



RESEARCH ARTICLE

DNA methylation at birth potentially mediates the association between prenatal lead (Pb) exposure and infant neurodevelopmental outcomes

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Abstract

Early-life lead (Pb) exposure has been linked to adverse neurodevelopmental outcomes. Recent evidence has indicated a critical role of DNA methylation (DNAm) in cognition, and Pb exposure has also been shown to alter DNAm. However, it is unknown whether DNAm is part of the mechanism of Pb neurotoxicity. This longitudinal study investigated the associations between trimester-specific (T1, T2, and T3) maternal blood Pb concentrations, gene-specific DNAm in umbilical cord

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blood, and infant neurodevelopmental outcomes at 12 and 24 months of age (mental development index, psychomotor development index, and behavioral rating scale of orientation/engagement and emotional regulation) among 85 mother–infant pairs from the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) study. In the mediation analysis for this pilot study, $P < 0.1$ was considered significant. DNAm at a locus in CCSE1 (probe ID cg02901723) mediated the association between T2 Pb on 24-month orientation/engagement [indirect effect estimate 4.44, 95% confidence interval (–0.09, 10.68), $P = 0.06$] and emotional regulation [3.62 (–0.05, 8.69), $P = 0.05$]. Cg18515027 (GCNT1) DNAm mediated the association of T1 Pb [–4.94 (–10.6, –0.77), $P = 0.01$] and T2 Pb [–3.52 (–8.09, –0.36), $P = 0.02$] with 24-month EMOCI, but there was a positive indirect effect estimate between T2 Pb and 24-month psychomotor development index [1.25 (–0.11, 3.32), $P = 0.09$]. The indirect effect was significant for cg19703494 (TRAPPC6A) DNAm in the association between T2 Pb and 24-month mental development index [1.54 (0, 3.87), $P = 0.05$]. There was also an indirect effect of cg23280166 (VPS11) DNAm on T3 Pb and 24-month EMOCI [2.43 (–0.16, 6.38), $P = 0.08$]. These associations provide preliminary evidence for gene-specific DNAm as mediators between prenatal Pb and adverse cognitive outcomes in offspring.

Key words: epigenetics; lead (Pb); developmental exposure; environmental exposure; developmental programming; neurodevelopment

Background

Lead (Pb) is an abundant element and environmental pollutant found in air, soil, and water that has been used for thousands of years due to the ease of extraction from ores, relative abundance on earth, and low cost. Examples of exposure sources common today include ingestion of contaminated water and lead-based paint in older homes. In some countries, such as Mexico, Pb absorbing into food from lead-glazed ceramics used for food preparation and storage is another major source of exposure (1). Pb is a cumulative toxicant that affects nearly all organs. Pb negatively impacts cognitive and behavioral functions, impairs learning and memory, decreases intelligence quotient (IQ), increases aggression, and may increase the risk for developing a variety of late-life neuropathological disorders (2–10). Despite major initiatives to reduce environmental exposures, epidemiological studies demonstrate that even low levels of Pb in blood affect IQ and behavior with major impacts on functioning (11–15).

During pregnancy, Pb from past exposures stored in maternal bone is released into the bloodstream, passes through the placenta, and reaches the developing fetus (16–18). Gestational Pb exposure has been associated with lower scores on neonatal and infant neurobehavioral tests, even when exposure levels are below 5 $\mu\text{g}/\text{dl}$ in maternal blood (5) and below 2 $\mu\text{g}/\text{dl}$ in cord blood (19). In the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) study, we previously reported that each unit of first trimester (T1) maternal blood Pb level (BLL, natural-log transformed $\mu\text{g}/\text{L}$) was associated with a decrease of 4.13 points on the Bayley Scales of Infant Development mental development index (MDI) among 24-month old children (10). Another report provided evidence that lower MDI scores as early as 6 months of age were associated with late pregnancy maternal Pb exposures below 5 $\mu\text{g}/\text{dl}$ (20). Mechanistically, the N-methyl-D-aspartate (NMDA) receptor is particularly sensitive to developmental Pb exposure due to its important role in hippocampal-dependent spatial learning and memory (21). Studies have shown that developmental Pb exposure in rats alters NMDA subunits and signaling, which are associated with impaired hippocampal long-term potentiation representing a biological mechanism by which Pb can impact learning and memory (22–24). Developmental exposures including Pb may also impact phenotype through alterations in gene regulation via epigenetic modifications, such as DNA methylation (25–27).

Epigenetics is the study of mitotically heritable and potentially reversible changes in gene expression that are independent of the DNA sequence. DNA methylation (5mC) is the addition of a methyl group covalently bound to the 5'-carbon of Cytosine. In mammals, a methylated cytosine is typically adjacent to a Guanine by a phosphate, referred to as a CpG site. The period of fetal development has been shown to be sensitive to prenatal exposures, mainly because of the dramatic DNA methylation changes and reprogramming that takes place during embryogenesis (25, 26). There is current evidence for epigenetic modifications related to deficits in language, cognition, motor skills, and other functional domains in various neurodevelopmental disorders (28–31). Pb-induced oxidative stress may alter DNA methylation, which could then alter gene expression and contribute to toxicity (32). A growing number of studies have provided evidence for gestational Pb exposure influencing offspring epigenetic programming in murine studies at candidate genes (33, 34) and epigenome-wide (35). Further, in human studies, epigenome-wide platforms have helped to identify candidate gene pathways that were potentially impacted by prenatal Pb exposure (36–39). Among these studies, we previously utilized the Infinium MethylationEPIC to profile DNA methylation at >850 000 CpG sites and reported statistically significant associations between maternal first- and third-trimester blood Pb levels and DNA methylation at several loci (38).

Despite the consistent evidence for the inverse association between prenatal Pb exposure and cognitive and behavioral outcomes in childhood (5, 10, 19, 20), whether perturbation of DNA methylation is a mediator of Pb-induced neurotoxicity is largely unexplored. We hypothesized that DNA methylation levels in previously identified prenatal Pb-associated genes at birth in umbilical cord blood (UCB) mediate the association of trimester-specific maternal Pb exposure with neurodevelopmental outcomes within the first 2 years of life. We utilized the ELEMENT longitudinal pregnancy cohort with rich data on trimester-specific Pb exposure and infant follow-up at 12 and 24 months of age to investigate this question. This longitudinal study builds upon our past ELEMENT study which reported an association between prenatal Pb exposure and reduced MDI score in infancy (10) and examines potential mediation of this association by DNA methylation.

