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Luisa Fernanda Sarmiento Date

Prenatal mercury and cadmium exposure and neurodevelopment

By

Luisa Fernanda Sarmiento Rodríguez
Master of Public Health

Environmental Health

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Committee Chair

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An abstract of
A thesis submitted to the Faculty of the
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Abstract

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By Luisa Fernanda Sarmiento Rodriguez

Prenatal exposures to toxic metals are associated with neurodevelopment effects in children. Heavy metals have different mechanisms of neurotoxic action including effects on the placenta. This organ plays an important role in the transference of the metal from the mother to the infant as well as in orchestrating appropriate development. The present study aims to investigate the potential association between exposure to cadmium and mercury and neurodevelopmental outcomes in the Rhode Island Child Health Study (RICHS). The sub-cohort included 316 participants that were assessed with the NICU Network Neurobehavioral Scale (NNNS) and have available biomarkers of exposure. Differences in the concentrations of metals across neurobehavioral profiles were initially examined, and followed by logistic regression to examine the association between metals exposure and membership in a neurobehavioral profile with the poorest performance. We found elevated odds of membership in a neurobehavioral profile characterized by increased excitability, poor quality of movement and reduced self-regulation associated with Cd and Hg exposures in models controlling for infant birthweight, gestational age, sex, maternal age, level of education and smoking status during pregnancy, but this relationship was not statistically significant. The use of the NNNS might not be suitable for populations with low exposures to heavy metals that do not display extreme neurobehavioral features but opens the possibility to further investigation related to the use of this assessment as a neurobehavioral predictive tool under the right conditions.

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PRENATAL MERCURY AND CADMIUM EXPOSURE AND NEURODEVELOPMENT

1. INTRODUCTION

Exposures to toxic metals during pregnancy pose a risk for deleterious effects on the fetus, including neurodevelopmental effects. During this stage of development the fetus is more vulnerable to any substance that can affect the placenta or can cross into the fetus and disrupt appropriate development (Punshon et al., 2016). Despite of the association of heavy metals to neurotoxic outcomes, the literature does not present conclusive results of the association of prenatal exposures to cadmium and mercury and early neurodevelopment and how the mechanisms of action of metals in the placenta compared to the fetus proper could affect the outcome. Early identification of potential neurodevelopmental deficits can provide an opportunity for early intervention. Therefore, the aim of the study is to evaluate the association between exposure to cadmium and mercury and early neurodevelopment, using the effect estimates for exposure in the placenta and in maternal toenails to identify how differences in the mechanisms of action of both metals in the placenta can affect neurodevelopment.

1.1. Prenatal exposure to cadmium and mercury

Mercury is an element present in nature and has three predominant forms, elemental mercury, ionic mercury (inorganic mercury) and organic mercury (UNEP, 2008). Mercury is known for its toxicity on humans, causing adverse impacts mainly in the central nervous system, including neurodevelopmental disorders and brain dysfunction (Snoj Tratnik et al., 2017). Exposure to mercury can occur by different routes such as ingestion, inhalation and dermal contact. The general population is mainly exposed through the ingestion of water, fish and seafood containing mercury, although humans can also be exposed to mercury through inhalation of inorganic mercury present in dental amalgam fillings or other resulting chemicals from industrial

activities. Other exposures to different forms of organic mercury are present as ethyl-mercury in some vaccines and pharmaceutical products (UNEP, 2008) (Gundacker & Hengstschläger, 2012).

In general, exposure to mercury from organic sources, predominantly as methylmercury, constitutes the major high health risk, particularly to the developing fetus during pregnancy (Chen et al., 2014). Maternal fish consumption increases exposure to methylmercury, and these exposures have been linked to long-term neurodevelopmental consequences (Fok et al., 2007).

Cadmium is a non-essential heavy metal naturally occurring in the environment. Cadmium exists in one oxidation state, and can form complexes with other substances. Sources of cadmium in the environment are primarily from activities such as mining, manufacturing, agriculture or waste disposal and incineration. Exposure to cadmium mainly occurs through inhalation and ingestion routes. Smoking is one of the major sources of exposure to cadmium through inhalation, as cadmium is taken up by leafy vegetables, including tobacco. Ingestion of vegetables, cereals and meat products contaminated with cadmium are another large source of exposure (ATSDR, 2012). Cadmium is toxic for humans resulting in harmful effects in different systems and organs such as kidneys, lungs and nervous system (ATSDR, 2012).

1.2. Placental transfer of cadmium and mercury

The placenta has a fundamental role in the development of the fetus through functions such as the transportation of nutrients, water, waste, and regulation of metabolism, endocrine functions and protection of the fetus from toxic exposures. Therefore, toxicants can impact fetal development by interfering with appropriate placental function (Marsit, 2016) .

The mechanisms of action of cadmium and mercury on neurodevelopment may differ based on the concentrations of these exposures which can reach the fetus proper. Chen (Chen et al., 2014), examined maternal exposure to cadmium, lead, selenium and mercury using plasma and red blood cells and assessed trans-placental passage from mother to fetus of the selected

elements in umbilical cord. In fact, according to (Gundacker & Hengstschläger, 2012) cord blood mercury levels are usually higher when compared to maternal blood levels. Results show higher levels of hemoglobin, hematocrit and plasma albumin in fetal blood compared to maternal blood increases the affinity of mercury to bind fetal blood cells, and there is active transport of the metal to the fetus. The results from these and other studies (Esteban-Vasallo, Aragonés, Pollan, López-Abente, & Perez-Gomez, 2012; Hinwood et al., 2013) demonstrate that there is transplacental transfer of mercury, selenium and lead from the mother to the fetus, denoted by strong correlations between maternal and cord blood concentrations of mercury, lead, and selenium, whereas cadmium presented a weaker maternal and cord blood correlation which can be attributed to a limited-transplacental passage.

