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*Maryam M. Abdullah, Agnes R. Ly, Wendy
A. Goldberg, K. Alison Clarke-Stewart,
John V. Dudgeon, Christopher G. Mull,
Tony J. Chan, et al.*

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Heavy Metal in Children's Tooth Enamel: Related to Autism and Disruptive Behaviors?

Maryam M. Abdullah · Agnes R. Ly · Wendy A. Goldberg ·
K. Alison Clarke-Stewart · John V. Dudgeon · Christopher G. Mull ·
Tony J. Chan · Erin E. Kent · Andrew Z. Mason · Jonathon E. Ericson

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Abstract To examine possible links between neurotoxicant exposure and neuropsychological disorders and child behavior, relative concentrations of lead, mercury, and manganese were examined in prenatal and postnatal enamel regions of deciduous teeth from children with Autism Spectrum Disorders (ASDs), high levels of disruptive behavior (HDB), and typically developing (TD) children. Using laser ablation inductively coupled plasma mass spectrometry, we found no significant differences in levels of these neurotoxins for children with ASDs compared with TD children, but there was marginal significance indicating that children with ASDs have lower manganese

levels. No significant differences emerged between children with HDB and TD children. The current findings challenge the notion that perinatal heavy metal exposure is a major contributor to the development of ASDs and HDB.

Keywords Autism · ADHD · Metal · Lead · Mercury · Manganese

Introduction

Children are vulnerable to the neurotoxic effects of heavy metal exposure because their brains are still developing (Rodier 1995). These vulnerabilities are especially prominent during the prenatal period, when there is an immature blood–brain barrier and neuronal growth, migration, and myelination processes occur on a specific and rapid schedule. Moreover, toxic substances can pass through the placental barrier and easily access the developing brain, interfering with these important processes, likely leading to adverse consequences (Costa et al. 2004). Indeed, prenatal factors and early environmental exposures have been implicated in adverse developmental sequelae for children, but progress in elucidating the role of chemicals and elements in the cause of these disorders has been slow (Grandjean and Landrigan 2006; Landrigan et al. 2006).

The current study provides an intensive examination of the associations between prenatal and very early childhood exposure to two widely studied neurotoxins, lead and mercury, and one less studied neurotoxicant, manganese, which have been implicated in the causation of neurodevelopmental disorders. Blood, urine, hair, and nails are the usual substances in which these neurotoxins have been examined; however, these biomarkers are limited to information about contemporaneous exposure. The

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M. M. Abdullah · A. R. Ly · W. A. Goldberg (✉) ·
K. A. Clarke-Stewart
Department of Psychology and Social Behavior, School of
Social Ecology, University of California, Irvine, 4201 Social and
Behavioral Sciences Gateway, Irvine, CA 92697-7085, USA
e-mail: wendy.goldberg@uci.edu

J. V. Dudgeon
Department of Anthropology, Idaho State University, Pocatello,
ID, USA

C. G. Mull
Department of Biological Sciences, Simon Fraser University,
Burnaby, BC, Canada

T. J. Chan · E. E. Kent · J. E. Ericson
School of Social Ecology, University of California, Irvine,
Social Ecology I, Irvine, CA 92697, USA

A. Z. Mason
Department of Biological Sciences, California State University,
Long Beach, Long Beach, CA, USA

objective of the current study was to examine concentrations of lead, mercury, and manganese in the shed teeth of children with and without a developmental disorder. Shed teeth, more than other biomarkers, are obtained noninvasively and provide a window into archival levels of heavy metal exposures during early periods of development. Below we review what is known about the effect of exposure to lead, mercury, and manganese in relation to autism spectrum disorders (ASDs) and behavioral problems.

In recent years, research attention has shifted toward investigation of the potential roles of various environmental agents, especially heavy metals (DeSoto and Hitlan 2010), as contributors to the development of ASDs. This interest has gained momentum given that genetic factors alone have not accounted for the number of cases or its varying clinical presentations and environmental factors have been far less studied. As Landrigan (2010) asserted, "...there is substantial imbalance between the extensive and highly sophisticated information on the genetics of autism and the scarcity of investigation into potential environmental causes" (p. 224). In fact, it is increasingly being recognized that both genetic and environmental factors play integrated roles in the etiology of ASDs through epigenetic factors (Grafodatskaya et al. 2010).

