Maternal blood lead concentrations, DNA methylation of MEG3 DMR regulating the DLK1/MEG3 imprinted domain and early growth in a multiethnic cohort

Monica D. Nye,1,2,3 Katherine E. King,4 Thomas H. Darrah,5 Rachel Maguire,6 Dereje D. Jima,6 Zhiqing Huang,1 Michelle A. Mendez,2,7 Rebecca C. Fry,2,3 Randy L. Jirtle,6,† Susan K. Murphy1,‡ and Cathrine Hoyo8,*,§

1Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Duke University, B226 LSRC, Box 91012, Research Drive, Durham, NC 27708, USA; 2Lineberger Comprehensive Cancer Center, The University of North Carolina at Chapel Hill, 450 West Street, CB 7295, UNC, Chapel Hill, NC 27599, USA; 3Department of Environmental Sciences and Engineering, Curriculum in Toxicology, The University of North Carolina at Chapel Hill, 135 Dauer Drive, CB 7431, UNC, Chapel Hill, NC 27599, USA; 4Environmental Public Health Division, U.S. Environmental Protection Agency and Department of Community and Family Medicine, Duke University, Durham, NC 27708, USA; 5School of Earth Sciences, The Ohio State University, Columbus, OH 43210, USA; 6Department of Biological Sciences, Center for Human Health and Environment, Campus Box 7633, NC State University, Raleigh, NC 27695, USA; 7Department of Nutrition, Gillings School of Public Health Fellow, Carolina Population Center, University of North Carolina at Chapel Hill, 2101G McGavran-Greenberg Hall Chapel Hill, NC 27599-7461, USA; 8Department of Biological Sciences, North Carolina State University, Raleigh, NC 27695, USA

*Correspondence address. Department of Biological Sciences, Center for Human Health and Environment, Campus Box 7633, NC State University, Raleigh, NC 27695, USA. E-mail: choyo@ncsu.edu
†Randy L. Jirtle, http://orcid.org/0000-0003-1767-045X
‡Susan K. Murphy, http://orcid.org/0000-0001-8298-7272
§Cathrine Hoyo, http://orcid.org/0000-0002-2466-8617

Abstract

Prenatal exposure to lead (Pb) is known to decrease fetal growth; but its effects on postnatal growth and mechanistic insights linking Pb to growth are not clearly defined. Genomically imprinted genes are powerful regulators of growth and energy utilization, and may be particularly vulnerable to environmental Pb exposure. Because imprinting is established early and maintained via DNA methylation, we hypothesized that prenatal Pb exposure alters DNA methylation of imprinted genes resulting in lower birth weight and rapid growth. Pb was measured by inductively coupled plasma mass spectrometry (ICP-MS) in peripheral blood of 321 women of the Newborn Epigenetic Study (NEST) obtained at gestation ~12 weeks. Linear and logistic regression models were used to evaluate associations between maternal Pb levels, methylation of differentially methylated regions (DMRs) regulating H19, MEG3, PEG3, and PLAGL1, measured by pyrosequencing, birth weight, and...
weight-for-height z score gains between birth and age 1 year, ages 1–2 years, and 2–3 years. Children born to women with Pb levels in the upper tertile had higher methylation of the regulatory region of the MEG3 DMR imprinted domain (β = 1.57, SE = 0.82, P = 0.06). Pb levels were also associated with lower birth weight (β = −0.41, SE = 0.15, P = 0.01) and rapid gains in adiposity (OR = 12.32, 95% CI = 1.25–121.30, P = 0.03) by age 2–3 years. These data provide early human evidence for Pb associations with hypermethylation at the MEG3 DMR regulatory region and rapid adiposity gain—a risk factor for childhood obesity and cardiometabolic diseases in adulthood.