These differences in passage may be related to the mechanisms by which metals can cross the placenta. Metallothionein is a protein whose functions are related to the regulation of essential metals, and cadmium-metallothionein binding is the mechanism that reduces the transfer of cadmium to the fetus. (Ronco, Arguello, Suazo, & Llanos, 2005). Mercury transfer across the placenta, on the other hand, depends on its chemical form. Methylmercury is actively transported by amino acid carriers (Gundacker & Hengstschläger, 2012), and ABC transporters to transfer across the placenta and reach the fetus (Straka et al., 2016).

1.3. Neurodevelopmental effects of cadmium and mercury

Cadmium and mercury are known for their action as neurotoxins with potential effects on neurodevelopment (Jiang, Hsi, Fan, & Chien, 2014). Epidemiological studies have presented associations between prenatal exposure to cadmium and lower full-score IQ, performance IQ and emotional symptoms. (Jeong et al., 2015; Rodríguez-Barranco et al., 2013; Sioen et al., 2013). Other studies have presented associations between higher maternal erythrocyte mercury levels and worse child test performance (Oken et al., 2008), and mercury blood levels and

attention deficit hyperactivity disorder(Bellinger, 2013), among others. Prenatal exposure to methylmercury is associated with neurodevelopmental disorders and subclinical brain dysfunction (Gundacker & Hengstschläger, 2012). (Grandjean, Weihe, White, & Debes, 1998) assessed prenatal methylmercury exposure and neurobehavioral performance in a birth cohort in the Faroe Islands. Results showed associations between language, attention, and memory and in a smaller extend motor functions in children prenatally exposed to high methylmercury levels compared to children exposed to low levels, suggesting that multiple domains of brain function can be affected by the exposure. Other studies have found an association between prenatal exposure to mercury and negative outcomes in cognition and whole neurodevelopment in infants, specially affecting cognition, language and motor skills (Snoj Tratnik et al., 2017). In fact, one study assessed the effects of prenatal low-level exposure to mercury and found that cord blood mercury level was significantly associated with adverse outcomes on passive muscle tone and active muscle tone (Wu, Ying, Shen, & Wang, 2014). Importantly, these effects of prenatal exposure have been observed later in childhood, and there has been little study of any early neurobehavioral phenotypes, which might suggest later neurobehavioral risk, linked to these exposures.

1.4. Effects on the Placenta

Mercury's transfer across the placenta may lead to disruption of placenta functions. This metal alters amino acid transfer, placental oxygen consumption, hormonal secretion and membrane fluidity in the placenta (Gundacker & Hengstschläger, 2012).

Cadmium accumulation in the human placenta could imply a risk for the development of adverse effects in the fetus through placental functional effects (Kippler et al., 2010). The presence of the metal in the placenta affects the transport of other elements such as calcium and zinc and alters different placental process such as cell migration (Gundacker &

Hengstschläger, 2012). Accumulation of cadmium in the placenta has been shown to begin as early as the first trimester (Kantola et al., 2000), which has implications in neurodevelopment since exposure to cadmium has been associated to a lower full-score IQ and performance IQ (Rodríguez-Barranco et al., 2013). Prenatal exposure to cadmium has been associated with a decline in infant's developmental quotients in a social domain (Wang et al., 2016).

1.5. Biomarkers used for cadmium and mercury detection

Diverse biomarkers have been used to reflect accurately exposure levels during pregnancy. Understanding the relationship between these heavy metals and the placenta allows predicting adverse outcomes. Biomarkers include maternal and neonate hair, cord blood, maternal and infant blood, maternal and neonate fingernails and toenails, and placental tissue (Grandjean et al., 1998; Sun et al., 2014). Sakamoto et al., conducted an epidemiologic study to compare samples of fingernails, toenails, maternal blood and maternal hair during early pregnancy. Mercury levels were measured in the specimen samples and compared to mercury levels in cord blood at parturition. They found that mercury levels in nails were strongly correlated to mercury levels in cord blood, thus reflecting fetal exposure (Sakamoto et al., 2015). In the same way, Punshon et al., used placental tissue to assess the effects of maternal metal concentrations, including cadmium, on infant metal concentrations; finding that there was no evidence of an indirect effect of maternal metal concentrations on infant metal concentrations through placental metal concentrations (Punshon et al., 2016).

1.6. Neurodevelopment Assessment

Neonatal neurologic and neurobehavioral assessment after birth can assess prenatal exposures and its immediate effects, before any outside influences for environment can affect the newborn. Neurodevelopmental assessments of newborns have changed over the years. Primarily, newborn neurobehavioral assessments were used to be assessed by describing and

defining the tone and reflexes of infants. Later, development of sensory function and the progression of central nervous system development began to be evaluated. The Neonatal Behavioral Assessment Scale (NBAS) was the first standardized scale to provide a comprehensive assessment of newborn neurobehavior. This scale focused on the interaction of the newborn with the surrounding environment, neurological responses and behavioral responses, and assumes that neonates are able to regulate their behavior. The evaluation scale comprises 28 behavioral and 18 reflex items based on four domains, autonomic physiological regulation, motor organization, state organization and regulation and attention or social interaction (Dietrich et al., 2005). The exam predicts behavioral problems in childhood with a sensitivity of 75% and specificity of 95 to 98% (El-Dib, Massaro, Glass, & Aly, 2011).