Methods

Study Population

The ELEMENT study consists of three longitudinal pregnancy cohorts used to investigate the influence of Pb exposure—in utero and in childhood—on health outcomes during sensitive periods of development. The current study includes mother–infant pairs with prenatal information from the second and third birth cohorts who were recruited at the Mexican Social Security Institute, Mexico City between 1997 and 2005. Eligibility and exclusion criteria are as previously described (40). Maternal variables collected include age, socioeconomic status, and IQ; child variables include sex, gestational age at birth, and neurodevelopmental and anthropometric assessments at multiple follow-up visits in infancy and childhood. For the current study, 85 ELEMENT participants were selected based on availability of prenatal blood Pb measures, archived UCB from which we could isolate DNA that passed quality control checks for DNA methylation analysis, and neurodevelopmental assessments at 12 and 24 months of age (38).

At time of recruitment, mothers were informed about the study and those who agreed to participate read and signed a letter of informed consent about the original study. Mothers also provided written, informed consent at each follow-up visit. The ethics committees of the National Institutes of Public Health of Mexico, participating hospitals, and the Internal Review Board at all participating institutions including the University of Michigan, approved the research study protocol and all amendments.

Pb Concentrations, DNA Isolation, and DNA Methylation Quantification

Concentrations of Pb were measured in maternal venous blood collected during each trimester as previously described (38). Infant venous blood was collected in trace metal-free tubes at ages 12 and 24 months and immediately refrigerated until Pb analysis using atomic absorption spectrophotometry (Model 3000; Perkin Elmer, Wellesley, MA, USA) at the Center for Environmental Health Research American British Cowdray Hospital Trace Metal Laboratory in Mexico City. For quality control, Pb analysis was repeated by the Wisconsin Laboratory of Hygiene, and precision and accuracy were demonstrated (Pearson $r > 0.98$; mean difference $< 1 \mu\text{g/dl}$ between inter-laboratory replicates).

DNA was isolated from UCB, bisulfite converted, and DNA methylation was quantified utilizing the Infinium MethylationEPIC BeadChip (Illumina) (41). Detailed description of the DNA isolation and data processing procedures is as previously described (38). The methylation level of each CpG site was calculated as beta-values ranging from 0 (unmethylated) to 1 (methylated). CpG sites from Rygiel *et al.* were selected to be tested as mediators in this analysis that fit the following criteria: CpG site was associated with a trimester-specific BLL with $P < 1E-5$ and effect size $> |0.01|$ and CpG site annotated to a gene related to neurodevelopment, fetal growth, or neuronal growth. The top five CpG sites meeting these criteria from each trimester of BLL were selected: Trimester 1: RAB5A (EPIC probe ID: cg17138393), EXT1 (cg00984923), KDM6B (cg16049335), GCNT1 (cg18515027), RPS29 (cg03724407); Trimester 2: CHTF18 (cg26820233), PRDM16 (cg12267948), TAPBP (cg20603557), TRAPPC12 (cg08025337), ICAM5 (cg10604476); Trimester 3: GORASP2 (cg02608914), TRAPPC6A (cg19703494), VPS11

(cg23280166), MTA1 (cg20482280), CCSER1 (cg02901723) (Supplementary Table S1).

Child Neurocognitive Assessment

Infant cognitive, motor, and behavioral development at 12 and 24 months was assessed by a trained personnel using the Bayley Scales of Infant Development II–Spanish version (BSID-II) (42) using a standardized protocol described in a previous study by our research group (43). It is the most widely used test to identify young children with potential developmental delays. Researchers performing the test were blinded to the infant's current and past Pb exposure results. Mental development index (MDI), psychomotor development index (PDI), and behavioral rating scale (BRS) of orientation/engagement (ORIEN) and emotional regulation (EMOCI) at 12 and 24 months of age were the outcomes included in this longitudinal study (MDI-12, MDI-24, PDI-12, PDI-24, EMOCI-12, EMOCI-24, ORIEN-12, and ORIEN-24). The BRS describes the child's attention, social engagement, orientation, and motivation, and may partly explain variations in individual performance based on behavior when assessing the MDI and PDI scales. MDI and PDI are classified using standardized scores. The deviation of an individual's score from that of the mean is used to classify developmental delay from this particular population: normal, mild delay, significantly delayed. Classification of developmental delay are as follows: normal (≥ 84), mild (≥ 75.4 and < 84), significant delay (< 75.4) for 12-month MDI and PDI scores; normal (≥ 85), mild (≥ 75.4 and < 85), significant delay (< 75.4) for 24-month MDI and PDI scores. ORIEN assesses an infant's state, affect, energy, interest, exploration, and responsiveness to an examiner, along with their behavior towards those measures. EMOCI is an assessment of the infant's range of affect and emotional response to both success and failure on the assessment. Raw scores were converted to percentiles for each factor within each age group. The BRS outcomes were scored as percentiles, where lower scores reflecting more adverse behaviors. Specifically, BRS scores can be categorized as Within Normal Limits (26th percentile or above), Questionable (11th to 25th percentile), and Non-Optimal (at or below the 10th percentile). Percentiles within normal limits were ≥ 70 for EMOCI-12, ≥ 72 for ORIEN-12, ≥ 82 for EMOCI-24, and ≥ 71 for ORIEN-24; questionable were between 49 and 69 for EMOCI-12, 57 and 71 for ORIEN-12, 33 and 81 for EMOCI-24, 27 and 70 for ORIEN-24; and Non-Optimal < 46 EMOCI-12, < 57 for ORIEN-12, < 33 for EMOCI-24, < 27 for ORIEN-24 for this particular study. To our knowledge, only one other study has assessed EMOCI and ORIEN in Pb exposed infants. Their study showed exposed infants had significantly lower scores in both EMOCI and ORIEN measures when compared to unexposed infants, but no other studies have used these specific measures (44). In our previous research with ELEMENT children (cohort 2), higher prenatal Pb exposure was associated with lower 24-month MDI scores (10); here, we expanded to include 24-month PDI, ORIEN and EMOCI, as well as 12-month MDI, PDI, ORIEN and EMOCI.

Covariates

Maternal IQ was measured using a Spanish translated version of the Information, Comprehension, Similarities, and Block Design subtests of the Wechsler Adult Intelligence Score (45). Additional information, such as infant weight, length, socioeconomic status, and other factors that could confound the relationship between Pb and infant development was collected.