**Key words:** DNA methylation; MEG3; lead; environmental exposures; epigenetics

**Introduction**

Exposures prior to birth can alter fetal development and lead to lifelong health effects [1]. In utero and early childhood exposure to environmental toxic metals including lead (Pb), increase risk of low birth weight [2–5] whether due to prematurity or growth restriction [6,7]. Pb exposure has also been associated with poorer cognitive function in childhood and adolescence [8–12]. In the postnatal period, the major source of human Pb exposure is lead-based paints in older housing and Pb-containing dust, and ingestion or inhalation are the main routes [13]. In utero, Pb readily crosses the placenta resulting in prenatal exposure [14,15].

Although protein disruption/misfolding, generation of oxidative stress, and endocrine disruption are known effects of toxic metal exposure, mechanisms causing these effects are still largely unknown [16–18]. A growing body of in vitro and in vivo evidence supports that environmental exposure to toxic metals, including Pb, can alter DNA methylation [19–26]. However, epigenetic targets are still not known.

In mice, [20] early life Pb exposure has been associated with hypermethylation of the regulatory sequences of H19, Igf2, and Igf2R. These imprinted genes are monoallelically expressed in a parent-of-origin dependent manner. Although DNA methylation alterations at these regulatory regions has been associated with overweight or obesity status in early childhood, human data linking Pb to epigenetic alterations and obesity are limited. These genes are functionally haploid and controlled by parental allele-specific differentially methylated regions (DMRs) established in gametes during early development and this methylation pattern is largely maintained in somatic tissues. Here we investigated the effects of maternal blood Pb levels on the DMRs of four imprinted genes (two maternally methylated: PEG3 and PLAGL1, and two paternally methylated: H19 and MEG3) three of which previously have been found to be associated with birth weight [27] and early life Pb exposure [22].

**Results**

Study participant characteristics are presented in Table 1. Although the study population is demographically diverse, maternal Pb levels were comparable by all demographic and physical characteristics (P > 0.05). The median maternal blood Pb level for participants overall was 0.36 μg/dl (range = 0–9.37 μg/dl of blood) and did not vary considerably in non-Hispanic Blacks (median = 0.34 μg/dl; range = 0–7.77 μg/dl), Hispanics (median = 0.82 μg/dl; range = 0–4.94 μg/dl), non-Hispanic Whites (median = 0.25 μg/dl; range = 0.04–9.37 μg/dl), and non-Hispanics of other race (median = 0.55 μg/dl; range = 0.07–2.50 μg/dl).

**Associations between Pb and DNA methylation**

Table 2 shows the regression coefficients for associations between maternal prenatal Pb levels and DNA methylation in the three DMRs previously associated with birth weight or prenatal and postnatal Pb exposure. Among the four DMRs examined, only the MEG3 DMR was associated with Pb levels (β = 1.96, SE = 0.81, P = 0.01) in adjusted models. After accounting for maternal race/ethnicity, education, maternal age at delivery, newborn’s gender and gestational age at birth, these associations were borderline significant (β = 1.57, SE = 0.82, P = 0.06).

**Associations between prenatal Pb levels and birth weight**

We examined the relationship between maternal blood Pb levels, birth weight, and changes in weight for height z (WHZ) scores at ages 1–3 (Table 3). When offspring of mothers in the highest and middle tertiles of Pb concentrations were compared to offspring born to women in the lowest prenatal Pb concentrations tertile, we found a strong association with lower birth weight (β = −0.24, SE = 0.15, P = 0.10 for middle Pb tertile and β = −0.41, SE = 0.15, P = 0.01 for highest tertile) (Table 3). These associations were adjusted for maternal age, race/ethnicity, education, smoking status, physical activity, pre-pregnancy BMI, antibiotic use, parity, breastfeeding 3 months or more, and the offspring’s sex and gestational age. Repeating these analyses in a subsample that excluded multiple gestation and preterm births (gestational age <37 weeks) did not significantly alter our findings (β = −0.41, SE = 0.16, P = 0.01 for the highest tertile of Pb).