The NICU Network Neurobehavioral Scales (NNNS) is a comprehensive neurobehavioral exam and stress assessment designed for term infants at risk and preterm infants, and it can be used for different purposes. The NNNS was developed to assess preterm infants and especially high risk infants focusing on exposure to drugs and preterm birth and examines the full range of neurobehavioral organization of the infant as well as neurologic reflexes, motor development, active and passive tone, infant stress, abstinence and withdrawal and neurologic functioning. 115 items are scored in the NNNS scoring form and used to compute 13 different summary scores. In this scale scores below the 10^{th} percentile or above the 90^{th} percentile are generally indicators of poor performance. This scale has been used in different studies especially in infants exposed to substances such as cocaine and alcohol(El-Dib et al., 2011) (Lester & Tronick, 2004). The scale summary scores focus on habituation, attention, handling, quality of movement, regulation, non-optimal reflexes, asymmetric reflexes, stress/abstinence, arousal, hypertonicity, hypotonicity, excitability and lethargy (Lester & Tronick, 2004). In order to reduce the testing burden when relating prenatal factors to the results of the NNNS, methods have been

developed to use the summary scores to assign infants membership into a latent profile derived from a combination of the individual summary scores using different methodologies as mentioned in (Appleton et al., 2016)and (Liu et al., 2010).

1.7. Rhode Island Birth Cohort

The data used in this study are derived from the Rhode Island Child Health Study, which recruited participants from 2009 to 2013. This hospital-based US cohort study enrolled mother and infant pairs following delivery at the Women and Infants Hospital of Rhode Island. Infants were oversampled for small and large for gestational age (SGA, LGA, respectively) infant, and appropriate for gestational age infants (AGA) were also enrolled matched on sex, gestational age and maternal age. The study enrolled 804 infants and 627 infants were assessed with the NNNS. Available information from the study included maternal health history, including diseases such as asthma, diabetes and pre/pregnancy obesity; pregnancy conditions, such as gestational diabetes, preeclampsia, depression, anxiety, obsessive compulsive disorder, and panic attacks; health behaviors, including smoking, alcohol use and physical activity; labor/delivery factors included cesarean delivery, labor induction, medication use during labor, fetal distress and breach birth; infant and maternal attributes were used as predictors of neurobehavior (Appleton et al., 2016).

2. AIM AND HYPOTHESIS

The specific aims of this study are to evaluate the association between exposure to cadmium and mercury and early neurodevelopment, using the NICU Network Neurobehavioral Scale (NNNS) in the Rhode Island Child health Study (RICHS). We aim to compare the effect estimates for exposure in the placenta and in maternal toenails to consider how the differences in the mechanisms of action of both heavy metals in the placenta can affect neurodevelopment. We hypothesized that exposure to both metals is associated with early neurodevelopmental

variation in children, and placental levels will show a stronger association for cadmium and neurobehavioral outcomes compared to maternal toenails.

3. METHODS

3.1. Study Population

Study participants were part of the Rhode Island Child Health Study (RICHS), which enrolled mother and infant pairs following delivery at the Women and Infants Hospital of Rhode Island from 2009 to 2013. Infants were selected based on their gestational age and birth-weight. Term infants (>37 weeks gestation) born small for gestational age (SGA, lowest 10th percentile) or large for gestational age (LGA, highest 10th percentile) were selected according to the Fenton growth chart; infants appropriately sized for gestational age (AGA) matched to the SGA and LGA infants on sex, gestational age (±3 days), and maternal age (±3 years) were also enrolled. Only singleton, viable infants were included in the study. Additional exclusion criteria were maternal age <18 or >40 years, congenital or chromosomal abnormality of the infant and a life-threatening medical complication of the mother. Eight hundred and forty infants were enrolled and 627 infants were assessed with the NNNS (Appleton et al., 2016).

3.2. Data Abstraction

1.1.1.Exposure Assessment

Within 2 hours of birth, full-thickness sections of placental parenchyma were taken approximately 2 cm from the umbilical cord-insertion site, free of maternal decidua. These sections were immediately frozen in liquid nitrogen and stored at –80°C until analyzed. A panel of trace metals was examined in the placental tissue using methods described in (Punshon et al., 2016).

First toenail clippings from all toes were requested from mothers and infants following discharge, and were available for a total of 187 infants. Collection was made by parents, who

provided their own and their children's toenail clippings and mailed them back in provided envelopes (Maccani et al., 2015). A panel of trace metals was examined in maternal and infant large toe samples as described in (Punshon et al., 2016).

1.1.2.Outcome Assessment

In order to analyze newborn neurobehavioral behavior, infants were examined with the NNNS (Lester and Tronick 2004). The NNNS was performed by one certified psychometrician blinded to the prenatal history. Assessments were conducted after the first 24 h of life, but before hospital discharge. As a result thirteen summary scores were derived from the exam (Maccani et al., 2015). In addition, using a model-based clustering algorithm, discrete neurobehavioral profiles were defined based on the individual summary scores as described in (Appleton et al., 2016).

3.3. Covariates

Covariate information was collected as part of the Rhode Island Child Health Study, via medical record abstraction and interviewer-administered questionnaire. In this cohort, pregnancy conditions were assessed including the presence or absence of gestational diabetes, depression, preeclampsia, and anxiety/obsessive compulsive disorder/panic attacks according to the medical record. Pregnancy weight gain was self-reported. Maternal health and pregnancy conditions were examined if the prevalence was >5%. Pregnancy health behaviors included any self-reported smoking, alcohol use, and hours of moderate/vigorous physical activity per week. Illicit drug use was not examined due to low prevalence (<1%). Infant attributes as listed in the medical record included birthweight, gestational age at birth, and Apgar score taken 1 and 5 min after birth (Appleton et al., 2016).