Z-scores by age and sex for length, weight and BMI were calculated by using the World Health Organization (WHO)/National Center for Health Statistics/CDC reference data (46, 47). For each UCB sample, estimates of cell-type proportions for T lymphocytes (CD4T, CD8T), B cells, NK cells, monocytes, granulocytes, and nucleated red blood cells were performed using an established method based on UCB cell-type specific differentially methylation regions (48).

Statistical Analysis

The R Project for Statistical Computing (version 3.6.1) was used to perform all statistical analyses. Descriptive statistics were calculated for continuous measures as means and standard deviations and for categorical measures as frequencies, including sex ratios, age of mothers, maternal IQ, socioeconomic status, gestational age, infant weight, infant length/weight/BMI-for-age Z-score, infant neurodevelopmental continuous measures (MDI, PDI, EMOCI, and ORIEN) and Pb biomarkers. Pb variables analyzed include maternal BLLs at each trimester (T1, T2, and T3) and infant BLLs at 12 and 24 months of age, which were all treated as continuous variables. The neurodevelopmental measures at 12 and 24 months of age were considered the primary dependent variables and models as continuous scores. Maternal BLL measures at the T1, T2, and T3 were the primary exposure biomarkers. The DNA methylation data within the fifteen differentially methylated CpG sites from the UCB EPIC analysis were the mediators (Supplementary Table S1).

Continuous variables' distributions were visually checked for normality. Current BLL and maternal trimester BLLs were natural-log transformed in all models to fit normality assumptions. We first determined relationships between prenatal Pb exposure variables and covariates (e.g. maternal age, offspring sex, length-for-age Z-score, weight, socioeconomic status, cell-types, etc.). We examined Spearman's rank-order correlations between continuous covariates including cell type estimates, Pb biomarkers, DNA methylation at each CpG site, and outcomes. We then chose potential confounding variables to include in final statistical mediation models that were associated with prenatal Pb, DNA methylation, and infant neurodevelopmental outcomes. Cell-types estimates were not correlated with the 15 CpG sites, exposures or outcomes; therefore, they were not included in downstream models. In final models, we included potential confounders in these relationships (infant sex, maternal age at birth, maternal IQ at birth) and covariates that influence the outcome (infant BLL, infant weight and length-for-age Z-scores, infant age at time of measurement).

Pairwise multivariable linear regression analyses were performed to test the outcome model (i.e. exposure → outcome; observed neurodevelopment outcomes scores at each timepoint given trimester-specific Pb exposure) and mediator models (i.e. exposure → mediator; DNA methylation per CpG-site given trimester-specific Pb exposure). This research is building upon an observed statistically significant relationship between maternal T1 BLLs and 24-month MDI score in a larger set of ELEMENT participants (10). Our previous EWAS paper (38) reported associations between Pb and the CpG-site specific DNA methylation at the fifteen selected CpG sites. In order to explore the potential for epigenetics to be mediator and to estimate effect sizes of mediation, this analysis was pursued even though the associations between exposure and outcome were not statistically significant at the 24-month timepoint in this

subset of the ELEMENT cohort (49). Not all ELEMENT participants included in the original publication had archived samples to isolate DNA from at birth. Thus, this pilot study has a smaller sample size and lower statistical power compared to the original study.

Mediation Models

Mediation analyses were conducted to examine whether DNA methylation at each of the CpG sites mediated the association between prenatal Pb exposure and each neurodevelopmental outcome measure (50, 51). The analysis was performed using the algorithms previously proposed in Imai *et al.* This incorporates the *mediate()* function within the mediation package (version 4.5.0) in R (52, 53). In this setting, we utilize the counterfactual framework to define causal effects within mediation analysis, which relies on the following assumptions to estimate parameters:

1. no unmeasured confounding in the relationship between exposure and outcome;
2. no unmeasured confounding in the relationship between the mediator and outcome after adjusting for the exposure variable;
3. no unmeasured confounding for the relationship between the exposure and mediator; and
4. no subsequent effects that the exposure might have (i.e. exposure-mediator interaction) that may confound any mediator and outcome relationship.

The total effect (β_{Total}), direct effect (β_{Direct}), average casual mediation effect (ACME) (i.e. indirect; β_{Indirect}), and the proportion mediated were calculated using quasi-Bayesian Monte Carlo method with 2000 simulations. The β_{Direct} represents the effect of prenatal Pb exposure on each neurodevelopmental outcome while holding DNA methylation constant; β_{Indirect} is the estimated effect of gestational Pb exposure operating through DNA methylation; β_{Total} is $\beta_{\text{Direct}} + \beta_{\text{Indirect}}$; the proportion mediated is the estimated proportion of the total effect of prenatal Pb exposure on each individual outcome measure due to the mediator, DNA methylation (Fig. 1). Regression estimates, 95% confidence intervals, and P-values were calculated for each effect estimate. Given the pilot nature of this study and current mediation methods having less power than typical statistical methods, ACME (i.e. β_{Indirect}) with P-values < 0.1 were considered statistically significant and discussed (54). If we were to adjust for multiple comparisons, considering our analysis to include 15 independent tests (for each CpG site; the multiple exposure biomarkers and outcomes are correlated with each other and therefore are not considered completely independent tests), a P-value < 0.003 would be considered statistically significant.

Since this mediation method assumes no exposure-mediator interactions, we performed a sensitivity analysis to test for exposure-mediator interactions. We re-ran all models with T2 BLLs as the predictor and EMOCI-24 as the outcome with an exposure-mediator (DNA methylation at each CpG site) interaction term included. We also re-ran any model that had a significant ACME with the interaction term included between the exposure and mediator. There was very limited evidence for interaction (i.e., $P > 0.1$ for effect estimate of all interaction terms except two), and as such only results from the primary model are reported here.

