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Prospective Associations of Early Pregnancy Metal Mixtures with Mitochondria DNA Copy Number and Telomere Length in Maternal and Cord Blood

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Background: Metal exposure during pregnancy influences maternal and child health. Oxidative stress and inflammation may mediate adverse effects of heavy metals, whereas essential metals may act as antioxidants. Mitochondrial DNA is a prime target for metal-induced oxidative damage. Telomere dysfunction is attributed to imbalances between reactive oxidant species and antioxidants.

Objectives: We evaluated individual and joint associations of prenatal metals with mitochondrial DNA copy number (mtDNAcn) and telomere length (TL) in maternal and cord blood as biomarkers of inflammation and oxidative stress.

Methods: We measured six nonessential metals (arsenic, barium, cadmium, cesium, lead, mercury) and four essential metals (magnesium, manganese, selenium, zinc) in first-trimester maternal red blood cells in Project Viva, a U.S. prebirth cohort. We measured relative mtDNAcn ($n = 898$) and TL ($n = 893$) in second-trimester maternal blood and mtDNAcn ($n = 419$) and TL ($n = 408$) in cord blood. We used multivariable linear regression and quantile g-computation to estimate associations between prenatal metals and the biomarkers. We used generalized additive models and Bayesian kernel machine regression to examine nonlinearity and interactions.

Results: A 2-fold increase in maternal magnesium was associated with lower maternal [$\beta = -0.07$, 95% confidence interval (CI): -0.10, -0.01] and cord blood ($\beta = -0.08$, 95% CI: -0.20, -0.01) mtDNAcn. Lead was associated with higher maternal mtDNAcn ($\beta = 0.04$, 95% CI: 0.01, 0.06). Selenium was associated with longer cord blood TL ($\beta = 0.30$, 95% CI: 0.01 0.50). An association was observed between the nonessential metal mixture and higher maternal mtDNAcn ($\beta = 0.04$, 95% CI: 0.01, 0.07). There was a nonlinear relationship between cord blood mtDNAcn and magnesium; maternal mtDNAcn and barium, lead, and mercury; and maternal TL and barium.

Discussion: Maternal exposure to metals such as lead, magnesium, and selenium was associated with mtDNAcn and TL in maternal second trimester and cord blood. Future work will evaluate whether these biomarkers are associated with child health. https://doi.org/10.1289/EHP9294

Introduction

Prenatal exposure to environmental chemicals is associated with adverse maternal and child health outcomes (as reviewed by Cohen Hubal et al. 2020; Varshavsky et al. 2020). The Developmental Origins of Health and Disease hypothesis postulates that environmental factors encountered *in utero* influence disease susceptibility and outcomes in adulthood (as reviewed by Wadhwa et al. 2009). Pregnancy is a period of heightened susceptibility to environmental exposures, because pregnancy comprises a series of tightly coordinated hormone-mediated events that alter maternal physiology in response to the developing fetus (as reviewed by Varshavsky et al. 2020).

Exposure to metals during pregnancy is ubiquitous (Callan et al. 2013). Certain metals, including the metalloid arsenic, can cross the placental barrier and accumulate in fetal tissue (as reviewed by Bommarito et al. 2017; Needham et al. 2011). For example, mercury is found in higher concentrations in cord blood, in comparison with maternal blood (Legler et al. 2015). Cadmium accumulates in the placenta and alters its biological pathways and function (as reviewed by Barr et al. 2007). Heavy metals such as arsenic, cadmium, lead, and mercury exert toxicity by generating reactive oxygen species (ROS), depleting antioxidants and bonding to sulfhydryl groups (as reviewed by Wu et al. 2016), leading to inflammation, oxidative stress, and DNA damage (Tchounwou et al. 2012). Essential metals at therapeutic levels play an important role in biochemical and physiological processes (Tchounwou et al. 2012), and they could mitigate the adverse effect of toxic metals by acting as antioxidants (as reviewed by Jan et al. 2015). For example, zinc is a cofactor for antioxidant enzymes, and a dietary supplement that included zinc alleviated oxidative stress from cadmium and lead exposure (as reviewed by Zhai et al. 2015).

Mitochondrial DNA copy number (mtDNAcn), a measure of mitochondrial genome abundance, is used as a marker of the mitochondria's dysfunction and response to oxidative stress (as reviewed by Castellani et al. 2020; Malik and Czajka 2013). Mitochondrial DNA is a prime target for metal-induced oxidative damage, because metals accumulate inside mitochondria via calcium transporters. Further, mitochondria are the main site of oxidative phosphorylation and the dominant cellular site for ROS

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generation (as reviewed by Meyer et al. 2017). Mitochondrial DNA lack histones and DNA repair capabilities present in nuclear DNA (Yakes and Van Houten 1997), making them more vulnerable to oxidative stress. Most somatic cells contain $10^2 - 10^4$ copies of mitochondrial DNA, although this varies by cell type and developmental state (as reviewed by Castellani et al. 2020). In a recent study, higher whole blood mtDNAcn was associated with prospective measures of greater DNAm-PhenoAge, an epigenetic aging biomarker of phenotypic age, and shorter leukocyte telomere length (TL), suggesting that it could be a biomarker of aging-related disease and mortality (Dolcini et al. 2020). After an acute chemical exposure and oxidative stress, mtDNAcn increases as a compensatory mechanism in response to decreased energy production. Under conditions of reduced antioxidant capacity, such as those resulting from chronic environmental exposure, oxidative stress may overwhelm the mitochondria and decrease mtDNAcn (Kupsco et al. 2019). However, there is limited research on the impact of blood metal concentration and mtDNAcn.

Telomeres are DNA-protein structures at the end of chromosomes that preserve chromosome stability and integrity (Blackburn 1991) and are a marker of cellular health and aging (Shammas 2011). Telomere dysfunction, induced by chronic inflammation, is attributed to an imbalance between the production of ROS and cellular antioxidant defenses (as reviewed by Welendorf et al. 2019). There is also evidence that telomere shortening is in part mediated by mitochondrial dysfunction and generates additional oxidative stress and inflammation (Passos et al. 2007). Initial TL and attrition during early life are primary predictors of TL across the life course (Cowell et al. 2020). Prior studies have found associations between late-pregnancy maternal metal mixture exposure and TL at birth (Cowell et al. 2020; Herlin et al. 2019), but there is limited evidence on the impact of early pregnancy metal mixture exposure.

The aim of our study was to test individual and joint associations between maternal first-trimester essential and nonessential metals and second-trimester maternal and cord blood mtDNAcn and TL. We hypothesized that prenatal metal exposure influences mtDNAcn and TL in both mothers and children with consistent associations in the two generations.

Methods

Study Population and Design

Between 1999 and 2002, pregnant persons were recruited to Project Viva, a prospective prebirth cohort study of prenatal exposures and child health, during their first prenatal visit at Atrius Harvard Vanguard Medical Associates, a multispecialty group practice in eastern Massachusetts (Oken et al. 2015). Eligibility criteria included fluency in English, gestational age less than 22 wk, singleton pregnancy, and plans to remain in the study area throughout gestation. Children were seen at in-person visits from birth to adolescence.

Of 2,128 mother–infant pairs, 28 mothers were enrolled more than once due to multiple pregnancies. We used data from first enrollment for this analysis ($n = 2{,}100$). We measured first trimester metals in maternal red blood cells (RBCs) among 1,407 mothers. Mercury was not measured in 17 of these participants. Of the 1,390 (65%) mother–infant pairs with all metal measurements, 893 out of 905 participants with samples available (Figure S1) and 898 out of 903 participants with samples available (Figure S2) were included in the maternal second trimester TL and mtDNAcn analyses, respectively. Of the mother–infant pairs with first-trimester metal measurements, 408 out of 428 participants with samples available (Figure S1) and 419 out of 425 participants with samples available (Figure S2) were included in the cord blood TL and mtDNAcn analyses, respectively.

Participants provided written informed consent at recruitment. The institutional review board of Harvard Pilgrim Health Care reviewed and approved all study protocols.

Maternal First-Trimester Metal Concentrations in Red Blood Cells

Upon recruitment (median: 10-wk gestation), a blood sample was obtained from every participating mother. The blood sample was centrifuged at 2,000 rpm for 10 min at 4°C to separate RBCs from plasma. All aliquots were stored at −70°C prior to metal analyses.

We measured concentrations of all metals, except for mercury, using inductively coupled plasma–mass spectrometry (ICP-MS). Briefly, 0.5 mL of stored packed RBCs was weighed and digested in 2 mL of ultrapure concentrated HNO_3 for 48 h and then further digested with 1 mL of 30% ultrapure hydrogen peroxide for 48 h, following dilution to 10 mL with deionized water. A triple quadrupole ICP-MS was used to analyze elements on a single run (Agilent 8800 triple quadrupole ICP-MS (ICP-QQQ; Agilent Technologies, Inc.) in tandem mass spectrometry (MS/MS) mode using appropriate cell gases and internal standards. Mercury concentrations were analyzed separately from RBCs using a Direct Mercury Analyzer 80 (Milestone, Inc.). Quality control (QC) measures included analysis of the initial and continuous calibration verification, procedural blanks, and repeated analysis of 2% of samples. Matrix-appropriate certified reference materials were analyzed once per study. Seronorm-Blood L3 was analyzed once daily as a QC sample to monitor accuracy. One sample each of the blind QC pools at high and low levels were run per batch, with 52 total duplicate samples included.

