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Prenatal exposure to mixtures of heavy metals and neurodevelopment in infants at 6 months

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ABSTRACT

Background: Exposure to mixture of neurotoxic metals such as lead, mercury and cadmium occurs at a specific point of time. When exposed to metal mixtures, one metal may act as an agonist or antagonist to another metal. Thus, it is important to study the effects of exposure to a combination of metals on children's development using advance statistical methods.

Objectives: In this study, we explored the effects of prenatal metal exposure including lead, mercury and cadmium in early pregnancy (12–20 weeks), late pregnancy (> 28 weeks), and at birth on neurodevelopment of infants at 6 months of age.

Methods: We included 523 eligible mother-child pairs from the mothers and children environmental health (MOCEH) study, a prospective birth cohort study in Korea. We used linear regression, Bayesian kernel machine regression (BKMR) and generalized additive models (GAM), to evaluate the effects of exposure to metal mixtures on neurodevelopment of infants aged 6 months. The Korean version of Bayley scale of infant and toddler development-II was used to measure the child's neurodevelopment.

Results: Linear regression models showed a significant negative effect of lead exposure during late pregnancy on the mental development index (MDI) [β = -2.51 (−4.92, −0.10)] scores of infants aged 6 months following co-exposure to mercury. Further, linear regression analysis showed a significant interaction between late pregnancy lead and mercury concentrations. BKMR analysis showed similar results as those obtained in linear regression models. These results were also replicated in the GAM. Stratification analysis showed that greater than 50 percentile concentration of mercury in late pregnancy potentiated the adverse effects of lead in late pregnancy on MDI [β = −4.33 (−7.66, −1.00)] and psychomotor development index (PDI) [β = −5.30 (−9.13, −1.46)] at 6 months of age. Prenatal cadmium exposure did not show a significant association with MDI and PDI at 6 months in the linear regression or BKMR analysis.

Conclusion: Based on all the statistical methods used, we demonstrated the effect of combined exposure to metals on the neurodevelopment of infants aged 6 months, with significant interaction between lead and mercury.

1. Introduction

Metals often co-exist, so humans are exposed to a combination of metals at a given point in time. The toxic effects of individual metals may depend on their interactions with another metal (Claus Henn et al., 2014). Evidence suggest toxic effects at very low concentrations of metals (Kortenkamp et al., 2007). Thus, exposure to mixtures of metals can lead to greater than additive health effects in children (Claus Henn

Abbreviations: MDI, Mental development index; PDI, psychomotor development index; BKMR, Bayesian kernel machine regression; GAM, generalized additive models; MOCEH, mothers and children environmental health; (K-BSID-II, Korean version of Bayley Scales of Infant Development II; PIPs, posterior inclusion probabilities; NMDAR, N- methyl-D-aspartate receptor

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et al., 2014). Prenatal exposure to heavy metals affects early stages of fetal and infant brain development. Placenta presents a weak barrier for heavy metals like lead, cadmium and mercury, which easily cross the placental barrier through passive diffusion (Caserta et al., 2013). Prenatal exposure to heavy metals can cause impaired cognitive development even at very low concentrations (Al-Saleh et al., 2011). The developmental origin of adult disease (DOAD) hypothesis suggests that early life environment may be associated with the development of disease in later life (Mathew and Ayyar, 2012). Lead, mercury and cadmium target the developing central nervous system. Prenatal exposure to heavy metals affects neurodevelopment in early childhood. These heavy metals can affect different functional domains like lead and mercury affects auditory, visual, motor and memory deficits and externalizing behavior, while cadmium affects visuomotor and externalizing behavior (Sanders et al., 2015; Riccio et al., 2010; Viaene et al., 2000). Also, there is a lack of sufficient data on prenatal exposures to metal mixtures at different time points and neurodevelopment in children. Hence, it is essential to determine the cognitive developmental effects on children resulting from exposure to metals during early pregnancy (12–20 weeks), late pregnancy (> 28 weeks) and at birth (cord blood). The individual effects of exposure to heavy metals on neurodevelopment of children are well-studied. However, there has been a knowledge gap on the potential toxic effects of metal mixtures on children's neurodevelopment.

The effects of co-exposure to metals were studied via traditional approaches such as multivariate linear regression, which is limited by multicollinearity, interaction, model specification and non-linear effects. Thus, it has been encouraged to apply various statistical approaches in the analysis of multiple metal mixtures (Taylor et al., 2016). In this study, we evaluated the effect of prenatal exposure to metal mixtures (lead, cadmium and mercury) on neurocognitive development of infants aged 6 months in a prospective birth cohort using three different statistical methods: linear regression, Bayesian kernel machine regression (BKMR) and generalized additive models (GAM).

2. Materials and methods

2.1. Study population

The study population comprises the subjects investigated in the mothers and children's environmental health (MOCEH) study, which is a prospective birth cohort study designed to explore the effects of prenatal and postnatal exposure to environmental pollutants on children's health and disease. The details of the study were described previously (Kim et al., 2009). Our study analyzed 523 mother-children pairs during early pregnancy, 467 mother-children pairs during late pregnancy, and 321 at the time of delivery. The selection of study subjects is outlined in Fig. 1. Informed consent was obtained from mothers and also on behalf of their children. The study protocol was approved by the Institutional Review Board of Ewha Womans University Hospital, Dankook University Hospital and Ulsan University Hospital.

2.2. Lead, mercury and cadmium exposure

We collected venous blood samples from pregnant women at early pregnancy (gestational age, 12–20 weeks), late pregnancy (gestational age, > 28 weeks) and from the umbilical cord (at birth). The samples were frozen and stored at −70 °C. The samples were brought to room temperature and vortexed after thawing. In total, 0.1 mL of blood was diluted in 1.8 mL of matrix modifier reagent composed of Triton X-100 and ammonium hydrogen phosphate dibasic for lead (Pb) analysis and Triton X-100 for the analysis of cadmium (Cd). The samples were vortexed and assayed using an atomic absorption spectrometer-graphite furnace (Analyst 900-Zeeman collection, PerkinElmer, Singapore) (Kim et al., 2013). The mercury (Hg) levels were measured in the blood sample initially dried in an oxygen stream passed through a quartz tube located inside a controlled heating coil. The combustion gases were decomposed further on a catalytic column at 750 °C. The Hg vapor was collected using a gold amalgamation trap and desorbed for quantification. The Hg content was determined via atomic absorption spectrometry (DMA-80; Milestone, Bergamo, Italy (Kim et al., 2017). Commercial reference materials were used for internal quality assurance and control (Lyphochek® Whole Blood Metals Control; Bio-Rad, Hercules, CA, USA). The precision and accuracy of the metal measurements were verified via regular monitoring using an external quality control program (inter-laboratory calibration exercises). The limits of detection (LOD) were 0.151 μg/dL for lead, 0.07 μg/L for total mercury and 0.098 μg/L for cadmium. No sample contained levels below the LOD for all the 3 metals.

