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Carmen A. Marable Date

Placental Genomic Imprinting, Toxic Metals, And Child Growth And Neurobehavioral Development

By

Carmen A. Marable
Degree to be awarded: MPH

Environmental Health

_____

Carmen J. Marsit, Ph.D
Committee Chair

_____

Paige Tolbert, Ph.D
Committee Member

Placental Genomic Imprinting, Toxic Metals, And Child Growth And Neurobehavioral Development

By

Carmen A. Marable

B.S
North Carolina Agricultural and Technical State University
2015

Thesis Committee Chair: Carmen J. Marsit, Ph.D.

An abstract of
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Abstract

Placental Genomic Imprinting, Toxic Metals, Child Growth And Neurobehavioral Development

By Carmen A. Marable

The placenta plays a critical role in fetal development, influencing growth and neurobehavioral development. Imprinted genes are highly regulated and play a key role in fetal development through, in part, their control of placental function. These genes are also thought to be sensitive to exposures to stressors and environmental toxicants. Using data from the New Hampshire Birth Cohort Study (NHBCS), we sought to first investigate the potential association between variation in imprinted gene expression and exposure to toxic metals in the placenta, including arsenic, mercury, lead, manganese, and cadmium, and secondly to investigate whether the identified pattern of expression of imprinted genes associated with metal exposure was associated with newborn and early childhood neurodevelopmental and growth outcomes. Neurodevelopment was assessed with using the Social Response Scales-2 (SRS-2) and growth by examining clinical measures at birth and through age 2 years. We identified associations only between cadmium exposures and imprinted gene expression, with increasing cadmium concentrations associated with reduced expression of *H19* (q=0.003), *IGF2* (q=0.043), and *IGF2AS* (q=0.028) and with increased expression of *MEST* (q=0.044), *DLX5* (q=0.043), *PHLDA2* (q=0.028), *GAA* (q=0.043) and *ABCA1* (q=0.044). Further, in linear models controlled for confounders, we noted associations between placental expression of *MEST* and the SRS-2 measure of social awareness and between expression of *PHLDA2* and social communication ($p<0.05$). We also noted, in multivariable linear models, associations between increasing expression of *MEST* and increased birthweight, as well as increased weight for age z-score and weight for age percentile at 12 months and 24 months (p=0.013, p=0. 036; p=0.012; p=0.023; p=0.004). Expression of placental PHLDA2 was also associated with weight for age percentile at 24 months. These analyses suggest that imprinted genes with variable expression associated to cadmium exposure are also related to child neurobehavioral and growth outcomes, in a prospective fashion, supportive of our hypothesis of the key role of imprinted genes in the placenta relative to neurobehavioral and growth outcomes. Understanding how altered expression of imprinted genes as critical regulators of development disrupts postnatal development may indicate the overall relevancy of this specific epigenetic mechanism.

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Background/Introduction

In utero exposures to a variety of trace metals, including arsenic, mercury, lead, manganese, and cadmium, can affect fetal, newborn, and infant health(Rahbar et al., 2015). Metals pose a major threat to children's health because there is a high probability that exposure will occur, and their exposure, individually and collectively, has been linked to adverse neurological and developmental effects(Henn, Coull, & Wright, 2014). Focusing on early life exposures hones in on the prenatal and perinatal periods which are susceptible developmental windows for potential environmental exposures placing the newborn at an increased risk for adverse health outcomes at birth and potentially throughout life (Sanders, Henn, & Wright, 2015). Susceptibility is not limited to altering or disturbing one system; the specific pathology may be differentiated by dose, timing, or type of exposure. The prevalence of arsenic (As), lead (Pb), cadmium (Cd), mercury (Hg), and manganese (Mn) exposures are a critical concern even at low levels especially during periods of fetal life and early childhood. During these periods, they can contribute to neurodevelopmental and behavioral outcome delays by disrupting the intended development of the central nervous system as well as leading to impacts in other critical newborn outcomes such as growth and even immune system development and function(Allen, 2015; Karagas et al., 2012; Sanders et al., 2015; Vahter, 2008).

Neurotoxic Effects of Metals

The prenatal neurotoxicity of the heavy metal is dependent on the permeability of the placental barrier and the blood brain barrier (BBB) to that heavy metal. Neurodevelopmental delays have been linked to transplacental exposure to heavy metals(Pigatto & Ronchi, 2013). Arsenic, mercury, and lead have been shown to increase BBB permeability(Sharma, Singh, & Siddiqi, 2014). Mercury (Hg) and lead (Pb) exposures have characterized neurotoxicity with the potential to damage or disrupt brain function through damage to the central nervous system, particularly the disruption of the formation of neuronal cells, and are well known to be associated with mental health outcomes including decreased

cognition (Grandjean, Weihe, Debes, Choi, & Budtz-Jorgensen, 2014; Lewis, Worobey, Ramsay, & McCormack, 1992) . Mercury exposures often resulting from emissions from power plants have led to loss of IQ in children, resulting in $1.3 billion being spent annually in the United States to treat methyl mercury toxicity (Trasande, Landrigan, & Schechter, 2005). Childhood lead exposure has been associated with decreased intelligence quotient and has led to an estimated annual cost burden of $61 billion in the United States(Sanders et al., 2015). Adverse outcomes as a result of lead exposure at levels as low as 10mg/dl have been found, particularly outcomes such as reduced intelligence quotient (IQ) and cognitive function, weakened fine-motor control and attention span, and academic deficits(Bellinger, 2008). These blood lead level associated outcomes have led to the Centers for Disease Control and Prevention's planning to reduce the screening guideline from 10mg/ dl(Liu & Lewis, 2014). Because of these significant public health concerns, environmental contributions to potential adverse neurobehavioral outcomes in newborns and children have led to great societal burden as it relates to economic losses. More recently, environmental exposures to other metals such as arsenic (As), cadmium (Cd), and manganese (Mn) (Green et al., 2015) have been suggested to pose putative neurodevelopmental risk including deficits in neurobehavioral function (Sanders et al., 2015). Manganese is an essential nutrient that can also exhibit neurodevelopmental toxicant properties following a potentially complicated dose-response, and in utero exposure has been linked to perturbations in children's intellectual function. Recent study findings have determined an association between early-life low-level environmental exposure to manganese and infant neurodevelopment as it relates to cognition, memory, motor function, and behavior (Henn et al., 2010). Once arsenic crosses the placenta it gains access to the blood-brain barrier (BBB) of the neonate which will allow it to directly influence the function of the central nervous system (Tolins, Ruchirawat, & Landrigan, 2014).In contrast, cadmium is mostly sequestered in the placenta, and so its effects on neurodevelopment may not be through impact on the central nervous system directly (Bhattacharyya, 1983) (Tolins et al., 2014).