**Association between prenatal Pb levels and change in WHZ scores**

We also examined the association between prenatal Pb levels and the rapidity of change in WHZ scores between birth and age 1 year, 1–2 years, and 2–3 years. In contrast to inverse associations found with birth weight, the highest tertiles of prenatal Pb levels were positively associated with the highest tertile of WHZ change between 0 and 1 years (OR = 2.31, 95% CI = 0.60–8.91, P = 0.22). By age 2–3 years, this positive association between prenatal Pb levels and WHZ change was significant (OR = 12.32, 95% CI = 1.25–121.30, P = 0.03) (Table 3). Repeating these analyses in a subsample that excluded multiple gestation and preterm births (gestational age <37 weeks) did not significantly alter our findings; the OR for WHZ change between 2 and 3 years = 11.23, 95% CI = 1.12–121.15, P = 0.04.

**Mediation of the relationship between MEG3 DMR methylation levels at birth and rapid early growth**

Structural equation models using R software were utilized to test if there was significant mediation of the relationship between Pb exposure and rapid gains in adiposity by hypermethylation of the regulatory sequences of the MEG3 DMR imprinted domain, after adjusting for breastfeeding, sex, prenatal cigarette smoking and ethnicity/race. We found no evidence for an
We found that, of the three DMRs previously associated with birth weight, [27] higher DNA methylation levels of the regulatory sequence of imprinted MEG3 DMR were significantly associated with Pb exposure. High levels of Pb in utero were also associated with lower birth weight, followed by accelerated change in adiposity during early childhood. While these findings require replication in larger studies with longer follow-up and a larger repertoire of regulatory DMRs, they provide early evidence for prenatal Pb exposure and risk of accelerated adiposity gain in humans. Rapid gain in adiposity is a strong and persistent risk factor for cardiometabolic diseases in adulthood [35]. Increasing the repertoire of regulatory DMRs for investigation has the potential to identify specific patterns, signatures, or pathways that are epigenetically altered by Pb exposure that not only change birth weight but also change the risk of cardiometabolic risk during the life course. MEG3 DMR methylation or prenatal Pb exposure could alter basal metabolism through DMRs regulating body weight and metabolism, such as GNAS, MEST, and NDN [36].

Our findings that prenatal Pb exposure increases obesity risk that is more apparent with increasing age is consistent with a previous report where in Agouti mice, Pb exposure altered glucose and obesity risk [37]. These findings are also consistent with human studies that reported associations between parental occupational Pb exposure and lower birth weight, [38,39] followed by rapid catch-up growth during early childhood and adolescence, [40] suggesting that Pb may contribute to altered utero and early postnatal growth and influence adult health. Saigal and colleagues showed that there are sex differences in catch-up growth and growth factor levels, with associations stronger in females.

Although our data were too limited for sex-specific analyses, we previously reported female-specific associations between Pb exposure and lower methylation of regulatory sequencing of IGF2/H19 imprinted domains [22]. In animal models, Faulk et al. have shown in their 2013 and 2014 studies that mice exposed to Pb in utero and in early life show sex-specific responses at imprinted loci and sex-specific change in weight gain following weaning and obesity in later life [41,37]. There are few human or animal studies that have investigated the effects of early life Pb exposure on adiposity gain at different ages during the life course, and mixed results have been reported [42,43,33]. It is possible that different molecular pathways are altered by Pb exposure depending on the sex of the offspring or timing of exposure; e.g., neurodevelopment in males and cardiometabolic risk in females. Pb exposure studies in mice have shown early-life susceptibility to Pb-associated adiposity, supporting gender-specific effects on later life obesity [44,33].

Pb is ubiquitous in the environment and enters the maternal or postnatal offspring blood supply via ingestion or inhalation of contaminated dust [45]. In circulation, it exists in the Pb\(^{2+}\) form and 99% is attached to erythrocytes [46]. Because the lifespan of erythrocytes is 120 days, prenatal Pb levels measured at \(\sim 12\) weeks gestation likely reflect fetal Pb exposure during peri-conception. Pb has been shown to readily cross the placenta in humans, evidenced by the similarity of Pb concentrations in maternal peripheral blood, placental and umbilical cord blood compartments [47,48,49]. Although the mechanisms by which Pb may influence rapid growth in early childhood are unknown and remain an active topic of investigation, Pb exposure in Agouti mice increases male body weight postnatally and induces methylation-mediated changes in coat color [41].