2.3. Neurodevelopmental outcomes

Neurodevelopment at 6 months of age was measured using the Korean version of Bayley Scales of Infant Development II (KBSID-II) (Bayley, 1993; Park and Cho, 2006). The KBSID-II showed excellent test-retest stability (Park and Cho, 2006). It is used to evaluate problem solving, memory, classification, vocalization, and language skills. The resulting score is known as the mental development index (MDI). The psychomotor development index (PDI) is scored according to the degree of muscle coordination, postural control, and finer skills of manipulation. Each test is standardized to produce a mean score of 100 and a standard deviation of 15. Here, the score compares the neurodevelopment of a typical Korean infant aged 6 months. Inter-rater reliability (kappa value > 0.8) was confirmed annually via rater training sessions and video monitoring of the examination. Each measurement was double-checked and confirmed via feedback between the examiner and the central coordinator.

2.4. Covariates

Demographic information related to participant's age, education, income and socio-economic status was collected using a structured questionnaire during prenatal visits. Gestational age was calculated using information derived from the last menstrual period. When the last menstrual period was unreliable, or involved a significant date variation (> 10 days) between ultra-sonographic estimates, the gestational age based on the first ultrasonography was used. Information related to infant's gender, birth weight, gestational age and birth order was collected using medical records at the time of delivery. Urinary cotinine in the pregnant women was measured during their prenatal visits (early and late pregnancy) to determine second-hand smoke exposure.

2.5. Statistical methods

Demographic characteristics of the study participants were reported using means and standard deviation and counts with percentages. Blood levels of heavy metals (Pb, Hg and Cd) were natural log-transformed. Urinary cotinine concentrations were natural log-transformed due to non-normal distribution and further adjusted for urinary creatinine. Correlations between heavy metals was explored using Spearman correlation co-efficient (Supplementary Table 1). The following co-variates were included in the analysis: mother's age, income, gender of the child, parity, birth weight and urinary cotinine concentration.

Each prenatal heavy metal with early and late pregnancy maternal blood, and cord blood in association with the MDI and PDI for 6 months was evaluated using multivariate regression analysis. The regression analysis included a single heavy metal model initially, followed by a model with two heavy metals and another model comprising all the three heavy metals. We further analyzed multiple interactions between the two and three heavy metals affecting neurodevelopment at 6 months using a linear regression method.

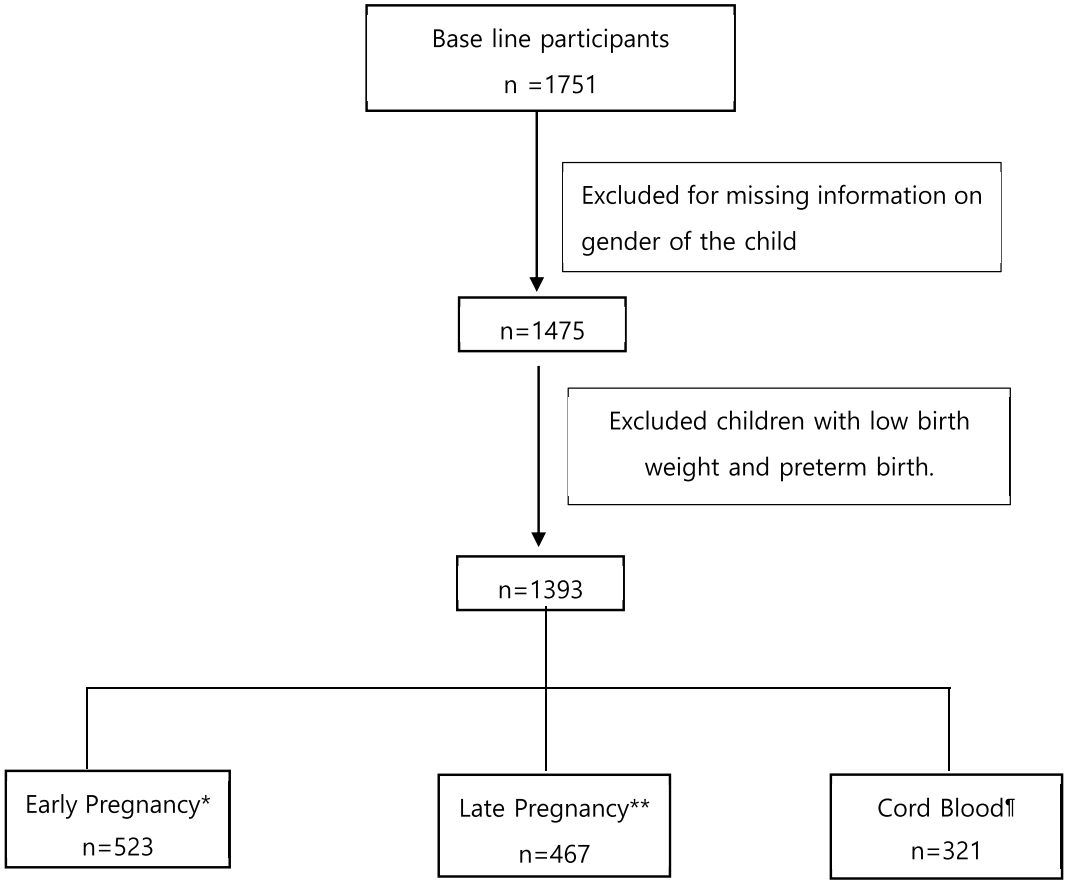

Fig. 1. Selection process of study participants, *study subjects selected having values for early pregnancy heavy metals (Pb, Hg and Cd), MDI/PDI scores at 6 months and selected co-variates (mother's age, income, gender of the child, parity, birth weight, cotinine concentration)** study subjects selected having values for late pregnancy heavy metals (Pb, Hg and Cd), MDI/PDI scores at 6 months and selected co-variates (mother's age, income, gender of the child, parity, birth weight, cotinine concentration) study subjects selected having values for cord blood heavy metals (Pb, Hg and Cd), MDI/PDI scores at 6 months and selected co-variates (mother's age, income, gender of the child, parity, birth weight, mother's late **pregnancy** cotinine concentration).

