RESEARCH ARTICLE

Cadmium exposure and MEG3 methylation differences between Whites and African Americans in the NEST Cohort

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Abstract

Cadmium (Cd) is a ubiquitous environmental pollutant associated with a wide range of health outcomes including cancer. However, obscure exposure sources often hinder prevention efforts. Further, although epigenetic mechanisms are suspected to link these associations, gene sequence regions targeted by Cd are unclear. Aberrant methylation of a differentially methylated region (DMR) on the MEG3 gene that regulates the expression of a cluster of genes including MEG3, DLK1, MEG8, MEG9 and DIO3 has been associated with multiple cancers. In 287 infant–mother pairs, we used a combination of linear regression and the Getis-Ord Gi* statistic to determine if maternal blood Cd concentrations were associated with offspring CpG methylation of the sequence region regulating a cluster of imprinted genes including MEG3. Correlations were used to examine potential sources and routes. We observed a significant geographic co-clustering of elevated prenatal Cd levels and MEG3 DMR hypermethylation in cord blood (P = 0.01), and these findings were substantiated in our statistical models (β = 1.70, se = 0.80, P = 0.03). These associations were strongest in those born to African American women (β = 3.52, se = 1.32, P = 0.01) compared with those born to White women (β = 1.24, se = 2.11, P = 0.56) or Hispanic women (β = 1.18, se = 1.24,
P = 0.34). Consistent with Cd bioaccumulation during the life course, blood Cd levels increased with age (β = 0.015 μg/dl/year, P = 0.003), and Cd concentrations were significantly correlated between blood and urine (ρ > 0.47, P < 0.01), but not hand wipe, soil or house dust concentrations (P > 0.05). Together, these data support that prenatal Cd exposure is associated with aberrant methylation of the imprint regulatory element for the MEG3 gene cluster at birth. However, neither house-dust nor water are likely exposure sources, and ingestion via contaminated hands is also unlikely to be a significant exposure route in this population. Larger studies are required to identify routes and sources of exposure.

**Key words:** maternal exposures; imprinted genes; offspring epigenetics; cadmium; CpG methylation; racial epigenetic outcome differences

### Introduction

Due to large industrial activity in the last two centuries, cadmium (Cd) is now a ubiquitous environmental contaminant that ranks in the top 10 chemicals of concern [1]. Once in the body, toxicity is amplified by bioaccumulation due to slow excretion rates [2]. This leads to its accumulation in target tissues such as the liver and pancreas, and in long-term storage compartments, including the kidney, neural tissues and bone [3]. The half-life of Cd in humans is estimated to be between 10 and 30 years [4, 5]. Emerging data suggest that ethnic minorities may be at higher risk of non-occupational exposure [6, 7], and co-exposure to other toxic metals, including arsenic (As) and lead (Pb) may interact with Cd to exacerbate health effects [1]. Although cigarette smoke is the main source of non-occupational exposure, environmental health agencies also point to contaminated dietary staples, water and pollution-contaminated air from which Cd can be ingested or inhaled. Recent data based on residential addresses of pregnant women also revealed geographic clustering of Cd and other toxic metals in an inner-city neighborhood, suggesting contaminated soils of some urban landscapes may be a source [6]. However, empirical data linking sources and routes to the body burden are limited.

Reported health effects of high occupational levels of Cd are consistent with target tissue injury (e.g. nephrotoxicity, neurotoxicity and carcinogenicity). In contrast, effects of exposure to lower non-occupational Cd and co-occurring toxic metals has been difficult to discern. The International Agency of Research on Cancer (IARC) has classified Cd as a probable carcinogen based on years of in vitro and in vivo evidence and inconsistent human data. However, in a recent comprehensive review, only half of the 57 human studies found that Cd exposure at levels experienced by the general population increased the risk of components of metabolic syndrome, an established risk factor for multiple cancers [8]. These inconsistencies may partially be due to an inability of prior studies to measure and account for co-occurring metals. Recent advances in inductively coupled plasma (ICP)-mass spectrometry (MS) have enabled the simultaneous measurement of multiple metals in smaller specimen quantities, characteristic in otherwise healthy humans [9].