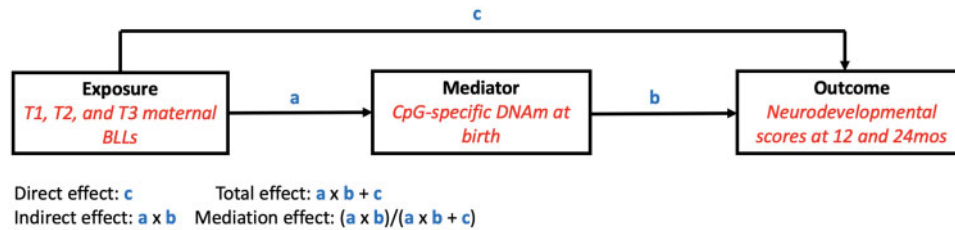


Figure 1: Diagram showing the associations tested in the mediation analyses. Direct effect refers to the association from maternal trimester-specific (T1, T2, and T3) Pb concentrations in blood and neurodevelopmental scores. Indirect effect refers to the association of maternal T1, T2, or T3 Pb BLLs on neurodevelopmental outcomes at 12 and 24 months of age through CpG site-specific DNA methylation at birth. The total effect is the sum of the direct and indirect effects. Mediation effect is a proportion of the indirect effect compared to the total effect. Covariates included in the final model were potential confounders in these relationships (infant sex, maternal age at birth, maternal IQ at birth) and covariates that influence the outcome (infant BLL, infant weight and length-for-age Z-scores, infant age at time of measurement). Note: T1, First trimester; T2, second trimester; T3, third trimester; BLL, blood lead level; CpG, cytosine-phosphate-guanine, DNAm, DNA methylation; mos, months of age.

Results

Population Characteristics

Participants include 85 mother-infant dyads from ELEMENT cohorts 2 and 3 with epigenetic, Pb exposure, and outcome data. Compared with all ELEMENT cohort 2 and 3 participants that completed the BSID-II and BRS assessments and had at least one prenatal blood Pb measure, the subset included in this study did not significantly differ on maternal age at birth, trimester BLLs, IQ, socioeconomic status, or offspring sex, weight, weight/length/BMI-for-Z-score, 12-month BLL, 12-month neurocognitive/behavioral outcomes, and 24-month neurocognitive outcomes; BLLs and BRS measures at 24 months were significantly higher in the subset (Table 1).

Among the 85 mothers included in this analysis, the mean age at delivery was 26.4 [standard deviation (SD) = 4.8] years (Table 1). About 66% of participants were either low-middle or middle socioeconomic status. Concentrations of Pb were measured in maternal blood during the first trimester (T1) with a geometric mean of 5.27 [geometric standard deviation (GSD) = 1.93] $\mu\text{g}/\text{dl}$, second trimester (T2) geometric mean of 4.74 (GSD = 1.96) $\mu\text{g}/\text{dl}$, and third trimester (T3) geometric mean of 4.98 (GSD = 1.93) $\mu\text{g}/\text{dl}$. Maternal BLLs between the trimesters were highly correlated ($r > 0.67$) according to a Spearman's rank-order correlation test. Infant whole blood Pb geometric mean was 3.92 (GSD = 1.80) at 12 months and 3.49 (GSD = 1.93) $\mu\text{g}/\text{dl}$ at 24 months of age, which were highly correlated ($r = 0.53$) with each other and moderately correlated ($r > 0.22$) with maternal trimester BLLs.

At 12 months of age, the mean weight was 9.35 kg (SD = 1.03), whereas the 24-month weight averaged 11.90 kg (SD = 1.40). The length-for-age Z-score was -0.004 (SD = 1.00) in 12-month children and 0.05 (SD = 0.97) in 24-month children. Thus, the distribution of the weight and stature of the analytic sample were within the normal range compared to the WHO reference population.

Mean maternal IQ was measured at 93.9 (SD = 19.5) points. At 12 months of age, mean MDI score was 95.1 (SD = 8.33) points and PDI was 90.1 (SD = 8.80) points; at 24 months of age, MDI mean was 91.1 (SD = 10.9) and PDI was 96.3 (SD = 8.37). Mean 12-month EMOCI percentile was 80.4 (SD = 22.7) and ORIEN percentile was 85.7 (SD = 19.1). At 24 months of age, mean EMOCI percentile was 80.9 (SD = 22.6) and ORIEN percentile was 77.9 (SD = 27.3). Each neurodevelopmental score or percentile was highly correlated between individuals at 24 months of age ($r > 0.49$), but only moderately correlated at 12 months ($r > 0.24$).

Pb, DNA Methylation, and Neurodevelopmental Outcomes

This study was conducted because we previously reported a significant inverse association between T1 BLL and MDI at 24 months in a larger set of ELEMENT children (10). In this smaller subset of 85 ELEMENT children who also have DNA methylation data from birth, we also report inverse associations between prenatal Pb and MDI (24 months), PDI (12 and 24 months), EMOCI (24 months), and ORIEN (24 months). While none of these associations were significant at $P < 0.05$ in this sub-cohort, we conducted pilot mediation analyses to estimate and report on effect sizes of the potential indirect effects from DNA methylation. Thus, the indirect effects are of most interest to this analysis, and indirect effects with $P < 0.1$ are considered statistically significant and discussed tentatively here. If adjusting for multiple-comparisons, $P < 0.003$ would be significant, and none of these pilot results meet that cut-off.

Exposure-outcome models estimated that at the 12-month neurodevelopment timepoint, each one-unit increase in ln-transformed Pb measured at T1, T2, and T3 was associated with decreases in PDI scores by 1.39, 2.01, and 1.83 points, respectively, but these estimates were not statistically significant (Supplementary Table S2, β_{Total}). This non-significant inverse relationship persisted at less magnitude at 24 months with the association between T2 Pb levels and PDI scores estimated to be -0.27 points (Supplementary Table S3, β_{Total}). T2 BLLs consistently had inverse associations with all neurodevelopmental outcomes at the 24-month timepoint. Specifically, the strongest associations measured a 6.04% ($P = 0.14$) decrease in EMOCI percentiles and a 5.20% ($P = 0.30$) decrease in ORIEN percentiles with on one-unit change ln-transformed T2 BLLs (Supplementary Table S3, β_{Total}). For additional exposure-outcome estimates (β_{Total}), see Supplementary Tables S2 (12-month outcomes) and S3 (24-month outcomes).