Environmental exposure to neurotoxicants has been implicated in the dramatic rise in the prevalence of ASDs in the past few decades (Landrigan 2010). A recent estimate in the U.S. indicate that one child out of every 100 is diagnosed with an ASD (Kogan et al. 2009). ASDs are a set of neurodevelopmental disorders considered to be one of the "new pediatric morbidities" (Landrigan et al. 2006). The specific interest in toxic metals and compounds stems largely from the controversy about possible links between ASDs and immunizations, particularly to thimerosal, a vaccine preservative derived from ethylmercury. Epidemiological studies conducted worldwide have found no empirical support for a causal link between vaccinations and ASDs (e.g., Heron et al. 2004; Honda et al. 2005; Madsen et al. 2002).

Neurotoxicant Exposure and Developmental Disorders

Extant environmental epidemiology studies have found links between the release of heavy metal pollutants into the atmosphere and diagnoses of ASDs. For example, associations were found between the amount and proximity to industrial releases of mercury in Texas and increased rates of diagnoses of ASDs and students needing special education (Palmer et al. 2006; Palmer et al. 2009). In a California study, higher amounts of heavy metals in the air near where pregnant women resided were associated with an increased

risk of children being diagnosed with an ASD (Windham et al. 2006). However, in another California study, exposure to high levels of lead, mercury, and manganese compounds did not differentiate children with ASDs or language impairments (Kalkbrenner et al. 2010). These conflicting findings may have methodological origins: The epidemiological analyses may be confounded by differences in the handling of data and the fact that ambient air pollution does not capture individual exposure levels (Lewandowski 2011). Recommendations have been made for more research using case-control methodology with individualized assessments of exposure (Lewandowski 2011).

Some non-epidemiological investigations have compared heavy metal exposure using specific samples taken from children with and without ASDs in order to ascertain individual exposure levels, but they, too, have produced mixed findings. Two studies conducted in Kuwait and India found elevated levels of lead and mercury in the hair and nails of children with ASDs, respectively (Fido and Al-Saad 2005; Lakshmi Priya and Geetha 2010). Recent studies conducted in the United States with urine and blood biomarkers found no elevated mercury levels in children with ASDs (Soden et al. 2007; Hertz-Picciotto et al. 2010). These conflicting findings may depend on the particular biomarker, suggesting that more research is needed.

In addition to ASDs, there is also evidence that exposure to neurotoxicants, particularly lead and manganese, is related to higher-than-normal levels of disruptive behaviors (HDB). Disruptive behaviors in childhood include disobedience, defiance, impulsivity, inattention, hyperactivity, and aggression. In extreme cases, high levels of these behaviors are diagnosed as disruptive disorders of which Attention Deficit/Hyperactivity Disorder (ADHD) is the best known (American Psychiatric Association 2000).

Prenatal and early postnatal lead poisoning and even elevated but sub-toxic concentrations of lead in blood, bone, and hair have been associated with higher frequencies of delinquent behaviors (Dietrich et al. 2001; Ris et al. 2004) as well as ADHD symptoms during childhood and adolescence (e.g., Froehlich et al. 2009; Nigg et al. 2008; Wang et al. 2008).

Prenatal exposure to manganese has also been linked to HDB. High concentrations of manganese in the blood of pregnant women have been associated with attention deficits in children at age three (Takser et al. 2003). Additionally, elevated levels of prenatal exposure to manganese measured in tooth enamel have been related to greater impulsivity, attention problems, and hyperactivity in children from 3 to 9 years of age (Ericson et al. 2007). High levels of manganese as measured in the hair of 6–15 year-old-children also have been linked to hyperactivity (Bouchard et al. 2007).

Biomarkers of Environmental Exposure

The choice of biomarker is critical to the measurement of metal exposure and the mixed types of biomarkers used make comparisons across studies difficult. Blood and bone specimens have been used but they are invasive sampling measures that only provide measures of contemporaneous rather than prenatal and early postnatal exposures. Hair and nails can provide information about exposure over a long period and are easily accessed; however, these keratinized structures can be subject to external sources of contamination such as shampoo (Bass et al. 2001) and are also not informative about prenatal exposure.