Toxic Metals and Fetal Growth

Birthweight is an important determinant of childhood morbidity and mortality and long-term health outcomes(Rahman et al., 2009).Fetal exposures to both cadmium and lead have been correlated to lower birth weight and birth size (Gundacker & Hengstschlager, 2012b). Environmental concentrations that are commonly seen as harmless can be detrimental to the overall developmental process during the intrauterine and early childhood periods. The developing evidence of arsenic exposure in utero suggests that high-level arsenic exposure in increases the developing fetus's risk of infant outcomes such as low birth weight, infant mortality, and birth defects (Fei et al., 2013). Both cadmium and arsenic have been shown to be inversely related to growth in children, specifically to infant size at birth (Gardner et al., 2013).

Sources of Toxic Metal Exposures

Exposure to mercury, cadmium, arsenic, and lead, for the general population, most often occurs via ingestion and inhalation, contributing to individual body burdens(Gundacker & Hengstschlager, 2012b). Mercury exposure mainly occurs through the ingestion of fish contaminated by methyl mercury from anthropogenic sources like power plants (Trasande et al., 2005). Exposure to lead can be heavily attributed to paint, dust, soil, and or water pipes contaminated with lead. Leaded gasoline, industrial emissions as well as occupational exposures also contribute to lead exposure, but have been lessened due to policies put into place to reduce exposures. Given that arsenic is the most commonly encountered environmental toxicant, there is concern about the widespread effects of this exposure, which can be found through natural drinking water contamination as well as through food sources such as rice and rice products(Gilbert-Diamond, Emond, Baker, Korrick, & Karagas, 2016). Cadmium exposures can result from eating contaminated foods, working in cadmium-contaminated work spaces, and smoking cigarettes. For example, cadmium is found in grains and seeds, crustaceans, potatoes, and leafy vegetables. Foods rich in cadmium such as: mushrooms, shellfish, cocoa powder, liver, mussels, and dried seaweed have been found to greatly increase to the concentration of cadmium in the body (Tchounwou, Yedjou,

Patlolla, & Sutton, 2012). Although manganese is an essential micronutrient critical to normal growth and development, excessive exposure to manganese causes an influx of manganese accumulation in the brain which results in neurotoxic effects. Sources of manganese include nuts, seafood, spinach and tea(Rahbar et al., 2015).

Importance of the Placenta

The placenta is essential to perpetuating fetal development as well as pregnancy, and largely controls the intrauterine environment (Fei et al., 2013) (Gueneuc, Deloron, & Bertin, 2017). The placenta is a transitional organ, serving as a semi-permeable barrier between the fetus and potentially toxic or harmful substances that may be transmitted from the blood of the mother. The placenta acts by mediating both nutrient and waste exchange and through metabolic and endocrine functions in order to regulate fetal growth, development and control intrinsic and exogenous exposures present in utero between the mother and fetus (Green & Marsit, 2015) (Tracy Punshon et al., 2015) (Gueneuc et al., 2017).

Placental Metal Concentrations

The placenta functions as a barrier to mercury, lead, and especially cadmium during the mother to fetus transfer process (Roels, Hubermont, Buchet, & Lauwerys, 1978), although it is an incomplete barrier to lead, mercury and arsenic which can cross the transplacental barrier and accumulate in the fetus (Fei et al., 2013). Cadmium, though, is for the most part sequestered in the placenta. Metal-specific placental transfer has been shown to be a contributor to adverse effects on neurodevelopment and intrauterine growth (Gundacker & Hengstschlager, 2012a). Metallothionein is essential to the regulation of metals in the placenta, and because it decreases in concentration as cadmium absorption increases it may be responsible for cadmium's difficulty in passing through the placenta as well as its storage within the placenta (Gundacker & Hengstschlager, 2012a). In recent studies, the placenta has been used as a pregnancy biomarker to identify exposure to lead, mercury, arsenic, manganese, and cadmium, demonstrating correlations between concentrations in the placenta and those in maternal and infant

samples (Tracy Punshon et al., 2015) (Tracy Punshon et al., 2015). As the placenta can reflect exposures experienced in utero as well as the consequences of those exposures, it can used as both a biomarker or exposure and early/intermediate effect.

