EPIGENETICS
2017, VOL. 12, NO. 8, 607–615
https://doi.org/10.1080/15592294.2017.1320637

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RESEARCH PAPER



# Prenatal exposure to neurotoxic metals is associated with increased placental glucocorticoid receptor DNA methylation

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**ABSTRACT**

Epigenetic alterations related to prenatal neurotoxic metals exposure may be key in understanding the origins of cognitive and neurobehavioral problems in children. Placental glucocorticoid receptor (*NR3C1*) methylation has been linked to neurobehavioral risk in early life, but has not been examined in response to neurotoxic metals exposure despite parallel lines of research showing metals exposure and *NR3C1* methylation each contribute to a similar set of neurobehavioral phenotypes. Thus, we conducted a study of prenatal neurotoxic metals exposure and placental *NR3C1* methylation in a cohort of healthy term singleton pregnancies from Rhode Island, USA (n = 222). Concentrations of arsenic (As; median 0.02 ug/g), cadmium (Cd; median 0.03 $\mu$g/g), lead (Pb; median 0.40 $\mu$g/g), manganese (Mn; median 0.56 $\mu$g/g), mercury (Hg; median 0.02 $\mu$g/g), and zinc (Zn; 145.18 $\mu$g/g) measured in infant toenails were categorized as tertiles. Multivariable linear regression models tested the independent associations for each metal with *NR3C1* methylation, as well as the cumulative risk of exposure to multiple metals simultaneously. Compared to the lowest exposure tertiles, higher levels of As, Cd, Pb, Mn, and Hg were each associated with increased placental *NR3C1* methylation (all $P<0.02$). Coefficients for these associations corresponded with a 0.71–1.41 percent increase in *NR3C1* methylation per tertile increase in metals concentrations. For Zn, the lowest exposure tertile compared with the highest tertile was associated with 1.26 percent increase in *NR3C1* methylation ($P=0.01$). Higher cumulative metal risk scores were marginally associated with greater *NR3C1* methylation. Taken together, these results indicate that prenatal exposure to neurotoxic metals may affect the offspring's NR3C1 activity, which may help explain cognitive and neurodevelopmental risk later in life.

**ARTICLE HISTORY**
Received 24 January 2017
Revised 17 March 2017
Accepted 10 April 2017

**KEYWORDS**
Metals; *NR3C1*; prenatal exposure; placenta

## Introduction

Early life exposure to heavy metals has long been linked to deleterious outcomes for children. In particular, many studies have shown that prenatal exposure to neurotoxic metals like arsenic, lead, and mercury, as well as under- or over-exposure to essential trace elements like zinc and manganese, are associated with perturbed fetal growth, adverse birth outcomes,[1] and cognitive and behavioral problems in later childhood.[2] Despite such mounting evidence showing the ill effects of gestational metals exposure, the biologic mechanisms linking such exposures to neurobehavioral outcomes are not well understood.

The fetal environment is regulated by the placenta, which plays an active immune-endocrine functional role in pregnancy, in addition to its role in nutrient, gas, and waste exchange.[3] The placenta is also involved in the development of the child's hypothalamic–pituitary–adrenal (HPA) axis, including the regulation of cortisol exposure to the fetus, through the actions of the glucocorticoid receptor (NR3C1) and downstream targets of its regulation.[4] A number of recent reports have also linked variation in the DNA methylation of *NR3C1* promoter region to fetal and newborn neurobehavioral phenotypes.[5-7] Neurotoxic metals can cross the placenta from mother to child during gestation,[8-11] and can also bioaccumulate in the placenta and cause functional damage, which may in turn impact fetal development due to the essential role of the placenta in development and fetal programming.[12,13] Moreover, research increasingly suggests that the placental epigenome is mediating the impact of environmental toxicant exposure in relation to child health.[14-18] The epigenome is thought to be most susceptible to environmental toxicant exposures during the earliest weeks of pregnancy,[18] which coincides with the development of core features of the central nervous system[19] and initiation of hormone activity of the HPA axis.[20] Thus, epigenetic modulation during gestation in response to neurotoxic metals exposure may be key in understanding the developmental origins of cognitive and neurobehavioral problems in children.

