Assessing the Mediating Role of Iron Status on Associations between an Industry-Relevant Metal Mixture and Verbal Learning and Memory in Italian Adolescents

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The authors declare they have nothing to disclose.

HIGHLIGHTS

- Metals have been linked with neurodevelopment, but less is known about the role of iron (Fe) status.
- We examined Fe status as a mediator of associations between a metal mixture and learning and memory.
- The mixture was associated with neurodevelopment; there was no evidence of mediation by Fe status.

ABSTRACT

Background: Metals, including lead (Pb), manganese (Mn), chromium (Cr) and copper (Cu), have been associated with neurodevelopment; iron (Fe) plays a role in the metabolism and neurotoxicity of metals, suggesting Fe may mediate metal-neurodevelopment associations. However, no study to date has examined Fe as a mediator of the association between metal mixtures and neurodevelopment.

Objective: We assessed Fe status as a mediator of a mixture of Pb, Mn, Cr and Cu in relation to verbal learning and memory in a cohort of Italian adolescents.

Methods: We used cross-sectional data from 383 adolescents (10-14 years) in the Public Health Impact of Metals Exposure Study. Metals were quantified in blood (Pb) or hair (Mn, Cr, Cu) using ICP-MS, and three metrics of Fe status (blood hemoglobin, serum ferritin and transferrin) were quantified using luminescence assays or immunoassays. Verbal learning and memory were assessed using the California Verbal Learning Test for Children (CVLT-C). We used Bayesian Kernel Machine Regression Causal Mediation Analysis to estimate four mediation effects: the natural direct effect (NDE), natural indirect effect (NIE), controlled direct effect (CDE) and total effect (TE). Beta (β) coefficients and 95% credible intervals (CIs) were estimated for all effects.

Results: The metal mixture was jointly associated with a greater number of words recalled on the CVLT-C, but these associations were not mediated by Fe status. For example, when ferritin was considered as the mediator, the NIE for long delay free recall was null (β=0.00; 95% CI=-0.22, 0.23). Conversely, the NDE (β=0.23; 95% CI=0.01, 0.44) indicated a beneficial association of the mixture with recall that operated independently of Fe status.

Conclusion: An industry-relevant metal mixture was associated with learning and memory, but there was no evidence of mediation by Fe status. Further studies in populations with Fe deficiency and greater variation in metal exposure are warranted.

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1. INTRODUCTION

Environmental exposure to metals, including lead (Pb), manganese (Mn), copper (Cu), chromium (Cr), and iron (Fe), is common among children, and can occur through air emissions, drinking water, and contact with contaminated soils (Agency for Toxic Substances and Disease Registry, 2020, 2012a, 2012b, 2004). Children living in close proximity to industry, such as the steel-producing ferroalloy industry, may be more highly exposed to metals, and prior research in the U.S., Canada, Brazil and elsewhere has reported higher biomarker concentrations of these metals among children living near ferroalloy plants (Boudissa et al., 2006; Butler et al., 2019; Haynes et al., 2012; Menezes-Filho et al., 2016, 2009; Riojas-Rodríguez et al., 2010). These findings are of public health concern because metals have been consistently associated with adverse neurodevelopment (Amorós et al., 2019; Caparros-Gonzalez et al., 2019; Carvalho et al., 2018; García-Chimalpopoca et al., 2019; Oulhote et al., 2014; Torres-Agustín et al., 2013; Wright et al., 2006; Yorifuji et al., 2011), and the ferroalloy industry is expanding globally ("Ferroalloy Market Share 2018-2025 Industry Growth Outlook Report," n.d.).

Children are exposed to multiple environmental metals simultaneously (Agency for Toxic Substances and Disease Registry, 2020, 2012b, 2012a, 2004), and many metals share similar neurotoxic pathways (e.g., dopaminergic toxicity, oxidative stress, mitochondrial disruption) (Ahamed and Siddiqui, 2007; Akinyemi et al., 2019; Gaetke et al., 2014; Kalita et al., 2018; Neal and Guilarte, 2013; O'Neal et al., 2014; Wise et al., 2022). These toxicological data suggest that concurrent exposure to multiple metals can induce joint or interactive neurodevelopmental effects. Findings in animals are generally consistent with studies in pediatric cohorts, which have reported cumulative effects of multiple metals on cognition (Chandra et al., 1981; Merced-Nieves et al., 2021; Shukla and Chandra, 1987; Stein et al., 2022). In the Public Health Impact of Metals Exposure (PHIME) study, we previously found that a mixture of Mn, Pb, Cu and Cr was jointly associated with indices of neurodevelopment, though the direction of effect varied by cognitive domain. For example, the metal mixture was adversely associated with verbal intelligence quotient (IQ) and visuospatial abilities (Bauer et al., 2020a; Rechtman et al., 2020), but beneficially

associated with verbal learning and memory measured on the California Verbal Learning Test for Children (CVLT-C) (Schildroth et al., 2023).

Recent epidemiological studies have investigated the role of Fe status, clinically measured through biomarkers like ferritin, hemoglobin, and transferrin (Gibson, 2005), as a modifier of the neurotoxicity of environmental metals in children (Schildroth et al., 2023, 2022b). Fe is a metal and essential nutrient required for cellular oxygen transport, neurotransmitter synthesis, and metabolic activity, and is therefore important for brain development and maturation (McCann et al., 2020). Perturbations to Fe status in gestation, early childhood, and adolescence have been consistently associated with worse scores on assessments of attention-related behaviors, memory, visuospatial abilities, IQ, and academic achievement (Halterman et al., 2001; Jáuregui-Lobera, 2014; Ji et al., 2017; Lukowski et al., 2010; Parkin et al., 2020; Roy et al., 2011; Tseng et al., 2018). A recent study of 922 adolescents observed reduced cognitive abilities among participants with lower brain Fe concentrations (measured using R2* relaxometry from magnetic resonance imaging) (Larsen et al., 2020). On the other hand, Fe overload has similarly been associated with decreased IQ scores in children, reflecting the neurotoxic impacts (i.e., oxidative stress) of Fe in excess (Salvador et al., 2011; Sammallahti et al., 2022). In addition, the toxicokinetics and toxicodynamics of Fe are similar to those of other metals. Fe and environmental metals like Pb and Mn share neurotoxic mechanisms (oxidative stress, mitochondrial disruption, altered neurotransmission and disruption of myelination) (Barkur and Bairy, 2015; Borchard et al., 2018; Fernsebner et al., 2014; Galaris et al., 2019; McCann et al., 2020; Neal and Guilarte, 2013; Soares et al., 2020; Walter et al., 2002), and also compete for transporters (e.g., the divalent metal transporter 1) in the duodenum, brain, and liver (Brain et al., 2006; Kordas, 2010; Kordas and Stoltzfus, 2004; Peraza et al., 1998). Environmental metals tend to deposit more into Fe-deficient, compared to Fe-replete, brain tissues in animal models, and this tendency has been further shown to impact neurotransmitter concentrations (Erikson et al., 2002; Kordas, 2010). These data support a body of evidence indicating that altered Fe status, in particular deficiency, impacts the neurotoxicity of environmental metals in children.