Teeth, in contrast, can be easily and non-invasively obtained as primary (baby) teeth are naturally shed when permanent teeth begin to emerge (approximately age 9–14 years). For decades, teeth have been noted as reliable indicators of element exposure. This is especially true for exposure to lead (Fergusson and Purchase 1987; Pocock et al. 1994; Farmer et al. 2006); however, teeth have also been used as indicators of exposure to other heavy metals (e.g., Tvinnereim et al. 1996).

For teeth to be considered accurate historical biomarkers of exposure at the point of tooth formation, it is necessary to examine enamel separate from the dentin component of teeth (Farmer et al. 2006). The formation of enamel in primary teeth begins in utero and ends approximately three months to one year after birth, depending on the type of tooth (Ash and Nelson 2003). A number of heavy metals are allowed access through the placental barrier into the fetal bloodstream, which deposits heavy metals into teeth during the mineralization process (Ash and Nelson 2003). Enamel, rather than whole teeth, provides an archival record of prenatal and early postnatal exposure because blood flow into enamel ceases upon its completion and is a non-renewable body tissue (Fergusson and Purchase 1987; Uryu et al. 2003). The neonatal line in the enamel delineates prenatal and postnatal regions of development. In contrast, dentin is living tissue constantly in exchange with the circulatory system. Hence, dentin reflects environmental exposure continually accumulated for the entire lifetime of the tooth rather than specifically during tooth formation (Farmer et al. 2006).

One previous study has used shed teeth as the biomarker and it was found that children with ASDs had significantly higher levels of mercury, but not lead, compared to typically developing children (Adams et al. 2007). However, the method of sample preparation involved the dissolution of whole teeth prior to analyses. The result of this method was data that combined the trace elements found in both enamel and dentin. Examination of the enamel of primary teeth may be able provide more accurate information about

exposure specifically during these very early periods of development.

The Current Study

The current study examined concentrations of lead, mercury, and manganese in tooth enamel of shed primary teeth. The specific aims were to compare levels of these key metals in: (1) children with ASDs compared to matched TD counterparts and (2) children with HDB compared to matched TD counterparts.

Method

Participants

Shed primary teeth were collected from 84 children (aged 9–14 years) in two large national research studies. Children in the ASD and comparison group were predominantly male (86%) and Caucasian (68%); the majority of mothers had attained at least a college education (59%) and were married (86%). Children in the HDB and comparison group were mostly male (80%) and Caucasian (85%); a minority of the mothers had attained a college education (30%) and the majority were married or living with a partner (80%). Children in this study were similar in demographics to the larger samples from which they were drawn (Goldberg et al. 2003; NICHD Early Child Care Research Network 2005). The families of the subsample of children for this study were contacted via mail and asked to mail back one molar tooth after it had been shed. Teeth were collected and stored in small, snap-lock, plastic boxes. Teeth from children diagnosed with an ASD ($n = 22$) and children with HDB ($n = 20$) were matched with teeth from typically developing children ($n = 42$) on child's gender and race, parents' education and marital status.

Children in the ASD group had been evaluated by a licensed clinical psychologist using two autism-specific assessments. The Autism Diagnostic Interview-Revised (ADI-R; Rutter et al. 2003) is a standardized, semi-structured investigator-based interview for caregivers of children for whom a diagnosis of an ASD is possible. The Autism Diagnostic Observation Schedule (ADOS: WPS Version; Lord et al. 1999) is a standardized observation designed to assess behaviors related to ASDs. Children in the HDB group were evaluated by their third grade teachers using the Disruptive Behavior Disorders (DBD) Rating Scale (Pelham et al. 1992). This 26-item instrument is adapted from the Diagnostic and Statistical manual of Mental Disorders (DSM-IV) items for Attention-Deficit/Hyperactivity Disorder and Oppositional Defiant Disorder.

The DBD yields a Total Disruptive Behavior score, an ADHD score, and scores for Inattention, Hyperactivity/Impulsivity, and Oppositional/Defiant.

Procedure

Sample Preparation

Teeth were cleaned in a sonicator and rinsed in distilled, deionized water. Cleaned and dried teeth were embedded in Buehler Epo-Thin epoxy and thin-sectioned and affixed to a microscope slide to visualize enamel structure and neonatal line under light microscope (see Fig. 1).