Several recent epigenome-wide association studies have linked prenatal exposure to arsenic, mercury, cadmium, and manganese to methylation across several loci,[21-28] including sites related to central nervous system development and behavioral disorders.[21,22] Moreover, patterning in methylation at these sites was prospectively linked to poor infant

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neurobehavior [22] and lower birthweight,[21] both considered sentinels of later life cognitive and behavioral problems.[29,30] Other studies of *in utero* exposure to arsenic, cadmium, and lead have found similar associations with LINE-1,[31,32] Alu,[32] and global DNA methylation.[26] Findings from animal models similarly indicate that gestational exposure to cadmium, mercury, manganese, and lead can modulate the mouse epigenome, particularly in regions related to neural crest cell migration, and in turn contribute to abnormal neurobehavior.[33] Also in mice, gestational zinc deficiencies have been linked to epigenetic dysregulation to metallothionein genes (a protein involved in the inactivation and detoxification of metals; a protective mechanism)[34]; animal work also suggests early life zinc deficiency contributes to increased DNA methylation to *BDNF* (a stress related gene modulated by the HPA axis), and subsequent cognitive impairment.[35] Taken together, these studies indicate that prenatal neurotoxic metals exposure may contribute to offspring epigenetic alterations indicative of future neurobehavioral morbidity.

Despite this accumulating evidence, few have explicitly considered gestational metals exposure as disrupting the offspring's HPA axis, the major neuroendocrine system that regulates body systems and responses to stress, including the regulation of glucocorticoids through the actions of the glucocorticoid receptor (NR3C1). Gestational dysregulation of the HPA axis, and of NR3C1 in particular, has been implicated in the pathophysiology of major neurobehavioral disorders.[36] Most studies considering gestational influences for *NR3C1* methylation have focused on maternal psychosocial factors, such as depression,[37] stress[5] and trauma[38] during pregnancy, given the potential for such exposures to directly overload and disrupt developing stress–response systems. No human studies have considered the role of neurotoxic metals as similarly disrupting *NR3C1*, despite the evidence linking prenatal metals exposure to a similar set of at-risk neurobehavioral phenotypes. Emerging animal work finds that prenatal arsenic exposure is associated with less Nr3c1 expression,[39] and lower levels of *Nr3c1* methylation in mouse brains,[40] suggesting prenatal programming to the glucocorticoid system may be attributable in part to arsenic exposure. Among humans, 2 recent studies of pregnant women found that lead,[41] and mercury in association with psychosocial stress,[42] were concurrently associated with dysregulated maternal salivary cortisol, suggesting heavy metals may disrupt HPA function during pregnancy. Taken together, these studies suggest that gestational neurotoxic metals exposure may contribute to poor childhood neurobehavioral outcomes via dysregulation of the HPA axis, and via *NR3C1* methylation in particular. However, we are aware of no study among humans that has considered this possibility explicitly. The present study fills this gap.

In this study, we examined whether prenatal exposure to a panel of neurotoxic metals would be associated with *NR3C1* methylation. Specifically, we hypothesized that prenatal exposure to higher levels of arsenic (As), cadmium (Cd), lead (Pb), manganese (Mn), and mercury (Hg), as well as deficiencies in zinc (Zn) would be associated with increased placental *NR3C1* methylation. We consider the independent effects of each metal in relation to placental *NR3C1* methylation, as well as the cumulative risk of exposure to multiple metals during pregnancy simultaneously. Also, because gestational programming effects are often sex-specific,[43,44] and sex differences in the neurotoxicity of metals has been observed,[45] we examined whether the prenatal metals exposure and *NR3C1* methylation associations differed by infant sex. To achieve these aims, we examined prospective data from 222 pregnant women who delivered healthy term infants, while controlling for potential social and biologic confounds.

## Results

### Descriptive statistics

Participant characteristics are listed in Table 1. Mothers were on average 31.5 y old, 79.3% were white, 11.7% had a high school education or less, 1.4% smoked, and 11.7% were depressed during pregnancy. Average pre-pregnancy BMI was 27.0. Half of the infants were male and birth weight was on average at the 59th percentile. Mothers with female infants