Analyses estimating the indirect mediating effect of locus-specific DNA methylation on the association between trimester-specific BLLs and four different neurodevelopmental outcomes at two timepoints indicated ten statistically significant ACME (β_{indirect} , $P < 0.1$) effect estimates (Fig. 2, Supplementary Fig. S1). For each one-unit increase in ln-transformed Pb exposure during T2, there was a 0.53% [95% confidence interval (CI): 0.23–0.83] increase in DNA methylation within a CCSE1 CpG site, cg02901723, at birth ($P < 0.001$; Fig. 3A β_{E-M}). The association between ln-transformed T2 BLLs and EMOCI score at 24 months of age (β_{Total}) was -6.05 ($-13.97, 0.11$) and the β_{Direct} (conditional on cg02901723 DNA methylation as a mediator) was -9.68 ($-18.37, -1.09$), thus showing that the magnitude of the

Table 1: Characteristics of ELEMENT mother–infant pairs included in the analysis with Pb biomarkers, DNA methylation data, and neurocognitive and behavioral outcome measures compared with characteristics from the rest of the participants in the same ELEMENT cohorts

Characteristics	ELEMENT subset for current study			ELEMENT Cohorts 2 and 3			P-value
	No.	Mean ± SD or percent (%)	Range	No.	Mean ± SD or percent (%)	Range	
Mothers							
Age (years) at offspring birth	85	26.4 ± 4.81	18.0 to 37.0	642	26.6 ± 5.43	14.0 to 44.0	0.56
Whole blood lead (μg/dl)							
First trimester*	69	5.27 ± 1.93	0.90 to 35.8	594	4.78 ± 1.89	0.00 to 35.8	0.23
Second trimester*	74	4.74 ± 1.96	0.80 to 38.2	616	4.23 ± 1.99	0.00 to 38.2	0.15
Third trimester*	76	4.98 ± 1.93	0.90 to 34.0	575	4.51 ± 1.92	0.00 to 38.1	0.13
Maternal IQ	82	93.9 ± 19.5	34.0 to 139.0	581	90.4 ± 21.6	34.0 to 182.0	0.06
Maternal SES	77			506			0.82
Lowest	12	15.6		85	16.8		
Low-middle	31	40.3		189	37.4		
Middle	20	26		150	29.6		
Middle-high	9	11.7		60	11.9		
Highest	5	6.49		22	4.3		
Offspring							
Male sex (%)	85	46		525	49.7		0.43
Whole blood lead (μg/dl)							
12 months*	78	3.92 ± 1.80	0.90 to 19.7	466	3.85 ± 1.88	0.00 to 20.4	0.87
24 months*	79	3.49 ± 1.93	0.80 to 17.5	522	3.98 ± 1.84	0.80 to 36.8	0.03
Neurological measures							
12 months							
MDI (patients)	85	95.1 ± 8.33	80.0 to 115.0	526	94.7 ± 8.95	60.0 to 116.0	0.83
PDI (patients)	85	90.1 ± 8.80	69.0 to 113.0	526	88.7 ± 8.75	50.0 to 113.0	0.34
EMOCI (%)	84	80.4 ± 22.7	10.0 to 99.0	526	78.7 ± 22.2	6.00 to 99.0	0.99
ORIEN (%)	84	85.7 ± 19.1	21.0 to 99.0	526	83.0 ± 20.4	9.00 to 99.0	0.34
24 months							
MDI (patients)	85	91.1 ± 10.9	72.0 to 122.0	569	86.8 ± 11.5	52.0 to 122.0	0.01
PDI (patients)	85	96.3 ± 8.37	80.0 to 117.0	569	93.1 ± 10.2	61.0 to 121.0	0.01
EMOCI (%)	85	80.9 ± 22.6	13.0 to 99.0	569	77.2 ± 23.1	11.0 to 99.0	0.10
ORIEN (%)	85	77.9 ± 27.3	10.0 to 99.0	569	74.9 ± 26.9	1.00 to 99.0	0.17
Weight (kg)							
12 months	85	9.35 ± 1.03	7.50 to 11.7	523	9.27 ± 1.10	6.30 to 14.8	0.62
24 months	85	11.9 ± 1.40	9.40 to 15.5	567	12.0 ± 1.52	8.50 to 19.5	0.58
Weight-for-age Z-score							
12 months	85	−0.04 ± 0.91	−1.93 to 2.13	523	−0.09 ± 1.00	−7.01	0.39
24 months	85	−0.04 ± 0.94	−1.89 to 2.32	567	0.09 ± 1.02	−7.3	0.78
Length-for-age Z-score							
12 months	85	−0.004 ± 1.00	−2.47 to 2.31	523	−0.08 ± 1.14	−8.09	0.35
24 months	85	0.05 ± 0.97	−2.07 to 2.55	567	0.28 ± 1.11	−8.43	0.38
BMI-for-age Z-score							
12 months	85	0.01 ± 1.01	−2.49 to 2.24	523	−0.09 ± 1.17	−8.48	0.35
24 months	85	0.12 ± 1.00	−2.08 to 2.62	567	0.35 ± 1.15	−8.74	0.33

*Geometric mean P-value < 0.05 considered statistically significant.

EMOCI, emotional regulation percentile; MDI, mental development index; ORIEN, orientation/engagement percentile; PDI, psychomotor development index; SD, standard deviation.

association between T2 BLLs on EMOCI score at 24 months of age became larger. β_{indirect} for the EMOCI percent at 24 months of age increased by 3.62% (−0.05, 8.69) for each one-unit change in ln-transformed T2 BLLs that was mediated through cg02901723 methylation ($P = 0.05$), and the estimate for proportion mediated between the exposure and outcome by cg02901723 DNA methylation was −49%. In other words, Pb exposure was associated with decreasing EMOCI score, while increasing DNA methylation at cg02901723 was associated with an increase in EMOCI score. When there is a larger magnitude of direct effect than total effect, or if the indirect effect is in the opposite direction as the direct effect, this suggests a suppressive

effect by the mediator on the direct effect between exposure and outcome (55). Similarly, cg02901723 DNA methylation mediated the effect of T2 Pb exposure on 24-month ORIEN score [$\beta_{\text{indirect}} = 4.44$ (−0.09, 10.68), $P = 0.06$]. The estimated percent of the total effect of T2 Pb exposure on 24-month ORIEN percentile due to the mediator, cg02901723 DNA methylation, is −54%.