We observed hypermethylation of the MEG3 DMR associated with prenatal Pb exposure. This finding is supported by several studies which have found biological relevance for hypermethylation at this regulatory region in a variety of human cancers

### Table 1. Characteristics of participants and maternal blood lead levels in 321 mother–child pairs

| N (%) | Lead (Pb; \(\mu g/dl\)) |
|-------|-----------------|
|       | Mean | SE | Min | Max |
| **Mother** |      |    |     |     |
| Age at delivery |      |    |     |     |
| 18–25 | 114 (35.5) | 0.90 | 0.13 | 0.00 | 7.77 |
| 26–35 | 165 (51.4) | 0.66 | 0.08 | 0.04 | 9.37 |
| >36  | 42 (13.1) | 0.82 | 0.11 | 0.04 | 2.67 |
| **Race/ethnicity** |      |    |     |     |
| NH White | 96 (29.9) | 0.79 | 0.14 | 0.04 | 9.37 |
| NH Black | 111 (34.6) | 0.70 | 0.11 | 0.00 | 7.77 |
| Hispanic | 102 (31.8) | 0.82 | 0.09 | 0.00 | 4.94 |
| Other | 12 (3.7) | 0.71 | 0.19 | 0.07 | 2.50 |
| **Education (years)** |      |    |     |     |
| 1–12 | 166 (52.2) | 0.86 | 0.10 | 0.00 | 7.77 |
| 13–15 | 42 (13.2) | 0.80 | 0.23 | 0.04 | 9.37 |
| 16 | 50 (15.7) | 0.64 | 0.11 | 0.05 | 3.64 |
| >17 | 60 (18.9) | 0.52 | 0.09 | 0.04 | 9.38 |
| **Pre-pregnancy BMI** |      |    |     |     |
| <25 | 135 (42.5) | 0.79 | 0.11 | 0.04 | 9.37 |
| 25–29.9 | 102 (32.1) | 0.66 | 0.07 | 0.00 | 3.66 |
| 30–34.9 | 46 (14.5) | 0.65 | 0.20 | 0.00 | 7.56 |
| ≥35 | 35 (11.0) | 1.08 | 0.26 | 0.07 | 7.77 |
| **Maternal smoking** |      |    |     |     |
| Smoked prior to pregnancy | 40 (12.9) | 0.64 | 0.14 | 0.00 | 3.66 |
| Smoked during pregnancy | 45 (14.5) | 0.78 | 0.15 | 0.00 | 5.52 |
| Never smoked | 226 (72.7) | 0.77 | 0.08 | 0.04 | 9.37 |
| **Physical activity** |      |    |     |     |
| No | 83 (26.4) | 0.73 | 0.12 | 0.04 | 7.77 |
| Yes | 231 (73.6) | 0.76 | 0.08 | 0.00 | 9.37 |
| **Antibiotic use during pregnancy** |      |    |     |     |
| No | 228 (73.3) | 0.70 | 0.07 | 0.00 | 9.37 |
| Yes | 83 (26.7) | 0.91 | 0.16 | 0.04 | 7.77 |
| **Child** |      |    |     |     |
| Gender |      |    |     |     |
| Female | 156 (48.6) | 0.82 | 0.10 | 0.04 | 7.77 |
| Male | 165 (51.4) | 0.71 | 0.09 | 0.00 | 9.37 |
| Gestational age (weeks) |      |    |     |     |
| <33 | 12 (3.7) | 1.34 | 0.40 | 0.15 | 5.43 |
| ≥34 | 309 (96.3) | 0.74 | 0.07 | 0.00 | 9.37 |
| Birth weight (g) |      |    |     |     |
| <2500 | 32 (10.0) | 0.85 | 0.17 | 0.11 | 5.43 |
| ≥2500 | 288 (90.0) | 0.76 | 0.07 | 0.00 | 9.37 |

The indirect effect of DNA methylation at the MEG3 DMR mediating the relationship between Pb and rapid growth (estimate = –0.02, 95% CIs –0.11 and 0.04, \(P = 0.44\)).