Part of the Stria of Retzius complex, the neonatal line is thought to be the result of physiological stress or metabolic alterations associated with birth and it distinguishes between the pre- and postnatal sections of enamel (Nanci 2008). The prenatal enamel is found between the dentin-enamel junction and the neonatal line; the postnatal enamel is found between the neonatal line and the surface of the tooth. Due to the difficulty of definitively locating the neonatal line in all individuals, a conservative approach was taken in which prenatal and postnatal enamel was sampled above the dentin horn (see Fig. 2 of SEM image of sampling sites). It is easier to distinguish between these regions at the top of the tooth (i.e., crown) than the bottom of the tooth (i.e., root).

Laser Ablation ICP-MS Analysis

LA-ICP-MS is an accurate procedure for examining trace element concentrations in dental enamel (Kang et al. 2004; Uryu et al. 2003). Laser ablation analyses were performed on prenatal and postnatal enamel near the most prominent cusp in the thin sections of the tooth. Enamel was ablated

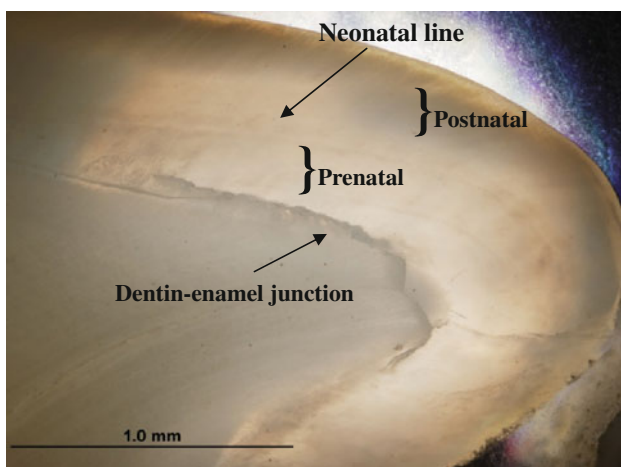


Fig. 1 Photo of the tooth enamel prenatal and postnatal regions near the crown of the tooth

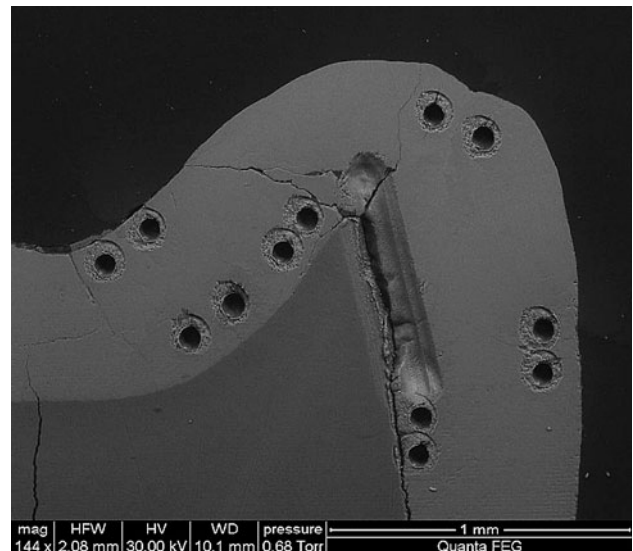


Fig. 2 BSE-SEM image of laser sampling sites

using a NewWave UP-213, 213 nm laser ablation system in imaged aperture mode. Ablated particulate material was analyzed by a Thermo X Series 2 Inductively Coupled Plasma-Mass Spectrometer using a dual-inlet (GC) interface, which allows the simultaneous addition of a liquid internal standard (Rh and Ir) to normalize for instrument drift and sensitivity suppression from matrix elements (Ca, P). Helium was used as the laser cell transport gas (0.33 l min^{-1}), which was blended at the dual inlet interface with argon and aspirated internal standard (0.70 l min^{-1}). Laser beam diameter is adjustable from 4 to 125 μm but was generally held at 75 μm , providing the optimal compromise between discreteness of ablation target and detection limit for most of the elements analyzed.

Experiment Standardization and Calibration

A combination of synthetic hydroxyapatite (HA) standards and USGS glass standards (GSC-1G, GSD-1G, GSE-1G) were used to calibrate the ICP-MS signal intensities of the unknown samples. We report averaged sample results for prenatal and postnatal manganese, mercury and lead (55Mn, 202Hg, 208Pb) for 42 case-control sample pairs. Individual calibration was performed on all specimens, with five synthetic apatite standards and three USGS standard glasses bracketing each unknown (Neff and Dudgeon 2006; Speakman and Neff 2005).