**Table 1.** Participant characteristics and bivariate associations with infant sex.

| | Full Sample | Females (n = 109) | Males (n = 113) | $P^{\wedge}$ |
|---|---|---|---|---|
| Maternal age, years (mean, SD) | 31.5 (4.4) | 31.1 (4.5) | 31.9 (4.2) | 0.18 |
| Maternal race, Black/Hispanic/Other (%) | 20.7 | 29.3 | 12.4 | 0.002 |
| Maternal race, white (%) | 79.3 | 70.6 | 87.6 | |
| Education, high school or less (%) | 11.7 | 10.1 | 13.3 | 0.46 |
| Education, more than high school (%) | 88.3 | 89.9 | 86.7 | |
| Maternal pre-pregnancy BMI, mean (SD) | 27.0 (6.6) | 26.0 (6.0) | 28.0 (7.1) | 0.02 |
| Tobacco use during pregnancy (yes), % | 1.4 | 1.8 | 0.88 | 0.54 |
| Tobacco use during pregnancy (no), % | 98.7 | 98.2 | 99.1 | |
| Depressed during pregnancy (yes), % | 11.7 | 10.1 | 13.3 | 0.46 |
| Depressed during pregnancy (no), % | 88.3 | 89.9 | 86.7 | |
| Infant birthweight percentile, mean (SD) | 58.7 (33.1) | 49.4 (33.5) | 67.8 (30.2) | <0.001 |
| Arsenic, $\mu$g/g, mean (SD) | 0.06 (0.11) | 0.05 (0.07) | 0.07 (0.14) | 0.25 |
| Cadmium, $\mu$g/g, mean (SD) | 0.08 (0.13) | 0.07 (0.10) | 0.09 (0.15) | 0.20 |
| Lead, $\mu$g/g, mean (SD) | 0.94 (2.1) | 1.18 (2.72) | 0.73 (1.04) | 0.11 |
| Manganese, $\mu$g/g, mean (SD) | 0.98 (2.8) | 1.16 (3.9) | 0.77 (0.83) | 0.30 |
| Mercury, $\mu$g/g, mean (SD) | 0.07 (0.10) | 0.08 (0.11) | 0.06 (0.08) | 0.29 |
| Zinc, $\mu$g/g, mean (SD) | 299.6 (798.5) | 379.2 (1113.7) | 222.8 (223.2) | 0.15 |
| Cumulative risk score, mean (SD) | 2.0 (1.2) | 2.0 (1.2) | 2.0 (1.3) | 0.79 |

$^{\wedge}$P-value corresponds to independent *t*-tests and $\chi^2$ tests with infant sex for continuous and categorical variables respectively.

**Table 2.** Metals concentrations according to tertile of the distributions[^].

| Metal | Low Tertile | Middle Tertile | High Tertile[*] |
|---|---|---|---|
| Arsenic, μg/g | 0.01 (0.01–0.02) | 0.03 (0.02–0.05) | 0.14 (0.06–0.98) |
| Cadmium, μg/g | 0.006 (0.003–0.01) | 0.04 (0.02–0.09) | 0.17 (0.09–0.86) |
| Lead, μg/g | 0.10 (0.008–0.21) | 0.40 (0.21–0.60) | 2.3 (0.60–17.7) |
| Manganese, μg/g | 0.13 (0.07–0.31) | 0.56 (0.33–0.85) | 2.2 (0.85–40.4) |
| Mercury, μg/g | 0.01 (0.006–0.02) | 0.03 (0.02–0.06) | 0.17 (0.07–0.80) |
| Zinc, μg/g | 94.2 (7.7–117.3) | 147.9 (117.5–188.9) | 648.4 (190.7–10,628.5) |
| Cumulative Risk Score | 0.78 (0–1) | 2.0 (2–2) | 3.5 (3–6) |

[^]Cell values are average metals concentration by tertile and (range).
[*]For each metal and cumulative risk score, contrasts between low and middle groups with the high tertile group are significantly different ($P<0.05$).

were significantly less likely to be white, and had lower body mass indices. Female infants had lower birth weight percentiles than males. No significant differences in metals concentrations by infant sex were observed.

Participants had an average metals cumulative risk score of 2 (a count of the high risk tertiles of exposure across all metals; range 0–6). Metals concentrations according by tertiles are listed in Table 2. For each metal and the cumulative risk score, the average concentration in the high tertile group was significantly greater when compared with the low and middle tertile groups (all $P<0.05$ from Tukey tests). This indicates that despite an overall lower level of exposure as would be expected in this healthy population, a higher risk group was identified. Moreover, while some correlations between metals concentrations were observed, exposures were largely independent with the following exceptions: Mn was positively correlated with Pb ($r = 0.60$, $P<0.001$) and Zn ($r = 0.19$, $P=0.005$); Zn was positively correlated with Pb ($r = 0.21$, $P=0.002$). No other correlations were observed between metals.

Table 3 lists descriptive information for *NR3C1* methylation. The average methylation extent across the *NR3C1* loci for the full sample was 1.95%. Such a relatively low degree of methylation was expected in this healthy cohort of term births and is similar to what has been previously reported.[6,46-48] Female infants had significantly higher overall methylation compared with males (2.17 vs. 1.73 percent methylated respectively), with higher levels of methylation evident for females at CpG sites 7, 10, 12, and 13.