3.4. Data Analysis

To analyze exposure to cadmium and mercury and its association with early neurodevelopment variation in children, bivariate analyses followed by logistic regression was used. Model choice was based on the distribution of the outcome (NNNS) variable. Wilcoxon one-way non-parametric test of variation across groups, known as the Kruskal Wallis test, was used to compare the concentration of cadmium and mercury in toenails and placenta by membership to each of the 7 defined neurobehavioral profiles. We then focused on neurobehavioral profile 7, which was chosen because this profile displayed the most extreme scores and presented below or above the average results for all NNNS Summary scores, which translates in a poor neurobehavioral performance. We grouped profiles 1 to 6 into one category (considered as not poor neurobehavioral performance) for this analysis, compared to profile 7 and employed logistic regression to examine the odds of membership in profile type 7 by metals biomarker concentrations, controlling for confounders. Covariates such as infant sex (Male-female), maternal education (high school degree or below – college equivalent or above), smoking status during pregnancy (yes – no), birthweight, gestational age and maternal age were included in the model. Any missing data were excluded.

4. RESULTS

4.1. Demographic characteristics of the participants

The demographic characteristics of the study population are summarized in Table 1. The total number of participants in this subcohort was 316, which includes infants with NNNS data, and measures of either toenail or placental metals. Mothers were on average 31 years old. The majority were Caucasian (69.9%) with high levels of maternal education; approximately 81% of the population obtained a college degree or higher. The sex ratio was similar having a slightly higher percentage of male infants (51.3%) compared to female infants (48.7%). Approximately

23% of the mothers reported smoking during pregnancy. Infants were born on average at a gestational age of 39.4 weeks and reported an average birthweight of 3599 g. Most of the infants were appropriately size for gestational age (55.4%).

Table 1. Demographic characteristics of the participants

Categorical variables						
Variable	**N**	**%**	**Std. dev**	**Median**	**Min**	**Max**
Participants	316	-	-	-	-	-
Growth status						
AGA	175	55.4	-	-	-	-
LGA	95	30.1	-	-	-	-
SGA	46	14.6	-	-	-	-
Infant gender						
Female	154	48.7	-	-	-	-
Male	162	51.3	-	-	-	-
Maternal Race						
Caucasian	243	76.9	-	-	-	-
African-american	23	7.3	-	-	-	-
Other	42	13.3	-	-	-	-
Unknown	8	2.5	-	-	-	-
Infant race						
Caucasian	221	69.9	-	-	-	-
African American	22	7.0	-	-	-	-
Other	65	20.6	-	-	-	-
Unknown	8	2.5	-	-	-	-
Maternal education level						
Less than High school	16	5.1	-	-	-	-
High school	45	14.2	-	-	-	-
College and above	255	80.7	-	-	-	-
Smoke during pregnancy						
NA	72	22.8	-	-	-	-
No	210	66.5	-	-	-	-
Yes	34	10.8	-	-	-	-
Continuous variables						
Variable	**N**	**%**	**Std. dev**	**Median**	**Min**	**Max**
Infant Birthweight (g)	316	3599.3	652.9	3600	2030	5465
Gestational Age (weeks)	316	39.4	0.9	39	37	42
Maternal Age (years)	316	30.6	5.1	31	18	40
Infant cadmium in toenails	180	-	0.20	0.06	0.001	1.28
Infant mercury in toenails	158	-	0.33	0.08	0.005	2.50
Placental cadmium	192	-	2.58	4.19	1.06	17.99
Placental mercury	192	-	2.66	1.66	0.00	26.35

4.2. NNNS Summary Scores

Table 2 presents the results obtained for each NNNS summary score. Most of the participants were assessed for all the items of the scale. The cohort did not register a lot of missing data, except for the habituation component that requires the infant to be asleep, and for the attention score. The habituation summary score registered n = 149 missing data and the attention summary score registered n = 34 missing values.

Table 2. NNNS descriptive statistics

NNNS Summary Score	N	Mean	Std. Dev.	Min	Max
non-optimal reflexes	316	5.70	2.14	0.00	11.00
Lethargy	316	6.40	2.47	1.00	14.00
Habituation	167	3.06	3.70	0.00	8.67
Handling	312	0.33	0.23	0.00	0.88
Excitability	316	4.57	2.83	0.00	13.00
Hypertonicity	316	0.42	0.77	0.00	5.00
Hypotonicity	316	0.54	0.70	0.00	4.00
Attention	282	3.54	1.97	0.00	7.71
Quality of movement	315	3.43	1.69	0.00	5.67
Regulation	313	4.45	1.59	0.00	7.14
Stress / Abstinence	316	0.16	0.07	0.00	0.41
Arousal	316	3.50	1.70	0.00	5.86
Asymmetric reflexes	316	1.63	1.30	0.00	6.00

(Appleton et al., 2016) developed a method in which different discrete groups of infants were identified. As a result they identified 7 different neurobehavioral profiles. A visual inspection was performed to verify the presence of significantly different summary scores across the profiles. In addition, a Kruskal-Wallis test was used to support the identification of differences. NNNS Summary Scores classified by neurobehavioral profile are presented in Table 3. NNNS Summary scores for arousal, Stress-abstinence, self-regulation, quality of movement, orientation, hypotonicity, hypertonicity, excitability, handling, lethargy and non-optimal reflex, were found to be significantly different across the 7 identified neurobehavioral profiles.