The paucity of mechanistic data linking molecular intermediates to Cd exposure and adverse health outcomes in humans has also limited our understanding of the effects of lower Cd doses, common in the general population, on health outcomes. Such mechanistic insights have the potential to inform early intervention strategies. Epigenetic regulation, a means by which gene expression is altered in response to environmental cues, including exposure to Cd during the prenatal period, provides flexibility in adapting to changing environments [10]. However, the sequence regions and signaling pathways affected by Cd exposure have been difficult to replicate. In part, this results from the majority of available data being measured using methylation arrays and other targeted regions for which the temporal stability of DNA methylation alterations are unknown. This temporal ambiguity between Cd exposure and methylation alterations complicates causal inference, especially for adult-onset diseases with suspected fetal origins, such as cancer. In contrast, methylation of CpG dinucleotides at regions that control genomic imprinting is differentially established in a sex-dependent manner in gametes and resists the wave of global demethylation that occurs after fertilization. As such, methylation marks of imprint regulatory sequences are temporally stable and have been proposed to serve as an archive of early exposures [11–14]. Recent research using whole-genome bisulfite sequencing with 12× to 15× coverage suggests that imprinted gene control regions are over-represented in differentially methylated regions (DMRs) of offspring and mothers exposed to Cd [15]. However, these findings have not been validated using more quantitatively accurate methods such as pyrosequencing.

Among the most studied cancer-associated imprinted gene, DMRs are those regulating the Delta-like 1 (DLK1)–Maternally expressed gene 3 (MEG3) domain at chromosome 14q32.2. This imprinted domain includes MEG3-IG (methylation established on the paternal allele in sperm) and MEG3 (methylation established on the maternal allele post-fertilization) DMRs. These DMRs regulate the imprinting and expression of a gene cluster that includes paternally expressed DLK1 and maternally expressed MEG3 and MEG8 (Fig. 1). The MEG3-IG DMR is located intergenically ~13kb upstream of the MEG3 transcription start site, while the MEG3 DMR is intragenic and overlaps with the MEG3 promoter. While the MEG3-IG is the main imprint control region that functions in the placenta to control proper allelic expression of transcripts in this region, the MEG3 DMR maintains the active (unmethylated) status at the IG-DMR on the maternal allele and functions in somatic tissues, allowing for expression of downstream genes. Hypermethylation of the maternally imprinted MEG3 DMR has been associated with multiple forms of cancer, many in organs targeted by Cd (reviewed in [16, 17]); however, an association between Cd and altered methylation of the MEG3 DMR has not been described.

In these analyses, we evaluate whether early prenatal Cd exposure associates with differential methylation of the MEG3 DMR control region in offspring. To inform public health policy decisions, we also assessed correlations between environmental routes of exposure including house dust, soil and water with Cd concentrations found in individuals in a geographic cluster with high Cd exposure.

### Results

The characteristics of n = 287 study participants included in these analyses are summarized in Table 1. African American
Methylation levels were higher in African Americans. Metals (Min, 25%, 50%, 75%, Max) obtained during the first trimester (mean gestational age, n = 287) were associated with higher MEG3 DMR hypermethylation. When stratified by ethnicity, associations between high maternal Cd exposure and MEG3 DMR hypermethylation were most apparent in offspring of African Americans (β = 1.84, P = 0.01) compared with Whites (β = 1.24, P = 0.56) and Hispanics (β = 1.18, P = 0.34) (Table 2). Tests for heterogeneity of regression coefficients for each ethnic group revealed that associations of Cd exposure and MEG3 hypermethylation were significantly higher in African Americans compared to both Whites (P = 0.02) and Hispanics (P = 0.03). Scatterplots of mean MEG3 methylation and maternal Cd exposure shown by race in Fig. 2 support that offspring of African American women had significantly higher methylation levels of the MEG3 DMR. Because the skewed Cd distribution that was not improved by log-transformation necessitated Cd to be dichotomized at the top quartile, we considered models where Cd was dichotomized at the top tertile or quintile, to ensure that our findings were not unduly influenced by how Cd was dichotomized. Our findings that Cd exposure and being born to an African American mother were independently associated with higher methylation of the MEG3 DMR (β = 1.94, P = 0.04).