Recoveries of QC standards were 90%–110% for all elements included in this analysis. Intraday coefficients of variation (CVs) were calculated using in-house QC pools at three different concentration levels before and after every 10 samples ($n = 7$). Intraday CVs were less than 5% for analytes included in the analysis, except for selenium (<10%). Interday CV was less than 15%, except for concentrations near the limit of detection (LOD) (Table S1).

We report results as metal concentrations in the RBC sample in nanograms per gram. All metals selected in this study had an intraclass correlation above 0.70 for duplicates and detection frequencies of 90% or above (Table S1). This approach resulted in the inclusion of five nonessential metals (barium, cadmium, cesium, lead, mercury), one nonessential metalloid (arsenic), and four essential metals (magnesium, manganese, selenium, zinc) in our analyses. For ease, we refer to all elements, including metal (loid)s, as metals when presenting results. Metal concentrations below the LOD were assigned the value of LOD/(square root of 2). Before statistical modeling, all metal concentrations were $\log_2$-transformed] to account for right-skewness. In addition, we examined correlation among metals to guide statistical modeling.

Relative Leukocyte TL

Relative leukocyte TL was assessed in maternal blood collected during the second trimester and in cord blood collected at birth. Venous umbilical cord blood collection was conducted in the delivery unit at the time of delivery. Whole blood was collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA), refrigerated for <24 h, and centrifuged at 2,000 rpm at 4°C for 10 min to separate plasma, nucleated cells [leukocytes and nucleated RBCs (nRBCs) in cord blood and leukocytes in maternal blood], and RBCs. Samples were aliquoted for storage

in liquid nitrogen. The TL assay was optimized based on previously described methods (Cawthon 2009; Pavanello et al. 2011). This assay uses duplex quantitative PCR (qPCR) to compare the relative amplification of TL repeat copy number to single gene (albumin) copy number. Genomic DNA was extracted from nucleated cells with commercially available Puregene Kits (Fisher, Catalog Nos. A407-4, A416-4; Qiagen, Catalog Nos. 158908, 158912, 158924). DNA samples were normalized to 2 ng/μL, and concentrations were confirmed using PicoGreen® quantification prior to amplification. The genomic primers are shown in Table S2. Additionally, iQ™ SYBR® Green Supermix, which contains an antibody-mediated hot-start iTaq™ DNA polymerase, was used, as well as a passive reference dye fluorescein. Samples were amplified according to the protocol shown in Table S3. Each sample was run in triplicate, and each plate contained a standard curve as a reference. The standard curve was made from a project-specific pooled sample comprising equal mass of total DNA from all maternal and cord blood samples analyzed. Two internal QC samples were run across all plates.

The TL/single copy gene ratio (T/S ratio) was calculated as the ratio of TL copy number relative to albumin copy number, both of which were estimated by the Bio-Rad software (Bio-Rad Laboratories) using the study-specific standard curve [$Cq = slope \times Log_{10}(Sq) + intercept$]. The ratio was calculated by dividing the starting quantity of telomere copy number by the starting quantity of albumin copy number. The T/S ratio was averaged across triplicates, and mean values were used for analyses.

We calculated the variance of each triplicate. Based on the spread of samples, a variance of 0.05 was set as the cutoff. If a sample variance was greater than 0.05, the sample was reanalyzed, and the T/S ratio based on the second run was recorded. Of the 1,402 analyzed samples for this assay (not limited to those included in the present analysis, which were included only if the corresponding participant also had metals measurements), 157 samples had variances over the cutoff and were reanalyzed. Of the samples that were rerun, 129 samples had variances within the expected range after the second run and were included in further analyses. Samples with variances above 0.05 after multiple reruns were excluded from the analyses. Samples contaminated during processing from plating malfunction were also excluded (Figure S1). TL is interpreted as the sample's TL relative to the population average of maternal and cord blood values in this study. Average T/S is proportional to the average TL per cell. Samples with a T/S greater than 1.0 have an average TL greater than that of the study average, whereas samples with a T/S less than 1.0 have an average TL shorter than that of the study average.

Relative mtDNAcn

mtDNAcn was assessed in blood collected from participants during the second trimester and in cord blood collected at birth. Venous umbilical cord blood collection was conducted in the delivery unit at the time of delivery. Whole blood was collected in vacutainer tubes containing EDTA, refrigerated for <24 h, and centrifuged at 2,000 rpm at 4°C for 10 min to separate plasma, nucleated cells, and RBCs. Samples were aliquoted for storage in liquid nitrogen. The mtDNAcn assay was optimized based on a previously described method (Andreu et al. 2009) that uses qPCR to compare the relative amplification of nuclear and mitochondrial segments of DNA. Genomic DNA was extracted from nucleated cells with commercially available Puregene Kits (Fisher, Catalog Nos. A407-4, A416-4; Qiagen, Catalog Nos.158908, 158912, 158924). DNA samples were normalized to 2 ng/μL, and concentrations were confirmed using PicoGreen® quantification prior to amplification. The genomic primers for the mitochondrial assay are shown in Table S4. For the nuclear assay, Life Technologies RNaseP Copy Number Reference Assay (PN 4316849), which contains two primers and a VIC® dye-labeled TAMRA™ probe to detect the genomic DNA reference sequence, was used. Samples were amplified according to the qPCR protocol in Table S5. Each sample was run in triplicate, and each plate contained a standard curve as a reference. The standard curve was made from a tissue-specific pooled sample comprising equal mass of total DNA from all maternal and cord blood samples analyzed in this study. Two internal QC samples were run across plates.

The starting quantity (nanograms) of mitochondrial and nuclear DNA for each sample was calculated based on the tissue-specific standard curve. The mtDNAcn was calculated by dividing the starting quantity of mtDNA by the starting quantity of nuclear DNA. Triplicates were averaged to determine mean mtDNAcn. If the CV for triplicate samples was above 0.15 and there was a clear outlier or a well that did not amplify, the outlier data point was removed, and the mean mtDNAcn was recalculated from the remaining duplicate samples. Samples that did not amplify or have sufficient DNA were excluded from analysis (Figure S2). The mtDNAcn is interpreted as the sample's mtDNAcn relative to the population average for both maternal and cord blood.

Covariate Assessment

Maternal demographics were obtained at baseline after each participant's initial clinical visit through a brief interview and questionnaire. To select confounders for adjustment in models, we used directed acyclic graphs (DAGs) (Figure S3) based on prior knowledge about covariates that may be related to prenatal metals exposure and/or maternal and cord blood mtDNAcn and TL (Breton et al. 2019; Clemente et al. 2019; Li et al. 2019; Liu et al. 2019; Marchetto et al. 2016; Pollack et al. 2018; Song et al. 2019; Valdes et al. 2005; Zhang et al. 2019). We included maternal race/ethnicity in the study design as a covariate, because it is a predictor of both metals exposure (Bulka et al. 2019) and TL (Lynch et al. 2016). Although there are limited studies characterizing whether race/ethnicity is a predictor of mtDNAcn, we observed differences in mtDNAcn by race/ethnicity in our study cohort. We think race/ethnicity could be a predictor of metals exposure and the biomarkers due to shared differences in unmeasured variables, such as experiences of racism and marginalization or diet and lifestyle. Confounders were consistent for all models and included maternal age at pregnancy (continuous), prepregnancy body mass index (BMI) in kilograms per square meter (continuous), education (less than college or college graduate), annual household income ($70,000 per year or less, or greater than $70,000 per year), self-identified race/ethnicity (White, Black, Asian, Hispanic, or more than one race), smoking status (never, former, or during pregnancy), and parity (nulliparous or greater than one). Child sex was also included in the cord blood TL and mtDNAcn models. All models were adjusted by sample plate to account for technical variation in the mtDNAcn (Figure S4) and TL (Figure S5) assays. Cell type and maternal diet were included in sensitivity analyses due to missing data. Delivery method and gestational age at birth were considered and not included in the models because they may be on the causal pathway.

Statistical Analyses: Multivariable Analyses for Individual Metals

We described our study sample using counts and percentages and examined TL and mtDNAcn across demographic characteristics using medians and interquartile ranges (IQRs). We used

unadjusted and adjusted linear regression models to test associations between individual maternal first-trimester metal concentrations and maternal and cord blood TL and mtDNAcn. We report estimates and 95% confidence intervals (95% CI) for difference in TL or mtDNAcn associated with a 2-fold increase in metal concentration, conditional on covariates.