Plan of Analysis

Because of the small sample size, nonparametric Wilcoxon Signed Rank Tests were used to compare the concentrations of lead, mercury, and manganese in the prenatal

enamel regions of teeth between children with an ASD and typically developing children (TD). Similarly, the concentrations of lead, mercury, and manganese in the postnatal enamel regions of teeth for children with an ASD and TD children were compared using Wilcoxon Signed Rank Tests. Likewise, the same case–control data analytic strategy was used to compare the concentrations of lead, mercury, and manganese in (1) the prenatal and (2) the postnatal enamel regions of teeth in children with HDBs (cases) and TD children (controls). For the comparisons of the concentrations in prenatal and postnatal enamel regions of teeth in children with an ASD and TD children, there was 99.8% power to detect large effects and 85.3% power to detect moderate effects. Similarly, there was 99.9% power and 88.6% power to detect large and moderate differences in the concentrations in prenatal and postnatal enamel regions of teeth of children with HDBs and TD children.

Results

Table 1 presents the means and standard deviations of concentrations (ppm) of lead, mercury, and manganese in the prenatal and postnatal enamel regions of the teeth and Wilcoxon Signed Rank Test comparisons between typically developing (TD) children and (a) children with ASDs and (b) children with HDB. In addition, effect sizes (*r*) are presented to demonstrate the magnitude of the observed effects (large: *r* = 0.5; moderate: *r* = 0.3; small: *r* = 0.1; Cohen 1988).

No significant differences between children with an ASD and TD children were observed in lead, mercury, and manganese concentrations in the prenatal or postnatal enamel regions. There was one marginally significant difference showing a lower level of postnatal manganese for

of children with ASDs compared with TD children. Likewise, there were no significant differences between children with HDB and TD children in lead, mercury, and manganese concentrations in the prenatal or postnatal enamel regions.

Discussion

The objective of the current study was to compare concentrations of lead, mercury, and manganese in a reliable and valid biomarker—shed primary teeth. Heavy metal concentrations were examined in the prenatal and postnatal enamel regions of teeth for children with an Autism Spectrum Disorder, children with higher-than-normal levels of disruptive behaviors, and typically developing children.

This is the first study, to our knowledge, to report concentrations of manganese in tooth enamel in children with ASDs. There was a marginally significant result indicating that children with an ASD had lower levels of manganese in the postnatal enamel region compared with their TD counterparts. Previous research on manganese levels in children with ASD has been very limited but one study found lower levels of manganese in children with ASDs compared to TD children using hair as the biomarker (Fido et al. 2002). However, other studies reported little or no difference in manganese in hair (Gentile et al. 1983). Given that this metal is implicated in causation of neurodevelopmental disabilities (Menezes-Filho et al. 2011; Wasserman et al. 2006; Wright et al. 2006), more research on manganese as a possible risk factor is needed (Ljung and Vahter 2007).

In the current study, no significant differences in lead or mercury concentrations were observed in the pre- or postnatal regions for children with ASDs or typical development. These null findings add to the contradictory

Table 1 Concentrations (ppm) of elements in the prenatal and postnatal regions of children’s deciduous teeth

	ASD (<i>n</i> = 22) ^a <i>M</i> (<i>SD</i>)	TD (<i>n</i> = 22) ^b <i>M</i> (<i>SD</i>)	<i>z</i>	<i>p</i>	<i>r</i>	HDB (<i>n</i> = 20) ^c <i>M</i> (<i>SD</i>)	TD (<i>n</i> = 20) ^b <i>M</i> (<i>SD</i>)	<i>z</i>	<i>p</i>	<i>r</i>
Prenatal lead	0.27 (0.27)	0.38 (0.59)	−0.86	0.39	−0.14	0.33 (0.33)	0.32 (0.36)	−0.15	0.88	−0.02
Postnatal lead	0.29 (0.29)	0.43 (0.61)	−0.80	0.43	−0.13	1.10 (3.47)	0.22 (0.23)	−0.45	0.65	−0.07
Prenatal mercury	1.42 (0.61)	1.90 (2.79)	−0.08	0.94	−0.01	1.27 (0.76)	1.82 (2.34)	−0.82	0.41	−0.12
Postnatal mercury	1.47 (0.77)	1.45 (0.90)	−0.18	0.86	−0.03	1.01 (0.45)	1.22 (0.70)	−0.82	0.41	−0.12
Prenatal manganese	1.41 (1.10)	1.63 (0.95)	−0.80	0.43	−0.13	1.62 (0.77)	1.58 (0.88)	−0.37	0.71	−0.06
Postnatal manganese	1.87 (2.01)	2.91 (2.43)	−1.74	0.08 [†]	−0.28	2.11 (2.22)	1.80 (1.70)	−0.41	0.68	−0.06