Further, a BKMR analysis was used to investigate the effects of exposure to metal mixtures based on a kernel function (Bobb et al., 2014). BKMR facilitates the study of multivariable exposure response function for non-linearity and interactions between exposures and outcome. This method also enables the study of effects to combined metal exposure (Valeri et al., 2017). A key feature of BKMR is that the exposure-response function is modeled flexibly and does not require a priori specification. The BKMR model is indicated by the following equation:

$$Yi = h\{Pbi,Hgi,Cdi\} + \beta^{q}Zi + ei \quad (1)$$

The function h {} in Equation (1) is an exposure–response function that accommodates nonlinearity and/or interaction between the different metal components (Pb, Hg and Cd) in the mixture, and $Z = Z_1, Z_2 \ldots \ldots Z_q$ for q potential confounders. The Gaussian kernel function was used, which is applicable to simulation studies and real-life scenarios (Valeri et al., 2017; Chiu et al., 2018). Models were run up to 20,000 iterations. The component-wise variable selection method that was used provides estimates of posterior inclusion probabilities (PIPs), which underscore the importance of each variable (Bobb et al., 2018). PIPs were investigated to determine the variable of "importance" using the PIP threshold of 0.5 (Barbieri and Berger, 2004; Coker et al., 2018). BKMR facilitates visual presentation of single-exposure health effects, and their interaction. First, the cumulative effect of exposure to the combination of three metals on the neurodevelopment at age 6 months was evaluated. Then, the effect of single exposure to a single heavy metal exposure was calculated when other heavy metals were fixed to their median value. Lastly, we studied the joint effect of 2 metals by plotting a dose-response relationship of single metal at different quantiles of another metal, based on the median value of the third metal.

A multivariable GAM was used to assess the reproducibility of results obtained from BKMR analysis and also to test the interaction between the metal mixtures. The GAM analysis is used to validate the assumptions of BKMR analysis in low-dimension settings. To determine the interaction, we used a tensor product smoother (ti) function. The GAM model included a term to measure the joint effect of lead and mercury [i.e., *ti(*Pbi,Hgi,*)*].

All statistical procedures were performed using R (version 3.5.0). BKMR was implemented with the R package bkmr and GAM was performed using R package mgcv. Statistical significance was considered at p-value < 0.05.

3. Results

3.1. Study population

Characteristics of the study subjects including mother-infant pairs are shown in Table 1. The mean age of mothers was 30 years. More than 50% of the participants had a total family income greater than 2 million Korean Won per month. Almost 50% of the pregnant women had education higher than high school. The prenatal cotinine exposure was 16.5 and 12.1 ng/mL during early and late pregnancy, respectively. Almost half of the newborns were girls. The average birth weight of the newborns was 3.3 kg. The mean values of MDI and PDI scores at 6 months were 96.8 and 96.9, respectively.

Table 1
Descriptive characteristics of study participants.

	n (%)	Mean ± SD
Mother's		
Age (years)	523	30.4 ± 3.4
Education		
< =High School	225 (46.8)	
> High School	255 (53.2)	
Income (10,000 KRW/month) ↑	–	
< 200	151 (28.9)	
200–400	278 (53.1)	
> 400	94 (18)	
Early pregnancy Cotinine Exposure (ng/ml)¶	523	16.5 ± 4.9
Late pregnancy Cotinine Exposure (ng/ml)¶	467	12.1 ± 4.2
Children's	–	
Gender	–	
Boy	283 (54.1)	
Girl	240 (45.9)	
Parity		
0	256 (48.9)	
1	224 (42.8)	
≥2	73 (8.3)	
Birth weight (kg)	523	3.3 ± 0.3
Gestational age (weeks)	523	39.0 ± 1.0
Bayley Score at 6 months		
MDI[a]	523	96.8 ± 10.9
PDI[b]	523	96.9 ± 14.1

↑1 USD = 1136 KRW as of 2019.03.30.
¶ , Geometric mean and geometric standard deviation.
Numbers do not always sum up to the same total due to missing values.
[a] MDI-mental development Index.
[b] PDI-psychomotor development index.

3.2. Prenatal metal concentration

The geometric means of lead concentration in early pregnancy, late pregnancy as well as cord blood were 1.33, 1.27 and 0.92 μg/dL, respectively. The cord blood mercury concentration was 5.21 μg/L higher than in early and late pregnancy. Prenatal cadmium exposure levels were 1.40, 1.52 and 0.68 μg/L for early pregnancy, late pregnancy, and cord blood, respectively (Table 2).

3.3. Multivariable linear regression analyses

Table 3 presents the effect of metal mixtures (Pb, Hg and Cd) on the neurodevelopment of infants at 6 months using multivariable linear regression models. In the model using one heavy metal at a time, exposure to lead during late pregnancy had a significantly negative effect on the MDI and PDI scores at 6 months in model 1 and 2, respectively. However, in late pregnancy, mercury and cadmium had no significant effect on MDI and PDI scores in models 3 to 6. Using two heavy metals at a time, the regression model showed that lead concentrations in late pregnancy marginally affected MDI and PDI scores at 6 months in models 7 and 8, respectively, in the presence of mercury exposure during late pregnancy. However, significant interaction was seen between lead and mercury in models 7 and 8. Models 9 and 10 showed that lead exposure in late pregnancy significantly negatively affected MDI and PDI scores in the presence of cadmium exposure. But, no significant interaction was seen in models 9 and 10. Models 11 and 12 did not show significant effects of exposure to late pregnancy mercury and cadmium on MDI and PDI at 6 months. In model using all three metals at a time, exposure to lead in late pregnancy significantly affected MDI scores at 6 months in model 13. While, exposure to lead in late pregnancy marginally affected PDI at 6 months in model 14. Early pregnancy and cord blood exposure to lead, mercury and cadmium metal mixtures did not significantly affect the MDI and PDI scores at 6 months.