Environmental metal exposure may also affect Fe dynamics in the body. The hematologic toxicity of certain metals has been established: Pb, for example, disrupts *heme* synthesis, transferrin expression, erythrocyte membranes, and erythropoietin production, particularly at concentrations >10 µg/dL (Agency for Toxic Substances and Disease Registry, 2020; McCann et al., 2020; Peters et al., 2021; Rossander-Hultén et al., 1991). Though the hematologic toxicity of metals has been shown primarily with respect to hemoglobin, there is some evidence that environmental metals adversely impact other metrics of Fe status (e.g., ferritin concentrations) even at low concentrations (e.g., blood Pb <5 µg/dL), which is supported by mechanistic data illustrating the ability of metals to interfere with Fe loading onto proteins (e.g., ferritin) (Liu et al., 2020); however, many prior studies in children were limited by methodological constraints (e.g., cross-sectional design) (Choi and Kim, 2005; Henríquez-Hernández et al., 2017; Jain et al., 2005; Kutllovci-Zogaj et al., 2014; Schildroth et al., 2022a; Wang et al., 2012; Weinhouse et al., 2017). This suggests that, in addition to acting as a modifier of metals-induced toxicity, Fe status may also mediate associations between environmental metals and neurodevelopment (**Figure 1,** see also: (Schildroth et al., 2022b)).

A mediator is a variable that lies on the causal pathway between an exposure and outcome of interest. Identifying mediating variables can help to 1) elucidate underlying biological mechanisms of the exposure(s) of interest and 2) identify relevant pathways for potential interventions (Hafeman and Schwartz, 2009). Therefore, characterizing Fe status as a mediator of metal-neurodevelopment associations is an important public health objective, particularly because Fe deficiency is the most common nutritional deficiency in the world (Stevens et al., 2013). Prior work assessing Fe status as a mediator of metals neurotoxicity is limited (Jeong et al., 2015), and no study to date has assessed Fe status as a mediator of environmental metals or of a complex metal mixture.

Our previous study in the PHIME cohort examined Fe status as a modifier of the associations between metal mixture exposure and verbal learning and memory (Schildroth et al., 2023); the goal of the current analysis was to expand on this work and evaluate Fe status as a potential mediator of the industry-relevant metal mixture. We used a novel statistical approach to evaluate associations of a mixture of Pb,

Mn, Cu and Cr with verbal learning and memory among adolescents, and to assess Fe status as a mediator of these associations. We hypothesized that the metal mixture was associated with altered Fe status (e.g., reduced ferritin concentrations), and that Fe status was a mediator of the associations between the metal mixture and neurodevelopment.

2. METHODS

2.1 Study Population

We used cross-sectional data from the Public Health Impact of Metals Exposure (PHIME) study, a cohort designed to assess the impact of metal exposures from ferroalloy industry on neurodevelopment among early adolescents (aged 10-14 years). The study population, recruitment procedures, and study protocols have been described in detail elsewhere (Lucchini et al., 2012a). In brief, we recruited 721 adolescents from three sites in Brescia, Italy with varying historical ferroalloy activity. The first site, Bagnolo Mella, had ferroalloy activity since 1974; the second site, Garda Lake, had no historical ferroalloy industry activity; and the third site, Valcamonica, had continuous ferroalloy activity that ended in 2001. Enrollment into PHIME occurred in two distinct waves that reflected two periods of funding: the first wave (2007 – 2010) enrolled 311 participants and the second (2010 – 2014) enrolled 410 participants. All study protocols were consistent between the study phases. During the second phase, we recruited participants from the Bagnolo Mella site, collected additional biomarkers (saliva, urine, nails), and additionally administered selected items from the Home Observation Measurement of the Environment (HOME) Short Form questionnaire (National Longitudinal Surveys, 1979).

Participants were eligible for enrollment into PHIME if they 1) were 10 – 14 years at the time of enrollment, 2) lived in the study region since birth and 3) were born into families that lived in the study region since the 1970s. Exclusion criteria were: 1) neurologic, hepatic, metabolic, endocrine, or psychiatric disease, or clinically relevant motor deficits that may have impacted testing, 2) use of medication with neurologic side effects, 3) clinically diagnosed cognitive or behavioral impairment, 4) visual deficits without corrective measures or 5) having ever received parenteral nutrition. Potential participants were given detailed information on all study protocols and gave informed consent prior to

enrollment. PHIME study protocols were approved by Institutional Review Boards at the Icahn School of Medicine at Mount Sinai, University of California Santa Cruz, and the Ethical Committee of Brescia.

2.2 Covariate Measurements

Information on covariates was collected by trained study staff either in person or via the phone using standardized questionnaires. We collected data on sociodemographic variables, including age (continuous, in years), biological sex (male or female), area of residence, birth order (first, second, third, or higher), parental occupation, and parental education level. Using World Health Organization and Italian National Institute for Statistics classifications (World Health Organization, 1988), education and occupation levels of both parents were classified as low, medium, or high. A socioeconomic status (SES) index (low, medium, or high) for each participant's family was constructed based on education and occupation levels for the parent with the highest educational and vocational attainment using previously developed methodology for Italian populations (Cesana et al., 1995; Lucchini et al., 2012b). A HOME score (range: 0 – 9) was calculated for each participant using ten selected items from the HOME Short Form (National Longitudinal Surveys, 1979).

2.3 Biomarker Collection and Measurement

Biomarkers were collected from participants at enrollment. We selected blood as the primary biomarker for Pb because it is the gold standard biomarker of exposure in the epidemiological literature and reflects total body Pb (Barbosa et al., 2005). There is a lack of consensus on the optimal biomarker for Mn, Cu, and Cr (Bertinato and Zouzoulas, 2009; Coetzee et al., 2016; Jursa et al., 2018; Lukaski, 1999). We selected hair as the primary biomarker for these metals because there was little missing hair data, hair has been used to characterize metals exposure previously in the literature (Bauer et al., 2020a), hair metal concentrations have been consistently associated (both beneficially and adversely) with cognitive outcomes in children (Bauer et al., 2020a; Coetzee et al., 2016), and hair metal concentrations have been correlated with environmental exposures (Bauer et al., 2020a; Coetzee et al., 2016).