Table 3. NNNS descriptive statistics across identified neurobehavioral profiles

	Profile 1	**Profile 2**	**Profile 3**	**Profile 4**	**Profile 5**	**Profile 6**	**Profile 7**	**P**
	n = 35	n= 57	n = 36	n = 53	n = 51	n = 51	n = 33	
NNNS Summary Score	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	
Non-optimal reflexes	6,23 (1,97)	4,98 (1,75)	7,36 (1,66)	5,58 (1,66)	5,59 (1,80)	5,45 (2,79)	5,30 (2,54)	<.0001
Lethargy	7,14 (2,39)	5,44 (1,93)	9,06 (2,57)	5,57 (1,96)	5,27 (2,61)	6,78 (1,98)	6,85 (1,86)	<.0001
Habituation	3,00 (3,79)	3,14 (3,81)	3,11 (3,73)	4,22 (3,98)	2,86 (3,72)	2,61 (3,57)	2,59 (3,56)	0.8982
Handling	0,21 (0,15)	0,33 (0,18)	0,21 (0,20)	0,19 (0,14)	0,37 (0,22)	0,53 (0,25)	0,48 (0,23)	<.0001
Excitability	2,71 (1,34)	3,86 (1,08)	2,53 (1,25)	1,38 (0,86)	6,25 (2,17)	7,47 (1,78)	8,00 (1,77)	<.0001
Hypertonicity	1,06 (0,24)	0,00 (0,00)	0,00 (0,00)	0,00 (0,00)	1,24 (1,24)	0,20 (0,66)	0,70 (0,47)	<.0001
Hypotonicity	0,63 (0,77)	0,47 (0,68)	1,06 (1,09)	0,30 (0,46)	0,43 (0,57)	0,49 (0,58)	0,67 (0,54)	0.0039
Attention	3,79 (1,81)	3,45 (2,06)	2,94 (1,56)	4,56 (2,07)	3,94 (2,06)	2,63 (1,56)	2,96 (1,65)	<.0001
Quality of Movement	3,63 (1,74)	3,39 (1,85)	3,70 (1,40)	3,85 (1,82)	3,26 (1,61)	3,35 (1,36)	2,75 (1,83)	<.0001
Regulation	5,10 (1,35)	4,84 (1,01)	4,87 (1,03)	5,31 (1,97)	4,06 (1,49)	3,51 (1,32)	3,26 (1,48)	<.0001
Stress, abstinence	0,14 (0,05)	0,14 (0,06)	0,15 (0,06)	0,11 (0,04)	0,18 (0,06)	0,20 (0,07)	0,21 (0,07)	<.0001
Arousal	2,95 (1,53)	3,59 (1,40)	2,99 (1,29)	2,82 (1,42)	3,51 (2,03)	4,36 (1,67)	4,25 (1,85)	<.0001
Asymmetrical reflexes	1,37 (1,14)	1,65 (1,29)	2,03 (1,48)	1,55 (1,32)	1,73 (1,37)	1,69 (1,33)	1,30 (1,05)	0.4235

Results show that Profile 2 characterizes for the presence of average results across the 13 measured scales of the NNNS Summary scores. Scores for neurobehavioral profiles types 1 and 3, generally presented a similar behavior. Infants classified with a neurobehavioral profile type 7 were more lethargic and excitable, they also required more handling and had greater levels of arousal, stress-abstinence hypertonicity and hypotonicity. These infants showed lower levels of habituation, attention, quality of movement and had the lowest scores for self-regulation and asymmetric reflexes. Infants with a neurobehavioral profile type 6 had similar behaviors to infants with a profile type 7 but their scores were not as extreme as the ones registered in profile type 7. While Infants in profile type 4 can be defined as infants with NNNS scores above the average, infants in profile type 7, usually displayed NNNS scores below the average. Approximately 18% of the infants exhibited a neurobehavioral profile type 2 (57 infants). Neurobehavioral profile 7 has the lowest percentage of classification with 10% of the infants.

The percentage of infants that were classified as part of the group with a neurobehavioral profile type 4 was 16.8%.

4.3. Heavy metal levels

Prenatal exposure to cadmium and mercury was analyzed using infant toenails and placenta as matrices of exposure to these heavy metals. Figure 1 to Figure 4 shows basic statistical measures for the heavy metal levels identified by each biomarker of exposure by NNNS profile.

1.1.3.Placental cadmium

There were 192 participants with measures of placental cadmium, and in the total cohort the mean levels of cadmium in the placenta were 0.14 µg/g. The distribution of placental levels of cadmium across the different profiles was similar (Pr> 0.47). Figure 1, presents a boxplot with the distribution of cadmium in the placenta according to each neurobehavioral profile. Profiles 4 and 6 had the highest mean levels of cadmium in placenta. The maximum registered levels of the heavy metal in the placenta were 17.99 µg/g.

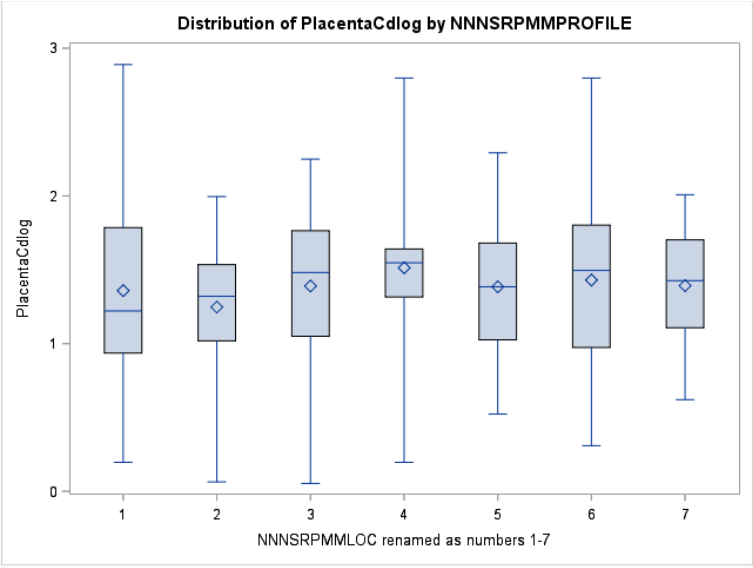

Figure 1. Distribution of placental cadmium across neurobehavioral profiles

1.1.4.Placental mercury

There were 192 participants with measures of placental mercury, whose mean levels were 0.19 µg/g. We also observed no significant differences in the placental levels of mercury across the different neurobehavioral profiles (Pr> 0.75). As shown in Figure 2, profile 5 registered the highest mean levels of mercury in the placenta.