When stratified by ethnicity, associations between high maternal Cd exposure and MEG3 DMR hypermethylation were most apparent in offspring of African Americans (β = 1.84, P = 0.01) compared with Whites (β = 1.24, P = 0.56) and Hispanics (β = 1.18, P = 0.34) (Table 2). Scatterplots of mean MEG3 methylation and maternal Cd exposure shown by race in Fig. 2 support that offspring of African American women had significantly higher methylation levels of the MEG3 DMR. Because the skewed Cd distribution that was not improved by log-transformation necessitated Cd to be dichotomized at the top quartile, we considered models where Cd was dichotomized at the top tertile or quintile, to ensure that our findings were not unduly influenced by how Cd was dichotomized. Our findings that Cd exposure and being born to an African American mother were independently associated with higher MEG3 DMR, remained unchanged, and heterogeneity test to determine whether the associations differed by race remained unaltered (data not shown).
Mediation by MEG3 DMR Methylation of Associations between Cd and Lower Birth Weight

Despite limited statistical power, given previously reported inverse associations of maternal Cd exposure and birthweight [18], we conducted mediation analyses to assess whether MEG3 methylation mediates this association. The direct effect of the highest quartile of maternal Cd burden on offspring birthweight was a loss of 174 g of birthweight ($b = -174; 95\%\ CI = (-310, -47.0); P = 0.01$). The tested mediation effect of MEG3 methylation was not significant in the full model with all ethnic groups ($b = 10.9; 95\%\ CI = (-37.0, 6.39); P = 0.23$), or when examined only for African Americans. In the fully adjusted model with all races, the proportion of this association explained by MEG3 methylation was 5.16% ($P = 0.24$). When examined only on African American mothers, the proportion increased to 28.4%, but was still not significant ($P = 0.18$).

MEG3 DMR Methylation and Gene Expression Cluster

To determine the functional significance of the MEG3 DMR hypermethylation that co-clustered with Cd and Pb exposure, we measured and correlated gene expression of three of the genes in the imprint gene cluster regulated by the MEG3 DMR (i.e. DLK1, MEG3 and DIO3) with DNA methylation in the cord blood of a subset of 22 offspring. We found negative correlations between both MEG3 imprint control regions and the expression of DIO3 and DLK1 (Supplementary Table S3), but these results were not significant and difficult to interpret due to small sample size.

Geospatial Cluster of MEG3 Hypermethylation

Figure 3 shows results of the geospatial cluster analysis (Getis-Ord Gi*) of offspring MEG3 hypermethylation dichotomized at the top quartile (>77% methylation), together with maternal blood concentration for Cd and Pb based on street address during the prenatal period of gestation. Among the 287 offspring, 32% of African Americans were in the top quartile, compared with 21% of Whites and 22% of Hispanics. We found evidence for geospatial clustering of MEG3 DMR hypermethylation with high confidence ($P < 0.001$). Intriguingly, this geographic clustering of MEG3 DMR hypermethylation coincided with elevated geographical Cd and Pb concentrations previously reported (Fig. 3) [6]. This spatial co-location of MEG3 DMR hypermethylation with elevated concentrations of Cd in the population supports the correlation between aberrant methylation and exposure.