289 Methodology for quantification of metals in each of these biomarkers has been described previously in
290 depth (Eastman et al., 2013; Lucas et al., 2015; Lucchini et al., 2012a; Smith et al., 2007). Briefly, 4mL
291 whole blood samples were collected using 19-gauge butterfly catheters and stored in lithium-heparin
292 Sarstedt Monovette Vacutainers. Hair samples were collected from the occipital region of the scalp with
293 stainless steel clippers and cleaned extensively using validated methods; exogenous metal contamination
294 was removed using Triton detergent, nitric acid, and sonification, and dried hair samples were then
295 digested using distilled nitric acid (Eastman et al., 2013; Lucas et al., 2015). Hair samples were dried
296 overnight and digested using nitric acid. All biomarker metal concentrations were quantified using
297 magnetic sector inductively coupled plasma mass spectrometry (Eastman et al., 2013; Lucchini et al.,
298 2012a; Smith et al., 2007). Limits of detection (LODs) were defined based on repeated measures of
299 procedural blanks (Butler et al., 2019); LODs ranged from 0.08 to 0.12 µg/g for hair Mn, Cr, and Cu, and
300 the LOD for blood Pb was 0.01 µg/dL.
301 Fe status was measured from blood samples using three clinically relevant biomarkers: ferritin
302 (ng/mL), hemoglobin (g/dL), and transferrin (mg/dL). Ferritin is considered a sensitive marker of Fe-
303 deficiency and reflects long-term Fe tissue storage; transferrin transports Fe to tissues and reflects Fe
304 availability; and hemoglobin reflects functional Fe status, where low concentrations are indicative of Fe-
305 deficient anemia (Gibson, 2005). Hemoglobin was measured in whole blood samples during a complete
306 blood count (CBC) with the Beckman Coulter LH Series (Beckman Coulter, Inc. Diagnostics Division,
307 CA, USA). Blood samples were collected into tubes containing EDTA coagulant. Ferritin and transferrin
308 were measured in serum using immunoassays with the Architect *i*2000*SR* – Abbott Laboratories (Abbott
309 Park, IL, USA) and ADVIA (Siemens Healthcare GmbH, Germany), respectively.

310 ***2.4 Cognitive Assessment***

311 The California Verbal Learning Test for Children (CVLT-C) was administered to PHIME
312 participants by trained neuropsychologists to assess verbal learning and memory (Delis et al., 1994). The
313 CVLT-C was only administered to participants during the second phase of PHIME (n= 403). The CVLT-
314 C consists of 5 recall trials (trials 1-5) of a list of 15 verbally presented words (List A). The 15 words

include 5 semantically related words from three separate categories (e.g., fruits). Participants then perform a recall trial from an interference list of 15 words (List B). This is followed by additional recall trials of List A, including free (i.e., not cued) and cued recall trials immediately following the interference list (short delay recall) and after 20-minutes (long delay recall). Lastly, participants complete a recognition trial, where they are asked to select target words (i.e., those on List A) from a written list of 44 words that includes both target and distraction words, including words from List B.

CVLT-C outcomes available for analysis included scores for the correct number of target words recalled on trial 1, trial 5, the inference list, the short (free and cued) delay trials, the long (free and cued) delay trials, and the recognition trial. Higher scores (i.e., a higher number of words correctly recalled) for these trials were indicative of better learning and memory. We calculated the number of intrusions, defined as the total number of non-target words recalled across all trials, and the number of perseveration errors, defined as the total number of target words repeated across all trials. Higher scores for both intrusions and perseverations were indicative of worse cognitive function. Lastly, we calculated a metric of forgetting by subtracting the number of correct words on the short delay free recall trial from the number of correct words on the long delay free recall trial (Kreutzer et al., 2011; Strauss et al., 2006). Negative scores for forgetting were indicative of worse memory performance, while positive scores reflected better performance (i.e., better retention or remembering). We *a priori* selected five CVLT-C outcomes for analysis in the current study, including trial 5 recall, long delay free recall, long delay cued recall, perseveration errors, and forgetting, because these outcomes represent varying aspects of learning and memory function (**Table S1**).

2.5 Statistical Analysis

All analyses were performed in R version 3.6.1.

2.5.1 Multiple imputation. The analytic sample for this study was restricted to adolescents with complete outcome data (n= 403). There was little missing data (<6% for covariates and biomarkers, **Table S2**). Missing biomarker and covariate data were imputed using Markov chain Monte Carlo methods (Zhou et al., 2001) with the *mice* package in R (Buuren and Groothuis-Oudshoorn, 2011), where

missing data were assumed to be missing at random. Twenty datasets were imputed using all covariate data, and one imputed dataset was randomly selected for all subsequent analyses.

2.5.2 Confounder selection. Confounders were identified *a priori* using knowledge of the literature, biologic plausibility, and directed acyclic graphs (DAGs) (Bauer et al., 2020b; Carvalho et al., 2018; Kordas, 2010; Torres-Agustín et al., 2013). All models were adjusted for age (continuous), biological sex (binary), HOME score (continuous) and socioeconomic status (ordinal, classified as low, medium, or high).

2.5.3 Summary statistics. Summary statistics, including medians, 25^{th} percentiles, 75^{th} percentiles, means and standard deviations (SDs), were calculated for all variables. Summary statistics for the randomly selected imputed dataset were similar to the complete data (**Table S3**). Distributions of all continuous variables were examined; based on visual inspection of histograms and boxplots, we identified several extreme values for metals concentrations. Participants with concentrations of any metal that were ±3 SDs from the mean (n= 20) were excluded from further analyses (final sample size= 383). All metals (Pb, Mn, Cu, Cr), ferritin, and one CVLT-C outcome (perseveration errors) were right-skewed, and we natural log-transformed these variables to reduce the influence of outlier values and to satisfy assumptions of normality of residuals for statistical modeling. Metals, Fe status metrics, and CVLT-C outcomes were z-standardized prior to regression modeling. We estimated Spearman correlation coefficients between the metals and between the CVLT-C outcomes.

2.5.4 Bayesian Kernel Machine Regression. We used Bayesian Kernel Machine Regression Causal Mediation Analysis (BKMR-CMA) as our primary model to investigate the mediating role of Fe status on associations between the metal mixture (Pb, Mn, Cr, Cu) and verbal learning and memory. BKMR is a highly flexible model that uses a kernel function to model the exposure response surface of the mixture, where individuals in the study population with similar exposure profiles are assumed to have similar neurodevelopment scores (Bobb et al., 2018, 2015). The flexible kernel function allows for non-linearity, metal-metal interactions, higher order interactions (e.g., interactions of an individual metal with the rest of the mixture), and estimation of cumulative effects (Bobb et al., 2018, 2015).