Imprinted Genes

Genomic imprinting is an epigenetic mechanism leading to parent-of-origin dependent gene expression; meaning that a subset of approximately 150 genes are expressed, consistently, from only either the maternal or paternal allele (Lawson, Cheverud, & Wolf, 2013). Imprinting plays a critical role in controlling sets of complex traits during fetal development as it may influence the transfer of nutrients to the fetus from the mother and control various cellular processes important in fetal development and growth. In addition, imprinted genes play an important role in neurobehavioral development (Reik & Walter, 2001). In evolutionary terms, imprinting arose with placental animals, and was thought to be driven by "parent conflict" wherein paternally expressed genes are involved in processes that increase nutritional intake of the fetus to maximize fetal growth, while maternally expressed genes are involved in processes that restrict fetal growth to preserve maternal fitness (Wolf, 2013). Complete loss of imprinting, resulting in either total loss of gene expression or biallelic expression due, in most cases, to genetic anomalies, can lead to highly penetrant, severe syndromic phenotypes often characterized by growth abnormalities, neurobehavioral deficits, and cancer susceptibility(Tycko & Morison, 2002) (O'Doherty, MacHugh, Spillane, & Magee, 2015). Recent data have linked subtle variation in placental imprinted gene expression to newborn birth weight (Kappil et al., 2015) and newborn neurobehavioral traits(Green et al., 2015). Studies investigating epigenetic influence in response to prenatal or neonatal exposures to environmental stimuli such as heavy metals have shown that different factors can affect gene expression in placental tissues (Green & Marsit, 2015). An experimental mouse model was used to investigate the effects of bisphenol A (BPA) exposure on genomic imprinting because imprinted genes are regulated by differential DNA methylation and unusual imprinting have been shown to disrupt fetal, placental, and postnatal development. Genome-wide

methylation levels were not reduced in the embryo. These findings suggest that abnormal development of the placenta and epigenetic changes in the embryo due to early environmental exposures to pollutants may disrupt fetal and postnatal health (Susiarjo, Sasson, Mesaros, & Bartolomei, 2013). Mechanistically being able to understand how imprinting contributes to gene expression regulation will allow us to begin to evaluate how the environment influences human health and disease (Jirtle, Sander, & Barrett, 2000). There may potentially be an association between in utero exposures and disease formation in adulthood because epigenetic modifications such as DNA methylation and imprinting elements have been found to be involved (Dolinoy, Das, Weidman, & Jirtle, 2007).

Study Objective

In summary, toxic trace metals can impact neurobehavioral development and growth, although the mechanism through which early life exposures to these metals lead to lifelong health risks remains incompletely understood. The placenta plays a critical role in fetal development, influencing growth and neurobehavioral development. Imprinted genes are highly regulated genes, which play a key role in fetal development through, in part, their control of placental function, and these genes are thought to be sensitive to exposures to stressors and environmental toxicants. Thus, the purpose of this study is to first investigate whether or not variation in imprinted gene expression is associated with exposure to toxic metals, and secondly to investigate whether or not the pattern of expression of imprinted genes that are altered by metals is also associated with neurodevelopmental and growth outcomes. Understanding how altered expression of imprinted genes as critical regulators of development disrupts postnatal development may indicate the overall relevancy of this specific epigenetic mechanisms (O'Doherty et al., 2015). We hypothesize that imprinted gene expression variation will be associated with toxic metal exposure, that the specific imprinted genes whose expression is altered by metals will be associated with neurobehavioral outcomes, and the strongest effects will be shown for cadmium because it poorly crosses the placenta. The first aim of the study will

address the association between toxic metals and gene imprinting, and the second aim of the study will investigate the relationship between the imprinted genes and neurodevelopment using the New Hampshire Birth Cohort Study(Green et al., 2016).

Methods

Study Population

The New Hampshire Birth Cohort Study (NHBCS) recruits pregnant women who obtain their prenatal care at clinics in New Hampshire, a state with detectable arsenic concentrations in private well water and exceeding the current maximum contaminant level (MCL) of 10 μg/L in over 10% of these wells. To be eligible for the study, women must be: a) currently pregnant, b) 18 to 45 years old, c) receiving routine prenatal care at one of the study clinics, d) consuming drinking water at their place of residence from a private well serving <15 households or 25 individuals, e) residing in the same place since their last menstrual period, using the same water supply, and f) not planning to move prior to delivery. The cohort, which began in 2009, is continuing to enroll, and to date over 1,500 women have enrolled in the study. For this analysis, we will examine individuals enrolled in the cohort between February 2012 and September 2013, with complete data on placental metals and imprinted gene expression (n=326). Relevant medical information is collected from structured medical record review and mothers are interviewed in-person by trained research assistants using structured questionnaires, which obtain extensive maternal socioeconomic indicators, lifestyle factors, medical histories, and exposure histories. Subsequent information on infant size is obtained from structured medical chart review from the delivery record and from pediatric well child visits. At age 3, parents are asked to complete the Social Responsiveness Scale-Second Edition (SRS-2), an instrument used to measure social behavioral deficits often associated with Autism Spectrum Disorder (ASD). The SRS-2 measuring scale consists of 65 items used for rating, and four rating forms are available spanning over three different age ranges. The form of interest for this study is the Preschool Form which covers ages 2-6 to 4-6 which can be rated by teachers and parents. Items on each form are scored on a 4-point Likert-type scale based on truth, ranging from 1=

not true to 4= almost *always true*. Results of the SRS-2 are reported as *T*-scores for subscale categories including: Social Motivation, Restricted Interest, Social Cognition, Social Awareness, and Repetitive Behavior. An overall score for the SRS-2 is also reported and is considered the most reliable measurement instrument for quantifying social deficits related to ASD (Bruni, 2014), and this will be the focus on our analysis.