Potential Sources of Environmental Chemical Exposure

To identify potential sources and routes of toxic metal exposure, we also examined correlations between levels of Cd in
blood, urine, soil, tap water and house dust in an independent sample (n = 37) of randomly selected children and adult residents in the geographic cluster. The range of measurements are summarized in Supplementary Figure S1. We found that concentrations of Cd in blood and urine were positively correlated (\( r = 0.47, P < 0.01 \)), and Cd blood concentrations were associated with age, as expected (\( \beta = 0.015 \, \text{mg/dl/year}, P = 0.003 \) and Table 3). However, Cd levels in soil, house dust and water were not significantly correlated with urine or blood Cd concentrations (Table 3). Removing two outliers (Supplementary Figure S1) did not materially alter these correlations (Supplementary Table S1).

**Discussion**

Exposure to non-occupational levels of Cd may be carcinogenic, and other common trace metals may exacerbate the effects [19]; however, until recently, the effects of other toxic metals were not easily measurable in limited quantities of source material, characteristic of human specimens. Although epigenetic responses to Cd exposure are the hypothesized mechanisms of action, the affected genomic regions are still unclear. We evaluated whether prenatal Cd exposure was associated with hypermethylation of the MEG3 DMR in offspring as similar patterns of MEG3 DMR methylation have been linked to the development or progression of multiple cancers (reviewed in [20]). Our key findings were that after adjusting for co-occurring toxic metals, As and Pb and other prenatal factors previously shown to alter CpG methylation at birth, exposure to Cd in utero was associated with hypermethylation of the MEG3 DMR and the magnitude of this effect was comparable to that of being born to an African American woman. These findings were corroborated by geospatial analysis demonstrating that hypermethylation of the MEG3 DMR (defined as quartile 4 or \( >77\% \) mean methylation) coincided geospatially with elevated Cd exposure. The MEG3 DMR effects are unlikely to be influenced by co-exposure to As or Pb. To our knowledge, we present the first evidence linking ethnic-specific hypermethylation of a region that controls a large imprinted gene domain to non-occupational Cd exposure, a common environmental toxicant in humans.

We found striking ethnic differences in the association between Cd exposure and methylation of MEG3. While these findings may be artifactual, and should be confirmed in larger studies, other studies have demonstrated epigenetic variability for both ethnicity and environmental exposures by ethnicity [21, 22]. Methylation pattern alterations in sequence regions
that regulate genomic imprinting in response to environmental exposures have been reported previously in humans. For example, both severe and moderate in utero caloric restriction during the 'Dutch Famine' and the Gambia [23, 24] was associated with hypomethylation of the imprint control region that regulates expression of the IGF2/H19 imprinted gene cluster in offspring six decades after birth [25]. Pb exposure in utero and in early life is associated with hypomethylation of regions regulating the IGF2/H19, PLAGL1/HYMA1 and MEG3 imprinted gene clusters among 30-year-old adults [26]. As adults, these individuals displayed decreased brain volume [27] and increased risk of neurological disorders and juvenile delinquency [28, 29]. MEG3 was first identified in mice as Gene Trap Locus 2 (Gtl2). Imprinting of the human ortholog of Gtl2, now referred to as Maternally Expressed Gene 3, or MEG3, was first reported by our group [30]. MEG3 encodes a long noncoding RNA (lncRNA) that is normally expressed in multiple tissues including placenta, brain, adrenal gland, pancreas, spleen and liver, and is reciprocally imprinted with paternally expressed DLK1. The expression of these genes is controlled by CpG methylation at the germline-derived intergenic differentially methylated DMR (MEG3-IG) located 13 kb upstream of MEG3, and at the MEG3 DMR that overlaps the promoter (Fig. 1) [30]. In mice, deletion of the MEG3-IG DMR on the maternal chromosome results in an epigenotype switch, where the domain starts exhibiting the transcriptional profile of the paternal chromosome [31, 32]. Aberrant hyper-methylation of the MEG3-IG or MEG3 DMRs is correlated with greatly decreased MEG3 transcripts, which in turn is associated with increased expression of the reciprocally imprinted DLK1, cell proliferation in vitro, more aggressive and higher grade human tumors including meningioma, colon, nepharyngeal, liver as well as leukemia [20]. Restoration of MEG3 expression suppresses tumor growth [33]. Since one function of the MEG3 DMR is to maintain an active (unmethylated) status in the MEG3 IG region, hypermethylation of MEG3 DMR in response to Cd has the potential to alter the expression of a large number of genes in this imprinted domain, including MEG3 and other imprinted noncoding RNA genes such as MEG8 and MEG9. These observations are consistent with our findings that hypermethylation of the MEG3 DMR was correlated, albeit not significantly, with the expression of DLK1, DIO3 and MEG3 (Supplementary Table S3).