The ACME (β_{indirect}) for cg18515027 in the association between T1 and T2 Pb exposure and EMOCI score at 24 months of age was statistically significant ($P = 0.01$ and 0.02). Further, cg18515027 was associated with T2 Pb and PDI scores at 12 months of age. Cg18515027 DNA methylation at birth increased by 0.42% (0.096, 0.75) with each one-unit increase in

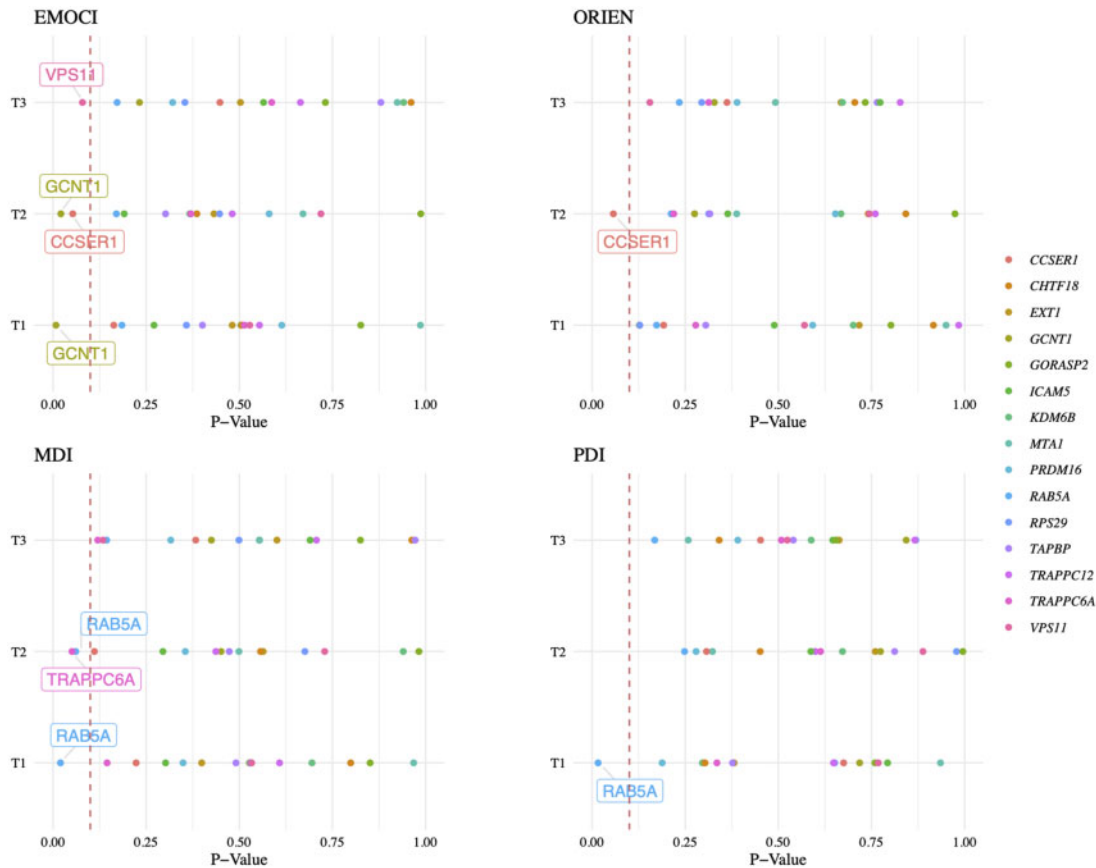


Figure 2: P-values for the average causal mediation effect (ACME) representing the influence of prenatal Pb exposure at each trimester on neurodevelopmental outcomes at 24 months of age through umbilical cord blood DNA methylation at each gene. Models control for offspring sex, current BLL, current weight, length-for-age Z-score, maternal IQ, and maternal age. Red dotted line is $P < 0.1$, which is considered statistically significant.

ln-transformed T2 BLLs (Fig. 3B β_{E-M} ; $P = 0.012$). Regressing 24-month EMOCI scores on cg18515027 DNA methylation showed an inverse association with EMOCI scores decreasing by 8.17 ($-14.67, -1.65$) for each one percent increase in DNA methylation ($P = 0.015$). Cg18515027 DNA methylation mediated the effects of T1 Pb exposure on 24-month EMOCI [$\beta_{\text{Indirect}} = -4.94$ ($-10.6, -0.77$), $P = 0.01$], and T2 Pb exposure [$\beta_{\text{Indirect}} = -3.52$ ($-8.09, -0.36$), $P = 0.02$]. The percent of the Pb-EMOCI relationship mediated by cg18515027 is estimated to be 69% for T1 BLLs and is reduced to 51% for T2 BLLs. In contrast, cg18515027 had a positive indirect effect between T2 Pb exposure and PDI scores at 24 months [$\beta_{\text{Indirect}} = 1.25$ ($-0.11, 3.32$), $P = 0.09$] with -38% mediated through DNA methylation of cg18515027 located within GCNT1.

The indirect effect was significant for cg19703494 DNA methylation located within TRAPPC6a in the association between maternal T2 Pb exposure and 24-month MDI scores [$\beta_{\text{Indirect}} = 1.54$ ($0, 3.87$), $P = 0.05$] with an estimated -31% mediated by cg19703494. There was a significant effect of cg23280166 DNA methylation located within VPS11 on the relationship between T3 maternal BLLs and EMOCI scores at 24 months of age [$\beta_{\text{Indirect}} = 2.43$ ($-0.16, 6.38$), $P = 0.08$] with -19% mediated. For additional mediation effect estimates (β_{Total} , β_{Indirect} , β_{Direct} , and proportion mediated) not mentioned above, see [Supplementary Tables S2](#) (12-month outcomes) and S3 (24-month outcomes).

Discussion

We conducted multiple mediation analyses in a subsample of 85 mother-child participants in the longitudinal study, ELEMENT, to begin to understand the potential for DNA methylation to be a mediator between gestational Pb exposure and offspring neurodevelopment. Specifically, we examined the association between maternal BLLs at each trimester with scores for four different domains of neurodevelopment at 12 and 24 months of age and whether UCB DNA methylation at 15 CpG sites statistically mediated these relationships (Fig. 1). This study expands upon previous ELEMENT research reporting that maternal trimester-specific BLLs predicted offspring MDI and PDI scores (10) to include EMOCI and ORIEN scores, as well as to investigate DNA methylation as a potential mediator. Using a significance cut-off of $P < 0.1$ for the ACME, we report suggestive evidence for locus-specific mediation, primarily for relationships between maternal blood Pb levels and 24-month outcomes. We provide preliminary evidence for both mediating and suppressive roles of DNA methylation in these relationships, depending on the locus. The latter of which can be interpreted as DNA methylation at a given gene suppressing the effect of Pb on a neurodevelopmental outcome (55).