Metal Mixtures

All models were adjusted for the same covariates as those used in the multivariable linear regression models for individual metals. In addition, the mixture models were adjusted for any metal not included in the mixture of interest. We estimated the overall associations of the metal mixtures on each outcome using quantile g-computation (Keil et al. 2020), a parametric, generalized linear model-based implementation of g-computation to estimate the association in each outcome for a one-quantile simultaneous increase in all of the exposures in a specified mixture, which is referred to as the mixture exposure–response. We obtained the variance with a nonparametric bootstrap ($B = 1{,}000$). Given a mixture association, we identified the most toxic or protective metals that contribute to the overall joint association. Unlike weighted quantile sum regression (WQS) (Carrico et al. 2015), quantile g-computation does not require directional homogeneity (Keil et al. 2020). We tested for nonlinearity using a square term for all metals or specific metals. We chose the linear models because they had the lowest Bayesian information criterion (BIC). The main models were implemented by categorizing all metals in quartiles and fitting a linear model. The quartile values are treated as continuous. The quantile g-computation estimator of the exposure–response is the sum of the regression coefficients across the included exposures. If all the regression coefficients are in the same direction, then the weight for a specific metal is the proportion of the effect due to that component, and all components sum to one. If metals have different directions of effect, then the weights are interpreted as the proportion of the positive (or negative) partial effect, and the positive and negative weights together sum to 2.0 (Niehoff et al. 2020). We examined associations between the overall metal mixture and biomarkers by incorporating all $\log_2$-transformed metal concentrations in the mixture (arsenic, barium, cadmium, cesium, lead, magnesium, manganese, mercury, selenium, zinc). We also conducted two additional mixture analyses based on nonessential (arsenic, barium, cadmium, cesium, lead, mercury) and essential (magnesium, manganese, selenium, zinc) metal classifications. We report estimates and 95% bootstrap CIs for difference in maternal and cord blood TL and mtDNAcn for 1-quartile increase in metal mixtures, conditional on covariates.

For all the exposure–outcome relationships, we ran sensitivity analyses with additional consideration for diet, which we *a priori* identified as a potential confounder. We did this by considering Mediterranean diet score (MDS) and Western diet score derived from a self-reported food frequency questionnaire, which was given during the first trimester. These dietary variables were previously associated with some metal concentrations in the first trimester (Lin et al. 2021) and could be an indicator for healthy or unhealthy dietary patterns. MDS (9-point) was calculated based on consumption of food groups associated with a traditional Mediterranean diet (Trichopoulou et al. 2009), including fish, dairy products, fruits, legumes, nuts, vegetables, whole grains products, red and processed meat, and unsaturated to saturated fat ratio. Alcohol consumption was not included, because it is not recommended for pregnant persons and because few pregnant persons drank alcohol in the study cohort (Lin et al. 2021). MDS was chosen as a proxy for healthy dietary pattern. In addition, we previously found that higher MDS was associated with arsenic, cadmium, mercury, and selenium (Lin et al. 2021), and many of these food items considered in the MDS could be related to the outcomes in this study. Sample size was reduced in these analyses due to 5%–7% missingness for dietary information ($n = 398$ for cord blood mtDNAcn, $n = 838$ for maternal mtDNAcn, $n = 386$ for cord blood TL, and $n = 832$ for maternal TL).

We also conducted sensitivity analyses for the cord blood exposure–outcome relationships, adjusting for the same covariates as the main models, as well as cell type. To estimate cell-type distribution in blood samples, a reference panel of nucleated cells were isolated from cord blood (Bakulski et al. 2016), which included CD8+T cells, CD4+T cells, natural killer (NK) cells, monocytes, B cells, granulocytes, and nRBCs (Wu et al. 2017). Sample size was reduced in these analyses due to 18%–21% missingness for cell type ($n = 342$ for cord blood mtDNAcn and $n = 333$ for cord blood TL).

Additionally, we graphically checked for nonlinearities and interactions among metals using Bayesian kernel machine regression (BKMR) with component-wise variable selection (Bobb et al. 2015, 2018). Because metals were low to moderately correlated, we chose component-wise variable selection, which treats each metal as exchangeable when entering the model. To complement visual inspection of nonlinearity among metals in BKMR, we used generalized additive models (GAMs), modeling all metal–outcome relationships as splines with four knots at the quartiles of each metal. To complement visual inspection of interaction among metals in BKMR, we used multivariable linear regression models with a multiplicative term. We tested interactions by including a cross-product term for just the metals that looked like they may be interacting based on BKMR results.

Based on results from the GAMs and BKMR, we conducted two additional sensitivity analyses for each overall metal–outcome relationship, in which we *a*) added a square term for the overall mixture and *b*) added a square term for metals in which we observed a nonlinear relationship in the GAMs. We used BIC for model selection. All statistical analyses were conducted in R (version 3.6.1; R Development Core Team).

Results

Population Characteristics

Table 1 describes the general characteristics of each subset of the study population used for the analysis of each outcome. Demographic characteristics were similar across subpopulations. The majority of mothers were White (74%), had at least a college education (72%), never smoked (68%), had normal prepregnancy BMI (18.5–25 $\mathrm{kg/m^2}$) (59%), lived in a household with an income greater than \$70,000 (62%), and were between 30 and 35 y old at enrollment in early pregnancy (50%). Table S6 describes the distribution of prenatal metals. Median RBC concentrations of all essential metals were within reference ranges, and median RBC concentrations of cadmium, lead, and mercury were similar to those in whole blood from a 2017–2018 subset of female National Health and Nutrition Examination Survey (NHANES) participants (16–45 y, $n = 1{,}308$; CDC 2020) (Table S7). As shown in Table 1, median relative maternal TL (0.64) was shorter than median relative cord blood TL (1.06), whereas median relative maternal mtDNAcn (0.99) was higher than median relative cord blood mtDNAcn (0.97).

The distribution of relative maternal and cord blood mtDNAcn and TL by participant characteristics is presented in Table 2. Median maternal TL was similar across age quartiles. Maternal and cord blood TL was higher in those with higher income and a college education. Median cord blood and maternal mtDNAcn differed by maternal race/ethnicity. Maternal and cord blood

Table 1. Sample characteristics of mother–child pairs from the Project Viva cohort included in each biomarker analysis.

Characteristic	Telomere length (TL)		Mitochondrial DNA copy number (mtDNAcn)	
	Maternal [*n* (%)]	Cord blood [*n* (%)]	Maternal [*n* (%)]	Cord blood [*n* (%)]
Overall[a]	893 (100)[b]	408 (100)[b]	898 (100)[c]	419 (100)[c]
Maternal age (y)				
Quartile 1 (16.9–<29.9)	226 (25.3)	102 (25.0)	228 (25.4)	105 (25.1)
Quartile 2 (29.9–<32.6)	223 (25.0)	102 (25.0)	221 (24.6)	107 (25.5)
Quartile 3 (32.6–<35.9)	221 (24.7)	103 (25.2)	226 (25.2)	103 (24.6)
Quartile 4 (35.9–<45.0)	223 (25.0)	101 (24.8)	223 (24.8)	104 (24.8)
Maternal prepregnancy body mass index				
Underweight (<18.5)	25 (2.8)	11 (2.7)	24 (2.7)	13 (3.1)
Normal (18.5–<25.0)	531 (59.5)	242 (59.3)	538 (59.9)	248 (59.2)
Overweight (25.0–<30.0)	198 (22.2)	98 (24.0)	197 (21.9)	100 (23.9)
Obese (≥30.0)	139 (15.6)	57 (14.0)	139 (15.5)	58 (13.8)
Maternal race/ethnicity				
Asian	33 (3.7)	14 (3.4)	33 (3.7)	14 (3.3)
Black	99 (11.1)	42 (10.3)	99 (11.0)	44 (10.5)
Hispanic	64 (7.2)	28 (6.9)	63 (7.0)	31 (7.4)
Mixed race	37 (4.1)	16 (3.9)	38 (4.2)	18 (4.3)
White	660 (73.9)	308 (75.5)	665 (74.1)	312 (74.5)
Maternal education				
Less than college	246 (27.5)	115 (28.2)	248 (27.6)	119 (28.4)
College graduate	647 (72.5)	293 (71.8)	650 (72.4)	300 (71.6)
Household income				
<$70,000 per year	338 (37.8)	161 (39.5)	340 (37.9)	167 (39.9)
≥$70,000 per year	555 (62.2)	247 (60.5)	558 (62.1)	252 (60.1)
Smoking				
Never	607 (68.0)	280 (68.6)	609 (67.8)	288 (68.7)
Former	183 (20.5)	85 (20.8)	185 (20.6)	86 (20.5)
During pregnancy	103 (11.5)	43 (10.5)	104 (11.6)	45 (10.7)
Parity				
Nulliparous	444 (49.7)	203 (49.8)	449 (50.0)	211 (50.4)
One or more	449 (50.3)	205 (50.2)	449 (50.0)	208 (49.6)
Infant sex				
Male	—	213 (52.2)	—	218 (52.0)
Female	—	195 (47.8)	—	201 (48.0)
Biomarker median (IQR)	0.64 (0.18)[b]	1.06 (0.48)[b]	0.99 (0.32)[c]	0.97 (0.29)[c]