While mechanisms elucidating how MEG3 DMR hypermethylation alters the trajectories of many cancers for the worse are still unclear, previous studies have indicated that MEG3 functions as a tumor suppressor through activation of p53. p53 is a crucial tumor suppressor involved in preventing cancer through its response to cellular stressors, acting as a transcription factor and inducing the expression of genes involved in senescence, apoptosis and cell cycle arrest. MEG3 has been shown to interact with the p53 DNA-binding domain at various p53 target genes [34]. Ectopic expression of MEG3 in human cancer cell lines restores tumor suppressive activities and results in accumulation of p53 protein, activation of p53 downstream targets and inhibition of cell proliferation by inducing cell cycle arrest and apoptosis [33, 35]. It is therefore plausible that Cd-induced decreases in MEG3 transcripts could result in loss of MEG3 tumor suppressive function and increase susceptibility to cancer.

The weak correlation observed between MEG3 DMR methylation and expression of the MEG3 transcripts may be because in humans, the MEG3 gene contains 10 exons and a number of RNA isoforms transcribed from human MEG3 [36, 37]. All isoforms contain common exons 1, 2, 3, 8 and 10, but exhibit variation in expression of exons 4–7 and 9 [36]. The ability to regulate p53 and cell proliferation varies among MEG3 isoforms and is dose dependent [34, 35], likely depending on the degree or pattern of methylation in this promoter region. Factors that influence MEG3 splicing could affect the ability of MEG3 RNAs to regulate p53 and subsequent cell cycle arrest, apoptosis and cancer suppression. Interestingly, 12 MEG3 isoforms are present in human fetal liver, but only a few are present in adult tissue, suggesting a difference in regulation of splicing mechanisms between adult and fetal tissues [36]. Thus, understanding the relationship between Cd, MEG3 DMR methylation and health outcomes will require an assessment of alternatively spliced MEG3 transcription in addition to CpG methylation.

We attempted to elucidate what environmental exposures are driving elevated Cd levels with home visits to 35 individuals in the geographical region identified in Fig. 3. The body burden of Cd measured in blood increased in a linear fashion with age. We had expected to identify routes of environmental exposures for body burden via correlations between environmental sources and human body fluid but were unable to do so. However, we cannot exclude the possibility that the lack of significant correlations may be due to the limited number of samples we assessed with home visits. Large studies are needed to identify sources and routes of Cd exposure that can guide intervention efforts.

This study has several strengths. The longitudinal design, with Cd measured in first trimester blood specimens and MEG3 DMR methylation measured in cord blood leukocytes enhances causal inference. Most Cd (99%) attaches to erythrocytes which have a lifespan of ~120 days, such that first trimester measurements are likely to provide an accurate representation of the levels of Cd present during epigenetic reprogramming that occurs during the periconceptional period. We have implemented multiplexed measurements of Cd, Pb and As with the