### Discussion

Prenatal exposure to Pb has been evaluated extensively in the context of neurotoxicity and its phenotypic outcomes such as attention deficit, [28] decreased brain volume, [29] and violent behavior [30,31] in humans and animal model systems. Developmental Pb exposure has been associated with lower birth weight in infants as well as altered DNA methylation and late onset obesity in mice [32,33].

We evaluated if prenatal exposure to Pb is associated with DNA methylation differences at four DMRs of imprinted genes previously associated with size at birth [27] and rapid early growth [34]. We found that, of the three DMRs previously associated with birth weight, [27] higher DNA methylation levels of
Table 2. Associations of Pb levels with DNA methylation at four DMRs

| Blood lead tertiles (ref = Low) | H19 | DLK1/MEG3 | PLAGL1 | PEG3 |
|-------------------------------|-----|-----------|--------|------|
|                               | β   | SE        | P      | N    |
| Biariate                      |     |           |        |      |
| Middle                        | –0.38 | 0.58 | 0.51 | 293 |
| High                          | –0.43 | 0.58 | 0.46 | 284 |
| Adjusted<sup>a</sup>          |     |           |        |      |
| Middle                        | 0.19 | 0.57 | 0.73 | 291 |
| High                          | 0.12 | 0.58 | 0.84 | 283 |
| DMR mean methylation         |     |           |        |      |

<sup>a</sup>Adjusted for maternal race/ethnicity, education, maternal age at delivery, newborn’s gender and gestational age at birth (days).

Table 3. Associations of Pb with birth weight and WHZ score change

| Pb Exposure level | Birth Weight<sup>b</sup> (β, SE, P-value) | WHZ change<sup>b</sup> (OR (95% CI), P-value) | WHZ from age 0 to 1 years (OR (95% CI), P-value) | WHZ from age 1 to 2 years (OR (95% CI), P-value) | WHZ from age 2 to 3 years (OR (95% CI), P-value) |
|-------------------|--------------------------------------------|---------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Reference = low   |                                            |                                             |                                               |                                               |                                               |
| N = 284           |                                            |                                             |                                               |                                               |                                               |
| Medium Pb         | –0.24 (0.15), 0.10                        |                                             |                                               |                                               |                                               |
| High Pb           | –0.41 (0.15) 0.01                         |                                             |                                               |                                               |                                               |
| Reference = medium|                                            |                                             |                                               |                                               |                                               |
| N = 114           |                                            |                                             |                                               |                                               |                                               |
| Lowest            | 0.87 (0.27–2.77) 0.81                      | 3.30 (0.68–15.99) 0.14                      |                                               |                                               |                                               |
| Highest           | 1.47 (0.48–4.52) 0.50                      | 1.72 (0.07–3.18) 0.45                      | 2.04 (0.21–19.45) 0.54                      | 1.61 (0.16–16.26) 0.69                      |
| Reference = High  |                                            |                                             |                                               |                                               |                                               |
| N = 62            |                                            |                                             |                                               |                                               |                                               |
| Lowest            | 1.70 (0.44–6.58) 0.45                      | 2.04 (0.21–19.45) 0.54                      |                                               |                                               |                                               |
| Highest           | 2.31 (0.60–8.91) 0.22                      | 2.04 (0.06–5.11) 0.59                      | 12.32 (1.25–121.30) 0.03                    |                                               |

<sup>b</sup>Adjusted for maternal age, race/ethnicity, education, smoking status, physical activity, pre-pregnancy BMI, gestational age at birth, antibiotic use, parity, and baby’s gender. Limited to infants gestational age of 34 weeks or greater.