Table 4 shows the results from the linear regression models for the associations between prenatal metals exposure categorized as tertiles and *NR3C1* methylation. For As, Cd, and Pb, comparable increases in methylation were observed at middle and higher levels of exposure. For Mn and Hg, high exposure, and not middle levels of exposure, was associated with increased *NR3C1* methylation. For Zn, the low exposure tertile was associated with increased *NR3C1* methylation compared with the highest exposure tertile. Higher cumulative risk scores were marginally associated with increased *NR3C1* methylation. Results were similar in unadjusted and adjusted models. The regression coefficients from the adjusted models for As, Cd, Pb, Mn, and Hg indicate that compared with the low exposed groups, higher levels of exposure corresponded with a 0.71-1.41 percent increase in *NR3C1* methylation. Low Zn exposure compared with high exposure was associated with 1.26 percent increase in methylation. Associations for As (mid and high tertiles), Cd (mid and high tertiles), Pb (high tertile), and Hg (high tertile) remain significant when applying the Bonferroni adjusted $\alpha$ of 0.004.

Table 5 lists the adjusted linear regression models for the association between prenatal metal exposure and *NR3C1* methylation stratified by infant sex. Associations for Pb, Mn, Zn were observed for females but not males in stratified models, though the interaction terms for these associations in subsequent models were not statistically significant (all $P>0.05$, data not shown). No interactions were observed between the other metals and infant sex in relation to *NR3C1* methylation.

**Table 3.** Descriptive information for *NR3C1* methylation for the full sample and stratified by infant sex.

| | | Mean % Methylated ± SD | | | |
|---|---|---|---|---|---|
| NR3C1 Exon 1F | Chromosomal location | Full sample | Females | Males | *P*[^] |
| CpG 1 | Chr 5: 142783592 | 1.25 ± 1.88 | 1.27 ± 2.07 | 1.23 ± 1.70 | 0.84 |
| CpG 2 | Chr 5: 142783599 | 0.93 ± 1.54 | 1.03 ± 1.77 | 0.83 ± 1.28 | 0.33 |
| CpG 3 | Chr 5: 142783602 | 1.11 ± 1.54 | 1.15 ± 1.61 | 1.06 ± 1.48 | 0.68 |
| CpG 4 | Chr 5: 142783608 | 0.88 ± 1.39 | 0.96 ± 1.49 | 0.80 ± 1.29 | 0.39 |
| CpG 5 | Chr 5: 142783611 | 1.15 ± 1.91 | 1.34 ± 2.34 | 0.97 ± 1.37 | 0.15 |
| CpG 6 | Chr 5: 142783501 | 1.11 ± 1.64 | 1.22 ± 1.87 | 1.01 ± 1.39 | 0.36 |
| CpG 7 | Chr 5: 142783503 | 3.84 ± 2.06 | 4.21 ± 2.49 | 3.52 ± 1.47 | 0.01 |
| CpG 8 | Chr 5: 142783513 | 1.84 ± 2.36 | 2.11 ± 2.99 | 1.58 ± 1.49 | 0.10 |
| CpG 9 | Chr 5: 142783519 | 2.81 ± 3.00 | 3.13 ± 3.80 | 2.50 ± 1.90 | 0.12 |
| CpG 10 | Chr 5: 142783533 | 2.73 ± 2.30 | 3.09 ± 2.87 | 2.39 ± 1.50 | 0.03 |
| CpG 11 | Chr 5: 142783555 | 2.12 ± 2.35 | 2.34 ± 2.64 | 1.90 ± 2.00 | 0.16 |
| CpG 12 | Chr 5: 142783570 | 3.09 ± 2.51 | 3.53 ± 3.09 | 2.67 ± 1.69 | 0.01 |
| CpG 13 | Chr 5: 142783573 | 2.47 ± 2.61 | 2.93 ± 3.20 | 2.03 ± 1.80 | 0.01 |
| Overall | | 1.95 ± 1.42 | 2.17 ± 1.66 | 1.73 ± 1.10 | 0.02 |

[^]*P*-value corresponds to independent *t*-tests with infant sex.