3.4. BKMR analyses

We used the BKMR method to further explore the effect of metal mixtures considering the limitations associated with linearity and interactions in the regression analysis. First, we explored the cumulative effect of metal mixtures on MDI/PDI, with concurrent changes in the components of all mixtures based on their median value. Second, the dose–response relationship of each component in the mixture and the potential interactions among the metals were determined by estimating the predicted change in MDI/PDI and setting the other metals at the median, 25th, or 75th percentiles. We explored the cumulative effect of the metal mixtures on the change in MDI and PDI scores at 6 months (Fig. 2A and B, Supplementary Figs. 1 and 3). Figure two displays the change in MDI and PDI scores with a simultaneous change following exposure to the three metals from a particular threshold, i.e., 25th to 75th percentile, when compared with each of the three metals at their median value, i.e., 50th percentile. We did not find significant changes in the cognitive development score of infants aged 6 months while estimating the cumulative effect of combined exposure to all three metals at different thresholds. Further, we estimated the univariate (single metal) exposure-response functions of prenatal exposure to lead, mercury and cadmium on MDI and PDI at 6 months (Fig. 3A and B, Supplementary Figs. 2 and 4). We found a linear relationship between exposure to lead, mercury, and cadmium in late pregnancy and MDI/PDI at 6 months. We further investigated and plotted bi-variate exposure response function of lead, mercury, and cadmium in late pregnancy and MDI and PDI (Fig. 4A and B). Fig. 4A and B (right 2nd panel) suggest the possible interaction between late pregnancy levels of lead and mercury, as the negative slope of lead was steeper and overlapped at higher levels of mercury. In the contrast, the negative slope of lead is not steeper (in the right 1st panel) and is parallel at different concentrations of cadmium.

To test the presence of interaction between late pregnancy lead and mercury affecting MDI and PDI at 6 months as seen in the BKMR analysis, the GAM was used. The GAM showed evidence of interaction between late pregnancy lead and mercury affecting MDI and PDI at 6 months ($p < 0.10$). Thus, GAM analyses corroborated the results obtained with the BKMR models of interaction between late pregnancy lead and mercury affecting MDI and PDI at 6 months of age.

Table 2
Distribution of prenatal heavy metals exposure.

	Early Pregnancy (n = 523)			Late Pregnancy (n = 467)			Cord blood (n = 321)		
	Lead (μg/dl)	Mercury (μg/l)	Cadmium (μg/l)	Lead (μg/dl)	Mercury (μg/l)	Cadmium (μg/l)	Lead (μg/dl)	Mercury (μg/l)	Cadmium (μg/l)
GM ± GSD	1.33 ± 1.51	3.30 ± 1.61	1.40 ± 1.41	1.27 ± 1.53	3.13 ± 1.73	1.52 ± 1.30	0.92 ± 1.59	5.21 ± 1.63	0.68 ± 1.31
10th percentile	0.78	1.90	1.00	0.76	1.73	1.13	0.53	2.96	0.49
25th percentile	1.01	2.44	1.17	1.00	2.25	1.30	0.71	3.97	0.57
Median	1.34	3.34	1.39	1.27	3.13	1.52	0.94	5.33	0.68
75th percentile	1.73	4.44	1.68	1.68	4.26	1.76	1.23	7.13	0.80
90th percentile	2.08	5.92	1.99	2.13	5.93	2.15	1.59	9.22	0.92

GM ± GSD: geometric mean and geometric standard deviation.

Table 3
Results from linear regression models of prenatal metal mixtures exposure on neurodevelopment of infants at 6 months.

			Early Pregnancy (n = 523)	Late Pregnancy (n = 467)	Cord blood (n = 321)
One heavy metal at a time	Model 1: Lead	MDI	0.18 (−1.94, 2.30)	**−1.91 (-3.64,-0.18)***	−0.16 (-2.76,2.42)
	Model 2: Lead	PDI	0.15 (−2.56,2.86)	**−2.40 (-4.59,-0.21)***	−1.04 (−4.37,2.27)
	Model 3: Mercury	MDI	0.11 (−1.79,2.02)	−0.13 (−0.61,0.34)	0.79 (−1.68,3.26)
	Model 4: Mercury	PDI	0.11 (−2.31,2.53)	−0.27 (−4.11,5.46)	−1.14 (−4.32,2.02)
	Model 5: Cadmium	MDI	1.51 (−1.40,4.43)	1.64 (−0.82,4.41)	−0.55 (−4.55,3.44)
	Model 6: Cadmium	PDI	−0.77 (-4.52,2.97)	0.36 (−2.76,3.50)	−1.02 (−6.15,4.11)
Two heavy metals at a time	Model 7: Lead	MDI	0.18 (−1.95,2.32)	**−2.21 (-4.61,0.18)#**[a]	−0.39 (−3.08,2.28)
	Mercury	MDI	0.01 (−1.91,1.91)	0.26 (−1.57,2.13)	0.79 (−1.76, 3.34)
	Model 8: Lead	PDI	0.13 (−2.59,2.87)	**−2.75(-5.79,0.28)#**[a]	−0.75 (−4.20,2.68)
	Mercury	PDI	0.15 (−2.29,2.60)	−0.23 (−2.56,2.10)	−1.07 (−4.35,2.20)
	Model 9: Lead	MDI	−0.03 (−2.34,2.39)	−2.52 (−4.85,-0.19)	−0.19 (−2.79, 2.40)
	Cadmium	MDI	1.51 (−1.44,4.46)	3.35 (−0.19,6.90)	−0.57 (−4.59,3.43)
	Model 10: Lead	PDI	0.02 (−2.93,2.98)	−3.05 (-6.00,-0.11)	−1.10 (−4.44, 2.23)
	Cadmium	PDI	−0.79 (−4.54,3.00)	2.14 (−2.33,6.63)	−1.14 (−6.30,4.00)
	Model 11: Mercury	MDI	−0.88 (−2.88,1.11)	0.01 (−1.83,1.84)	0.65 (−1.85, 3.16)
	Cadmium	MDI	1.49 (−1.43,4.41)	2.98 (−0.83,6.81)	−0.45 (−4.56, 3.65)
	Model 12: Mercury	PDI	−0.33 (−2.90,2.22)	−0.48 (−5.25,5.50)	−1.39 (−4.61, 1.82)
	Cadmium	PDI	−0.78 (−4.53,2.97)	1.55 (−1.30,8.62)	−1.57 (−6.84, 3.70)
All three heavy metals at a time	Model 13: Lead,	MDI	0.05 (-2.26,2.37)	**−2.51 (-4.92,-0.10)***	−0.40 (−3.09,2.28)
	Mercury	MDI	−0.89 (−2.89,1.11)	0.18 (−1.65,2.02)	0.74 (−1.83,3.33)
	Cadmium	MDI	1.47 (−1.48,4.44)	3.59 (−0.26,7.44)	−0.47 (−4.59,3.64)
	Model 14: Lead	PDI	0.05 (−2.92,3.03)	**−2.93 (-6.00,0.13)#**	−0.79 (−4.24,2.65)
	Mercury	PDI	−0.34 (−2.92,2.23)	−0.26 (−2.61, 2.07)	−1.22 (−4.53, 2.09)
	Cadmium	PDI	−0.79 (−4.60,3.01)	2.25 (−2.65, 7.16)	−1.61 (−6.90, 3.66)

MDI; mental development index, PDI; psychomotor development index.
Linear regression model adjusted for mother's age, income, child's gender, birth weight, parity and prenatal cotinine exposure.
*p-value < 0.05, #p-value < 0.10.
[a] Significant multiplicative interaction between lead and mercury.

