>0.119</b> | -2.00 (0.91)        | <b>0.028</b> | 1.16 (1.27)         | 0.361        |
| <b>PEG10/SGCE (mean, se)</b> | 44.8% (0.37)        |              |                     |              |                     |              |
| Quartile 2                   | -0.22 (0.32)        | 0.489        | -0.08 (0.34)        | 0.820        | <b>-0.83 (0.61)</b> | <b>0.174</b> |
| Quartile 3                   | <b>-0.44 (0.32)</b> | <b>0.178</b> | 0.23 (0.35)         | 0.518        | <b>-2.08 (0.56)</b> | <b>0.000</b> |
| Quartile 4                   | -0.14 (0.33)        | 0.661        | -0.11 (0.36)        | 0.769        | <b>-1.12 (0.58)</b> | <b>0.054</b> |
| <b>MEG3-IG (mean, se)</b>    | 49.9% (0.69)        |              |                     |              |                     |              |
| Quartile 2                   | -0.54 (0.60)        | 0.372        | 0.04 (0.71)         | 0.953        | <b>-2.36 (1.19)</b> | <b>0.048</b> |
| Quartile 3                   | -0.67 (0.60)        | 0.263        | -0.43 (0.75)        | 0.566        | -1.31 (1.04)        | 0.206        |
| Quartile 4                   | -0.68 (0.61)        | 0.269        | -0.50 (0.75)        | 0.504        | -0.92 (1.16)        | 0.426        |
| <b>PLAGL1 (mean, se)</b>     | 50.0% (0.46)        |              |                     |              |                     |              |
| Quartile 2                   | 0.20 (0.39)         | 0.607        | 0.62 (0.50)         | 0.209        | <b>-0.44 (0.69)</b> | <b>0.521</b> |
| Quartile 3                   | -1.01 (0.40)        | <b>0.011</b> | 0.01 (0.53)         | 0.988        | <b>-1.86 (0.62)</b> | <b>0.003</b> |
| Quartile 4                   | -0.08 (0.41)        | 0.851        | 0.55 (0.53)         | 0.305        | <b>-1.04 (0.64)</b> | <b>0.105</b> |
| <b>PEG3 (mean, se)</b>       | 36.0% (0.26)        |              |                     |              |                     |              |
| Quartile 2                   | 0.43 (0.22)         | <b>0.031</b> | -0.12 (0.28)        | 0.679        | <b>1.66 (0.38)</b>  | <b>0.000</b> |
| Quartile 3                   | 0.22 (0.23)         | 0.294        | -0.24 (0.30)        | 0.428        | <b>1.01 (0.34)</b>  | <b>0.003</b> |
| Quartile 4                   | 0.06 (0.23)         | 0.602        | -0.28 (0.29)        | 0.339        | <b>0.63 (0.37)</b>  | <b>0.089</b> |
| <b>PEG1/MEST (mean, se)</b>  | 43.7% (0.48)        |              |                     |              |                     |              |
| Quartile 2                   | <b>-0.57 (0.42)</b> | <b>0.177</b> | -0.13 (0.55)        | 0.816        | <b>-1.51 (0.60)</b> | <b>0.013</b> |
| Quartile 3                   | -0.09 (0.43)        | 0.835        | 0.40 (0.57)         | 0.483        | <b>-1.38 (0.58)</b> | <b>0.017</b> |
| Quartile 4                   | 0.39 (0.44)         | 0.367        | 0.56 (0.58)         | 0.333        | 0.01 (0.60)         | 0.984        |
| <b>H19 (mean, se)</b>        | 46.0% (0.37)        |              |                     |              |                     |              |
| Quartile 2                   | -0.03 (0.32)        | 0.920        | 0.37 (0.35)         | 0.287        | <b>-2.17 (0.74)</b> | <b>0.003</b> |
| Quartile 3                   | -0.20 (0.33)        | 0.545        | 0.04 (0.37)         | 0.908        | <b>-0.99 (0.67)</b> | <b>0.138</b> |
| Quartile 4                   | 0.09 (0.33)         | 0.777        | 0.26 (0.37)         | 0.484        | -0.77 (0.71)        | 0.278        |
| <b>IGF2 (mean, se)</b>       | 51.0% (0.49)        |              |                     |              |                     |              |
| Quartile 2                   | -1.22 (0.42)        | <b>0.004</b> | <b>-1.17 (0.50)</b> | <b>0.020</b> | <b>-1.39 (0.80)</b> | <b>0.083</b> |
| Quartile 3                   | -0.47 (0.43)        | 0.271        | <b>-1.06 (0.52)</b> | <b>0.041</b> | 0.64 (0.74)         | 0.388        |
| Quartile 4                   | -0.04 (0.43)        | 0.929        | 0.07 (0.52)         | 0.886        | -0.43 (0.78)        | 0.586        |

Relationships adjusted for maternal race/ethnicity, cigarette smoking, delivery route, gestational age at blood draw, gestational age and delivery, physical activity, pre-pregnancy BMI. Referents are infants born to women with folate levels in the lowest quartiles. Maternal folate concentrations are associated with DNA methylation at all DMRs for either all subjects, those born to obese or non-obese women-associations are nonlinear.