Note: —, no data available; IQR, interquartile range.
[a]Data was complete for all participants.
[b]TL interpreted as the sample's TL relative to the population average of maternal and cord blood values in this study.
[c]mtDNAcn interpreted as the sample's mtDNAcn relative to the population average for both maternal and cord blood for this study.

mtDNAcn was lower in those with higher income and a college education. After regressing the plate effects from each of the biomarkers and examining correlations among residuals, cord blood TL and cord blood mtDNAcn were negatively correlated ($r = -0.13$; $p = 0.01$), whereas cord blood mtDNAcn and maternal mtDNAcn were positively correlated ($r = 0.15$; $p = 0.004$). Correlations among the other biomarkers were not statistically significant (Figure 1). Cell-type composition was moderately correlated with cord blood mtDNAcn and mildly correlated with cord blood TL (Figure S6). Correlations among CD4+T cells and B cells, natural killer cells and CD4+T cells, cord blood mtDNAcn and CD8+T cells and nRBCs, as well as cord blood TL and B cells, CD4+T cells, CD8+T cells, natural killer cells, and nRBCs were not statistically significant (Figure S6).

Metal Correlations

Figure 1 shows the Spearman correlation coefficients for metals included in the analyses. Metals with positive correlations are blue, whereas those with negative correlations are red. Metals showed low to moderate intercorrelations. Arsenic and mercury had the highest correlation ($r = 0.53$; $p < 0.001$), followed by magnesium and selenium ($r = 0.43$; $p < 0.001$), magnesium and zinc ($r = 0.37$; $p < 0.001$), and selenium and zinc ($r = 0.37$; $p < 0.001$). Barium and zinc had the highest negative correlation ($r = -0.23$; $p < 0.001$). Barium was negatively correlated with all other metals. The correlations between mercury and cadmium, magnesium, manganese, and zinc were not statistically significant (Figure 1). Arsenic was weakly correlated with cord blood B cells ($r = 0.12$; $p = 0.03$), zinc was mildly correlated with CD4+T cells ($r = 0.11$; $p = 0.03$ magnesium and zinc were mildly negatively correlated with monocytes ($r = -0.12$; $p = 0.02$). Correlations among the other metals and cell types were not statistically significant (Figure S6).

Individual Metal Analyses

Table S8 shows multivariable linear regression results for associations between $\log_2$-transformed first-trimester RBC concentrations of each metal individually and relative maternal and cord blood TL and mtDNAcn. Figure 2 shows the corresponding Forest plots of estimates and 95% CIs. A 2-fold increase in maternal magnesium was associated with lower maternal ($\beta = -0.07$, 95% CI: −0.10, −0.01) and cord blood ($\beta = -0.08$, 95% CI: −0.20, −0.01) mtDNAcn. In contrast, lead was associated with higher maternal mtDNAcn ($\beta = 0.04$, 95% CI: 0.01, 0.06). Selenium was associated with longer cord blood TL ($\beta = 0.30$, 95% CI: 0.01, 0.50).

A 2-fold increase in maternal manganese was marginally associated with lower cord blood mtDNAcn ($\beta = -0.03$, 95% CI: −0.06, 0.004). A 2-fold increase in maternal barium was marginally associated with lower cord blood mtDNAcn ($\beta = -0.01$, 95% CI: −0.03, 0.001). A 2-fold increase in arsenic ($\beta = 0.04$, 95% CI: −0.01, 0.09) and zinc ($\beta = 0.20$, 95% CI: −0.05, 0.50) was marginally associated with longer cord blood TL. A 2-fold increase in maternal mercury was marginally associated with

Table 2. Sample characteristics of mother–child pairs in the Project Viva cohort and median (IQR of relative maternal and cord blood TL and mtDNAcn) by participant characteristics.

	Telomere length (TL)		Mitochondrial DNA copy number (mtDNAcn)	
Characteristic	Maternal median (IQR)[a]	Cord blood median (IQR)[a]	Maternal median (IQR)[b]	Cord blood median (IQR)[b]
Overall	0.64 (0.18)	1.06 (0.48)	0.99 (0.32)	0.97 (0.29)
Maternal age (y)				
Quartile 1 (16.9–< 29.9)	0.65 (0.18)	1.03 (0.43)	0.98 (0.34)	1.01 (0.24)
Quartile 2 (29.9–< 32.6)	0.65 (0.19)	1.11 (0.66)	0.99 (0.31)	0.98 (0.33)
Quartile 3 (32.6–< 35.9)	0.63 (0.15)	1.04 (0.56)	0.97 (0.34)	0.95 (0.25)
Quartile 4 (35.9–< 45.0)	0.64 (0.17)	1.03 (0.37)	1.00 (0.30)	0.98 (0.24)
Maternal body mass index				
Underweight (<18.5)	0.65 (0.13)	1.07 (0.48)	0.99 (0.33)	0.97 (0.29)
Normal (18.5–< 25.0)	0.65 (0.18)	1.00 (0.40)	1.02 (0.33)	0.95 (0.31)
Overweight (25.0–< 30.0)	0.63 (0.19)	1.09 (0.51)	0.97 (0.30)	0.98 (0.29)
Obese (≥30.0)	0.64 (0.15)	1.04 (0.29)	0.87 (0.23)	1.12 (0.27)
Maternal race				
Asian	0.67 (0.15)	1.20 (0.41)	0.95 (0.17)	1.02 (0.20)
Black	0.63 (0.19)	1.06 (0.54)	1.11 (0.38)	1.07 (0.30)
Hispanic	0.63 (0.13)	1.09 (0.42)	1.04 (0.42)	0.98 (0.38)
Mixed race	0.64 (0.18)	1.10 (0.46)	1.06 (0.39)	1.11 (0.31)
White	0.64 (0.18)	1.04 (0.49)	0.97 (0.30)	0.96 (0.28)
Maternal education				
Less than college	0.63 (0.18)	1.05 (0.45)	1.01 (0.34)	1.00 (0.31)
College graduate	0.65 (0.18)	1.06 (0.51)	0.98 (0.32)	0.96 (0.28)
Household income				
<$70,000 per year	0.64 (0.19)	1.04 (0.47)	1.00 (0.34)	1.00 (0.29)
≥$70,000 per year	0.65 (0.17)	1.08 (0.48)	0.98 (0.32)	0.96 (0.28)
Smoking				
Never	0.64 (0.18)	1.04 (0.42)	0.98 (0.33)	0.98 (0.28)
Former	0.65 (0.17)	1.11 (0.60)	1.00 (0.33)	0.97 (0.27)
During pregnancy	0.62 (0.22)	1.07 (0.52)	1.00 (0.29)	0.97 (0.31)
Parity				
Nulliparous	0.65 (0.19)	1.07 (0.49)	0.99 (0.31)	0.96 (0.30)
One or more	0.63 (0.16)	1.04 (0.45)	0.98 (0.33)	0.99 (0.27)
Infant sex				
Male	—	1.07 (0.44)	—	0.97 (0.30)
Female	—	1.04 (0.58)	—	0.98 (0.28)

Note: —, no data available; IQR, interquartile range.
[a]TL is interpreted as the sample's TL relative to the population average of maternal and cord blood values in this study.
[b]MtDNAcn number is interpreted as the sample's mtDNAcn to the population average for both maternal and cord blood for this study.

shorter maternal TL ($\beta = -0.008$, 95% CI: −0.02, 0.003), whereas a 2-fold increase in maternal zinc was marginally associated with longer maternal TL ($\beta = 0.05$, 95% CI: −0.01, 0.10). However, none of these marginal associations excluded the null with 95% confidence. No associations were observed between cadmium or cesium and the outcomes. Adjustment for cell type in cord blood in our models did not alter associations between the prenatal metals and cord blood mtDNAcn or TL (Table S9). Adjustment for Mediterranean diet did not alter associations between prenatal metals and cord blood and maternal mtDNAcn or TL, except for the association between prenatal magnesium and maternal second trimester mtDNAcn was no longer statistically significant ($\beta = -0.04$, 95% CI: −0.10, 0.02; Table S10). However, this difference was due to wider CIs from reduced sample size and not the diet adjustment, because there was still no association when removing diet from the model but keeping the reduced sample size ($n = 398$).