3.5. Stratification analyses

Further, stratification analysis by median concentrations of mercury in late pregnancy was performed. Lead exposure in late pregnancy significantly affected MDI and PDI at age 6 months in the group of mothers exposed to greater than median concentration of mercury in late pregnancy, shown in Table 4.

4. Discussion

In this study we found that joint exposure to lead and mercury during late pregnancy affected MDI and PDI scores at 6 months in infants. We also found potential evidence of interaction between lead and mercury levels in late pregnancy affecting MDI and PDI at 6 months. Further, the stratified analysis showed that mercury in late pregnancy resulted to be a potentiator to the toxic effects of late pregnancy lead on infant's neurodevelopment at 6 months of age. Higher concentrations of mercury in late pregnancy significantly increased the harmful effects of lead in late pregnancy on MDI and PDI at 6 months. No significant results of lead exposure were observed during early pregnancy and in cord blood on the neurodevelopment of infants at 6 months. Also, we did not find any significant effects on neurodevelopment in infants aged 6 months following prenatal exposure to mercury and cadmium.

In this study, three different statistical approaches were used to analyze the effect of prenatal metal mixtures: lead, mercury and cadmium on the neurodevelopment of infants at 6 months. The linear regression approach included models with a single metal at a time, two metals at a time, and all the three metals adjusted together. Linear regression analysis using single metal at a time showed that lead exposure in late pregnancy affected MDI and PDI at 6 months significantly. Using two metals (Pb and Hg) at a time, the linear regression model revealed a marginal effect of late pregnancy lead on MDI and PDI and a significant interaction between lead and mercury. When the model was mutually adjusted for all the three metals together, lead in late pregnancy significantly reduced MDI scores at 6 months. These results are comparable to previous studies evaluating the effect of heavy metal exposure on neurodevelopment in children (Bellinger, 2013; Braun et al., 2012).

Children and fetuses are highly susceptible to heavy metal exposure even at lower levels (Bellinger et al., 2017). The geometric mean level of blood lead in our study was less than the recent CDC reference value of 5 μg/dL (Centers for Disease Control and Prevention, 2012). Our results show, lead exposure during late pregnancy affected neurodevelopment in infants. Heavy metals such as lead, mercury and cadmium cross the placental tissues and accumulate in fetal tissue. Lead stores in bone are mobilized during pregnancy. Lead easily crosses the placental barrier by passive diffusion. Mercury is transferred through the placenta via passive transport and amino acid carriers whereas the placental transfer of cadmium is low, as protein metallothionein in placenta partly restricts the transfer of cadmium leading to its accumulation in the placental tissue (Caserta et al., 2013; Kippler et al., 2010). The distance of maternal-fetal diffusion in early pregnancy is about 20–38 μm. While, the maternal-fetal diffusion distance is reduced up to 4 μm during late pregnancy (Gundacker and Hengstschläger., 2012). Further, the toxicokinetic studies have reported vigorous placental transport of lead during the last trimester (Liu et al., 2014).

Blood concentration of heavy metals: lead, mercury and cadmium measured in our study reflect recent exposure (weeks/months). The half-life of blood lead is 35 days (Hu et al., 2006), blood mercury is 48–53 days (Boerleider et al., 2017) and blood cadmium is 3 months (Järup and Åkesson, 2009). So, it can be said that heavy metals in blood affect neurodevelopment concurrently. Further, we conducted sensitivity analysis to explore the effect of cumulative exposure of early and late pregnancy heavy metals on MDI and PDI of infants at 6 months. After controlling for early pregnancy heavy metals exposure, we found that late pregnancy lead exposure significantly affected MDI in infants at 6 months (data not shown). Therefore, our study demonstrated late pregnancy as a critical window of lead exposure significantly influencing neurodevelopment in infants.

The fetal uptake of lead depends on the total external exposure. The total external exposure to fetus depends on various factors like mother's exposure to contaminated air and food. Other factors that can affect