**Table 4.** Regression coefficients for associations between offspring DMR methylation at the 9 regions and birth weight

| DMR         | $\beta$ SE           | P value      |
|-------------|----------------------|--------------|
| *MEG3       | <b>-10.92 (4.12)</b> | <b>0.008</b> |
| NNAT        | -7.57 (4.04)         | 0.062        |
| *PEG10/SGCE | <b>18.11 (5.77)</b>  | <b>0.002</b> |
| MEG3-IG     | 0.42 (9.13)          | 0.963        |
| *PLAGL1     | <b>12.33 (3.78)</b>  | <b>0.001</b> |
| PEG3        | 1.36 (8.97)          | 0.879        |
| PEG1/MEST   | -5.60 (5.68)         | 0.325        |
| *H19        | <b>20.25 (6.76)</b>  | <b>0.003</b> |
| IGF2        | 6.10 (5.71)          | 0.286        |

Referents are infants with methylation levels in the fourth quartile. Models are adjusted for maternal race, sex, cigarette smoking, gestational age at delivery, gestational age at blood draw, physical activity, pre-pregnancy BMI, and delivery route. \*Significant associations are noted between birth weight and methylation fractions at DMRs regulating *SGCE*, and *PLAGL1*, and one of two DMRs regulating the *IGF2/H19* and *DLK1/MEG3* imprinted domains.

## Methods

### Study participants

Study participants were enrolled as part of the Newborn Epigenetics Study (NEST). A description of study enrollment procedures is detailed elsewhere.<sup>75</sup> Briefly, between 2009 and 2011, English or Spanish speaking pregnant women aged 18 y and older were identified from clinic logs of five prenatal clinics in Durham County, NC, USA. Women intending to relinquish custody of the offspring resulting from the index pregnancy were excluded, as were those positive for human immunodeficiency virus, due to the limited research on the relationships between HIV, its treatment, and DNA methylation in the offspring. Of 2,548 women who met eligibility criteria, 1700 (66.6%) consented and were enrolled. The 848 women who declined were similar to those who consented in age ( $P = 0.70$ ) although more likely to be Asian and Native American ( $P < 0.001$ ). Of the 1304 (76.7%) women who remain enrolled in the study, erythrocyte folate concentrations were measured from maternal venous blood among the first 528, 63% ( $n = 330$ ) of which was drawn during the first trimester. These analyses are restricted to 496 singleton infant-mother pairs in whom birth weight, pre-pregnancy weight and height data were available. The study protocol was approved by the Duke University and Durham Regional Hospital Institutional Review Boards (IRB).

### Data collection

Maternal age, marital status, parity, weight and height at last menstrual period, sex of offspring, prenatal obesity, gestational diabetes and ethnicity/race were self-reported and also verified with abstracted medical records. Maternal age at delivery was computed as the difference between date of birth and delivery date. General health during pregnancy, cigarette smoking, alcohol use, and maternal education were self-reported via a questionnaire. Parturition data including birth weight, gestational age at

birth, infant sex, delivery mode and any infection present during delivery were abstracted by trained personnel from medical records after delivery. Gestational age at birth was divided into two categories: preterm  $< 37$  wk gestation and term  $\geq 37$  wk gestation. Delivery mode was defined as either vaginal delivery or Caesarian section (C-section).

### Specimen collection and handling

Following enrollment at the first prenatal visit, pregnant women donated peripheral blood specimens in a 10 ml lavender top vacutainer tube from which 1 ml was removed and frozen for erythrocyte folate measurements. Infant cord blood specimens were collected at birth, also in EDTA-treated vacutainer tubes. The samples were centrifuged using standard protocols to allow for collection of plasma and buffy coat for DNA extraction (Qiagen). All samples were stored at  $-80^{\circ}\text{C}$  prior to use.