ppm Parts per million

[†] *p* < 0.10

^a Autism spectrum disorders

^b Typically developing

^c High levels of disruptive behaviors

evidence regarding lead and mercury exposure and ASDs (e.g., Adams et al. 2007; Fido and Al-Saad 2005; Hertz-Picciotto et al. 2010; Windham et al. 2006). The mixed conclusions may be attributable to differences in sample type (e.g., teeth, hair), means of exposure (e.g., ingested, inhaled), and timing of exposure (distal vs. proximal to the onset of symptoms). Given that there are many known adverse developmental consequences of exposure of lead, further investigation of heavy metals in different biomarkers and timing of exposure may provide valuable information in the etiology of disordered neurobehavioral development.

In the comparisons between children with HDB and typical development, there were no significant differences in levels of lead, mercury, or manganese in prenatal or postnatal regions. In previous studies indicating a link between HDB and lead, researchers selected children who were clinically diagnosed with ADHD (Braun et al. 2006; Froehlich et al. 2009; Nigg et al. 2008) and measured lead levels in their blood, bone, and hair. Some of the HDB children in the current study were subclinical diagnostically and this may be why a significant association did not emerge. Alternatively, later rather than very early exposure to lead may be a meaningful contributor to HDB, at least at these subclinical levels. These null findings are consistent with some past studies using maternal and child hair samples to examine links between mercury and externalizing behaviors (Davidson et al. 1998).

Our findings are discrepant with a correlational study in which higher levels of manganese in tooth enamel were associated with behavioral disinhibition and externalizing symptoms in children (Ericson et al. 2007). The discrepancy may be attributed to methodological differences between the techniques used to assess levels of manganese in shed teeth (LA-ICP-MS laser sampling in this study and secondary ion mass spectrometry (SIMS) in the other study). Additional research is needed to pursue the effect of manganese exposure during early development.

Limitations

The current study was limited by the small samples (for ASD, HDB, and TD). The analyses had sufficient power to detect moderate and large effects but were underpowered for the detection of small effects. Although participants were matched on geographic location, child gender, ethnicity, parents' education, and parental marital status, it would be useful in future studies to control for potential covariates such as maternal age at birth, access to health care during pregnancy, and child's age at shedding of the tooth. Lastly, LA-ICP-MS has demonstrated sensitivity in its detection of trace metals specifically in tooth enamel (Kang et al. 2004; Uryu et al. 2003) but that does not

preclude the possibility of measurement imprecision due to the placement and size of sampling points.

Conclusions

Despite the limitations, a major contribution of the current study is the use of enamel as a biomarker; to our knowledge, this is the first time this technique has been used to study children with ASDs. Other contributions were the separation of enamel from dentin to probe early development and the analyses of prenatal and early postnatal exposures to distinguish between these two periods (Farmer et al. 2006; Fergusson and Purchase 1987). Both ASDs and HDB pose substantial threats to children's well-being; therefore, identifying environmental factors that contribute to their development is crucial (Landrigan 2010). Findings from the current study indicate that there are no significant differences in prenatal and early postnatal levels of lead, mercury, and manganese between groups; thus, they do not support an association between heavy metal exposure very early in life and the etiology of ASDs and HDB. The study does not rule out the possibility of adverse effects from exposure to neurotoxicants beyond the early postnatal developmental period. The method and null findings of the current study highlight the need to conduct additional research utilizing biomarkers that can provide extensive pre- and postnatal exposure assessments. Given the use of diverse methods in the current literature, comparisons across studies using similar biomarkers obtained at identical developmental points would be a fruitful direction for future syntheses and investigations of associations between heavy metal exposure and developmental disorders.

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