MEG3 is expressed in normal tissues, but its loss of expression in multiple cancer cell lines inhibits tumor cell proliferation suggesting it functions as a tumor suppressor [51]. Located at human chromosome 14q32.2 is the germline-derived primary DLK1/MEG3 intergenic DMR (MEG3-IC) and the postfertilization-derived secondary MEG3 DMR in addition to other imprinted genes. This domain produces multiple alternatively spliced long non-coding RNA (lncRNA) transcripts. The imprinted expression of the genes within the DLK1/MEG3 domain plays an important role in the development and growth [52,53]. A recent study of MEG3 RNA’s ability to bind regions of TGF-beta family of proteins and regulate their expression demonstrates the importance of this interaction in cancer and also for normal growth and development [53].

The importance of regulation via methylation of the DLK1/MEG3 imprinted region has been studied by other groups and shown to play role in birth weight/early childhood obesity, diabetes, and neurobehavioral development. In Temple syndrome, infants experience intrauterine growth retardation, low birth weight, premature puberty with corresponding truncal obesity; this syndrome results from aberrant methylation within the MEG3-IC/MEG3-DMR region [54]. Temple syndrome results from hypomethylation, although at a region different than our investigation, it demonstrates the effects of aberrant methylation within this region. DLK1/MEG3 hypermethylation has been shown to play a role in human diabetes and impaired glucose tolerance in mice [55,56]. Overall, our finding that only the MEG3 DMR showed higher methylation in relation to prenatal Pb exposure suggest specificity in epigenetic response to prenatal Pb exposure.

A major strength of our study is its prospective design in early life, and our ability to measure Pb in whole blood very early in pregnancy, a period likely to include the periconceptional period, as we have examined Pb exposure before birth and the effects up to 3 years later. Because we measured Pb in erythrocytes at gestational age <12 weeks, we likely captured the periconceptional period [57] during which many DNA methylation marks are erased and stably re-established. Consequently, DNA methylation measured in mixed cell populations obtained from peripheral blood samples provides a reasonable estimate of DNA methylation status across tissue types for imprinted genes. We have previously studied methylation in a large panel of human conceptal tissues and found that for MEG3-IC and MEG3 DMR methylation did not greatly differ across most tissue examined [58]. Another strength is the design of the study which contains a multiethnic cohort where the three major ethnic groups in the USA, non-Hispanic Whites, non-Hispanic Blacks, and Hispanics are well represented. Unfortunately, we were unable to analyze Pb effects within race/ethnic groups due to small sample size when subdividing into racial categories by Pb exposure levels and our mediation analysis was likely under-powered.

The main limitation of our study is the small size which limited our statistical power to see mediation by the MEG3 DMR, of the association between Pb and rapid growth. We also had limited power to examine race/ethnic group and/or sex-specific associations. Additionally, we only obtained one measure of Pb levels in maternal blood and thus we were unable to determine if other developmental windows, in utero, when Pb substantially increases risk during the gestational period. However, these limitations are unlikely to negatively influence our findings. Pregnancy as a whole is associated with higher levels of Pb from previous exposure stored in long-term physiological deposits including bone and tissue, and released throughout gestation, resulting from an increase in bone reabsorption [59].
In summary, we found higher DNA methylation of the regulatory sequence of MEG3 DMR related to prenatal exposure to Pb. We also found associations between prenatal Pb exposure and lower birth weight, and a steeper growth trajectory from birth to age 3 years. Larger studies examining associations between Pb, epigenetics, and growth trajectories are required.

Methods
Study Participants
Pregnant women were recruited at Duke University obstetrics care-related facilities in Durham County, North Carolina as part of the Newborn Epigenetic Study (NEST), which focuses on the influence of early life environmental exposures on children’s health. Detailed methods for study participant enrollment have been previously reported [27,60]. Briefly, between 2009 and 2011, pregnant women 18 years and older who planned to give birth at Duke University or Durham Regional Hospitals were recruited from perinatal clinics within the Durham County area of North Carolina. Interviews or self-administered questionnaires were obtained at the time of enrollment and medical records were abstracted to obtain relevant information about the pregnancy. Of the 2584 women approached 1700 (66.7% response rate) consented and enrolled; HIV-1 infected women were excluded. At 1 year of age follow-up, 76.7% (1,304) of those consented and enrolled remained in the study. The present analyses are limited to the first 321 infant–mother pairs where maternal blood Pb levels were measured.