### DNA methylation analysis

DNA was extracted using Puregene reagents (Qiagen) and quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). 800 ng of genomic DNA was treated with sodium bisulfite using the Zymo EZ DNA Methylation kit (Zymo Research), to convert unmethylated cytosines to uracils, leaving methylated cytosines unchanged. Pyrosequencing was performed using a Pyromark Q96 MD pyrosequencer (Qiagen) to measure DNA methylation at nine imprint regulatory regions in mothers and newborns. Pyrosequencing assay design, genomic coordinates, assay conditions and assay validation have been previously described.<sup>73,76</sup> Percent methylation for each CpG cytosine was determined using Pyro Q-CpG Software (Qiagen). DMRs in which DNA methylation was measured include two regions regulating the *IGF2/H19* imprinted domain (one upstream of the imprinted *IGF2* promoters and one upstream of the *H19* locus at the imprint center), two regions regulating the *DLK1/MEG3* imprinted domain (the imprint center intergenic to *DLK1* and *MEG3* and the DMR within the *MEG3* promoter region, the maternally methylated *PEG3* promoter region, the maternally methylated *MEST* promoter region, the maternally methylated *PEG10* promoter region from which *SGCE* is also transcribed in the opposite direction, the maternally methylated *NNAT* promoter and the maternally methylated *PLAGL1* promoter.

### Measurement of erythrocyte folate concentrations

Maternal whole blood samples were sent to Craft Technologies for measurement of erythrocyte folate using a commercial kit, ID-Vit Folic acid (Immundiagnostic-ALPCO; Salem, NH, Ref KIF005) which uses the folate dependent strain *Lactobacillus rhamnosus* (taxon id 47715) in a 96 well format.<sup>77</sup> Erythrocyte folate levels were calculated using the following formula: RBC folate = (whole blood folate(ng/ml))-(plasma folate ng/ml) (1-hematocrit/100))/ [hematocrit levels] as previously described.<sup>78</sup>

### Statistical analysis

Birth weight was normally distributed, and folate concentrations were slightly right-skewed. Folate was therefore categorized into quartiles. Maternal obesity was treated as a continuous variable in adjustment, but also was dichotomized at BMI  $\leq 30$  to determine if associations varied by maternal obesity. Self-reported maternal physical activity was used as a continuous variable. The following variables were treated as categorical variables:



maternal age 18–20, 21–30, 31–39 and 40+ years, maternal education as less than high school; high school to less than college, college and graduate school education, parity as none, one, two and three or more, delivery mode as vaginal or Cesarean section, and finally, gestational age at birth stratified as less than, equal to, or more than 37 wk. Because DNA methylation fractions at some CpG dinucleotides within the *PLAGL1*, *PEG3*, *MEG3*, and *PEG10/SGCE* DMRs were not normally distributed as assessed via Kolmogorov-Smirnov tests, analyses were also confirmed using quartiles of DNA methylation levels and Wilcoxon rank sum tests.

Mean differences in birth weight were compared within each folate quartile using generalized linear models to adjust for potential confounding by maternal age, gestational age at blood draw, gestational age at delivery, cigarette smoking (smoked before pregnancy, during pregnancy and none), sex of offspring, education, and maternal race/ethnicity. Because the functional significance of DMR methylation marks at imprinted loci and folate concentrations vary considerably by pre-pregnancy obesity and ethnicity/race,<sup>59</sup> we also explored associations between folate and birth weight, restricted to Non-Hispanic Whites, Non-Hispanic Blacks and Hispanics and BMI < 30 vs. BMI ≥ 30 kg/m<sup>2</sup>. Folate concentrations were measured in blood that was collected < 120 d gestation (approximating the average lifespan of a red blood cell) in 80% of women, 15% were measured in blood collected between 120–170 d, and the remainder in blood samples collected after 180 d gestation. Hence, gestational age at blood draw, computed as the difference between date of delivery and date of blood draw, was included in multivariable analyses. In

exploratory analyses, we also restricted refined models to specific trimester of maternal blood draw.

To examine whether DNA methylation at sequences regulating imprinted genes may explain some of the observed association between folate and birth weight, we identified DMRs that were associated with maternal folate levels using mixed models to allow for unconstrained model entry of individual CpGs, adjusting for factors previously shown to influence DNA methylation.<sup>11,46,79</sup> DMRs significantly associated with folate were then examined for associations with birth weight; the subset statistically significantly associated with maternal folate levels and birth weight ( $P \leq 0.05$ ) were included in refined models to determine the extent to which the main effects were attenuated. Finally, structural equation models were then used to evaluate mediation of the relationship between maternal folate and BW by the subset of DMRs that attenuated the main effects. These models quantify mediation as the product of the regression coefficients for folate, relevant DMR and birth weight.<sup>80</sup>

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#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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