Quantile g-Computation

In the quantile g-computation mixtures approach (Table 3), a null association was observed between the overall metal mixture and each of the outcomes. An association was observed between the nonessential metal mixture and higher maternal mtDNAcn ($\beta = 0.04$, 95% CI: 0.01, 0.07). A marginal association was observed between the essential metal mixture and lower maternal mtDNAcn ($\beta = -0.02$, 95% CI: −0.04, 0.003). A marginal association was observed between the nonessential metal mixture and shorter maternal TL ($\beta = -0.03$, 95% CI: −0.06, 0.005). Weights are presented in Figure S7, and the regression coefficients used to determine the weights are shown in Table S11. After adjustment for cell type, the overall prenatal metal mixture was associated with lower cord blood mtDNAcn ($\beta = -0.04$, 95% CI: −0.07, −0.002) (Table S12). After adjustment for MDS, the prenatal metal mixture was marginally associated with higher maternal mtDNAcn ($\beta = 0.03$, 95% CI: −0.005, 0.07; Table S13).

Metal Mixture Analyses: Interaction and Nonlinear Relationships

Mixture results from BKMR models showed trends similar to those of linear regression and quantile g-computation models (Figures S8–11). Potential interactions were visualized using BKMR and tested in multivariable linear regression models, using a multiplicative term. There was a marginal interaction observed between arsenic and lead and maternal mtDNAcn ($p_{int} = 0.06$), between arsenic and magnesium and maternal TL ($p_{int} = 0.09$), and between mercury and zinc and maternal TL ($p_{int} = 0.10$). Using GAMs, we observed evidence of nonlinear relationships for the association between cord blood mtDNAcn and magnesium ($p = 0.04$), maternal mtDNAcn and barium ($p = 0.02$), maternal mtDNAcn and mercury ($p = 0.06$), maternal mtDNAcn and lead ($p = 0.003$), and maternal TL and barium ($p = 0.05$) (Figure 3). The remaining nonsignificant GAM plots are shown in Figures S12–S15.

Based on GAM and BKMR results, we conducted sensitivity analyses in which we included square terms in quantile

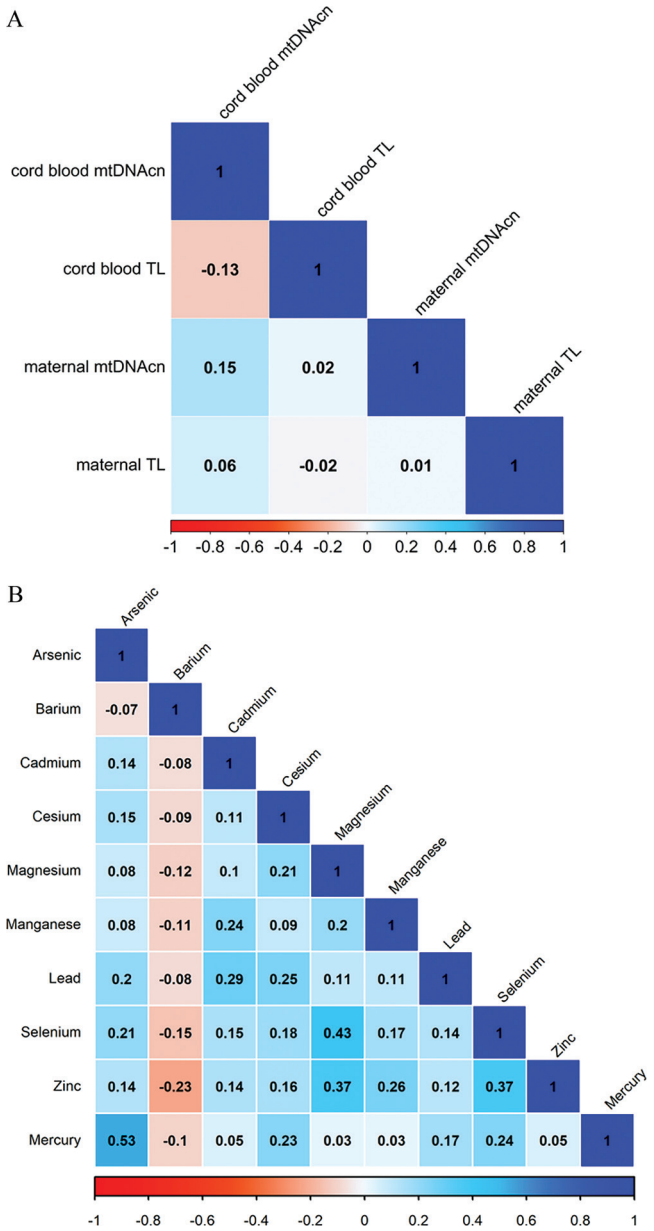

Figure 1. Spearman correlation coefficients among (A) cord blood and maternal mtDNAcn and TL residuals after regressing out plate effects ($n = 402$), and (B) Spearman correlation coefficients between maternal metal concentrations in red blood cells ($n = 1{,}390$). Note: mtDNAcn, mitochondrial DNA copy number; TL, telomere length.

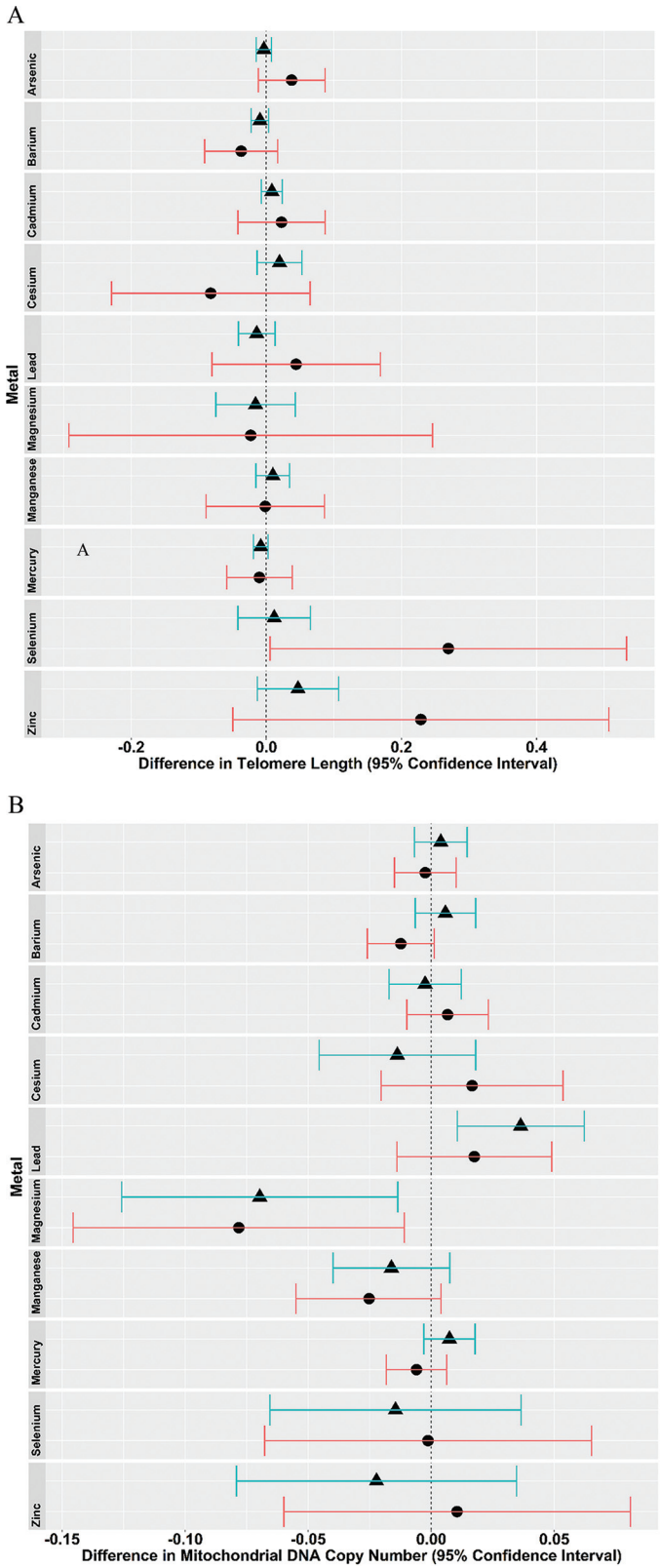

Figure 2. Multivariable linear regression results for adjusted association between first trimester red blood cell concentrations of individual metals and maternal (top blue/triangle) and cord blood (bottom red/circle) (A) TL ($n = 893$ and $n = 408$, respectively) and (B) mtDNAcn ($n = 898$ and $n = 419$, respectively). Estimates are difference in TL or mtDNAcn associated with a 2-fold increase in metal concentration, adjusted for sample plate, maternal age, prepregnancy body mass index, race/ethnicity, education, income, smoking during pregnancy, parity, and child sex at birth (cord blood models only). Table S8 shows numerical summary of the data. Note: mtDNAcn, mitochondrial DNA copy number; TL, telomere length.

g-computation to select between linear and nonlinear mixture models. The linear models had the lowest BIC, and thus they were favored over the nonlinear models for the relationship between all the prenatal metals and maternal and cord blood mtDNAcn and cord blood TL. However, for the relationship between the overall prenatal metal mixture as well as the nonessential metal mixture and maternal TL, the lowest BIC was observed in the model in which a squared term was added to barium. The overall nonlinear mixture finding was likely driven by barium because the mixture exposure–response followed a trend similar to that observed in the GAM (Figure 3).