Ethics Statement
The Duke University Institutional Review Board (IRB) approved this study protocol, and written informed consent was obtained from all study participants.

Data Collection
Pb levels were measured using blood samples collected at enrollment from pregnant women (n = 321) for the NEST project (mean gestational age, 12 weeks). Ten milliliter of peripheral blood was collected into EDTA tubes with 1 ml subsequently stored whole, and the remainder centrifuged to obtain plasma and the buffy coat layer. We measured covariate data (maternal race, education, cigarette smoking, parity, and obesity status prior to pregnancy) using a combination of medical records and a questionnaire. This subsample of the cohort was comparable to the larger cohort in factors previously associated with DNA methylation and/or poor birth outcomes, maternal age, education, race, pre-pregnancy BMI, or smoking status (P > 0.05).

DNA methylation analysis
Genomic DNA from buffy coat specimens obtained from umbilical cord blood was isolated using the QiaGen DNeasy Blood and Tissue Kit (QiaGen; Valencia, CA) and then treated with sodium bisulfite using the Zymo EZ DNA Methylation Kit (Zymo Research, Irvine, CA). Bisulfite treatment modifies the DNA by converting unmethylated cytosines to uracils and leaving methylated cytosines unchanged. Pyrosequencing was performed using a QiaGen Pyromark Q96 MD Pyrosequencer. Assay design and validation for the DMRs analyzed herein: H19 (4 CpGs), MEG3 (8 CpGs), PEG3 (10 CpGs), and PLAGL1 (6 CpGs) have been previously reported [58,61]. The percent methylation for each CG dinucleotide in the target sequence was calculated using PyroQ CPG Software (QiaGen). We evaluated between 4 and 10 CpGs per DMR and there was a high correlation between the values of CpGs within a DMR site (Cronbach’s alphas for these regions were 0.95–0.99) allowing us to use the mean methylation value for all interrogated CpG sites within a given DMR. [62] Values in the statistical models represent the mean methylation of the CpG sites contained within the region analyzed.

Measurement of Pb
Maternal blood Pb levels were measured in whole blood in nanograms per gram (ng/g), and then converted to μg/dl before statistical analysis to allow comparison with other studies. Pb concentrations were measured using well-established solution-based inductively coupled plasma mass spectrometry (ICP-MS) methods [63,64,65]. One milliliter of frozen (~80°C) maternal blood was equilibrated to room temperature for approximately 4 h, then homogenized with a GlobalSpec laboratory slow shaker (GlobalSpec, East Greenbrush, NY) for 30 min. From these samples, a ~0.2 ml aliquot was pipetted into a trace-metal-clean test tube and verified gravimetrically to ≥0.001 mg using a calibrated mass balance.

Samples were then spiked with internal standards consisting of known quantities (10 and 1 ng/g, respectively) of indium (In) and bismuth (Bi) (obtained from SCP Science), used to correct for instrumental drift. The solutions were then diluted using water purified to 18.2 MΩ/cm resistance (by a Milli-Q water purification system, Millipore, Bedford, MA, USA) and acidified using ultra-pure 12.4 mol/l hydrochloric acid to result in a final concentration of 2% hydrochloric acid (by volume). All standards, including aliquots of the certified NIST 955c, and procedural blanks were prepared by the same process.