Across trimesters, higher T2 maternal BLLs were most consistently associated (total effect) with lower childhood cognitive abilities at 24 months of age, though most were not statistically

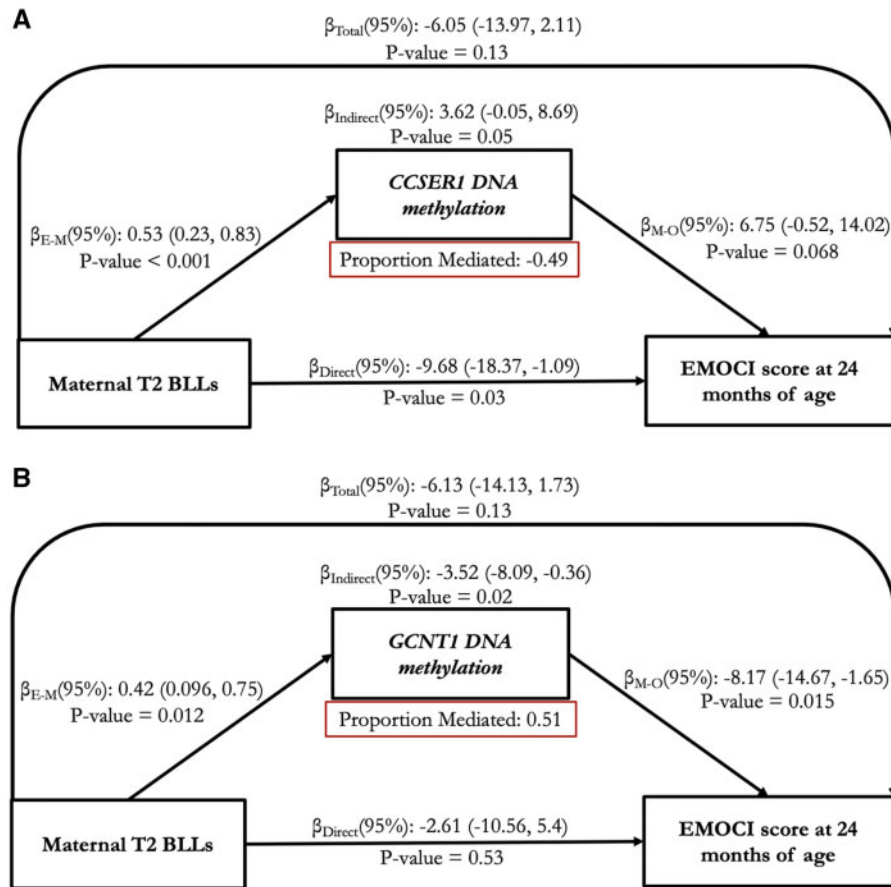


Figure 3: Relationship between ln-transformed T2 maternal BLLs, DNA methylation at (A) cg02901723 (CCSER1) and (B) cg18515027 (GCNT1), and 24-month-old infant EMOCI scores controlling for infant sex, current BLL, current weight, length-for-age Z-score, maternal IQ and maternal age. P-value < 0.1 is considered statistically significant. Note: T2, second trimester; BLL, blood lead level.

significant, likely due to the sample size. Statistically significant indirect (mediation) effects by DNA methylation of cg02901723, cg18515027, and cg19703494 were also observed in models of T2 BLLs with neurodevelopmental outcomes. The majority of these indirect effects were in the opposite direction of direct effects (i.e. suppressive). When added to the model, cg02901723 DNA methylation suppressed the association between prenatal Pb exposure during T2 and ORIEN and EMOCI scores at 24 months of age. Cg02901723 (in the gene CCSER1, alias FAM190A) is frequently altered in human cancers, but recent studies have verified CCSER1 expression in the cerebellum is associated with attention deficit hyperactivity disorder (56, 57).

DNA methylation of cg18515027 mediated associations between T1 and T2 BLL and EMOCI-24 (Fig. 3B). In contrast, there was a suppressive effect of cg18515027 on the relationship between T2 BLLs and PDI-12 scores. The protein product of the gene associated with cg18515027 (GCNT1) behaves like a cell surface marker to indicate whether a T-cell has received Notch signals (58). Notch signaling stimulates proliferative signaling during neurogenesis and plays an important role in regulation of embryonic development (59, 60). DNA methylation within cg19703494 negatively mediated the association between T1 BLLs and T2 BLLs with MDI-24. The gene that this CpG site is in—TRAPPC6a—is part of the TRAPP complex that plays a major role in endoplasmic reticulum-Golgi transport, but little is known about 6a specifically. Interestingly, within the TRAPP family, TRAPP9 is associated with intellectual disability as well

as microcephaly and problems with speech, and TRAPP11 is involved in movement disorders, ataxia (nervous system degeneration), intellectual disability, and muscular dystrophy (61). Cg23280166 was a statistically significant mediator between Pb at T3 and EMOCI-24. This site is annotated to the gene VPS11, which encodes an essential protein of the endosomal pathway, and it is hypothesized that in neuronal cells abnormal VPS11 functioning would attenuate the degradation of plasma membrane receptors thereby contributing to progressive developmental delay (62).

During early gestation, neurogenesis occurs at an astonishing rate starting on embryonic day 42 and ending in mid-gestation (63, 64). By Week 8, neuronal proliferation and migration begins. During T2, axons form branches (i.e. dendrites) and synapses with a cortical plate where cortical circuits are then organized (65–69). This timing is in line with inverse associations between T1 and T2 Pb exposure and neurodevelopment measures in children up to 24 months of age that have been reported by us and others (5, 10). By the end of T2 and into T3, the human brain contains billions of neurons (69–71). In T3, these new cells can communicate, and the brain starts to exhibit neuronal differentiation, axonal elongation, synapse formation, and myelination (63, 64, 72–74). The timing of these developmental processes may explain why Pb exposure during late pregnancy also correlates with early-life lower cognition scores (19, 20).