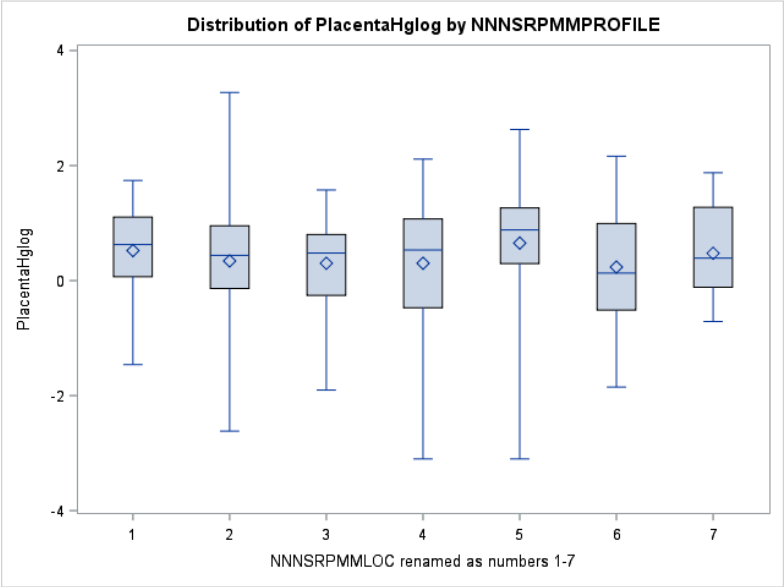

Figure 2. Distribution of placental mercury across neurobehavioral profiles

1.1.5.Cadmium in infant toenails

Mean levels of cadmium in infant toenails were 0.14 µg/g, available from 180 participants. As with placental levels, we observed no significant differences in cadmium concentrations across the different profiles (Pr> 0.75). As shown in Figure 3, profile 7 registered the highest mean levels of cadmium in toenails.

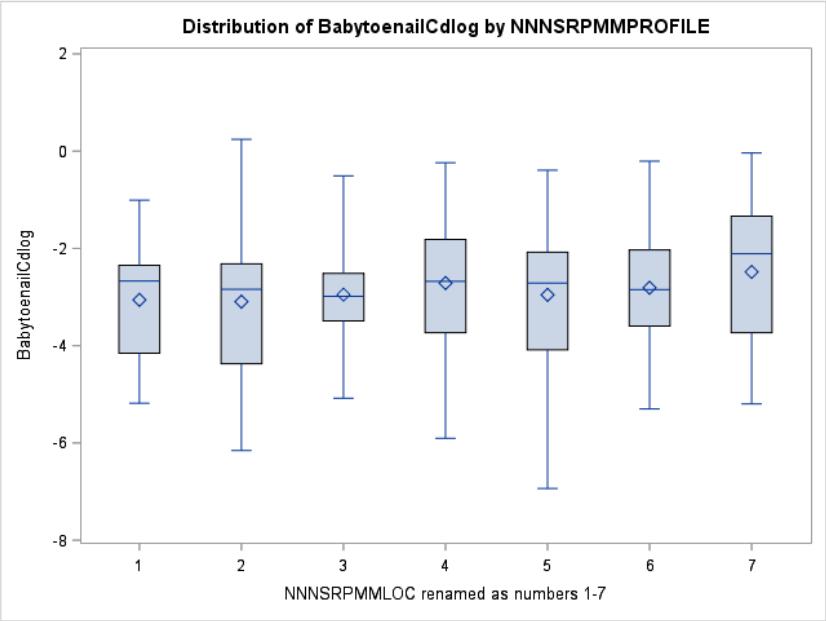

Figure 3. Distribution of cadmium in infant toenails across neurobehavioral profiles

1.1.6.Mercury in infant toenails

A total of 158 infants had measures of mercury in toenails and there were no differences in the concentrations of this metal across the NNNS profiles (Pr> 0.50). As shown in Figure 4, profile 1 registered the highest mean levels of mercury in toenails.

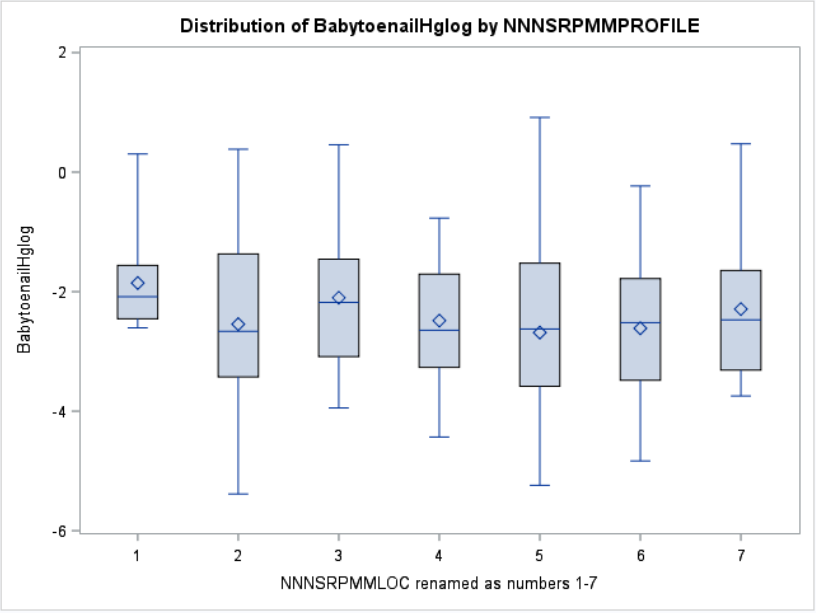

Figure 4. Distribution of mercury in infant toenails across neurobehavioral profiles