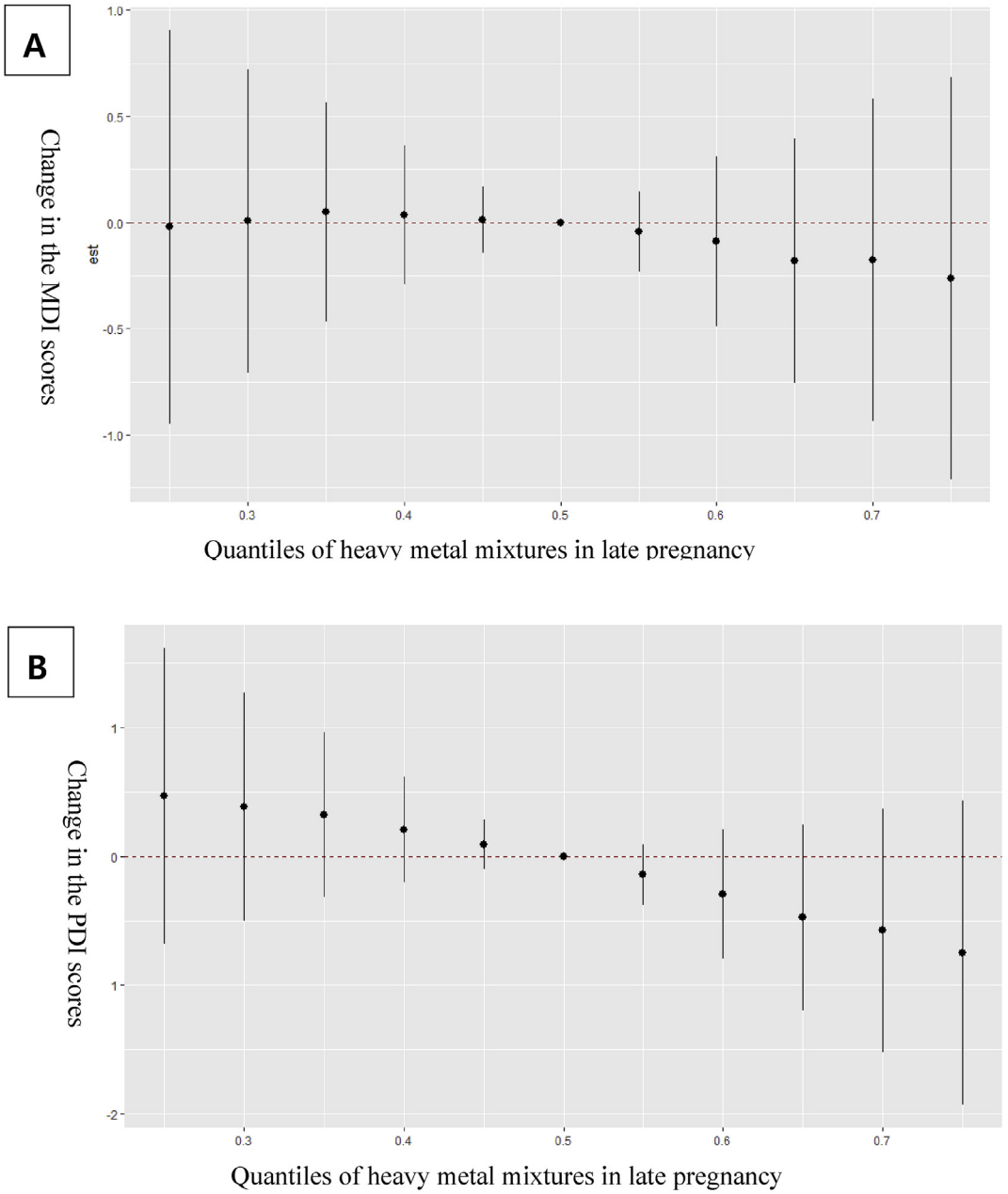

Fig. 2. Cumulative effect of late pregnancy metal mixture on neurodevelopment of infants at 6 months, Cumulative effect of the mixture of 3 heavy metals (lead, cadmium and mercury) exposure in late pregnancy (estimates and 95% credible intervals) by Bayesian kernel machine regression. This plot compares the estimated change in neurodevelopment score when all the 3 heavy metal exposures are at a particular quantile (x-axis) compared to when all 3 metal exposure are at 50th percentile. A: MDI B: PDI at 6 months.

total lead exposure are calcium, iron and nutrition status of the pregnant women (Torres-Sánchez et al., 1999). Thus, we further explored the cumulative effect of early and late pregnancy lead exposure, mother's calcium, iron and total calorie intake, as exposure factors affecting cord blood lead concentration. The cumulative exposure analysis (data not shown) showed that early and late pregnancy lead had a significant positive impact on the cord blood concentration. Significant positive correlation between early and late pregnancy and cord blood lead concentration were observed. But, the insignificant results of cord blood lead concentration on cognitive development of infants could be due to very low level of cord blood lead in our study participants (Reddy et al., 2014).

We next applied the BKMR analysis, a novel approach to test the joint and individual effect of prenatal heavy metals exposure on infant's neurodevelopment. BKMR analysis also revealed late pregnancy lead as the most important contributor to MDI and PDI at 6 months of age. Linear and negative association was seen between late pregnancy lead and MDI and PDI at 6 months. Significant interactions between late pregnancy lead and mercury affected MDI and PDI at 6 months. Lead toxicity in late pregnancy affected MDI and PDI at 6 months of age substantially and had more than additive effect. The results obtained via linear regression and BKMR analysis were reproducible. The BKMR approach not only facilitates the evaluation of cumulative effects of combined metal exposure but also the effect of individual heavy metals on neurodevelopment at 6 months of age at different exposure levels of other metals. Additionally, BKMR provides analyses of non-linearity and interaction between different elements, for example, combined exposure to lead and mercury. Further, GAM analysis verified the results obtained via linear regression and BKMR analysis showed a linear inverse association between late pregnancy lead and MDI at 6 months.