Development of the brain and nervous system *in utero* is a highly complex process, in which epigenetics plays a critical

role. During gestation, a global reprogramming of epigenetic modifications in the embryo occurs making it the most vulnerable period to environmental perturbations (75). These changes can be propagated across germ layers within the developing fetus. It has been shown that during early neuronal differentiation, DNA methylation occurs at promoters to repress germ-line specific genes, while methylation loss at other promoters activates neuronal-specific genes (76–78). How these widespread reprogramming events relate to specific pathways of fetal neurodevelopment remains a key gap in research. It is particularly challenging, both scientifically and ethically, to research fetal development, but it is essential we enhance the understanding of mechanistic processes in order to both improve infant and maternal health, particularly in analyses of environmental exposures during pregnancy. Prenatal and early postnatal Pb exposure have been shown in rodent and human studies to be associated with epigenetic changes in the offspring including in the brain (79, 80) and blood (33, 34, 38, 81, 82). Metals' exposure, such as to Pb, causes indirect reactive oxygen species formation with thiol depletion resulting in oxidative stress, which might be a route to explain the DNA methylation changes by Pb (83–85). Pb-induced oxidative stress could result in oxidative damage of methylated cytosines and decrease the level of methylation.

Gestational epigenomic changes would be expected to propagate across all tissues, yet it is still important to consider tissue-specificity when conducting differential methylation research. UCB was the available tissue for at-birth DNA methylation profiling, but the most relevant tissue would ideally be brain, specifically the cortex, cerebellum, or hippocampus. We utilized a publicly available database with matched blood and adult brain DNA methylation profiles to infer the level of similarity between blood and brain at the CpG sites included in our study (86). Of the 15 CpG sites analyzed, 8 CpGs were available in the database. Of these, 5 CpGs were moderately to highly correlated between blood and the prefrontal cortex (cg14911689, cg26654770, cg01201512, cg06657917: $r > 0.72$; cg26371957: $r = 0.44$) and entorhinal cortex (cg14911689, cg26654770, cg01201512, cg06657917: $r > 0.79$; cg26371957: $r = 0.48$), and 3 CpGs between blood and cerebellum (cg14911689, cg26654770: $r > 0.70$; cg01201512: $r = 0.52$). Blood DNA methylation of 3 CpG-sites was not correlated with any brain regions (cg25353752, cg00002033, and cg03463208). This comparison is limited since samples in the database were from adults and our study focuses on UCB and infants. Even so, correlations between blood and brain at five of the eight CpGs within this study suggest they may be able to serve as biomarkers of effects in other tissues, and this should be validated in future studies.

This pilot study is one of a growing number to consider DNA methylation as a mediator in the association between prenatal exposures and childhood phenotypes. For example, one study identified a positive mediating effect of gene-specific methylation on the association between non-syndromic cleft lip and/or palate and in utero Pb exposure. Pb exposure mediated 9.3% of the relationship between Pb and the outcome with a statistically significant indirect effect (odds ratio) of 1.26 (95% CI: 1.05–1.97) (87). Another study reported that gene-specific differential methylation explained 12–19% of the 202 g lower birthweight among offspring of women who smoked during pregnancy versus non-smokers (88). Finally, another study identified negative mediating effects of DNA methylation at 66 CpG sites on cognitive appraisal predicting high C-peptide secretion with 54 of those CpG sites being hypomethylated and 12 CpGs hypermethylated; DNA methylation explained 5.2–32.0% of the variance,

depending on the site (89). In terms of this negative (i.e. suppressive) effect, we hypothesize that DNA methylation changes in response to some exposures may serve to protect against the impacts of that exposure. For example, while changes at some genes and/or biological pathways may be part of the mechanism leading to Pb toxicity, changes at another set of genes may mitigate some of the toxic impacts. We cannot prove causality based on the design of our study and its' pilot nature. Future studies should confirm this hypothesis.

There are several strengths within this study, including the longitudinal design with both exposure and outcome having multiple timepoint measurements allowing the assessment of windows of susceptibility during prenatal into postnatal neurodevelopment. The selection of genes was hypothesis-driven based on associations between prenatal Pb exposure and DNA methylation at birth in the same cohort in an epigenome-wide study (38); although, this could also be a limitation since additional genes that influence cognitive development were not examined here. DNA methylation was assessed in cord blood instead of brain. Due to the sample size limited by availability of DNA methylation data, we were unable to test whether sex differences in the exposure-outcome or exposure-mediator-outcome relationships exist. Further, the small sample size limits our statistical power; therefore, we cannot conclude that the results presented here are not by chance alone as they did not withstand correction for multiple hypothesis testing. In a previous ELEMENT study by Hu *et al.*, the association between first trimester maternal BLLs and 24-month MDI scores was statistically significant ($n = 146$). While we did not achieve statistical significance with the smaller subset included in this present study, the effect estimates (i.e. the total effects for the T2 and T3 BLL on MDI) were similar. The generalizability of this study may be limited to populations with similar racial-ethnic and socioeconomic backgrounds. Replication with a larger and more diverse study population is needed.

Conclusion

The results of this pilot study show that DNA methylation at several genes statistically mediates the association between T2 Pb exposure and neurodevelopmental scores at 24 months of age. This effect was independent from that of postnatal Pb exposure, and the strongest evidence for mediation was for DNA methylation of cg02901723 and cg18515027. At cg02901723, increasing methylation suppressed the influence of Pb on the outcome; whereas at cg18515027, increasing methylation mediated the association. Pb remains a global environmental problem. The majority of preventative measures focuses on early childhood and often ignores prenatal exposure during embryonic development as an additional critical period affecting long-term health. Our study builds upon previous research on prenatal Pb exposure and its association with decreased childhood IQ, MDI, and adverse neurobehavioral outcomes; we provide preliminary data to consider epigenetic alterations as one of the biological mechanisms contributing to these long-term effects. As designed, our study cannot prove causality, and we suggest future research on this topic that includes the genes that showed evidence for mediation (CCSER1, GCNT1, TRAPP6A, and VPS11). Identifying genes and their corresponding biological pathways involved in Pb's neurotoxic effects is an important step in developing interventions to disrupt or reverse toxicity.

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Data Availability

Epigenetic data is available through the NIH HHEAR Data Repository located at: <https://hheardatacenter.mssm.edu> (doi: 10.36043/1431_392). All other data are available upon reasonable request.

Ethics Statement

The research protocol and all amendments to the study were approved by the Ethics Committees of the National Institutes of Public Health of Mexico, participating hospitals, and the Internal Review Board at participating institutions including the University of Michigan. This information is provided in the manuscript.

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Conflict of interest statement. None declared.

Supplementary Data

Supplementary data are available at *EnvEpig* online.

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