1.1.7.Correlations between metals

Table 4 presents the spearman correlation coefficients for the different matrices metal concentrations in the entire cohort. Placental cadmium concentrations were moderate positively correlated to placental mercury concentrations. Similarly, placental mercury concentrations showed a moderate and positive correlation with maternal toenail mercury concentrations, and placental mercury concentrations had a positive correlation with maternal toenail concentrations of cadmium. It is important to highlight that mercury and cadmium concentrations in infant toenails were moderately and positively correlated with cadmium and mercury concentrations in maternal toenails.

Table 4. Spearman Correlation Coefficients for metal concentrations

	Placenta Hg	Placenta Cd	Infant toenail Hg	Infant toenail Cd	Maternal toenail Hg	Maternal toenail Cd
Placenta Hg	1.00	0.30	0.06	0.12	0.45	0.29
		<.0001	0.65	0.40	0.0003	0.02
Placenta Cd	0.30	1.00	0.23	0.01	0.08	0.18
	<.0001		0.08	0.96	0.53	0.17
Infant toenail Hg	0.06	0.23	1.00	0.31	0.30	-0.12
	0.65	0.08		<.0001	0.0002	0.15
Infant toenail Cd	0.12	0.01	0.31	1.00	0.13	0.29
	0.40	0.96	<.0001		0.10	0.0001
Maternal toenail Hg	0.45	0.08	0.30	0.13	1.00	0.08
	0.0003	0.53	0.0002	0.10		0.31
Maternal toenail Cd	0.29	0.18	-0.12	0.29	0.08	1.00
	0.02	0.17	0.15	0.0001	0.31	

4.4. Logistic regression model

Neurobehavioral profile type 7 was defined as the event of interest given that in the exploratory analysis, this profile displayed most extreme scores and performed below the average results for all NNNS Summary Scores as described in the methods section.

A non-parametric test was performed aiming to find if there was a statistically significant difference in the median levels of each biomarker in infants with a neurobehavioral profile type 7 compared to all the other neurobehavioral profiles. In these examinations, we observed a

borderline difference in the median concentrations of cadmium in the infant toenails, with infants in profile 7 having a median of 0.122 while those in the other profiles a median of 0.060 (P=0.14). There were no differences in the concentrations of the other metals biomarkers in Profile 7 vs. the other profiles.

Table 5. Odds ratios (95% confidence intervals) for the association between prenatal exposure to cadmium and mercury and NNNS Neurobehavioral profiles

	OR, 95% CI			
Covariates	**Mercury in toenails**	**Mercury in placenta**	**Cadmium in toenails**	**Cadmium in placenta**
Metals Exposure (Log ug/g)	1.42 (0.82,2.46)	1.04 (0.56,1.92)	1.15 (0.73,1.80)	1.69 (0.51,5.58)
Sex (Male)	0.87 (0.24,3.14)	0.98 (0.31, 3.06)	0.84 (0.24,2.98)	0.99 (0.32,3.08)
Birthweight, g	1.00 (1.00,1.00)	1.00 (1.00,1.00)	1.00 (1.00,1.00)	1.00 (1.00,1.00)
Gestational age, wks	0.85 (0.39,1.87)	1.91(0.97,3.75)	0.83 (0.38,1.8)	1.86 (0.94,3.67)
Maternal age, yrs	0.99 (0.82,1.19)	0.99 (0.88,1.12)	0.99 (0.83,1.19)	0.98 (0.87, 1.10)
Maternal education (High School or less)	2.02 (0.21,19.80)	5.07 (0.76,33.87)	2.45 (0.25,23.68)	5.06 (0.75, 33.88)
Smoking (Smokers)	2.79 (0.44, 17.65)	2.29 (0.45,11.81)	2.54 (0.41,15.78)	2.23 (0.47, 10.71)

*** None of the p values were statistically significant***

Table 5, presents the results of the logistic regression. The applied logistic models confirmed the univariate findings, showing that there was a not significant relationship between metal level biomarkers and infant membership to neurobehavioral profile type 7. As described in table 6, the odds ratios were over 1. Mercury Toenail measures in infants and cadmium placental measures showed elevated odds ratios for an association between metals exposure and membership of neurobehavioral profile 7. Results show that one log increase in the concentration of mercury in toenail resulted in an elevated odds ratio for membership in Profile 7 of 1.42 (95% CI 0.82, 2.46) while a one log increase in placental cadmium concentrations resulted in an elevated odds ratio for membership in profile 7 of 1.69 (95% CI 0.51, 5.58),

controlling for birthweight, gestational age, sex, maternal age, education and smoking status, but the associations are considered not significant, as the confidence intervals include the null.

5. DISCUSSION

The results of this study showed there was not a significant difference of the metal concentrations in the different matrices across neurobehavioral profiles. Nevertheless, it is important to highlight that in general, when the neurobehavioral profile with the poorest performance was compared to the other neurobehavioral profiles as one category, the concentrations of metals were slightly elevated. The lack of statistical significance in these results could be a consequence of the sample size. In this population, the presence of infants with neurobehavioral profile type 7 was limited and given the analysis conditions, final comparison groups with measures of metals and neurobehavioral profiles had a small number of participants. Consequently, the sample size of each group for analysis might not provide the statistical power necessary to identify a difference at the level of effect observed.