Placenta Samples

Following delivery, the placenta was biopsied adjacent to the cord insertion (to minimize heterogeneity), removing any maternal decidua, placed either in a trace metal free tube or immediately in RNAlater (Life Technologies, Carlsbad, CA) and frozen at –80°C within 24 hr. A panel of trace metals was examined in placenta samples as described in (T. Punshon et al., 2016) using inductively-coupled plasma dynamic reaction cell mass spectrometry. For imprinted gene analyses, RNA were extracted by using the RNA/DNA extraction kit (Norgen Biotek, Thorold, ON) subsequently quantified using the Qubit Fluorometer (Life Technologies) and stored at –80°C until profiled. Placental RNA was quantified using a custom-designed code-set containing 110 imprinted genes (Nanostring Technologies, WA) following the methods previously described (Kappil et al Epigenetics 2015). The NanoString Norm package (Waggott et al., 2012) in R was used to normalize the nCounter data following recommendation by the manufacturer. This included initial normalization against the geometric mean of spike-in controls to account for differences in hybridization and recovery, and then by the geometric mean of standard housekeeping genes (*GAPDH, RPL19, RPLP0*) to account for differences in sample content. Finally, the background threshold of detection was set at two standard deviations above the mean of the included negative controls, and the data was log2 transformed for analysis.

Data Analysis

Analysis began by constructing frequency and percent distributions for the New Hampshire Birth Cohort study in order to generate descriptive values for study participant demographics. The association between

placenta imprinted gene expression and placenta-specific metal levels were determined using linear regression models, with each imprinted gene modeled as the dependent variable, and individual trace metal levels for As, Hg, Pb, Cd, and Mn used as independent variables controlled for the appropriate confounders. The potential covariates adjusted for in the model included: gestational age, maternal body mass index (BMI), maternal education, maternal smoking status, and infant sex. Birthweight was not adjusted for because it is a growth outcome of interest. For every model, each metal was log10 transformed and the minimum concentration that was greater than zero was added to the respective model for that metal. To assess for differences in the relationship between imprinted gene expression and placenta metals concentration based on infant sex, we examined differences in the expression of the identified genes by sex, testing this difference using a Student's T-test. As this is performing a large number of tests, we controlled Type I error using the Benjamini and Hochberg False Discovery Rate method. For those genes with FDR $q<0.1$ for their association with trace metals, we examined their association with outcomes.

Separately, the association between the identified imprinting gene expression values found to be significantly associated with metals exposure and the SRS-2 and growth outcomes were assessed using linear regression models, with SRS-2 and growth outcomes serving as dependent variables and the identified imprinting genes as independent variables. Variables measured from the SRS-2 assessment were: total raw score, raw score, social awareness, social cognition, social communication, social motivation, restricted and repetitive behavior, social communication and interaction under the DMS-5 ASD compatibility criteria, and restricted and repetitive behavior under the DMS-5 ASD compatibility criteria. Additional outcomes variables used for the outcome assessment consisted of: birthweight, placenta weight, weight for age z score at 6 months, weight for age z score at 12 months, weight for age z score at 24 months, weight for age percent at 6 months, weight for age percent at 12 months, and weight for age percent at 24 months. Potential covariates adjusted for in the model included: gestational age, maternal body mass index (BMI), maternal education, maternal smoking status, and infant sex. We used Pearson's correlation statistical tests were to measure the strength of association between the 8 placental

metal specimens of interest, and to examine the association between the SRS-2 variables and the identified imprinting signatures in a bivariate analysis. Statistical analyses were performed using R Studio.

Results

Descriptive Characteristics of the Cohort

As shown in Table 1, the study population was predominantly white (98%) and college educated (69%), with an average age of 31.5 (4.91), a mean body mass index (BMI) of 26.4 (5.92), and primarily gave birth to full-term infants (>37 weeks, mean gestational age of 39.4 weeks) with an equal male/ female distribution (53% male and 47% female) and most birthweights ≥ 2500g (95%). Arsenic had the lowest average placental concentration (0.955 μg/g) and manganese had the highest average concentration (70.33μg/g) (Table 1).

Correlations between Metals in the Placenta

Placental metal concentrations were generally positively correlated (Table S1). The strongest correlations were between Hg and As, between Mn and Pb, and between Pb and Hg. Conversely, the weakest correlations were between As and Pb, between Cd and Hg, and between Cd and Pb. The most significant correlations were between Mn and Pb. Correlations with Hg were the weakest in placentas overall.

Imprinted Genes

After examining the association between individual placental metal concentrations and the panel of roughly 100 imprinted genes, we identified 8 genes whose expression was significantly associated with placenta cadmium (Table 2). The full list of these genes and their associations with individual metals can be found in Supplemental Table 2. Mean gene expression between males and females were not significantly different (Figure 1). The most significant ($p< 0.01$) alteration in gene expression following cadmium exposure was shown for *H19* (q=0.003). Paternally expressed *IGF2* (q=0.043), *IGF2AS*

(q=0.028) also showed reduced expression, while *MEST* (q=0.044), maternally expressed *DLX5* (q=0.043) and *PHLDA2* (q=0.028), and unknown maternally/paternally expressed genes *GAA* (q=0.043) and *ABCA1* (q=0.044) were all significantly increased with increasing Cd concentrations ($p<0.05$).

Neurodevelopment Outcome Relative to Gene Expression

To examine if Cd associated alterations in expression of imprinted genes is associated with SRS-2 Treatment Subscales and proposed DSM-5 Criteria for ASD, which are used to identify and monitor neurodevelopmental outcomes, we tested the potential variance of social awareness, social cognition, social motivation, restricted interests and repetitive behavior, and social communication and interaction all relative to the expression of the identified imprinted genes *IGF2, IGF2AS, MEST, DLX5, H19, PHLDA2, GAA*, and *ABCA1*. We observed differences a significant positive correlation between *PHLDA2* expression and Social Communication ($p<0.01$) and a negative correlation between *MEST* expression and Social Awareness ($p<0.05$) (Table 3). Going beyond the univariate analysis, linear models controlled for confounders also demonstrated the association between *MEST* and decreased social awareness (Table 4). Likewise in models controlled for confounders, *PHLDA2* expression was positively associated with the score for social communication (Table 4).

Growth Outcome Relative to Gene Expression

In bivariate analyses, increasing *H19* expression was significantly associated with an increase in birthweight ($p= 0.013$, Table 5), but this associate was not upheld in a multivariable model controlled for confounders (Table 6).