**Table 4.** Multivariable linear regression models for the association of prenatal metals exposure and placental DNA methylation to *NR3C1*.

| | Unadjusted | Adjusted |
|---|---|---|
| Arsenic | | |
| Low | *Ref* | *Ref* |
| Mid | 0.71 (0.04) | 0.74 (0.04) |
| | <0.001 | 0.001 |
| High | 0.69 (0.04) | 0.71 (0.04) |
| | <0.001 | 0.0002 |
| Cadmium | | |
| Low | *Ref* | *Ref* |
| Mid | 0.71 (0.04) | 0.71 (0.04) |
| | <0.001 | <0.001 |
| High | 0.73 (0.04) | 0.74 (0.04) |
| | <0.001 | <0.001 |
| Lead | | |
| Low | *Ref* | *Ref* |
| Mid | 0.80 (0.04) | 0.81 (0.04) |
| | 0.02 | 0.02 |
| High | 0.74 (0.04) | 0.77 (0.04) |
| | <0.001 | 0.004 |
| Manganese | | |
| Low | *Ref* | *Ref* |
| Mid | 0.89 (0.04) | 0.92 (0.04) |
| | 0.21 | 0.38 |
| High | 0.77 (0.04) | 0.80 (0.04) |
| | 0.006 | 0.02 |
| Mercury | | |
| Low | *Ref* | *Ref* |
| Mid | 0.96 (0.04) | 1.01 (0.04) |
| | 0.62 | 0.90 |
| High | 1.39 (0.04) | 1.41 (0.04) |
| | <0.001 | <0.001 |
| Zinc | | |
| Low | 1.22 (0.04) | 1.26 (0.04) |
| | 0.04 | 0.01 |
| Mid | 1.04 (0.04) | 1.10 (0.04) |
| | 0.65 | 0.32 |
| High | *Ref* | *Ref* |
| Cumulative Risk | | |
| Low | *Ref* | *Ref* |
| Mid | 1.03 (0.04) | 1.02 (0.04) |
| | 0.7862 | 0.81 |
| High | 0.83 (0.04) | 0.86 (0.04) |
| | 0.04 | 0.09 |

Top cell entries $\beta$ (SE), bottom cell entry *P*-value. Adjusted models control for maternal age, race, education, pre-pregnancy BMI, prenatal tobacco use, prenatal depression, infant gender, and birthweight percentile. High, Mid, and Low levels of exposure correspond to tertiles of the distributions for each metal.

**Table 5.** Sex-specific multivariable linear regression models for the association of prenatal metals exposure and placental DNA methylation to *NR3C1*.

| | Females (n = 109) | Males (n = 113) |
|---|---|---|
| Arsenic | | |
| Low | *Ref* | *Ref* |
| Mid | 0.73 (0.07) | 0.75 (0.05) |
| | 0.04 | 0.01 |
| High | 0.66 (0.06) | 0.72 (0.05) |
| | 0.005 | 0.007 |
| Cadmium | | |
| Low | *Ref* | *Ref* |
| Mid | 0.67 (0.07) | 0.68 (0.05) |
| | 0.008 | 0.001 |
| High | 0.65 (0.07) | 0.81 (0.04) |
| | 0.005 | 0.03 |
| Lead | | |
| Low | *Ref* | *Ref* |
| Mid | 0.78 (0.06) | 0.84 (0.05) |
| | 0.09 | 0.14 |
| High | 0.65 (0.07) | 0.87 (0.05) |
| | 0.007 | 0.22 |
| Manganese | | |
| Low | *Ref* | *Ref* |
| Mid | 0.84 (0.07) | 1.01 (0.05) |
| | 0.25 | 0.90 |
| High | 0.69 (0.07) | 0.89 (0.05) |
| | 0.01 | 0.34 |
| Mercury | | |
| Low | *Ref* | *Ref* |
| Mid | 0.93 (0.06) | 1.11 (0.05) |
| | 0.61 | 0.34 |
| High | 1.56 (0.06) | 1.34 (0.05) |
| | 0.003 | 0.01 |
| Zinc | | |
| Low | 1.42 (0.07) | 1.08 (0.05) |
| | 0.02 | 0.53 |
| Mid | 1.17 (0.07) | 1.02 (0.05) |
| | 0.34 | 0.89 |
| High | *Ref* | *Ref* |
| Cumulative Risk | | |
| Low | *Ref* | *Ref* |
| Mid | 1.00 (0.06) | 1.00 (0.05) |
| | 0.99 | 0.97 |
| High | 0.75 (0.07) | 0.91 (0.05) |
| | 0.06 | 0.41 |

Top cell entries $\beta$ (SE), bottom cell entry *P*-value. Adjusted models control for maternal age, race, education, pre-pregnancy BMI, prenatal tobacco use, prenatal depression, and birthweight percentile.