BKMR-CMA is an extension of BKMR used to quantify the mediation of the mixture (Pb, Mn, Cr, Cu) by a third variable, where the mediating variable is also allowed to interact with the mixture by including the mediator (i.e., ferritin, transferrin, hemoglobin) in the kernel function (Devick et al., 2022). Three BKMR models were fit to estimate mediation effects: [1] the outcome model, which quantified the association of the mixture (Pb, Mn, Cr, Cu) with the CVLT-C outcomes when Fe status was included in the kernel function; [2] the mediator model, which quantified the association of the metal mixture (Pb, Mn, Cr, Cu) with Fe status; and [3] the total effects model, which quantified the association of the metal mixture (Pb, Mn, Cr, Cu) with the CVLT-C outcomes when Fe status was excluded from the model. These models took the following form:

[1] $CVLT\ score = h(Mn_i,\ Pb_i,\ Cr_i,\ Cu_i,\ Fe\ status\ metric_i) + \beta_1 * Sex_i + \beta_2 * Age_i + \beta_3 * SES_i + \beta_4 * HOME\ score_i + e_i$

[2] $Fe\ status\ metric = h(Mn_i,\ Pb_i,\ Cr_i,\ Cu_i) + \beta_1 * Sex_i + \beta_2 * Age_i + \beta_3 * SES_i + \beta_4 * HOME\ score_i + e_i$

[3] $CVLT\ score = h(Mn_i,\ Pb_i,\ Cr_i,\ Cu_i) + \beta_1 * Sex_i + \beta_2 * Age_i + \beta_3 * SES_i + \beta_4 * HOME\ score_i + e_i$

Where h represents the kernel function, e_i is the error term, and confounders were assumed to have linear associations with the outcomes.

Each of these models was fit utilizing the default non-informative prior specifications and 50,000 iterations with a 50% burn-in. The component-wise variable selection option was used to estimate posterior inclusion probabilities (PIPs), which describe the relative importance of each component in the kernel function with the outcome while accounting for multiple testing.

Using Equations [1-3], we estimated four mediation effects using previously developed methodology (Devick et al., 2022): the controlled direct effect (CDE), natural direct effect (NDE), natural indirect effect (NIE) and total effect (TE). The CDE described the association of the metal mixture (Pb, Mn, Cr, Cu) with each CVLT-C outcome for an increase in the mixture from its 25th to 75th percentile, holding the mediator (e.g., ferritin) at its 25th, 50th, and 75th percentiles. The NDE quantified the direct association (i.e., not mediated through Fe status) of the mixture (Pb, Mn, Cr, Cu) with each CVLT-C

399 outcome for an increase in the mixture from its 25th to 75th percentiles, holding the mediator constant at
400 the level it would take if the mixture was held at its 25th percentile. The NIE described the indirect
401 association (i.e., mediated through Fe status) of the mixture (Pb, Mn, Cr, Cu) with each CVLT-C outcome
402 when the mixture was held at its 75th percentile, and Fe status was set to the concentration it would have
403 taken when the mixture was set at its 25th percentile compared to its 75th percentile. The TE reflected the
404 sum of both the direct (NDE) and indirect (NIE) pathways. These mediation effects are visualized in
405 **Figure 2A**. Beta coefficients and 95% credible intervals (CIs) were estimated for all mediation effects.
406 We also estimated summary measures to describe associations of the mixture (Pb, Mn, Cr, Cu, Fe
407 status metric) with each CVLT-C outcome (using Equation [1]) and the association of the metal mixture
408 (Pb, Mn, Cr, Cu) with Fe status metrics (using Equation [3]). These summary measures included 1)
409 exposure-response profiles for each exposure variable included in the kernel function, holding all other
410 exposure variables at their medians; 2) exposure-response profiles of each exposure variable included in
411 the kernel function at the 25th, 50th, and 75th percentile of a second exposure variable, while holding other
412 exposure variables at their 50th percentiles; 3) the association for a percentile increase in all exposure
413 variables simultaneously, compared to the 50th percentile of all exposure variables; and 4) the association
414 for each exposure variable included in the kernel function for an increase from its 25th to 75th percentiles,
415 holding all other exposure variables at their 25th, 50th, or 75th percentiles.
416 ***2.5.5 Sex-stratified analyses.*** Epidemiological evidence suggests that associations of metals with
417 neurodevelopmental outcomes can be sex-specific, and that females are more susceptible to Fe deficiency
418 in adolescence (Bauer et al., 2017; Kounnavong et al., 2020; Llop et al., 2013; Rechtman et al., 2020;
419 Shaw, 1996; Zhu et al., 2021). In exploratory analyses, we fit BKMR-CMA models for each CVLT-C
420 outcome in sex-stratified datasets to examine potential sex-specific effects.
421 ***2.5.6 BKMR-CMA sensitivity analyses.*** We investigated the sensitivity of our findings to the
422 specification of the priors by fitting the BKMR-CMA models 1) using the gamma prior distribution
423 instead of the default inverse uniform distribution, and 2) changing the degree of smoothness of the *h*
424 function from the default (100) to 50 and 1000 (Bauer et al., 2020a; Valeri et al., 2017). We also

performed a complete case analysis, where we fit BKMR-CMA models for one *a priori* selected CVLT outcome (trial 5 recall) in a restricted sample that included only adolescents with complete data for all biomarkers, CVLT outcomes, and confounders (n= 329).

2.5.7 Generalized additive models (GAMs) and multivariable linear regression. To investigate the robustness of our BKMR-CMA findings, we additionally used a regression-based causal mediation approach to examine mediation of the association of the individual metals by Fe status. There was limited evidence that the Fe status markers (ferritin, hemoglobin, transferrin) were associated with CVLT-C scores on any subtest, or of mediation of the overall mixture by Fe status, in BKMR-CMA models. We therefore present findings from regression-based approaches only for the association of Cu with trial 5 because Cu was found to drive the beneficial association of the metal mixture with the recall trial outcomes in our previous work (Schildroth et al., 2023).

Because there was evidence of non-linear associations of the metals with Fe status metrics in BKMR-CMA models, we first used Generalized Additive Models (GAMs) to visually inspect the shape of the associations between each metal with the CVLT-C outcomes, each metal with the Fe status metrics, and each Fe status metric with the CVLT-C outcomes. These models were fit using penalized splines (knots= 4) to allow for non-linear associations, and likelihood ratio tests (LRTs) were used to compare fits between the models with and without the penalized splines. GAM plots and LRTs confirmed non-linear associations of the nutrient transition metals (Mn, Cu, Cr) with markers of Fe status observed in the BKMR-CMA models, which is consistent with prior literature (Schildroth et al., 2022a). These essential metals were therefore modeled using GAMs with penalized splines (knots= 4) to account for non-linearity in the mediator regression models that characterized the association between the individual metals and markers of Fe status [Equation 4]. Pb was modeled continuously in these models. Although non-linear associations of the nutrient metals with neurodevelopmental outcomes have been reported previously in the literature (Bauer et al., 2020a; Claus Henn et al., 2010), there was no evidence of non-linear associations of any of the metals or Fe status markers with the CVLT scores. Therefore, all biomarker concentrations were modeled continuously in the outcome linear regression models in Equation [5].

Regression models were fit using the regression-based approach in the *CMAverse* package in R (Shi et al., 2021; Valeri and VanderWeele, 2013). Mediation effects for the regression-based approach were estimated by fitting an outcome model and a mediator model, specified in Equations [4] and [5], respectively. These models were fully adjusted for all *a priori* selected confounders, and adjusted for all other metals (Pb, Mn, and Cr).