On the other hand, bivariate analyses suggested that *MEST* gene expression was also associated with increased birthweight, as well as with weight for age z-score and weight for age percentile at 12 months and 24 months ($p=0.013$, $p=0. 036$; $p=0.012$; $p=0.023$; $p=0.004$, Table 5). In multivariable models controlled for confounders, the association between *MEST* and weight for age z score at 12 months and 24 months remained significant (Table 6). Bivariate analysis also demonstrated that weight for age percentile

at 24 months was significantly associated with *PHLDA2* expression (p= 0.034, Table 5) and that association was also held in models controlled for confounders (Table 6).

Discussion

This study demonstrates the potential for imprinted gene expression to serve as an intermediate between placental exposure to toxic metals and neurodevelopmental and growth outcomes. Validating imprinted gene expression as a pediatric biomarker of exposure for neurodevelopmental and growth outcomes can help monitor growth prenatally and throughout child development to provide optimal preventative care and treatment. Comprehending the relevance and usefulness of epigenetic mechanisms such as imprinted genes can potentially expedite identification and treatment of multi-causal adverse neurodevelopmental and growth outcomes that plague pediatric health.

Our first key finding was the association between the expression of *IGF2, IGF2AS, MEST, DLX5, H19, PHLDA2, GAA*, and *ABCA1*and placental exposure to cadmium. We hypothesized the strongest effects would be shown for cadmium due to its sequestration in the placenta. Two of the three paternally expressed genes *IGF2* and *IGF2AS* yielded negative effect estimates denoting reduced expression (Table2). *IGF2* is a growth factor that acts by restricting both placental and fetal growth (Dilworth et al., 2010). *IGF2AS* is non-coding RNA that also effect growth and has been implicated in Beckwith-Wiedemann syndrome (Okutsu et al., 2000). Maternally expressed genes *H19* and *PHLDA2* are both associated with placental overgrowth while the placental function of *DLX5* is unknown (Rugor et al., 2015) (Table 7).

A second key finding in this study was the association between the pattern of expression of imprinted genes altered by metals and neurodevelopmental and growth outcomes. *MEST* is one of the most upregulated genes during early development and is vital to neuronal differentiation and is influenced by both genetic and environmental variability(Mesman, van Hooft, & Smidt, 2016). Phenotypically, *MEST* affects neural development and behavior and has been linked to ASD, and also affects growth resulting in fetal growth restriction and smaller placentas (McMinn et al., 2006). The neurodevelopmental effects of

MEST were supported in the correlation observed between social awareness and *MEST*. Although H19 is most often characterized relative to growth as reflected by its association with birthweight we did not observe a significant association between H19 and birthweigh in covariate controlled models. We did observe an association between *PHLDA2* and weight for age percent at 24 months, as well as between placental *MEST* expression and weight for age z score at 12 months and 24 months. This would suggest a potential prospective utility in the examination of these genes in the term placenta, and further that variation in placental expression of these critical growth regulators may have long-term programming effects. Understanding the underlying mechanisms of this trend of association may be useful in monitoring growth beyond the postnatal period (Table 7).

Conclusions and Recommendations

In conclusion, we have found that placental cadmium exposure is associated with varied expression of a number of specific imprinted genes in the placenta. We further demonstrate that placental expression of *PHLDA2* was associated with social communication, and placental expression of *MEST* influences the association between MEST and social awareness measures at age 3 years. *PHLDA2* also was found to be associated with weight for age percentile at 24 months, while the expression of placental *MEST* was associated with weight for age z scores at 12 months and 24 months. This study provided preliminary data and evidence of associations needed to formally indicate imprinted gene expression a pediatric biomarker and as an intermediate between placental metal exposure and neurodevelopmental and growth outcomes. Future studies are needed to replicate and confirm these results and the utility of imprinted gene expression as a pediatric biomarker.

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Tables & Figures

Table 1: Characteristics of study population, New Hampshire Birth Cohort

Variables	**Total (n=326)**
Maternal Characteristics	
Age, years, mean (SD)	31.54 (4.91)
BMI before pregnancy, kg/m^2, mean (SD)	26.37 (5.92)
Enrollment age, years, mean (SD)	31.54 (4.92)
Tobacco use during Pregnancy, N (%)	
No	264 (80.98)
Yes	36 (11.04)
Race, Caucasian, N (%)	
No	6 (1.89)
Yes	319 (97.85)
Education, N (%)	
< High school	33 (11.34)
Some college	57 (19.59)
College or more	201 (69.07)
Infant Characteristics	
Gestational age, weeks, mean (SD)	39.38 (1.56)
Infant sex, N (%)	
Male	173 (53.07)
Female	153 (46.93)
Birthweight, g, mean (SD)	3435 (520.50)
<2500g N (%)	13(3.99)
≥ 2500g N (%)	309(94.8)

Placenta Metal Concentration (μg/g placenta)

Mercury (Hg)		Lead (Pb)		Arsenic (As)		Cadmium (Cd)		Manganese (Mn)	
Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
1.793	0.003-19.76	2.195	0.171-53.60	0.955	0.011-16.49	3.613	0.108-22.31	70.33	0.00 - 180.4

Note: Not all N values equal xxx because of missing values. Abbreviations: SD is standard deviation, BMI is body mass index, NA is not assessed.

Table 2: Imprinted genes demonstrating significant (q<0.05) association between their placental expression and placenta Cd concentration.