Pb concentrations were measured using a Perkin Elmer DRC II (Dynamic Reaction Cell) axial field ICP-MS at the University of Massachusetts-Boston [63,66]. To clean sample lines and reduce memory effects, sample lines were sequentially washed with 18.2 MΩ cm resistance (by a Milli-Q water purification system, Millipore, Bedford, MA, USA) water for 90 s and a 2% nitric acid solution for 120 s between analyses. Procedural blanks were analyzed within each block of 10 samples, to monitor and correct for instrumental and procedural backgrounds. Calibration standards used to determine Pb in blood included aliquots of 18.2 MΩ cm resistance H2O, NIST 955c SRM, and NIST 955c SRM spiked with known quantities of Pb in a linear range from 0.025 to 10 ng/g. Standards were prepared from 1000 mg/l single element standards obtained from SCP Science, USA. Pb was measured in nanogram per gram of blood (1000 ng/g of blood ~1000 ng/μl) [63].

For all sample analysis from reported data, the external precision was less than 3.6%. Isobaric corrections were performed online using ICP-MS software. Five duplicate analyses (n = 5) were performed for each sample solution. Limits of detection (LOD) and limits of quantification (LOQ) according to Long and Winefordner [67] were less than 0.0052 and 0.014 ng/g for Pb, respectively. Method detection limits were calculated according to the two-step approach using the t0.05,1,000 method at 99% CI (t = 3.71). The MDL for Pb yielded a value of 0.053 ng/g (53 pg/g or ppt).

Statistical Analysis
We used linear regression models to examine associations of Pb levels in pregnant women, categorized into tertiles, with DNA methylation at four DMRs of imprinted genes that have
previously been associated with variations in birth weight. We first present descriptive statistics for mothers, with a range of Pb exposure in the sample. We excluded three participants (1%) with high circulating blood Pb levels (69.64, 109.17, and 259.39 μg/dl), with a final sample size of 321 mother–child pairs. Pb was categorized into tertiles because the natural log did not normalize the distribution of data. Tertile cutoffs (in μg/dl) were low Pb = 0–0.217, medium Pb = 0.218–0.619, and high Pb = 0.62–9.37. We then computed WHZ scores using Centers for Disease Control (CDC) growth charts. [68] Weight for height Z score change was calculated by subtracting the weight for height Z score of the previous year from the current year weight for height Z score. Logistic regression was used to model the association between the change in weight for height Z score and maternal blood lead levels.

Differences between WHZ scores for consecutive ages were computed, resulting in a continuous value that was categorized into tertiles of slow (< 5.21 to 0.04 at age 0–1; 2.73 to < 0.72 at age 1–2; > 1.68 to < 0.42 at age 2–3), normal (0.05 to 1.62 at age 0–1; 0.71 to 0.95 at age 1–2; 0.41 to < 0.19 at age 2–3), and very rapid (1.63 to 5.65 at age 0–1; 0.10 to 2.87 at age 1–2; 0.20 to 2.36 at age 2–3) growth. We then used linear and logistic regression to evaluate whether prenatal Pb exposure was associated with birth weight, modeled as continuous, and changes in WHZ at ages 0–1 years, 1–2 years, and 2–3 years, modeled as categorical. We found no significant differences between study participants with initial epigenetic and birth weight data (n = 310) and the subset that remained in follow-up by the time the children were 3; except for gestational age (P < 0.004) and birth weight (P = 0.057), because the WHZ score models were limited to those over 34 weeks gestational age.

Logistic regression models were used to evaluate the relationship between Pb tertiles and normally distributed birth weight or DNA methylation levels, and categorical adiposity gain tertiles, respectively. Covariates evaluated for potential confounding were selected based on previous studies, and included cigarette smoking, categorized as never smoked, smoked during pregnancy, or quit smoking before pregnancy, [69] antibiotic use (during pregnancy or not), [63] physical activity dichotomized as ever or never, [62] socioeconomic status estimated by maternal years of schooling, and race/ethnicity defined as self-reported non-Hispanic Black, Hispanic, and non-Hispanic Other vs. non-Hispanic Whites, [70] and pre-pregnancy BMI. [71] We also included maternal age and sex as animal and human studies suggest that the effects of environmental Pb exposure may be sex-specific. [37,22] We used SAS V9.03 for data analysis (SAS Institute Inc.) and the R package.

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