[4] $Fe\ status\ metric = \beta_0 + \beta_1 * s(Cu) + \beta_2 * s(Cr) + \beta_3 * s(Mn) + \beta_4 * Pb + \beta_5 * Sex + \beta_6 * Age + \beta_7 * SES + \beta_8 * HOME\ score$

[1]These models were fit using GAMs; s() denotes a penalized spline (knots= 4)

[5] $Trial\ 5\ recall = \beta_0 + \beta_1 * Cu + \beta_2 * Cr + \beta_3 * Mn + \beta_4 * Pb + \beta_5 * Sex + \beta_6 * Age + \beta_7 * SES + \beta_8 * HOME\ score + \beta_9 * Fe\ status\ metric$

[1]These models were fit using linear regression

As we have previously reported (Schildroth et al., 2023), there was evidence of an interaction between Cu and ferritin for trial 5 recall. Allowing for exposure-mediator interaction is pertinent for correctly specifying the outcome model (VanderWeele, 2016). Therefore, we included an interaction term between Cu and ferritin in the outcome linear regression model when ferritin was considered the mediator.

In the regression-based approach, the total effect (TE) is decomposed into the pure natural direct effect (PNDE) and the total natural indirect effect (TNIE). The PNDE reflects the direct association of the exposure with the outcome when the mediator is set to its natural value at low concentration levels of the exposure, and the TNIE reflects the indirect association of the exposure with the outcome due to both mediation and mediator-exposure interaction (Shi et al., 2021; Valeri and VanderWeele, 2013; Vanderweele, 2014). We report the PNDE and TNIE for the association of Cu with trial 5 when ferritin, transferrin, and hemoglobin were considered the mediators. We also report the CDE for these associations, setting the mediators at their 50th percentiles. These mediation effects are visualized in **Figure 2B**.

3. RESULTS

About half the participants in the analytic sample were male (53%), lived in the Bagnolo Mella study site (53%) and came from families characterized as medium SES (53%, **Table 1**). The mean age was 12.3 years (SD: 1.0) and the mean HOME score was 6.0 (SD: 1.7). Median (25^{th} – 75^{th} percentile) hair concentrations for Mn, Cu, and Cr were 0.07 µg/g (0.04 – 0.12 µg/g), 9.4 µg/g (6.6 – 14.8 µg/g), and 0.04 µg/g (0.03 – 0.06 µg/g), respectively. The median blood Pb concentration was 1.3 µg/dL (1.0 – 1.7 µg/g). Females had higher median concentrations of hair Cu (10.3 µg/g; 25^{th} – 75^{th} percentiles: 7.6 – 16.5 µg/g) compared to males (8.6 µg/g; 25^{th} – 75^{th} percentiles: 6.3 – 13.7 µg/g), but males had higher median concentrations of blood Pb (1.4 µg/dL; 25^{th} – 75^{th} percentiles: 1.1 – 2.0 µg/dL) compared to females (1.1 µg/dL; 25^{th} – 75^{th} percentiles: 0.9 – 1.4 µg/dL). Median concentrations of Mn and Cr were similar in males and females (**Table S4**). Spearman correlation coefficients between the metals were weak and ranged from 0.01 (blood Pb – hair Cu) to 0.35 (hair Mn – hair Cr).

Median concentrations for serum ferritin (32.0 ng/mL; 25^{th} – 75^{th} percentile: 21.0 – 44.0 ng/mL), serum transferrin (283.0 mg/dL; 25^{th} – 75^{th} percentile: 260.0 – 308.0 mg/dL), and blood hemoglobin (13.8 g/dL; 25^{th} – 75^{th} percentile: 13.2 – 14.4 g/dL) were within normal clinical ranges for adolescents (Gibson, 2005). Males had higher median concentrations of ferritin (33.0 ng/mL vs. 30.0 ng/mL), transferrin (284.0 mg/dL vs. 282.5 mg/dL) and hemoglobin (14.0 g/dL vs. 13.6 g/dL) (**Table S4**) compared to females, which is typical during the adolescent period (Gibson, 2005).

3.1 Associations of the Metal Mixture with Fe Status

The metal mixture was inconsistently associated with concentrations of Fe status markers. Compared to the 50^{th} percentile of the overall mixture, setting the metal mixture (Pb, Mn, Cr, Cu) to its 90^{th} percentile was associated with lower serum ferritin concentrations (β= -0.19, 95% CI= -0.46, 0.07). Conversely, the 90^{th} percentile of the mixture was associated with a 0.33 SD increase (β= 0.33, 95% CI= 0.03, 0.63) in hemoglobin concentrations, compared to when the metal mixture was set to its 50^{th} percentile. The metal mixture was not materially associated with transferrin concentrations (**Figure 3**).

505 The association of the mixture with ferritin was driven by Pb (PIP= 0.83), where a change in Pb
506 from its 25th to 75th percentiles was associated with a 0.33 SD decrease (95% CI= -0.58, -0.07) in serum
507 ferritin concentrations when the rest of the mixture was held at its 50th percentile (**Figure 4**). The
508 association of the mixture with hemoglobin concentrations was also driven by Pb; however, a change in
509 Pb from its 25th to 75th percentiles was associated with higher concentrations of hemoglobin (β= 0.43,
510 95% CI= 0.18, 0.68) when the mixture was held at its 50th percentile (**Figure S1**). None of the metals in
511 the mixture were materially associated with transferrin concentrations (**Figure S2**).
512
513 ***3.2 Associations of the Metal Mixture with CVLT-C Outcomes***
514 Consistent with our previous findings in PHIME (Schildroth et al., 2023), the metal mixture was
515 jointly associated with better performance on the recall trials, especially long delay free recall, when each
516 of the Fe status metrics was included in the mixture model (**Figures S3-S11, panel C**). Compared to the
517 50th percentile, the 90th percentile of the mixture was associated with higher scores for long delay free
518 recall scores when ferritin (β= 0.20, 95% CI= -0.15, 0.55), transferrin (β= 0.22, 95% CI= -0.12, 0.55), and
519 hemoglobin (β= 0.22, 95% CI= -0.08, 0.53) were included in the model, respectively (**Figure S3**). Similar
520 associations were observed for trial 5 recall and long delay cued recall (**Figures S6-S11, panel C**), though
521 the beta coefficients were smaller in magnitude. The positive association of the mixture with the recall
522 trials was driven primarily by copper (**Figures S3-S11**).
523 The metal mixture was also associated with higher scores for perseveration errors, reflecting
524 worse cognitive performance, when each of the Fe status metrics was included as a component of the
525 mixture. The 90th percentile of the mixture, compared to the 50th percentile, was associated with a 0.25
526 SD increase (95% CI= -0.07, 0.56), 0.23 SD increase (95% CI= -0.15, 0.60), and 0.22 SD increase (95%
527 CI= -0.10, 0.54) in ln-transformed perseveration errors when ferritin, transferrin, and hemoglobin were
528 included in the model, respectively. These associations were primarily driven by the association between
529 Pb and higher number of perseveration errors (**Figures S12-S14, panel C**).
530