Imprinted Gene	Estimate	Std.Error	t-value	p-value	q-value
Cadmium (Cd)					
H19	-0.66	0.16	-4.20	3.46E-05	0.003
IGF2AS	-0.48	0.14	-3.41	0.001	0.028
PHLDA2	0.44	0.13	3.31	0.001	0.028
GAA	0.38	0.12	3.08	0.002	0.043
DLX5	0.41	0.14	3.02	0.003	0.043
IGF2	-0.58	0.19	-2.97	0.003	0.043
ABCA1	0.40	0.14	2.92	0.004	0.044
MEST	0.36	0.13	2.88	0.004	0.044

Note: All models adjusted for maternal age, maternal BMI, maternal education, tobacco use, gestational age, and infant sex.

Table 3: Pearson's correlation (r) between of SRS-2 Treatment Subscales and proposed DSM-5 Criteria for ASD, and identified imprinted genes altered by Cd exposure.

	SSR-2 Summary Score Variables					***DSM-5*–Compatible Variables**	
	Social Awareness	Social Cognition	Social Communication	Social Motivation	Restricted Interests and Repetitive Behavior	Social Communication And Interaction	Restricted Interests and Repetitive Behavior
H19	0.18*	0.09	-0.03	0.04	0.01	0.07	0.01
IGF2AS	0.04	-0.005	-0.11	0.03	-0.08	-0.03	-0.08
PHLDA2	-0.12	0.09	0.23**	-0.03	0.10	0.09	0.10
GAA	-0.09	0.05	0.07	-0.01	-0.005	0.02	-0.005
DLX5	-0.004	0.03	0.001	-0.01	0.05	0.02	0.05
IGF2	0.14	0.05	-0.006	0.05	0.01	0.07	0.01
ABCA1	-0.12	-0.008	0.04	0.01	-0.06	-0.001	-0.06
MEST	-0.21*	-0.01	0.05	-0.04	-0.08	-0.05	-0.08

Note: [a]p-values obtained from Pearson's correlation.
*Significant at level p<0.05, ** Significant at level p<0.01.

Table 4: Association between SRS-2 Treatment Subscales and proposed DSM-5 Criteria for ASD, and identified imprinted genes associated with Cd exposure.

Association Between PHLDA2 and Social Communication					**Association Between MEST and Social Awareness**				
Birth	Estimate	Std. Error	t value	Pr(>|t|)	**Birth**	Estimate	Std. Error	t value	Pr(>|t|)
PHLDA2	1.87	0.75	2.48	0.0148 *	*MEST*	-2.48	1.08	-2.30	0.0237*
Maternal BMI	0.02	0.08	0.27	0.79	Maternal BMI	0.12	0.12	1.04	0.30
Gestational Age	0.16	0.34	0.47	0.64	Gestational Age	-0.51	0.49	-1.03	0.31
Maternal Education: Less than or equal to high school	2.24	1.91	1.17	0.25	Maternal Education: Less than or equal to high school	3.70	2.73	0.35	0.18
Maternal Education: Some college or beyond	1.57	1.29	1.22	0.22	Maternal Education: Some college or beyond	0.70	1.86	0.38	0.71
Ever Smoker: Yes	2.81	1.74	1.62	0.11	Ever Smoker: Yes	-1.93	2.49	-0.78	0.44

Note: Linear models were adjusted for maternal age, maternal BMI, maternal education, tobacco use, gestational age, and infant sex.
*Significant at level $p<0.05$, ** Significant at level $p<0.01$, *** Significance at level $p<0.001$

Table 5: Bivariate Growth outcome associations with Cd-associated placenta imprinted gene expression.

	IGF2		IGF2AS		MEST		DLX5	
Factor	**Coeff**	**p- value**	**Coeff**	**p- value**	**Coeff**	**p-value**	**Coeff**	**p-value**
Birthweight	-13.34	**0.633**	**31.92**	**0.412**	**107.3**	**0.013***	**2.502**	**0.949**
Placenta Weight	10.28	**0.332**	**12.44**	**0.403**	**4.059**	**0.821**	**-1.093**	**0.944**
Weight/Age z-score 6months	**0.016**	**0.815**	**0.055**	**0.580**	**0.141**	**0.166**	**-0.009**	**0.928**
Weight/Age z-score 12months	**0.024**	**0.734**	**0.167**	**0.101**	**0.209**	**0.036***	**-0.106**	**0.269**
Weight/Age z-score 24months	**0.004**	**0.962**	**0.097**	**0.451**	**0.321**	**0.012***	**-0.072**	**0.564**
Weight/Age percent 6months	**0.810**	**0.696**	**1.398**	**0.637**	**4.594**	**0.126**	**0.348**	**0.904**
Weight/Age percent 12months	**0.849**	**0.674**	**5.252**	**0.072**	**6.499**	**0.023***	**0.907**	**0.314**
Weight/Age percent 24months	**-0.466**	**0.858**	**2.583**	**0.473**	**10.37**	**0.004****	**-1.593**	**0.652**
	H19		**PHLDA2**		**GAA**		**ABCA1**	
Factor	**Coeff**	**p- value**	**Coeff**	**p- value**	**Coeff**	**p-value**	**Coeff**	**p-value**
Birthweight	**7.758**	**0.013***	**27.45**	**0.507**	**78.49**	**0.073**	**-51.49**	**0.1863**
Placenta Weight	**23.04**	**0.075**	**-10.30**	**0.538**	**-6.358**	**0.724**	**-28.52**	**0.066**
Weight/Age z-score 6months	**0.048**	**0.5733**	**-0.040**	**0.691**	**-0.095**	**0.370**	**-0.139**	**0.144**
Weight/Age z-score 12months	**0.021**	**0.800**	**0.065**	**0.509**	**-0.017**	**0.873**	**-0.073**	**0.437**
Weight/Age z-score 24months	**-0.042**	**0.708**	**0.236**	**0.063**	**0.109**	**0.427**	**0.029**	**0.821**
Weight/Age percent 6months	**1.670**	**0.504**	**-0.525**	**0.860**	**-2.101**	**0.503**	**-3.346**	**0.235**
Weight/Age percent 12months	**0.741**	**0.759**	**2.338**	**0.409**	**0.245**	**0.935**	**-2.352**	**0.385**
Weight/Age percent 24months	**-2.289**	**0.468**	**7.531**	**0.034***	**3.763**	**0.329**	**0.877**	**0.808**

*Significant at level $p<0.05$, ** Significant at level $p<0.01$.