## Discussion

In this study, we found that gestational exposure to higher levels of As, Cd, Pb, Mn, Hg, and lower levels of Zn each may contribute to DNA methylation to placental *NR3C1*, even after controlling for relevant biologic and social confounds. These findings were particularly noteworthy as the overall level of metals exposure was low in this population, and most associations survived a conservative Bonferroni adjusted $\alpha$ level. Also, we found that cumulative exposure to multiple metals during gestation may jointly modulate *NR3C1* methylation, and some sex specific patterning was observed. Our findings build on prior work with this sample that has shown associations between prenatal Hg exposure and high-risk neurobehavior in infancy,[22] and linkages between placental *NR3C1* methylation and multiple domains of infant neurobehavior.[7,47,48] Taken together, these findings suggest that prenatal exposure to neurotoxic metals may affect the offspring's developing HPA axis, which may help explain cognitive and neurodevelopmental risk later in life.

Our finding that exposure to multiple metals simultaneously during pregnancy was associated with greater placental *NR3C1* methylation warrants further discussion. While our simple summary score to assess cumulative exposure does not indicate which combination of metals may be jointly affecting methylation extent, it does suggest that there is may be an accumulated epigenetic impact of exposure to metal mixtures during pregnancy. This finding is novel as few have considered accumulated burden, co-action between metals, or complex mixtures in association with gestational epigenetic programming. One study that has done so found the pair-wise interaction between prenatal Hg and As to predict cord blood hypermethylation among the top ranked CpG islands and South shore regions in an epigenome wide examination.[24] Several studies have considered the joint action of metal mixtures in the perinatal period in relation to child neurodevelopmental and behavioral

outcomes, suggesting synergies exist between metals to potentiate risk.[2,49] The present study is congruent with both of these lines of inquiry and suggests that exposure to multiple metals during gestation may jointly or cumulatively modulate *NR3C1*. We encourage future research to build on our simple cumulative risk approach and conduct more comprehensive examinations of gestational metals mixtures exposure, epigenetic regulation, and neurobehavioral outcomes.

Some sex-specific associations were observed in stratified models suggesting that prenatal exposure to higher levels of Pb, Mn, and lower levels of Zn may contribute to *NR3C1* methylation for females and not males, although the interactions were not statistically significant. No sex patterning in methylation was observed for As, Cd, or Hg. Previous work has shown some epigenetic patterning by sex in relation to prenatal As, Cd, and Pb exposures and DNA methylation,[26,40,50,51] though these studies reflect a heterogeneous mix of epigenome wide association studies, candidate gene examinations, and mouse models, rendering it difficult to compare our findings with published research. Thus, we suggest cautious interpretation of our sex-specific analysis and advise replication of these results.

NR3C1 is the nuclear receptor to which glucocorticoids like cortisol bind. Cortisol circulates in the bloodstream, binds to these receptors, and in so doing, reduces HPA activity and further cortisol secretion. *NR3C1* methylation can reduce gene expression, which reduces the number of receptors available for cortisol to bind to, thus potentially resulting in larger degrees of circulating cortisol levels in the blood. In the context of the fetal environment, such NR3C1 silencing could result in an over exposure of glucocorticoids during development, thus programming and disrupting the offspring's cortisol regulation system and contribute to neurobehavioral risk. Several studies have tested this hypothesis and show that increased *NR3C1* methylation is associated with reduced gene expression and neurobehavioral risk in infancy.[7,47,48,52,53] Our study builds on this burgeoning area of research and suggests that *NR3C1* methylation and neurodevelopmental sequelae may be due in part to neurotoxic metals exposure during pregnancy.

This study has some limitations. First, in restricting study enrollment to healthy, term pregnancies, we may have constrained the variance of the exposure variables and thus have underestimated the effects of metals exposure. Moreover, given the sample size and relatively lower levels of exposure in the population, we cannot determine whether the associations with methylation are linear or nonlinear. We advise future work to test these associations among larger samples with wider distributions of metals exposure. We also were unable to determine the source of metals exposure (e.g., dietary, water, home environment). Additionally, we relied on newborn infant toenail samples to provide general measures of prenatal exposure that were collected on average 2.8 months after birth. As such, metals concentrations may reflect a combination of prenatal exposures and postnatal external environmental influences. However, validation work in this sample and another birth cohort has shown metals concentrations during pregnancy assessed via maternal toenail and urine are moderately correlated with concentrations assessed in newborns infant toenails,[54] which somewhat mitigates this concern. Nonetheless, we suggest that future research utilize additional biomarker based assessments of exposure during pregnancy, coupled with questionnaire information on source, to help overcome these difficulties. In addition, the sex-specific analyses may have lacked statistical power. These limitations notwithstanding, this study has several strengths. It is among the first to consider the association between neurotoxic metals exposure and placental *NR3C1* methylation. We focused on a healthy population of infants from uncomplicated pregnancies, thereby mitigating concerns that associations could be confounded by maternal or infant illness. Moreover, we controlled for a set of prospectively assessed maternal and infant covariates (e.g., smoking, depression, birthweight) further strengthening our control of confounding. Finally, as prior work tends to focus on isolating the effect a single metal in relation to epigenetic alterations, we considered the independent and cumulative effects of multiple metals exposures occurring during pregnancy. We encourage future work to replicate and extend these finding among larger, more heterogeneous samples, and also to consider *NR3C1* methylation extent beyond this region of exon 1F, as well as to other potentially important control regions of this gene.