3.3 Mediation of the Metal Mixture by Fe Status

Figure 5 shows the mediation effects of Fe status for trial 5, long delay free, and long delay cued recall in BKMR-CMA models. Notably, there was no evidence of mediation by Fe status: the NIE, which reflects the indirect association of the mixture with neurodevelopment mediated through Fe status, was null across all three Fe status metrics for trial 5 (**Figure 5, panel A**), long delay free (**Figure 5, panel B**), and long delay cued (**Figure 5, panel C**) recall. Conversely, the NDE, which reflects the direct association of the mixture (for a change from its 25th to 75th percentiles) not mediated by Fe status, was positive for all three recall trials when ferritin, hemoglobin, or transferrin were considered the mediators (**Figure 5, panels A-C**), suggesting better cognitive performance with increasing metal concentrations, independent of Fe status. The strongest associations were observed for LDFR: the NDE was estimated to be 0.23 (95% CI= 0.01, 0.44), 0.23 (95% CI= 0.02, 0.44), and 0.23 (95% CI= 0.01, 0.45) when ferritin, hemoglobin, and transferrin, respectively, were considered the mediators (**Figure 5, panel B**). These findings suggest that associations of the metal mixture with the recall trials in these data operate exclusively through the direct pathway and are not mediated by Fe status.

There was also no evidence of mediation by any Fe status marker for the association between Cu and trial 5 in the regression-based approach (**Table S5**). For example, when ferritin was considered the mediator, the TNIE was null (β= 0.00, 95% CI= -0.01, 0.01), while the PNDE suggested a beneficial association of Cu with trial 5 (β= 0.21, 95% CI= 0.11, 0.30) that operated exclusively on the direct pathway. Similar associations were also found when hemoglobin or transferrin were considered the mediator (**Table S5**).

The NIE was also null for perseveration errors, suggesting no evidence of mediation by any Fe status metric (**Figure 6**). However, the NDE (for a change in the mixture from its 25th to 75th percentiles) was positive for perseveration errors, indicating adverse associations with cognitive performance. The NDE was estimated to be 0.13 (95% CI= -0.08, 0.37), 0.14 (95% CI= -0.08, 0.38), and 0.13 (95% CI= -0.08, 0.36) when ferritin, transferrin, or hemoglobin were considered the mediator. As with the recall

trials, these findings suggest that the adverse association of the mixture with perseveration errors operated exclusively via the direct pathway.

The mixture was not materially associated with forgetting, and there was similarly no evidence of mediation by any of the Fe status markers (**Figure S15**).

3.4 Sex-stratified Analyses: Associations of the Metal Mixture with Fe Status

In sex-stratified BKMR-CMA models, the negative association of the mixture (Pb, Mn, Cr, Cu) at its 90th percentile, compared to the 50th percentile, with ferritin was stronger in males (β= -0.21; 95% CI= -0.69, 0.28) compared to females (β= -0.08; 95% CI= -0.44, 0.29, **Figure 7, panels A and B**). The positive association of the mixture at its 90th percentile, compared to the 50th percentile, with hemoglobin was also stronger in males (β= 0.34; 95% CI= -0.03, 0.70) compared to females (β= 0.16; 95% CI= -0.26, 0.59, **Figure 7, panels C and D**). Higher concentrations of the mixture (Pb, Mn, Cr, Cu) were not materially associated with transferrin in males (at the 90th percentile, compared to the 50th percentile: β= -0.09; 95% CI= -0.46, 0.28) or females (at the 90th percentile, compared to the 50th percentile: β= -0.12; 95% CI= -0.52, 0.28, **Figure 7, panels E and F**). As with our main findings, the joint associations of the mixture with ferritin and hemoglobin among males were driven primarily by Pb (**Figure S16**).

3.5 Sex-stratified Analyses: Associations of the Metal Mixture with CVLT-C Outcomes

As we have previously reported (Schildroth et al., 2023), there was evidence of sex-specific associations of the mixture with our indices of neurodevelopment, though these associations tended to be imprecise. Notably, joint increases in the overall mixture were associated with better recall scores only among females. For example, the 90th percentile of the mixture (compared to the 50th percentile) was associated with a 0.25 SD increase (95% CI= -0.39, 0.90) in trial 5 recall scores among females, but a 0.14 decrease (95% CI= -0.63, 0.36) in males when ferritin was included in the model. Similar associations were observed for the other recall trials, and these sex-specific associations were driven primarily by stronger associations of Cu with the recall trials among females (data not shown). As in the main findings, there was no evidence of mediation by any Fe status metric in either sex (**Figures S17-S21**).

3.6 BKMR-CMA Sensitivity Analyses

We performed a series of sensitivity analyses to examine the robustness of our main BKMR-CMA findings: 1) a complete case analysis, where we restricted the analytic sample to adolescents with no missing data (n= 329); 2) an analysis changing the default uniform prior distribution to a gamma distribution; and 3) an analysis changing the default smoothness of the *h* function (100) to 50 and 1000.

The mediation effects for trial 5 recall, including the NDE, NIE, TE, and CDEs, from sensitivity analyses that used the gamma prior distribution and changed the smoothness of the *h* function to 50 or 1000 were consistent with our main findings (**Figures S22-S24**). Findings for the mediation effects for the complete case analysis were also similar to our main findings, though associations tended to be stronger (**Figure S25**). For example, the NDEs for trial 5 in the complete case analysis for ferritin, transferrin, and hemoglobin were 0.19 (95% CI= -0.04, 0.41; main model: β= 0.12; 95% CI= -0.09, 0.34), 0.18 (95% CI= -0.05, 0.40; main model: β= 0.14; 95% CI= -0.08, 0.35), and 0.18 (95% CI= -0.04, 0.41; main model: β= 0.14; 95% CI= -0.08, 0.36), respectively. There was no evidence of mediation by any Fe status metric in any of the sensitivity analyses.

Associations of the metals with ferritin, transferrin, and hemoglobin also tended to be similar in sensitivity analyses (**Figures S26-S37**), with one notable exception: the association of Pb with ferritin that we observed in the main model (**Figure 3**) was null in the analysis that used the gamma distribution (**Figure S26**) and in the complete case analysis (**Figure S35**).

4. DISCUSSION

In this study of Italian adolescents residing near ferroalloy production sites, we found that a mixture of Pb, Mn, Cu, and Cr was jointly associated with aspects of verbal learning and memory. As we have previously reported, the associations of the overall mixture with the recall trials and perseveration errors were driven primarily by Cu (beneficial) and Pb (adverse), respectively (Schildroth et al., 2023). The metal mixture was also associated with markers of Fe status, (i.e., ferritin and hemoglobin), but there was no evidence that Fe status mediated the association of the metal mixture with neurodevelopment.