Table 6: Five-Predictor Model Data Statistics relative to the association between growth outcomes and Cd-associated placenta imprinted gene expression.

Association Between MEST and Weight for Age Z Score					**Association Between MEST and Weight for Age Z Score**				
12 Months	Estimate	Std. Error	t value	Pr(>|t|)	**24 Months**	Estimate	Std. Error	t value	Pr(>|t|)
MEST	0.209	0.099	2.11	0.0363*	MEST	0.321	0.126	2.54	0.0121*
Maternal BMI	0.031	0.012	2.52	0.0127*	Maternal BMI	0.028	0.0148	1.89	0.0603
Gestational Age	0.025	0.046	0.552	0.582	Gestational Age	0.007	0.056	0.125	0.901
Maternal Education: Less than or equal to high school	-0.203	0.246	-0.827	0.409	Maternal Education: Less than or equal to high school	-0.004	0.325	-0.012	0.99
Maternal Education: Some college or beyond	-0.283	0.181	-1.57	0.119	Maternal Education: Some college or beyond	-0.209	0.235	-0.889	0.375
Ever Smoker: Yes	-0.043	0.230	-0.188	0.851	Ever Smoker: Yes	0.063	0.301	0.208	0.836
Association Between H19 and Birthweight					**Association Between PHLDA2 and Weight for Age Percent**				
Birth	Estimate	Std. Error	t value	Pr(>|t|)	**24 Months**	Estimate	Std. Error	t value	Pr(>|t|)
H19	7.76	33.8	0.23	0.819	PHLDA2	7.53	3.52	2.14	0.0343*
Maternal BMI	5.21	4.64	1.12	0.262	Maternal BMI	0.936	0.419	2.24	0.0269*
Gestational Age	148	17.8	8.31	4.14E+15***	Gestational Age	-0.3	1.57	-0.191	0.849
Maternal Education: Less than or equal to high school	73	90.2	0.81	0.419	Maternal Education: Less than or equal to high school	-2.68	9.17	-0.292	0.771
Maternal Education: Some college or beyond	-36.7	68.9	-0.533	0.594	Maternal Education: Some college or beyond	-7.32	6.64	-1.1	0.273
Ever Smoker: Yes	-125	460	-0.272	0.786	Ever Smoker: Yes	3.02	8.5	0.355	0.723

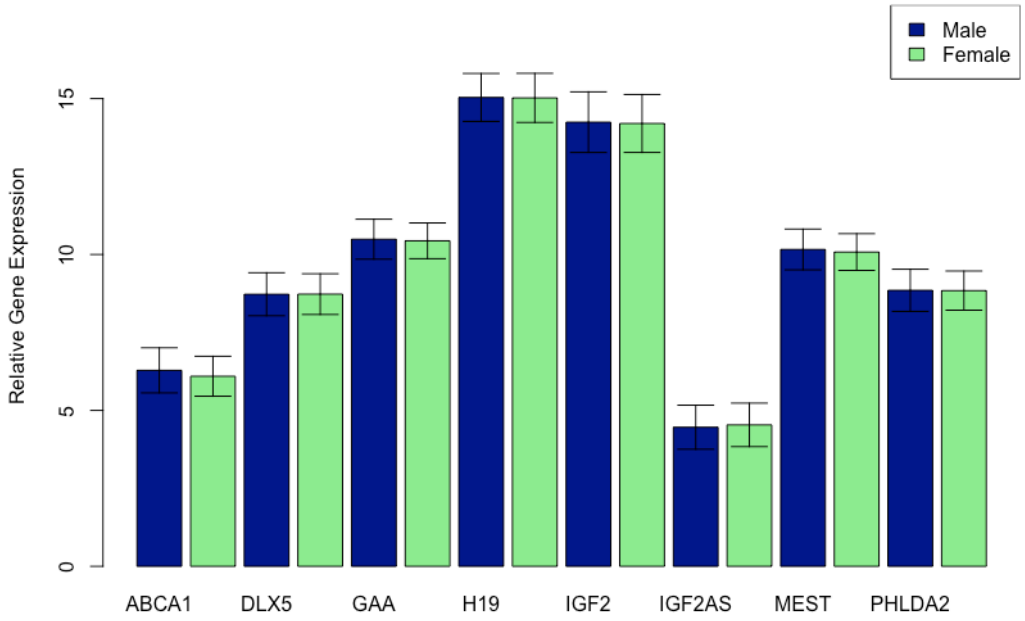
Note: Linear models were adjusted for maternal age, maternal BMI, maternal education, tobacco use, gestational age, and infant sex.
*Significant at level p<0.05, ** Significant at level p<0.01, *** Significance at level p<0.001.

Table 7: Description of genes associated with placenta cadmium concentration.