Discussion

In this prospective U.S. prebirth cohort, we observed a negative association between maternal first-trimester magnesium concentration

Table 3. Quantile g-computation estimates for difference in maternal and cord blood TL and mtDNAcn for one-quartile increase in metal mixtures determined *a priori*, conditional on the covariates.

		Difference in TL [β (95% bootstrap CI)]		Difference in mtDNAcn [β (95% bootstrap CI)]	
Model[a]	Mixture	Maternal ($n = 893$)	Cord blood ($n = 408$)	Maternal ($n = 898$)	Cord blood ($n = 419$)
All metals[b]	As, Ba, Cd, Cs, Hg, Mg, Mn, Pb, Se, Zn	−0.01 (−0.04, 0.02)	0.04 (−0.10, 0.20)	0.02 (−0.01, 0.05)	−0.03 (−0.07, 0.01)
Essential[c]	Mg, Mn, Se, Zn	0.01 (−0.01, 0.04)	0.05 (−0.06, 0.20)	−0.02 (−0.04, 0.003)	−0.02 (−0.05, 0.01)
Nonessential[d]	As, Ba, Cd, Cs, Hg, Pb	−0.03 (−0.06, 0.005)	−0.01 (−0.2, 0.2)	0.04 (0.01, 0.07)	−0.01 (−0.04, 0.03)

Note: This table corresponds to Table S11 and Figure S7 in the supplemental material. As, arsenic; Ba, barium; BMI, body mass index; Cd, cadmium; CI, confidence interval; Cs, cesium; Hg, mercury; Mg, magnesium; Mn, manganese; mtDNAcn, mitochondrial DNA copy number; Pb, lead; Se, selenium; TL, telomere length; Zn, zinc.
[a]Metals were $\log_2$-transformed in the analyses.
[b]Difference in each outcome for one-quartile increase in all metals, conditional on the covariates sample plate, maternal age, maternal prepregnancy BMI, maternal race/ethnicity, education, income, smoking during pregnancy, parity, and child sex at birth (cord blood only).
[c]Difference in each outcome for one-quartile increase in essential metals, conditional on the covariates sample plate, maternal age, maternal prepregnancy BMI, maternal race/ethnicity, education, income, smoking during pregnancy, parity, child sex at birth (cord blood only), and the nonessential metals.
[d]Difference in each outcome for one quartile increase in nonessential metals, conditional on the covariates sample plate, maternal age, maternal prepregnancy BMI, maternal race/ethnicity, education, income, smoking during pregnancy, parity, child sex at birth (cord blood only), and the essential metals.

and both maternal second-trimester and cord blood mtDNAcn, as well as a positive association between maternal first-trimester lead concentration and second-trimester maternal blood mtDNAcn. A positive association was observed between maternal first-trimester selenium concentration and cord blood TL. Additionally, we observed an association between the nonessential metal mixture and higher maternal mtDNAcn. We also observed a nonlinear relationship between cord blood mtDNAcn and magnesium; maternal mtDNAcn and barium, lead, and mercury; and maternal TL and barium, which indicates the complexity of both the metals and the biomarker. These exposures are relevant to public health due to the ubiquitous nature of the metals included in our analysis, as well as the potential role of altered TL and mtDNAcn in aging, oxidative stress, and chronic disease in the life course.

Blood biomarkers of mtDNAcn and TL are implicated in age-related and chronic disease susceptibility. In a recent study, higher whole-blood mtDNAcn was associated with prospective measures of greater DNAm-PhenoAge and shorter leukocyte TL, suggesting that it could be a biomarker of aging-related disease and mortality (Dolcini et al. 2020). Variation in mtDNAcn has also been linked to chronic kidney disease, cancer, neurodegenerative diseases, and liver disease (as reviewed by Castellani et al. 2020). Both mtDNAcn and TL may also be predictors of cardiovascular (Yue et al. 2018), psychiatric (Aas et al. 2019; Otsuka et al. 2017), metabolic (Al-Kafaji et al. 2018; Meng et al. 2016; Skuratovskaia et al. 2019; Tian et al. 2017; Valdes et al. 2005), and immune-mediated disease (Irvin et al. 2018; Lee et al. 2017; Lee and Bae 2018; Peng et al. 2019). Specifically, in the presence of decreased mtDNAcn, cells enter a state of energy crisis, leading to changes in nuclear gene expression that may increase the risk of chronic disease, cancer, or premature aging (as reviewed by Castellani et al. 2020). One study found an inverse association between blood mtDNAcn and all-cause mortality (Ashar et al. 2015). Similarly, chronological age is correlated with TL due to the gradual erosion of telomeres during the life course (Vaiserman and Krasnienkov 2021).

Median maternal TL in our study was similar across age quantiles, likely due to the narrow age range of participants. Consistent with literature, maternal and cord blood TL was higher in those with higher income and a college education (Mitchell et al. 2014). Although cord blood TL and cord blood mtDNAcn were negatively correlated and cord blood mtDNAcn and maternal mtDNAcn were positively correlated, there was no significant correlation between maternal and cord blood TL. A prior study found that cord blood and maternal blood TL were weakly correlated ($r = 0.20$; Herlin et al. 2019), but it did not account for sample plate.

Few studies have examined mtDNAcn in relation to individual metal concentrations. In a prospective cohort study of 762 mother–newborn pairs in Wuhan, China, a doubling of maternal urinary aluminum concentration during the second and third trimesters was associated with a 3.2% (95% CI: 0.9, 5.5) and 4.2% (95% CI: 1.6, 6.8) difference in newborn mtDNAcn, respectively, although the association between maternal urinary aluminum concentration during the first trimester and newborn mtDNAcn was not significant (Liu et al. 2019). In the Programming Research in Obesity, Growth, Environment, and Social Stressors (PROGRESS) prospective birth cohort study of 410 mother–infant pairs in Mexico City, Mexico, associations were observed between maternal blood lead concentrations, second and third trimester, and cord blood mtDNAcn. A positive association was observed between cord blood mtDNAcn and lead concentration measured in mothers in the second trimester ($\beta = 0.06$, 95% CI: 0.01, 0.1), mothers in the third trimester ($\beta = 0.05$, 95% CI: 0.002, 0.1) and cord blood levels ($\beta = 0.05$, 95% CI: 0.004, 0.1) (Sanchez-Guerra et al. 2019). In our study, we did not observe a significant association between lead concentration in the first trimester and cord blood mtDNAcn, although the magnitude and direction of association was consistent with the prior study from PROGRESS. However, we did observe a positive significant association between lead concentration in the first trimester and maternal mtDNAcn with a similar magnitude and direction of association, as well as evidence of nonlinearity. Although the PROGRESS study measured metals in cord blood and at several time points during pregnancy, our study relied on a single measurement during the first trimester. Average blood lead concentration at all time points was higher in the PROGRESS study, compared with that in our study. For example, mean ± standard deviation (SD) blood lead concentration in maternal second trimester blood was 3.8 ± 2.6 μg/dL, whereas mean ± SD blood lead concentration in maternal first trimester blood in our study was 2.0 ± 0.9 μg/dL.

In the PROGRESS cohort, a positive association was reported between manganese in the third trimester and manganese in cord blood with cord blood mtDNAcn ($\beta = 0.05$, 95% CI: 0.01, 0.08 and $\beta = 0.04$, 95% CI: 0.01, 0.06, respectively). It was hypothesized that maternal iron deficiency might act in synergy with higher manganese to increase oxidative stress, which could manifest as changes in cord blood mtDNAcn. Anemia modified the association between cord blood mtDNAcn and manganese in the second trimester (Kupsco et al. 2019). Namely, a positive association was observed between second-trimester manganese and cord blood mtDNAcn in mothers with normal hemoglobin, and a negative association was observed among those with low hemoglobin (Kupsco et al. 2019). In contrast, we did not find associations between maternal manganese and maternal or cord blood mtDNAcn in our study. Exposure to manganese during late pregnancy in comparison with early pregnancy may have stronger associations with newborn mtDNAcn, but more studies are needed to explore differences from varying windows of exposure. In addition, we did not evaluate iron deficiency as a confounder