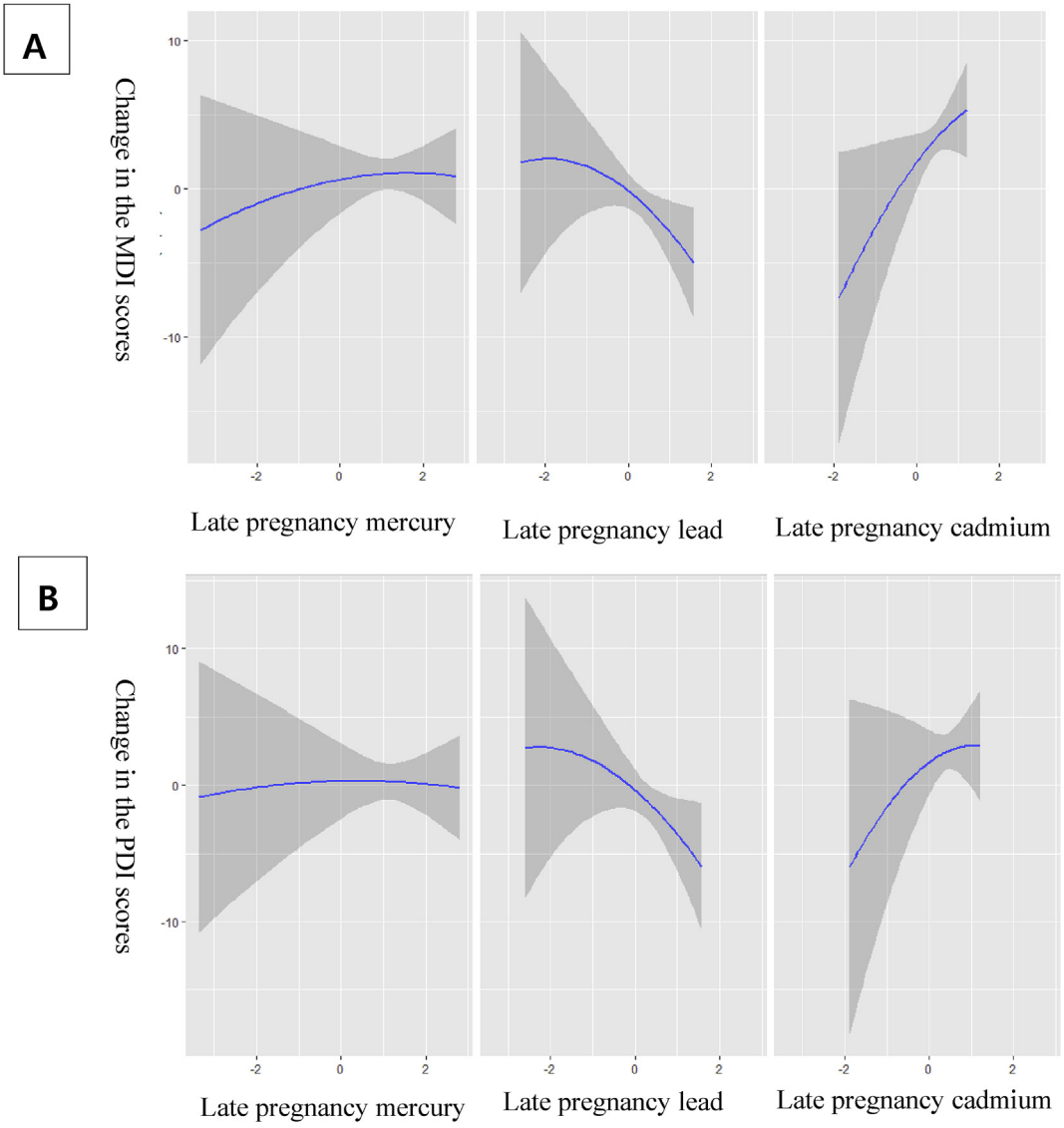

Fig. 3. Effect of exposure to single heavy metal in late pregnancy on cognitive development of infants at 6 months, Univariate exposure-response functions and 95% confidence bands for association between single heavy metal exposure in late pregnancy and MDI and PDI at 6 months when other heavy metals exposure in late pregnancy are fixed at the median. These results were estimated by Bayesian kernel machine regression. Models adjusted for mother's age, income, child's gender, birthweight and parity and prenatal cotinine exposure.

Evidence of interaction between late pregnancy lead and mercury was observed affecting MDI and PDI at 6 months by GAM analysis.

Our study shows synergistic interaction between lead and mercury. Linear regression stratified according to mercury concentrations in late pregnancy shows significant impact of lead exposure in late pregnancy on MDI and PDI at 6 months only in the presence of mercury at higher than median concentrations. Thus, mercury can be a “potentiator” of lead. Lead toxicity on cognitive and psychomotor development of infants aged 6 months may be increased by higher concentrations of mercury. A study by Boucher et al. showed that lead exposure effects in children intensified with higher concentrations of mercury or polychlorinated biphenyls exposure (Boucher et al., 2011). However, another study by Yorifiji et al. found less than additive effects of prenatal lead and mercury on children's neurodevelopment (Yorifuji et al., 2011). Very few studies have explored the effects of mixtures of metals on children's neurological development, suggesting a significant knowledge gap.

Lead affects myelination and differentiation of nerve cells. Lead interferes with neurotransmitter release and affects neuro transmission by altering the synaptic activity of neurons (Lidsky & Schneider, 2003). Lead also disrupts with the organization of human cortex and intraneural regulatory mechanism (Webb et al., 2001; Chen et al., 2012). Mercury also impedes nerve cell proliferation and migration, which are required for normal neural development (Solan and Lindow, 2014). Mercury also affects cortical organization in fetal brain (Choi et al., 1978). As, neuronal migration and myelination of the neural tracts begun by 25 weeks in the developing fetal brain (Schnaas et al., 2006; Herschkowitz, 1988). While the deep cortical layer gets developing clearly from 28 weeks (Larsen and Schneider, 2001). Thus, exposure to lead and mercury in late pregnancy affects neurodevelopment in infants. While, cadmium affects neurodevelopment causing oxidative stress in astrocytes (Wang and Du, 2013). Cadmium interferes with the calcium and zinc dependent process and induces apoptosis (Méndez-Armenta and Ríos, 2007). Cadmium affects the balance of synaptic transmission and alters the growth of developing neuron by amending cell signaling/gene expression systems (Gulisano et al., 2009).

Various mechanisms of lead and methyl mercury mediating neurotoxicity have been reported (Wigle, 2003). However, one possible mechanism may involve dysregulation of neurochemicals, especially, glutamate and its N-methyl-D-aspartate receptor (NMDAR), which are