| Table 3: environmental and body burden of Cd concentrations |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| N               | Blood           | Urine           | Soil            | Water           | Dust (W)        | Dust (V)        | Age             |
| Blood           | 36              | 0.47 (<0.01)    | −0.12 (0.49)    | 0.18 (0.32)     | −0.04 (0.83)    | 0.17 (0.54)     | 0.58 (<0.01)    |
| Urine           | 36              | 0.31 (0.08)     | −0.06 (0.75)    | 0.31 (0.08)     | −0.06 (0.75)    | 0.42 (0.10)     | 0.16 (0.37)     |
| Soil            | 35              | −0.14 (0.43)    | 0.01 (0.94)     | −0.14 (0.43)    | 0.01 (0.94)     | 0.12 (0.66)     | −0.09 (0.62)    |
| Water           | 35              | −0.06 (0.74)    | 0.14 (0.60)     | −0.06 (0.74)    | 0.14 (0.60)     | 0.08 (0.63)     |               |
| Dust (W)        | 37              | −0.36 (0.16)    | −0.17 (0.31)    | −0.36 (0.16)    | −0.17 (0.31)    |               |               |
| Dust (V)        | 17              | 0.26 (0.32)     |               |               |               |               |               |
| Age             | 37              |               |               |               |               |               |               |

Spearman correlations (P-values) are shown for the levels of Cd measured in blood (ng/g), urine (ng/g), soil (µg/g), household water (µg/l), house dust wipe (W, µg/g), house dust vacuumed (V, µg/g) and age for youngest household occupant. Two measurements were removed due to value > mean + 3 standard deviations (see Supplementary Methods).
use of ICP-MS, which enabled statistical adjustment for these co-occurring toxic metals in statistical analyses. Further, we utilized geospatial analyses to assess the overlap of geographical distribution of MEG3 DMR hypermethylation with environmental exposure to corroborate our statistical models. These analyses support that levels of Cd, and not those of Pb or As, associate with MEG3 DMR hypermethylation. The multi-ethnic composition of our cohort enabled stratification by ethnicity, revealing considerably large effects in offspring of African Americans that were not apparent in Whites or Hispanics. While the significance of these ethnic differences is unclear, they suggest that, at a minimum, race-specific analyses are warranted in human epigenetic studies as well in the assessment of Cd exposure and imprinted gene methylation. The latter will require a concerted effort in characterizing the human imprint regulatory network of genes. Although this study solely focused on the regulatory region of MEG3, this region regulates a large number of imprinted genes, including DLK1, MEG8, MEG9, MEG11 and DIO3 and similar patterns of methylation have been associated with multiple cancers. The follow-up of the cohort is short, limited only to age five years for a small number of children. Thus, the associations between Cd exposure, MEG3 DMR methylation and phenotypes that are likely to be maintained into adulthood have as yet to be shown in humans.

Despite these limitations, our geospatial data and linear regression analyses provide novel evidence for Cd, and not for Pb or As, associating with MEG3 DMR hypermethylation, with the potential to alter gene expression for a large number of paternally and maternally expressed genes in the imprinted domain. Similar methylation in this regulatory region has been associated with the initiation of aggressiveness of multiple human tumors. Larger studies are needed to identify routes of exposure and to verify associations in order to inform public health intervention efforts, and to identify the array of genes and specific pathways perturbed by Cd via altered CpG methylation that elevate risk of chronic diseases including cancer.

**Methods**

**Study Participants**

Participants derived from the Newborn Epigenetics Study (NEST), a cohort of pregnant women and their children whom, at enrollment, intended to receive obstetric care at one of two facilities in Durham County, NC, between 2009 and 2011. All participants provided informed consent. Accrual protocol details have been provided elsewhere [38]. Briefly, 1700 pregnant women were enrolled consecutively. Inclusion criteria were pregnancy, aged 18+ years or older and English or Spanish speaking. We excluded those planning to relinquish custody of the child and those whom, despite intent, did not deliver at one of the two facilities due to our inability to obtain umbilical cord blood. These analyses are restricted to 287 infant-mother pairs in whom at least Cd, As and Pb were measured in maternal blood (i.e. the first 310 enrolled) and for whom CpG methylation was measured in umbilical cord blood leukocytes. This study was approved by Duke University School of Medicine’s Institutional Review Board. A total of 36 randomly selected households in the geographic clusters, where the Cd geospatial cluster was identified, were re-contacted between December 2014 and February 2016, to confirm the body burdens of Cd, As and Pb and identify potential sources of exposure. Blood and urine samples were obtained from the youngest member of the household, for a total of 10 children under the age of 20 years and 26 aged 20–64 years. Hands and floor samples were obtained using hand and dust wipes on wooden floors and vacuums on carpeted floors. This study was approved by North Carolina State University Institutional Review Board.