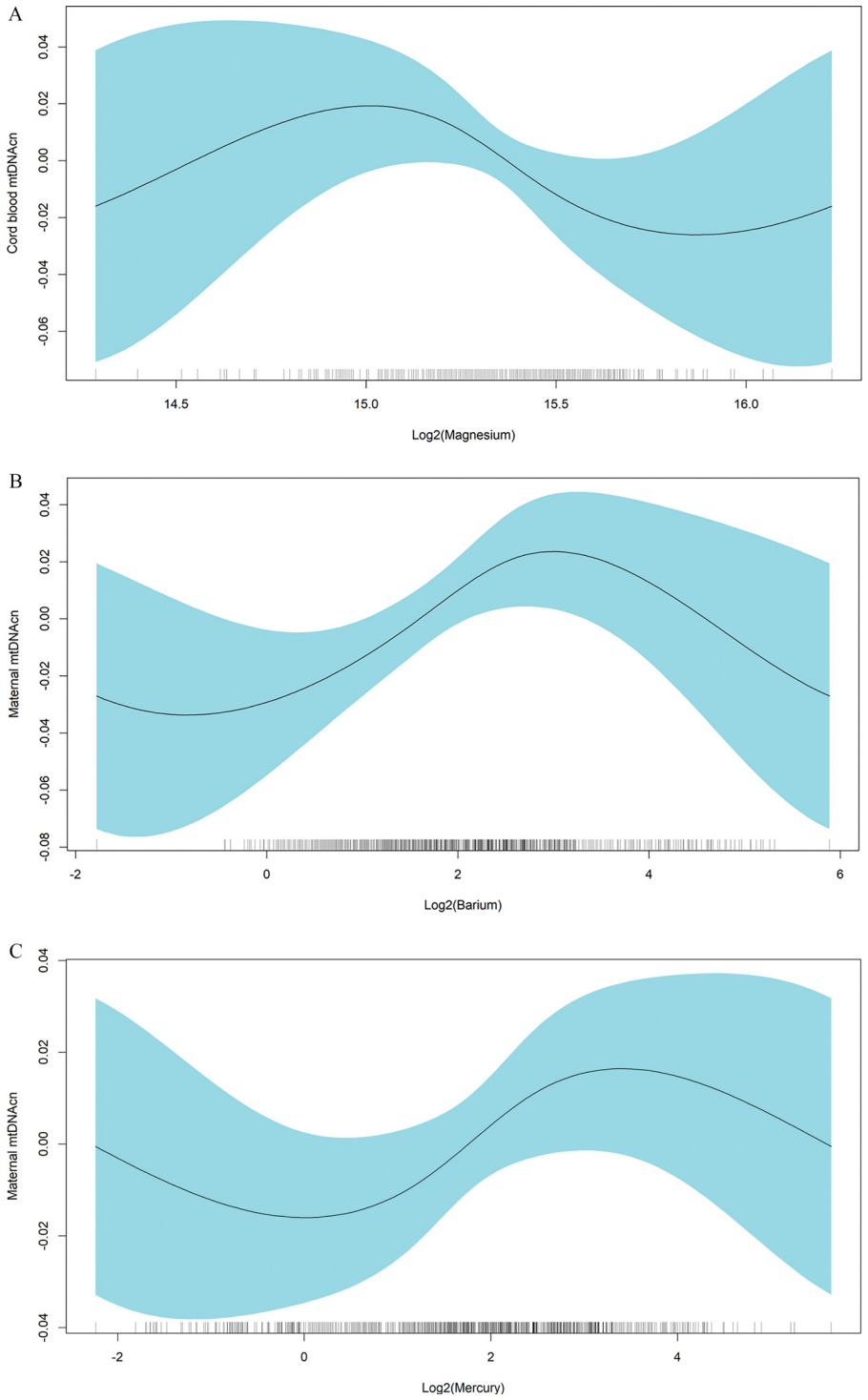

Figure 3. Nonlinearity detected in generalized additive models for the association between (A) maternal first-trimester magnesium and cord blood mtDNAcn ($n = 419$), (B) maternal first-trimester barium and maternal second-trimester mtDNAcn ($n = 898$), (C) maternal first-trimester mercury and maternal second-trimester mtDNAcn ($n = 898$), (D) maternal first-trimester lead and maternal second-trimester mtDNAcn ($n = 898$), and (E) maternal first-trimester barium and maternal second-trimester TL ($n = 893$). Note: mtDNAcn, mitochondrial DNA copy number; TL, telomere length.

or effect modifier in this study because it was not measured at the same time point in all study participants.

Similarly, few studies have examined associations between prenatal metals and TL. One study examined associations between maternal urinary manganese concentration in each trimester of pregnancy and cord blood TL (Bi et al. 2021). A positive association was observed between maternal urinary manganese concentration in the second trimester and cord blood TL, but no association was observed between maternal urinary manganese concentrations in the first and third trimesters and cord blood TL (Bi et al. 2021). Similarly, we observed no association between first trimester manganese and cord blood TL.

Another study examined whether selenium is protective against heavy metal–induced TL shortening in 408 mother–infant pairs who were enrolled in a prospective cohort study in Myanmar (Wai et al. 2020). Maternal spot urine samples were collected during the third

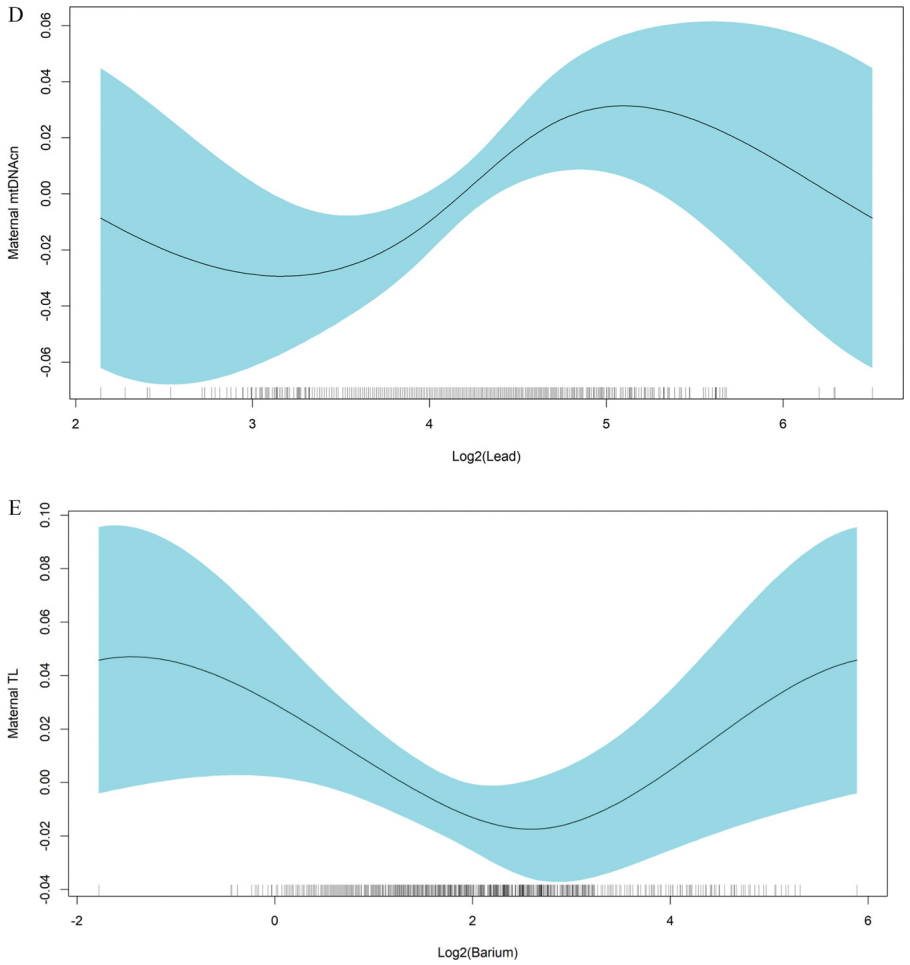

Figure 3. (*Continued.*)

trimester to measure metal concentrations, and blood samples were collected from the umbilical cord at birth to measure TL. In linear regression models, arsenic, cadmium, and lead levels had inverse associations with TL, but the associations were attenuated after adjustment for selenium level, especially for lead (unadjusted $\beta = -0.10$, 95% CI: 0.18, −0.01; adjusted $\beta = -0.03$, 95% CI: −0.13, 0.05). After stratifying the data by selenium concentration, there was no significant association between cadmium or lead and TL in the high selenium group (Wai et al. 2020). In our study, first-trimester selenium was associated with longer cord blood TL ($\beta = 0.30$, 95% CI: 0.01, 0.50). There was no significant association between cadmium or lead and cord blood TL, although the metals had positive regression coefficients. There was a suggestive positive association between maternal arsenic and cord blood TL in our study.

Another study examined the association between urinary cadmium concentration in mothers at birth and cord blood TL in 410 mother–newborn pairs from a prospective birth cohort study in Wuhan, China. In the multivariable linear regression model that adjusted for maternal confounders, as well as selenium and zinc concentration, a 2-fold increase in maternal urinary cadmium concentration was associated with 6.83% shorter relative cord blood TL (95% CI: −11.44%, −1.97%). Models with adjustment for selenium and zinc showed a stronger inverse association between cadmium and TL, suggesting negative confounding and a potential beneficial effect from selenium and zinc (Zhang et al. 2019). In our study, we did not observe associations between cadmium and cord blood TL.