Evidence is accumulating that prenatal metal exposure contributes to epigenetic alterations among offspring. Our study adds to this emerging evidence base and demonstrated that gestational exposure to multiple neurotoxic metals, alone and in combination, was associated with increased *NR3C1* methylation, a key regulator of HPA activity. We encourage future research to replicate these findings and explicitly test *NR3C1* methylation as a linking mechanism between gestational metals exposure and childhood cognitive and behavioral phenotypes. Doing so will enhance our understanding of the developmental and epigenetic origins of neurodevelopmental risk.

## Materials and methods

### *Study population*

Study subjects were part of the Rhode Island Child Health Study, which enrolled healthy mother and infant pairs following delivery at the Women and Infants Hospital of Rhode Island (Providence, RI, USA). The parent aims for the original study were to identify the environmental and epigenetic determinants of fetal growth. Thus, term infants born small for gestational age (lowest 10th percentile), or large for gestational age (highest 10th percentile), based on birth weight and gestational age and calculated from the Fenton growth chart[55,56] were prioritized for enrollment; in addition, infants appropriate for gestational age matched on sex, gestational age ( $\pm$ 3 days), and maternal age ( $\pm$ 3 years) were also enrolled as control infants. Only singleton, viable infants were included in the study. Other exclusion criteria were maternal age ($<$ 18 or $>$40 y excluded), a life-threatening medical complication of the mother, and congenital or chromosomal abnormality of the infant. Following the birth but before hospital discharge, an interviewer-administered questionnaire was used to obtain information on maternal characteristics, and exposure histories. A structure chart review was conducted to obtain information on maternal morbidities and infant characteristics. Toenail clippings were collected from mothers and

infants after discharge and assayed for metals concentrations. The analytic sample for the current study includes 222 infants who had complete metals, placental *NR3C1* methylation, and covariate information. Study protocols were approved by the Institutional Review Boards for Women and Infants' Hospital and Dartmouth College.

### Metals exposure

A first toenail clipping from infants was requested following hospital discharge.[57] Parents were asked to collect their own and their children's toenail clippings from all toes and mail back the samples in provided envelopes. Average time from birth to collection was 2.8 months (range 0.3–7.1 months). Six metals were examined in this analysis: arsenic (As), cadmium (Cd), lead (Pb), manganese (Mn), mercury (Hg), and zinc (Zn). Infant toenail clippings were analyzed for $\mu$g of each metal per g of toenail material following methods described previously [58] in the Dartmouth Trace Element Analysis laboratory. Samples were read in 6 batches. Within each batch, samples with concentrations below the limit of detection were assigned a value equal to half of the lowest observed value in that batch.[21] Similar to previous reports from this study population,[22] the number of samples with concentrations below the minimal detection levels varied for each metal, ranging from 1% (Zn) to 68% (As).

To avoid assuming a linear response and to also avoid bias due to detection limits, a 3-level categorical variable was derived for each metal reflecting tertiles of its distribution (i.e., low, middle, high levels of exposure). For As, Cd, Pb, Mn, and Hg, we hypothesized that higher levels of exposure would associate with more *NR3C1* methylation and thus treated the low tertile group as the reference category in analysis. As some Zn exposure is necessary for healthy fetal brain development,[59,60] we hypothesized that low levels of Zn exposure would be associated with increased *NR3C1* methylation. Thus, the high tertile group was the reference category for Zn analyses.

To assess the overall risk of exposure to multiple metals during pregnancy, a cumulative risk score was derived. Using composite variable construction methods described in prior epigenetic programming research,[61] the cumulative risk score was a count of the high risk tertiles of exposure across all metals; range 0–6). Because no metal was hypothesized to exact a greater effect than another, component parts of the composite were not additionally weighted, resulting in a simple and conservative summary of the cumulative exposure to metals during pregnancy. Higher scores reflect greater prenatal metals exposure risk.