When considering the association of the overall mixture with verbal learning and memory, we found that a joint increase in all components of the mixture (Pb, Mn, Cu, Cr, marker of Fe status) was positively associated with the recall trials when any of the three markers of Fe status (ferritin, hemoglobin, or transferrin) was included in the mixture. These associations were primarily driven by Cu, which we have reported on previously (Schildroth et al., 2023), and may reflect the role of Cu as an essential nutrient needed for catecholamine synthesis, cellular respiration, formation/maintenance of myelin, and long-term potentiation (Gaetke et al., 2014; Opazo et al., 2014). It should also be noted that, although we observed beneficial associations of Cu with cognition, previous studies have reported beneficial, adverse and nonlinear associations of Cu with neurodevelopment outcomes (Amorós et al., 2019; Bauer et al., 2020a; Liu et al., 2018; Zhou et al., 2015), likely reflecting differences in dose, among other factors. Conversely, the overall mixture was concurrently associated with increased perseveration errors, reflecting worse cognitive performance. This association was driven primarily by Pb, which is consistent with the known toxicological mechanisms of Pb (e.g., disruption of neurotransmitter release and neuronal plasticity) and previous research in pediatric cohorts (Neal and Guilarte, 2013; Sanders et al., 2009).

We further found that the mixture was jointly associated with lower concentrations of serum ferritin, and that this association was primarily due to Pb. Increased blood Pb concentrations have previously been associated with decreased concentrations of markers of Fe status in children (Choi and Kim, 2005; Hegazy et al., 2010; Jeong et al., 2015). The hematologic toxicity of Pb is well established: Pb shares mechanisms of uptake and transport with Fe, disrupts enzymes involved in *heme* synthesis, interferes with the expression of transferrin, disrupts cellular membranes, and has been associated with decreased concentrations of erythropoietin (EPO), the primary hormone that stimulates the production of erythrocytes (Kordas, 2010; Levander, 1979). Although most studies have found Pb-induced hematologic toxicity at concentrations >10 µg/dL, several studies with blood Pb concentrations ≤10 µg/dL have reported decreased platelet counts and inhibition of δ-ALAD, an enzyme active in the production of *heme* (Agency for Toxic Substances and Disease Registry, 2020). These studies suggest that Pb hematologic

toxicity may occur at Pb concentrations <10 µg/dL; however, most prior studies were cross-sectional in design and conducted in adults or in populations with small sample sizes. Other studies in children have alternatively reported higher blood Pb concentrations among children who were Fe-deficient (Ataur Rahman et al., 2012; Bradman et al., 2001; Khan et al., 2011). Because the toxicokinetics and toxicodynamics of Pb are closely related to those of Fe (Agency for Toxic Substances and Disease Registry, 2020), further research in larger pediatric cohorts with longitudinal designs, particularly in populations with low environmental Pb exposure, is warranted.

Conversely, increasing percentiles of the mixture were jointly associated with higher concentrations of hemoglobin in the current analysis, and this association was again driven by Pb. However, given the cross-sectional nature of this study and known hematologic toxicity of Pb, this finding should not be interpreted as a beneficial association of Pb on hematologic function. One alternative explanation of this finding is that EPO production was higher in response to low-level Pb exposure in our Fe-replete population. EPO is secreted by the kidneys, and concentrations increase under certain conditions (e.g., low oxygen, blood loss, damaged or malfunctioning erythrocytes) (Agency for Toxic Substances and Disease Registry, 2020; Suresh et al., 2020). This suggests that EPO may increase following toxicant exposure as a compensatory mechanism against the hematologic toxicity of Pb (e.g., disruption of *heme* synthesis). Although we did not have EPO concentrations to further investigate this hypothesis in the PHIME cohort, a compensatory mechanism was observed in one study of 5-year-old Fe-replete children, where environmental Pb exposure was associated with increased EPO concentrations (Factor-Litvak et al., 1998). Thus, the hypothesized compensatory mechanism could explain the positive association of blood Pb with Hb in our analysis. Alternatively, this finding could be explained by residual confounding by other nutrients (e.g., vitamin A, vitamin B6, vitamin B12, riboflavin, or folic acid) or dietary factors that have been associated with both Fe status and metals exposure (Al-Attar, 2011; Ferri et al., 2015, 2012; Fishman et al., n.d.; Levander, 1979). For example, we have previously shown that Pb was detectable in soils and Fe-rich vegetables (e.g., spinach) grown in gardens near ferroalloy industry, suggesting diet may be an important confounder in this population (Ferri et al., 2015, 2012). It is also

possible that this is a spurious statistical correlation given the cross-sectional nature of our data and the known kinetics of Pb in the body (e.g., >90% of whole blood Pb is bound to hemoglobin) (Collin et al., 2022), highlighting the need for additional studies with longitudinal designs.

Although the overall mixture was associated with indicators of neurodevelopment and markers of Fe status, there was no evidence that Fe status mediated the associations between the metal mixture and verbal learning and memory. These results were consistent across three clinical markers of Fe status. We are aware of only one prior study that considered metals and Fe status in a mediation analysis of neurodevelopmental outcomes. Using cross-sectional data from a population of 5-year old Korean children, Jeong et al. found that blood Pb was a partial mediator of the positive association between ferritin and verbal IQ, whereby exposure to Pb attenuated the beneficial effect of ferritin on neurodevelopment (Jeong et al., 2015). We likely did not observe any mediation by Fe status in the current analysis because Fe status was, on average, sufficient and not strongly related to the neurodevelopmental outcomes in this study population. Fe-deficiency has been associated with worse neurodevelopment in other pediatric cohorts because Fe is required for processes of neuronal development and function (e.g., metabolism, myelination, neurotransmitter synthesis) (Halterman et al., 2001; Jáuregui-Lobera, 2014; Ji et al., 2017; Lukowski et al., 2010; Parkin et al., 2020; Roy et al., 2011; Tseng et al., 2018; Wang et al., 2019, 2017). Jeong et al. classified nearly half of their participants as having either low (<15 ng/mL) or low-normal ferritin levels (15.0 <30.0 ng/mL) (Jeong et al., 2015), whereas the PHIME population had minimal indication of Fe deficiency (median serum ferritin: 32.0 ng/mL). This likely explains, at least in part, the null mediation findings in our study. However, because Fe-deficiency, and specifically anemia, has been consistently associated with neurodevelopment in children (Halterman et al., 2001; Jáuregui-Lobera, 2014; Ji et al., 2017; Lukowski et al., 2010; Parkin et al., 2020; Roy et al., 2011; Tseng et al., 2018; Wang et al., 2019, 2017), mediation by Fe status is still possible, and other prospective studies with larger variations in Pb and Fe levels are warranted.