In line with the bivariate tests, the obtained odds ratios from logistic models controlled for confounders showed a trend in the expected direction. Placental concentrations of metals displayed a stronger association for cadmium and neurobehavioral outcomes compared to infant toenails. The effect estimate of cadmium was greater for placental concentrations of the metal than for the concentration in toenails. These results could be explained by the tendency of cadmium to accumulate in placental tissue. In the case of mercury exposure, the effect estimate was greater for the concentrations of the metal in toenails than for the concentrations in the placenta, which may be explained by the fact that mercury has the ability to cross the placental barrier and there is a transference of the heavy metal from the mother to the fetus, having a greater effect in the forming fetus. Cadmium in the placenta showed the highest

concentrations of metals across all matrices. This result could support the hypothesis of an accumulation of the metal in the placenta, as it was expected.

There are a number of factors that could be considered limitations for this study. As it was previously mentioned, sample size plays an important role in the statistical analysis of the data, without the necessary sample size, it is not possible to identify robust results unless the effect estimates are very large. Given that this cohort is relatively healthy, we would not expect to see extreme effects. Another important factor is that this specific cohort was only exposed to low levels of cadmium and mercury. These low levels of exposure may not be large enough to detect a marked effect. Other environmental exposures to substances that can impact neurobehavioral effects were not taken into account in this study, such as concentrations of pesticides, lead or polycyclic aromatic hydrocarbons.

The selected matrices for the analysis were placenta and toenails. Toenails were selected given that people are more prone to provide their own toenails and their infants' toenails since they are easy to collect, preserve, manipulate and send for analysis. Additionally toenail collection is a non-invasive process. The correlation results for the biomarkers displayed a significant positive correlation of maternal concentrations of cadmium and mercury and infant concentrations of both metals in toenails. There was a significant positive correlation of maternal concentrations of mercury in the placenta and maternal concentrations of mercury in toenails. Supporting the feasibility of the use of this biomarker to indicate prenatal levels of exposure to mercury. Toenail mercury concentrations can be used as an independent indicator of exposure. (Sakamoto et al., 2015) showed that Hg concentrations in toenails at parturition can be a useful biomarker of prenatal Hg exposure to mothers and fetuses, especially referring to the third semester of gestation. Nevertheless, it mentions that the concentrations of mercury in other matrices are lower at parturition compared to early pregnancy and can be explained by the

transfer of mercury from the mother to the fetus. This may suggest that another matrix such as fetal cord blood can be a better matrix to measure mercury at parturition. For cadmium, the literature does not present a conclusive example in which cadmium levels in toenails have been consistently used as biomarkers of exposure to the metal. The use of toenails can be limiting, given that toenails in infants are small and possibly the amount of the matrix is not enough to provide an accurate estimate.

The use of the NNNS Summary scores showed significant differences for all the summary scales across the different profiles. The assessment is sensitive to detect extreme profiles of neurobehavior that have been associated to high-risk prenatal exposures such as alcohol and cocaine (Tronick & Lester, 2013). The method has been used in other studies among infants that have not been exposed to high-risk conditions and has showed significant neurobehavioral differences among the participants. Nevertheless, there is a possibility that the method is not as sensible to detect neurobehavioral deficiencies among small groups of infants with low pre-natal exposures to heavy metals that do not show extreme behaviors. (Fink, Tronick, Olson, & Lester, 2012) showed the suitability of the method in a healthy population under conditions classified as low risk, but stressed the existence of significant differences in neurobehavior associated with perinatal factors, in addition to other aspects such as ethnicity that were associated with clear neurobehavioral performance variations. During the statistical analysis of the current study, race was not found to be a significant predictor of neurobehavioral performance, and therefore was not included given the low ethnic variation of the sub-cohort. The inclusion of other perinatal factors that can affect neurobehavioral outcomes in this sub-cohort should be included in further research.

Even when this is a low risk sub-cohort, given the socioeconomic status and habits of its participants, among infants with a membership to neurobehavioral profile type 7 there was a

poor neurobehavioral performance. This could suggest the need for further research in this area in order to predict neurobehavioral effects that can provide the possibility of early interventions. NNNS assessment was performed in newborn infants, and neurobehavioral functions can change during growth and can be improved, supporting the importance of assessing the feasibility of this method as a predictor of neurobehavior even in low risk populations to develop early interventions. Other studies such as the one developed by (Liu et al., 2010) used the NNNS results as a predictor of negative medical and behavioral findings at an older age. In the same way, given the appropriate sample size, the results from a study like this could be used as predictors of further neurobehavioral outcomes in populations with low exposures to metals and can be compared with future assessments at a more advance neurodevelopmental age in which marked differences in development can be identified.

6. CONCLUSION AND RECOMMENDATIONS

We found elevated odds of membership to a neurobehavioral profile that exhibited more extreme characteristics associated with prenatal exposure to cadmium and mercury, nevertheless the relationship was not statistically significant. Further research on how to provide a better estimation of prenatal exposure to cadmium is needed, as well as research on the use of other matrices such as fetal cord blood to indicate the concentrations of mercury at parturition. We recommend exploring other methods of neurobehavioral assessment aiming to detect subtle differences in infants that might be exposed to low concentrations of metals. The study of other birth cohorts with more diverse demographic characteristics is recommended, in order to make the results more generalizable and confirm the predictive values of the neurobehavioral assessment in diverse populations.

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