### Placenta sample collection, nucleic acid extraction and bisulfite modification

For each subject and within 2 h of delivery, 12 samples of placental tissue, 3 from each of quadrant (totaling approximately 8–10 g of tissue) were excised. All samples were full thickness sections of the placenta, 2 cm from the umbilical cord insertion site, free of maternal decidua. The samples were placed immediately in RNAlater (Life Technologies, Grand Island, NY) and stored at 4°C. At least 72 h later, placenta samples were removed from RNAlater, blotted dry, snap-frozen in liquid nitrogen, homogenized by pulverization using a stainless steel cup and piston unit (Cellcrusher, Cork, Ireland), and stored at −80°C until needed for examination. DNA was extracted from the placenta samples using the Qiagen DNAeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA). Purified DNA was quantified using a ND-2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA), and DNA samples (500 nanograms) were bisulfite-modified using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA.) and stored at −20°C. To prevent batch effects from bisulfite treatment interfering with the analysis, samples were randomized across conversion batches.

### Bisulfite pyrosequencing DNA methylation analysis

The DNA methylation status for the *NR3C1* exon 1F promoter region was assessed using quantitative bisulfite pyrosequencing as described previously.[46] Bisulfite conversion assessments were included on each sequencing read. In order for the sample's DNA methylation extent to be called, the bisulfite conversion rate must be >93%, and for all samples examined the conversion rate was >95%, as suggested by the instrument manufacturer. All samples were sequenced in triplicates from the same bisulfite converted DNA template, and if any of the individual repeats differed by >10% of the triplicate mean the sample was repeated. In each pyrosequencing batch, fully methylated and fully unmethylated control samples (Qiagen, Valencia, CA) as well as a DNA sample from peripheral blood that was not bisulfite modified to assess non-specific amplification were run.

### NR3C1 methylation extent

Since methylation of the individual CpG sites were correlated and as prior reports have linked average methylation across the region sequenced with reduced expression,[46] methylation across each of the CpG sites was averaged to obtain an overall measure of methylation extent. As the distribution was skewed, we applied a $\log_{10}$ transformation to approximate a normal distribution. *NR3C1* was treated continuously in analysis.

### Covariates

Maternal age, race, education, prenatal tobacco use, depression during pregnancy, infant sex, and birth weight percentile were included as covariates in multivariable models based on their potential to confound metals exposure and placental DNA methylation associations.[62] Race/ethnicity was dichotomized as white and black/Hispanic/other based on the distribution of race/ethnicity in the sample. Education was dichotomized according to the highest level attained (high school or less vs. more than high school). Prenatal tobacco use was assessed dichotomously (yes/no) as recorded in the medical record. Maternal depression during pregnancy was recorded as present or absent in the medical record and treated dichotomously in analysis. Maternal age and birth weight percentile were continuous variables.

### Statistical analysis

Descriptive statistics for participant characteristics, prenatal metals exposure, and *NR3C1* methylation were generated, and bivariate relations with infant sex were examined via $\chi^2$ and independent *t* tests. To test whether the metals tertile groupings signified different levels of exposure, ANOVA and Tukey tests were conducted to assess the contrasts between high, middle, and low groups. Correlations between the continuously measured metals concentrations were also examined. Next, unadjusted and adjusted linear regression models assessed the association of each metal and cumulative risk score with placental *NR3C1* methylation. To correct for multiple comparisons, a Bonferroni adjusted $\alpha$ was applied (adjusted $\alpha$: 0.05/14 = 0.004). Then, sex-specific associations were assessed via stratification of adjusted linear models, and fitting interaction terms. Finally, as *NR3C1* was $\log_{10}$ transformed, coefficients were back-transformed and percent change in methylation per unit change in exposure were reported. Statistical significance was determined by *P*-values lower than 0.05 and 95% confidence intervals.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

## Funding

Dr. Appleton is supported by a JPB Environmental Health Fellowship award granted by The JPB Foundation and managed by the Harvard T.H. Chan School of Public Health. This work was supported by the National Institutes of Health [NIH-NIMH R01MH094609, NIH-NIEHS R01ES022223, and NIH-NIEHS P01 ES022832] and by the United States Environmental Protection Agency [US EPA grant RD83544201]. Its contents are solely the responsibility of the grantee and do not necessarily represent the official views of the US EPA. Further, the US EPA does not endorse the purchase of any commercial products or services mentioned in the presentation.

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