**Measurements**

**MEG3 DMR Methylation**

Trained phlebotomists drew 10 ml of pregnant women’s blood into ethylenediaminetetraacetic acid (EDTA)-containing vacutainer tubes at enrollment. DNA for pyrosequencing was extracted from umbilical cord blood buffy coat using Puregene reagents. Pyrosequencing was performed using a Qiagen Pyromark Q96 MD Pyrosequencer. Primers and PCR conditions have been previously described in detail [39, 40] as well as in Supplementary Methods. The methylation fraction for each CpG dinucleotide was calculated using PyroQ CpG Software (Qiagen). The methylation fraction was analysed at multiple CpG sites for the MEG3 DMRs.

**Blood Cd, Pb and As**

From the 10 ml of peripheral blood obtained from pregnant women in EDTA-containing vacutainer tubes, 1 ml of whole blood was removed and stored. About 200 µl aliquots were used to measure Cd, Pb and As concentrations using a Perkin Elmer Dynamic Reaction Cell (DRC) II axial field ICP-MS at the University of Massachusetts, Boston as previously described [9, 41, 42]. To clean sample lines and reduce memory effects, sample lines were sequentially washed with 18.2 MΩ cm resistance water (Milli-Q water purification system, Millipore, Bedford, MA, USA) for 90 s and a 2% nitric acid solution for 120 s between analyses. Procedural blanks were analysed within each block of 10 samples, to monitor and correct for instrumental and procedural backgrounds. Calibration standards used to determine metal in blood included aliquots of Milli-Q water, and NIST 955c SRM spiked with known quantities of each metal in a linear range from 0.025 to 10 µg/dl. Standards were prepared from 1000 mg/l single element standards (SCP Science, USA). Method detection limits (MDLs) were calculated according to the two-step approach using the twoSLM method (USEPA, 1993) at 99% CI (t = 3.71) [43]. The MDLs yielded values of 0.006, 0.005 and 0.071 µg/dl for Cd, Pb and As, respectively. Limits of detection (LOD) were 0.002, 0.002 and 0.022 µg/dl for Cd, Pb and As, respectively, and limits of quantification (LOQ) (according to Long and Winefordner, 1983) were 0.0007, 0.0006 and 0.0073 µg/dl for Cd, Pb and As, respectively. The number of samples below the LOD for Cd, Pb and As were 2, 1 and 1, respectively. Correlations between each metal are reported in Supplementary Table S2.

**House Dust and Hand Wipe Cd and Pb**

In water samples, trace metals were analysed after calibration using serial dilutions of NIST 1643e standard spiked with U and Th. Ghost wipe samples were digested with ultra-pure HNO3 (Fisher Optima) and double-distilled water (resistivity >18.2 MΩ cm). For measurement of total Pb, Cd and other trace elements, 0.034 g of soil and dust floor samples were digested. Reference materials used for instrument calibration and standardization included USGS certified geochemical reference materials G2, W2, BIR, SCO, SDO, AGV, RGM and SDC. All digestion processes of the dust samples were prepared using ultrapure HNO3 and HF reagents (Fisher Optima) and double-distilled water. All plastic polyethylene bottles and tubes were soaked.
and cleaned with 1 N HCl and 1 N HNO₃, and then rinsed with deionized water. Trace elements were analysed via a VG Plasmaquad 3 ICP-MS at Duke’s Nicholas School of the Environment.