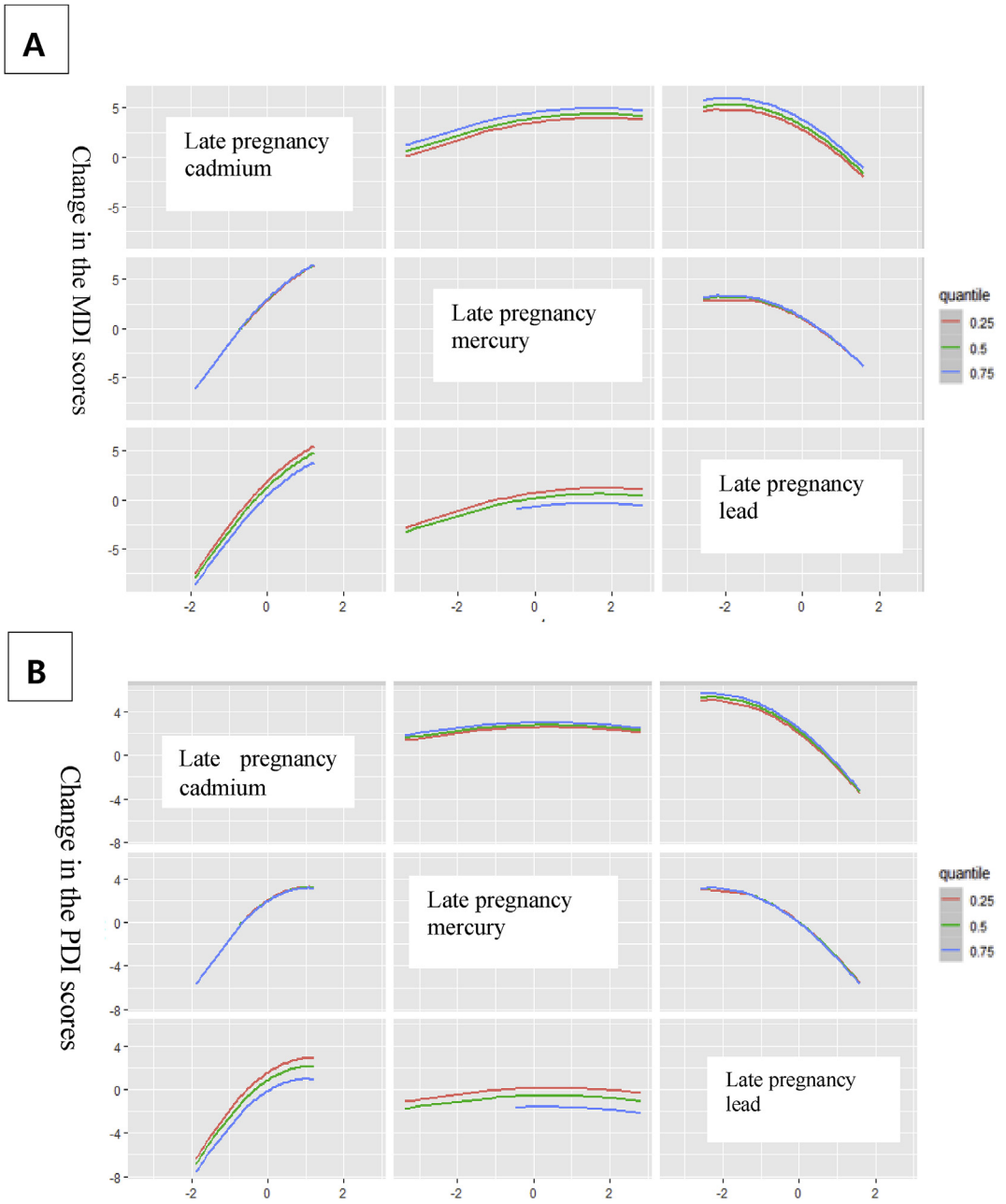

Fig. 4. Bi-variate exposure response functions of metal mixtures in late pregnancy and cognitive development of infants at 6 months, Bi-variate response function for late pregnancy lead when late pregnancy cadmium is fixed at 25, 50, 75 percentiles and mercury is fixed at their median value (right 1st panel). Bi-variate response function for late pregnancy lead when late pregnancy mercury is fixed at 25, 50, 75 percentiles and cadmium is fixed at their median value (right 2nd panel). (A) = MDI scores at 6 months and (B) = PDI scores at 6 months. Results were estimated by Bayesian kernel machine regression. Models adjusted for mother's age, income, child's gender, birth weight parity and prenatal cotinine exposure.

of particular interest (Fitsanakis and Aschner, 2005). Studies have reported that lead and methylmercury inhibit glutamate uptake by astrocytes from synapse (Struzyńska, 2000), which may cause cell death. Furthermore, lead and methyl mercury may also alter NMDAR expression (Basu et al., 2007; Toscano and Guilarte, 2005). NMDAR is related to important neurodevelopmental function, in terms of learning and memory (Toscano and Guilarte, 2005), suggesting that its alteration may result in lead- and methyl mercury-induced cognitive deficits. Therefore, interaction between lead and mercury may have synergistic effects on neurodevelopment in six-month-old infants.

4.1. Limitations

In this study, due to few measurements of cotinine in the neonatal urine samples at the time of birth, we used late pregnancy cotinine measurement in maternal urine for the linear regression analysis to evaluate the effect of cord blood heavy metal exposures on neurodevelopment of infants. We did not find a significant association between cord blood lead exposure and cognitive development in infants at 6 months. In our study, late pregnancy lead was highly correlated ($r = 0.54$, p-value < 0.05) to cord blood lead. Strong correlation suggests that lead transfers from mother to fetus through placenta. But, the extreme low level of cord blood lead in our study participants may be the reason why we attained insignificant results for cord blood lead

Table 4
Effect of late pregnancy exposure to metal mixture (lead and cadmium) on cognitive development of 6 month old infants stratified by mercury concentration.

		Mercury exposure (n = 233) < median value	Mercury exposure (n = 234) > =median value
MDI	Lead	−0.89 (−4.48,2.68)	−4.33 (−7.66,-1.00)*
	Cadmium	4.13 (−1.76,10.09)	2.78 (−2.41,7.99)
PDI	Lead	−0.39 (−5.49,4.70)	−5.30 (−9.13,-1.46)*
	Cadmium	3.57 (−4.86,12.01)	0.97 (−5.01,6.97)

Each model included lead and cadmium exposure adjusted for mother's age, income, child's gender, birth weight, parity and prenatal cotinine exposure.
*p < 0.05.

exposure (Reddy et al., 2014). We may not be able to accurately access the effect of early and late pregnancy heavy metals exposure on neurodevelopment of infants. As, a biological marker, blood metal concentration can reflect, a mixture of both external and internal exposure with almost no ability to distinguish between either (Hu et al., 2006). But, sensitivity analysis showed that late pregnancy lead concentration significantly affected cognitive development in infants at 6 months after adjusting for early pregnancy heavy metal exposure. Measurements of postnatal heavy metal exposure at 6 months of age were not performed in the present study, which may have biased our results.

4.2. Strengths

In this study we identified late pregnancy as a susceptible window of exposure to prenatal metal (lead) and metal mixtures (lead and mercury) affecting neurodevelopment in infants at 6 months of age. We have used a new statistical method BKMR, which facilitated the exploration and visualization of joint effects and the interaction between different metals during the exposure. BKMR enables evaluation of the potential non-linearity and synergistic effects of prenatal exposure to metal mixtures on cognitive growth of infants at 6 months of age (Valeri et al., 2017). Using the BKMR method, we have overcome the limitations of traditional approaches in studying the effects of exposure to different metal combinations and their interactions.

5. Conclusion

Combined exposure to lead and mercury during late pregnancy affects neurodevelopment in infants at 6 months. Exposure to lead in late pregnancy was found to result in most neuro-toxic effects. BKMR is a valuable tool to explore exposures to large number of metals or chemical mixtures. Even though the lead concentrations were below the reference value, our results emphasize the need for strategies to prevent exposure to metals individually and in combination during pregnancy. Thus, additional studies are needed to confirm the health effects of metal mixtures in low exposed population of pregnant women and children.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2020.109122.

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Role of the funder

The funders had no role in the conduct of the study, collection and analysis of the data.

The authors declare they have no actual or potential competing financial interest.

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