Gene	Name	Function	Phenotypic Effects	Reference
Paternally Expressed Genes				
IGF2	Insulin Like Growth Factor 2	Growth Factor	Placental and fetal growth restriction.	(Dilworth et al., 2010)
IGF2AS		Non-coding RNA	Implicated in Beckwith-Wiedemann syndrome and Wilms tumor	(Okutsu et al., 2000)
MEST	Mesoderm Specific Transcript	Neuronal differentiation	Affects neural development and behavior, fetal growth restriction, and maternal behavior; smaller placentas.	(McMinn et al., 2006)
Maternally Expressed Genes				
DLX5	Distal-less homeobox 5	Developmental transcription factor and is crucial for bone development. Unknown placental function.	Early embryonic lethality, significantly upregulated in preeclamptic placenta.	(Rugor et al., 2015)
H19		Non-coding RNA. Controls imprinting of IGF2	Fetal overgrowth and placentas, with larger glycogen storage spaces.	(Esquiliano, Guo, Liang, Dikkes, & Lopez, 2009)
PHLDA2	Pleckstrin homology-like domain family A member 2	Cytoplasmic protein with pleckstrin-homology domain	Placental overgrowth; fetal growth remains unchanged.	(Jensen, Tunster, & John, 2014)
Isoform Specific or Unknown Maternal/Paternal Expression				
GAA	Glucosidase Alpha, Acid	Encodes lysosomal alpha-glucosidase	Defects in this gene are the cause of glycogen storage disease II, also known as Pompe's disease.	(Beratis, LaBadie, & Hirschhorn, 1978)
ABCA1	ATP binding cassette subfamily A member 1	Transports cholesterol and certain fats across the cell membrane to the outside of the cell	Mutations in the *ABCA1* gene can cause familial HDL deficiency, which leads to reduced levels of HDL in the blood and may result in early-onset cardiovascular disease	(Lindegaard et al., 2008)

Note: Table includes both known and putative imprinted genes with significant cadmium expression levels in the placenta.

Figure 1: Sex differences in placental imprinted gene expression.



Note: Mean gene expression is shown (±SEM) in male (N=173) vs. females (n=153) placentas. Difference in expression was tested using independent samples t-test, and no significant differences were observed (all $p > 0.05$).

Supplemental Tables

Table S1: Matrix of correlations (r)[a] between individual metals.

Exposure	Mercury (Hg)	Lead (Pb)	Arsenic (As)	Cadmium (Cd)	Manganese (Mn)
Mercury	1.0	0.1	0.2	0.004	0.09
Lead	0.1	1.0	-0.009	0.02	0.25**
Arsenic	0.2	-0.009	1.0	0.07	0.08
Cadmium	0.004	0.02	0.07	1.0	0.05
Manganese	0.09	0.25**	0.08	0.05	1.0

Note: Values were obtained using the Bonforoni probability matrix to evaluate the strength and significance of the correlations among the independent variables (individual metals). [a] Pearson's product-moment correlation coefficient and corresponding p-values.
*Significant at level $p<0.05$, ** Significant at level $p<0.01$.

Table S2: Association between gene expression and individual metals. Increase in individual metal levels for the panel of ~100 imprinted genes assessed using placenta tissue samples.

Imprinted Gene	Estimate	Std.Error	t-value	p-value	q-value
Cadmium (Cd)					
H19	-0.66	0.16	-4.20	3.46E-05	0.003**
IGF2AS	-0.48	0.14	-3.41	0.001	0.028*
PHLDA2	0.44	0.13	3.31	0.001	0.028*
GAA	0.38	0.12	3.08	0.002	0.043*
DLX5	0.41	0.14	3.02	0.003	0.043*
IGF2	-0.58	0.19	-2.97	0.003	0.043*
ABCA1	0.40	0.14	2.92	0.004	0.044*
MEST	0.36	0.13	2.88	0.004	0.044*
Mercury (Hg)					
SHANK2	-0.15	0.05	-2.90	0.004	0.092
GPR1	0.13	0.06	2.27	0.024	0.092
LDB1	-0.07	0.03	-2.22	0.027	0.094
CTNND2	0.10	0.05	2.17	0.031	0.149
RB1	0.08	0.04	2.06	0.041	0.162
TRAPPC9	-0.10	0.05	-1.86	0.064	0.198
KCNQ1OT1	0.10	0.05	1.83	0.069	0.242
SHANK2	-0.15	0.05	-2.90	0.003	0.092
Lead (Pb)					
ITUP1/MIMT1_(Usp29)	0.23	0.11	2.18	0.030	0.997
TRAPPC9	0.22	0.13	1.67	0.096	0.997
CPXM2	-0.50	0.31	-1.60	0.111	0.996
PEG3	0.20	0.13	1.53	0.126	0.997
L3MBTL	0.25	0.18	1.41	0.160	0.997
DLX5	0.17	0.13	1.33	0.183	0.997
CDKAL1	0.12	0.09	1.27	0.205	0.997
ZNF331	0.15	0.13	1.21	0.226	0.997
Arsenic (As)					
NHP2L1	NHP2L1	NHP2L1	NHP2L1	NHP2L1	NHP2L1
INPP5F	INPP5F	INPP5F	INPP5F	INPP5F	INPP5F
SDHD	SDHD	SDHD	SDHD	SDHD	SDHD
SGCE	SGCE	SGCE	SGCE	SGCE	SGCE
ZBTB8B	ZBTB8B	ZBTB8B	ZBTB8B	ZBTB8B	ZBTB8B
CTNND2	CTNND2	CTNND2	CTNND2	CTNND2	CTNND2
UBE3A	UBE3A	UBE3A	UBE3A	UBE3A	UBE3A
Manganese (Mn)					
HYMAI	0.63	0.32	1.98	0.049	0.993
IGF2R	0.24	0.12	1.89	0.060	0.993
NNAT	-0.32	0.20	-1.59	0.113	0.993
LOC253039	-0.32	0.20	-1.58	0.114	0.993
GPR1	0.27	0.18	1.46	0.145	0.993
ILK	0.11	0.08	1.32	0.189	0.993
H19	-0.25	0.20	-1.29	0.196	0.993
FAM50B	-0.18	0.14	-1.28	0.202	0.993

Note: All models adjusted for maternal age, maternal BMI, maternal education, tobacco use, gestational age, and infant sex. *Significant at level $p<0.05$, ** Significant at level $p<0.01$.