To our knowledge, only one prior study has examined associations between prenatal metal mixtures and cord blood TL. The study implemented WQS regression to examine associations between urinary metal mixtures in late pregnancy and cord blood TL in 100 mother-newborn pairs in the Boston and New York City-based Programming of Intergenerational Stress Mechanisms (PRISM) pregnancy cohort. In adjusted models, a nonessential metal mixture [arsenic, barium, cadmium, nickel, lead] was associated with lower cord blood TL ($\beta = -0.50$, 95% CI: −0.78, −0.21). The top metals contributing to the negative association included barium (weight: 35.4%), cadmium (24.5%), and lead (26.9%). In models stratified by antioxidant intake, which included the essential metals magnesium, selenium, and zinc estimated from food frequency questionnaires, the significant inverse association between metals and TL remained only among mothers with low antioxidant intake [low: $\beta = -0.92$, 95% CI: −1.53, −0.30; high: $\beta = 0.03$, 95% CI: −0.58, 0.52; (Cowell et al. 2020)]. Differences in study findings may be attributed to differing timing of exposures, as we sampled metals from the first trimester, whereas PRISM measured metals closer to delivery (average of 32 wk). Different biological exposure matrices by which metals were measured could also explain the differences, because we measured metals in RBCs and PRISM quantified urinary metals. In addition, different metals were included in the mixtures, because PRISM only measured arsenic, barium, cadmium, and lead. In PRISM, there was evidence that dietary antioxidants modified the association, and the cohort was sampled from urban lower income individuals. In contrast, participants in

our population were majority high income and college educated, and thus they were expected to have access to high quality prenatal care and nutritional options.

In our study, we observed a nonlinear relationship between first-trimester barium concentration and second-trimester mtDNAcn and TL. Another recent study in the Project Viva cohort observed an inverse U-shaped association between first-trimester RBC barium and maternal glucose concentrations measured 1 h after nonfasting 50-g glucose challenges from clinical gestational diabetes screening at 26–28 wk gestation (Zheng et al. 2021). The nonlinear association between barium, lead, magnesium, and mercury and mtDNAcn is consistent with the hypotheses that acute chemical exposure would result in increased mtDNAcn to compensate for decreased energy production and that chronic environmental exposure would overwhelm the mitochondria and result in decreased mtDNAcn (Kupsco et al. 2019). Consistently, increased concentration of barium, lead, magnesium, and mercury, was associated with increased mtDNAcn at low levels of exposure and decreased mtDNAcn at high levels of exposure. Inconsistencies in prior study findings could be due to nonlinear relationships between certain metals and the biomarkers if different populations have relatively low or high blood metal concentrations. Although linear regression still captured the overall trend between the metals and biomarkers in the present study, potential nonlinearity should be considered in future studies for mtDNAcn and TL and metal toxicology.

Key strengths of this study include a relatively large sample size for each outcome, measures of both nonessential and essential metals in participants during early pregnancy, and measures of TL and mtDNAcn measured subsequently in participants during the second trimester and at birth in cord blood. Using RBCs for measuring prenatal measures is another strength for many of our metals. Blood cadmium is the most valid marker of recent exposure (Järup and Åkesson 2009). RBC cadmium is also a useful measure of long-term exposures in subjects without exposures other than dietary, especially among nonsmokers (Lin et al. 2021). Blood lead is the primary biomarker for lead exposure and reflects recent exposure (Barbosa et al. 2005). In addition, RBCs are a good biological matrix for measuring lead, because the majority of lead is bound to RBCs (Lin et al. 2021). RBC magnesium is often cited as the preferable measurement for magnesium deficiency, in comparison with serum or plasma, due to their higher magnesium content (Workinger et al. 2018), and blood manganese is a reasonable indicator of recent environmental exposure (Andrade et al. 2015).

However, one limitation of our study is that metals measured in maternal RBCs reflect more recent exposure and may not be the ideal biomarker for all the measured metals. For example, most absorbed arsenic has a short half-life in blood. In addition, the RBC analysis of arsenic captures total arsenic concentrations and does not provide information on the form of arsenic absorbed. As a result, urinary inorganic arsenic and its metabolites would be a more sensitive measure (NRC 1999). Similarly, RBC mercury reflects total mercury and does not differentiate between elemental, inorganic, and organic (the more toxic form) of mercury, although it is still a good proxy for organic mercury, because more than 90% of organic mercury in blood is bound to RBCs (Berglund et al. 2005). Although this is a limitation of our study, metal concentrations in RBCs may be most predictive of what transports into fetal circulation during pregnancy (Chen et al. 2014). Other studies have used urine or toenail metal measurements as alternatives, which can limit comparability among studies and reflect different exposure windows (as reviewed by Gutiérrez-González et al. 2019; Sommar et al. 2014; NRC 1999). The utility of RBC metal measurements has been discussed previously (Lin et al. 2021).

In addition, our cohort includes individuals who are majority White, have high income and educational levels, and speak English, which limits external generalizability to other populations. For example, a majority White population may limit external generalizability, because the median blood metals distribution may be shifted to a higher level in non-White populations disproportionately burdened with high heavy metals exposure (Davis et al. 2016). Results can be generalized only to populations with similar metal concentrations. The metal measurements are still comparable to the general U.S. population, and geometric mean essential metal concentrations were within the clinical reference range for RBC measurements.

A final limitation of our study includes the lack of cell-type data for maternal TL and mtDNAcn, as well as complete cell-type data for cord blood TL and mtDNAcn. Our sensitivity analyses, in which we adjusted for cord blood cell type in a subset of individuals with this covariate information, suggested that our lack of adjustment for cell type in cord blood did not influence our exposure–response findings. However, we cannot rule out outcome misclassification, because it is well documented that blood samples used to measure mtDNAcn and TL will contain a variable number of different cell types and cell-type ratios, and different immune cells may have different mtDNAcn and TL (Hurtado-Roca et al. 2016; Lin et al. 2019). We do not think prenatal metals would influence cell type, although this has not been evaluated in prior literature comprehensively. Similarly, mtDNAcn was estimated from genomic DNA derived from buffy coat, which contains leukocytes and platelets that were not differentiated. Platelet contamination is a potential source of outcome misclassification in this study, because we did not have data on platelet count in the maternal or cord blood samples, which can lead to overestimation of leukocyte mtDNAcn measurements, because platelets contain high levels of mtDNA but no nuclear DNA (Hurtado-Roca et al. 2016; Urata et al. 2008). However, we are unaware of literature that would support a role for metals inducing higher platelet counts, and we therefore do not believe prenatal metal concentration would influence platelet count in otherwise healthy children. One recent study in a prospective prebirth cohort from Wuhan, China, found that higher urinary arsenic, cadmium, and manganese was associated with lower platelet counts across pregnancy in repeated measures analyses and that higher cadmium was associated with decreased platelet count cross-sectionally during all three trimesters of pregnancy. However, this study collected metals in urine, rather than RBCs, and did not have data on prepregnancy platelet count status (Bao et al. 2020).

Residual confounding is possible when using lower degrees of granularity for socioeconomic status and income. Other sources of residual confounding may include coexposure to other toxic chemicals not included in this study, or genetic variation that could influence the metal level in RBCs and the outcomes, such as those related to ion transporters or cell membrane receptors.

Study findings can help elucidate the effect of metals on oxidative stress and inflammatory pathways, even at relatively low-level exposure. Additional studies should examine prenatal metals at multiple time points during pregnancy and their association with these biomarkers in different study populations.

Conclusion

In this U.S. prebirth cohort, certain prenatal metals and mixtures of metals were associated with mtDNAcn and TL in maternal second-trimester and neonatal cord blood. Not all associations between metals and the biomarkers followed expected trends, in that metals that were classified as either essential or nonessential

did not always have associations with the biomarkers in the same direction as other metals in the same classification. Observed associations were relatively small, which could be due to the narrow range of exposure in this population. Results of this study can only be used to infer associations in populations with similar metal exposure ranges and population characteristics, which could determine the potential of metals to promote oxidative stress and inflammation. This is especially true, because different metals concentrations may behave differently in the context of a mixture, and essential metals are therapeutic within a certain range, but deficiency or excess can have detrimental effects. In addition, we observed some nonlinear associations, making it difficult to extrapolate outside the observed exposure range. In our study, we hypothesize that the overall effect of the metal mixtures seemed to balance out the individual associations observed between metals and mtDNAcn and TL. The research highlights the potential complex role of metal mixtures in pathways of disease, as well as the complexity of the mitochondrial–telomere axis. Future work will evaluate the extent to which these biomarkers during pregnancy and in early life are associated with health outcomes and child development.

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