Expression Data
Expression of DLK1, DIO3 and MEG3 were assessed using Taqman® gene expression assays (Hs00171584_m1, Hs00956431_s1 and Hs00292028_m1 respectively) using 18S (Hs99999901_s1) as the housekeeping gene. Protocols were followed according the manufacturer’s instructions. In brief, delta Ct values net of 18S were then subtracted from mean delta Ct value for each measured transcript by individual to get an index of low to high expression individuals (Supplementary Data S1).

Covariate Data
In addition to other toxic metals, factors that may influence the association between Cd exposure and CpG methylation include maternal obesity, parity, gestational age, ethnicity and offspring sex. Maternal obesity was estimated from self-reported maternal pre-pregnancy anthropometric measurements, including current height (m) and usual pre-pregnancy weight (kg) and BMI that was expressed as kg/m². Participants were defined as non-obese (BMI < 30 kg/m²) and obese (BMI ≥ 30 kg/m²) according to the World Health Organization classifications. Ethnicity was self-reported as African American, White, Hispanic or Other. Parity, gestational age at delivery and sex of offspring were reported and verified using medical records.

Statistical Analyses
Cluster Analysis
MEG3 DMR methylation percentages were normally distributed, and ranged from S6 to 85%, IQR = 69–77%, with mean and median of 72.3 and 72.9%, respectively. A MEG3 DMR methylation fraction at or exceeding the top quartile (Q3/Q4 = 77% methylation) was defined as MEG3 DMR hypermethylation. To ensure that an appropriate denominator was used, these analyses were limited to the subset of women living in Durham County at the time of blood draw used for metal measurements. To minimize the effects of cigarette smoking in this cluster analysis, smokers were also excluded.

The Hot Spot Analysis and Kernel Density Tools within ArcGIS 10.2.2 for cluster mapping and analysis was used to generate geographical heat maps from infant MEG3 DMR methylation at birth, yet de-identify the specific prenatal address of study participants. The MEG3 DMR hypermethylation density was calculated over a 1 km² grid and reported as ng/g/km² and the Getis-Ord Gi* statistic was calculated for each offspring. This statistic is based on the MEG3 DMR hypermethylation value, a spatial weight between neighboring features and the total number of features. The Gi* statistic generated a Z-score and corresponding P-value that tested the hypothesis that a given pattern was the result of randomness in MEG3 DMR methylation fractions. For this analysis, a fixed-distance band of 1500 m was used to calculate the spatial influence of neighboring points. Points falling outside the 1500 m band were considered non-influential in the calculation of the Gi*. The resulting significance levels (P-value) were then plotted using a heat map to show high (P < 0.01) and low cluster probabilities for a given methylation fraction.

Linear Regression Models
We used general linear regression models to evaluate the relationship between Cd exposure during the first trimester and MEG3 DMR methylation at birth. Because of the severely right-skewed distribution of Cd concentrations that could not be improved by log-transformation, Cd exposure was dichotomized as upper quartile of blood concentration (0.513 ng/g) compared with all others. These models were adjusted for maternal co-exposure to Pb and As, race/ethnicity (African American, White, Hispanic and Other), cigarette smoking during gestation (yes or no), maternal obesity before pregnancy (continuous BMI), weeks of gestation (continuous) as a general indicator of fetal distress, offspring sex and maternal education, a stable indicator of socioeconomic status. Refined models were stratified separately by race/ethnicity and estimates for race were tested for homogeneity in SAS 9.4.

Mediation analysis
We used the mediation [44] package in R to test if mean MEG3 methylation acted as a mediator on previous work showing associations between MEG3 hypermethylation and low birthweight.

Supplementary Data
Supplementary data are available at EnvEpig online.

Conflict of Interest Statement
None declared.

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