In sex-stratified models, the beneficial associations of the mixture with the recall trials were stronger among females. Sex-specific associations of Cu with neurodevelopment have been previously

688 reported, such that Cu was more strongly associated with adverse cognitive scores in males (Amorós et
689 al., 2019; Zhou et al., 2015). Animal data are inconsistent: studies have reported higher susceptibility to
690 Cu toxicity among both males and females (Chen et al., 2006; Lamtai et al., 2020). These findings may
691 relate to differences in hormonal modulation of Cu-induced neurotoxicity (Lamtai et al., 2020). In the
692 current study, sexual dimorphic findings may also be due to differential Cu concentrations between the
693 sexes (median: females, 10.3 µg/g; males, 8.6 µg/g). However, further research is needed to better
694 understand sex-specific impacts of Cu on neurodevelopment.
695 We also found sexual dimorphic associations between the metal mixture and markers of Fe status:
696 the association of the overall mixture, driven by Pb, with lower ferritin concentrations and higher
697 hemoglobin concentrations was stronger in males compared to females. These findings are contrary to a
698 previous epidemiological study in adolescents that found stronger adverse associations of metals (Mn, Pb,
699 cadmium, selenium) or their mixture in females (Schildroth et al., 2022a). However, stronger associations
700 of metals with Fe status among females in this prior analysis were driven primarily by Mn and cadmium,
701 and biomarker concentrations of these metals tended to be higher in females compared to males
702 (Schildroth et al., 2022a). In contrast, the sex-specific associations in the current analysis were driven by
703 Pb, where median Pb concentrations were modestly higher in males (1.4 µg/dL) than in females (1.1
704 µg/dL). This might partly explain why we observed stronger associations of the metal mixture with
705 markers of Fe status in males compared to females in our study. As with our main analysis in the full
706 cohort, there was no evidence that Fe status mediated the association of the metal mixture with
707 neurodevelopment in sex-stratified analyses.
708 This analysis had several strengths. This analysis is among the first to use the novel BKMR-CMA
709 approach to examine mediation of any chemical mixture, and this study was the first to assess mediation
710 of any metal or a metal mixture by Fe status in relation to neurodevelopmental outcomes. We were also
711 able to examine mediation by multiple clinically relevant biomarkers for Fe status that each quantify
712 different aspects of Fe status with various sensitivities for reflecting Fe deficiency (Gibson, 2005). We
713 further quantified metals (Pb, Mn, Cr, and Cu) using biomarkers that have been consistently utilized in

previous epidemiological studies of neurodevelopment (Bauer et al., 2020a; Caparros-Gonzalez et al., 2019; Carvalho et al., 2018; Oulhote et al., 2014; Torres-Agustín et al., 2013; Wright et al., 2006; Yorifuji et al., 2011). Our study also focused on the adolescent period, an understudied yet critical period for neuronal maturation, physical growth, and possible Fe deficiency (Anttila et al., 1997; Arain et al., 2013; Das et al., 2017; Mesías et al., 2013; Shaw et al., 2020). Lastly, we utilized the CVLT-C to assess neurodevelopment, which is an objective and commonly used test of verbal learning and memory in children and adolescents (Lezak et al., 2012).

The primary limitation of this analysis was its cross-sectional design, where the metals, Fe status, and neurodevelopment were assessed concurrently. Reverse causation is therefore possible, and longitudinal studies are needed to confirm findings. Residual confounding is also possible as we were missing data on key covariates that may be associated with metals, Fe status, and neurodevelopment. For example, we were not able to control for biomarkers of inflammation (e.g., C-reactive protein) that may impact levels of Fe status markers (Gibson, 2005), other co-exposures (e.g., nickel), or past exposures (e.g., in the prenatal and early childhood periods) that have similarly been associated with neurodevelopment in adolescence (Bauer et al., 2021; Lamtai et al., 2018; Rechtman et al., 2022). We had a limited sample size (n= 383), which likely impacted the precision of our estimates.

5. CONCLUSION

In conclusion, we found that an environmentally relevant metal mixture was associated with Fe status and aspects of verbal learning and memory, though mediation of the mixture by Fe status was not observed. Fe status should nonetheless be considered as a possible mediator of metal mixtures in future studies of neurodevelopment, especially in populations with Fe deficiency or higher environmental metal exposure given the known hematologic toxicity of metals like Pb, and the mechanistic overlap of Fe with other metals.

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Conflicts of Interest. The authors declare no conflicts of interest.

Abbreviations

Pb= lead, Mn= manganese, Cu= copper, Cr= chromium, Fe= iron, PHIME= Public Health Impact of Metals Exposure, IQ= intelligence quotient, CVLT-C= California Verbal Learning Test for Children, SES= socioeconomic status, HOME= Home Observation for Measurement of the Environment, LOD= limit of detection, CBC= complete blood count, DAG= directed acyclic graph, BKMR-CMA= Bayesian Kernel Machine Regression Causal Mediation Analysis, PIP= posterior inclusion probability, CDE= controlled direct effect, NDE= natural direct effect, NIE= natural indirect effect, TE= total effect, CI= credible interval, GAM= generalized additive model, LRT= likelihood ratio test, PNDE= pure natural direct effect, TNIE= total natural indirect effect, SD= standard deviation.

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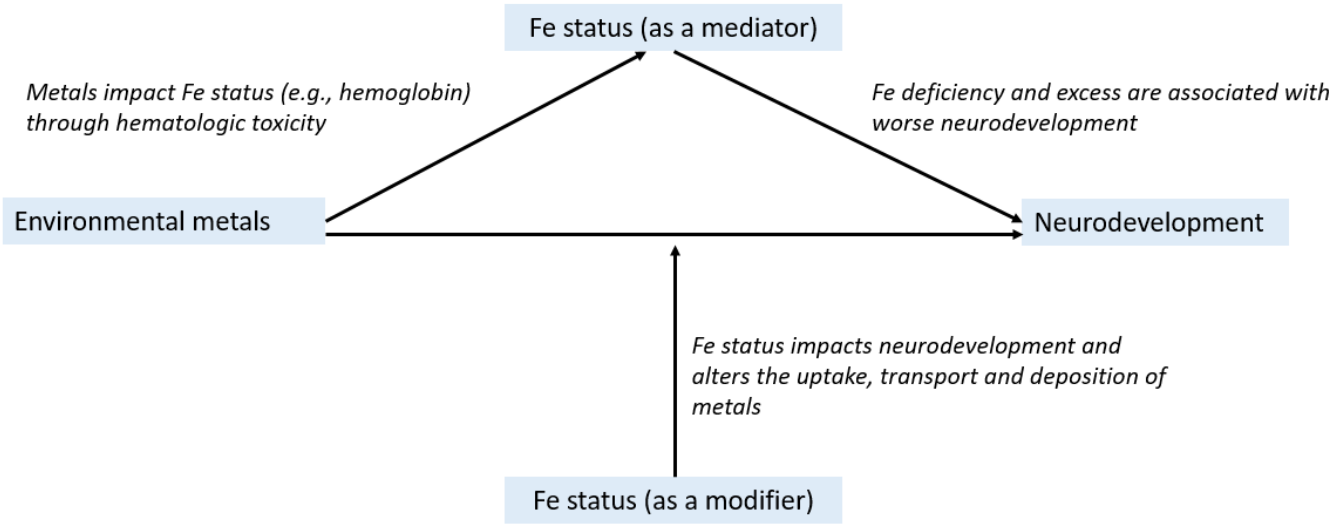
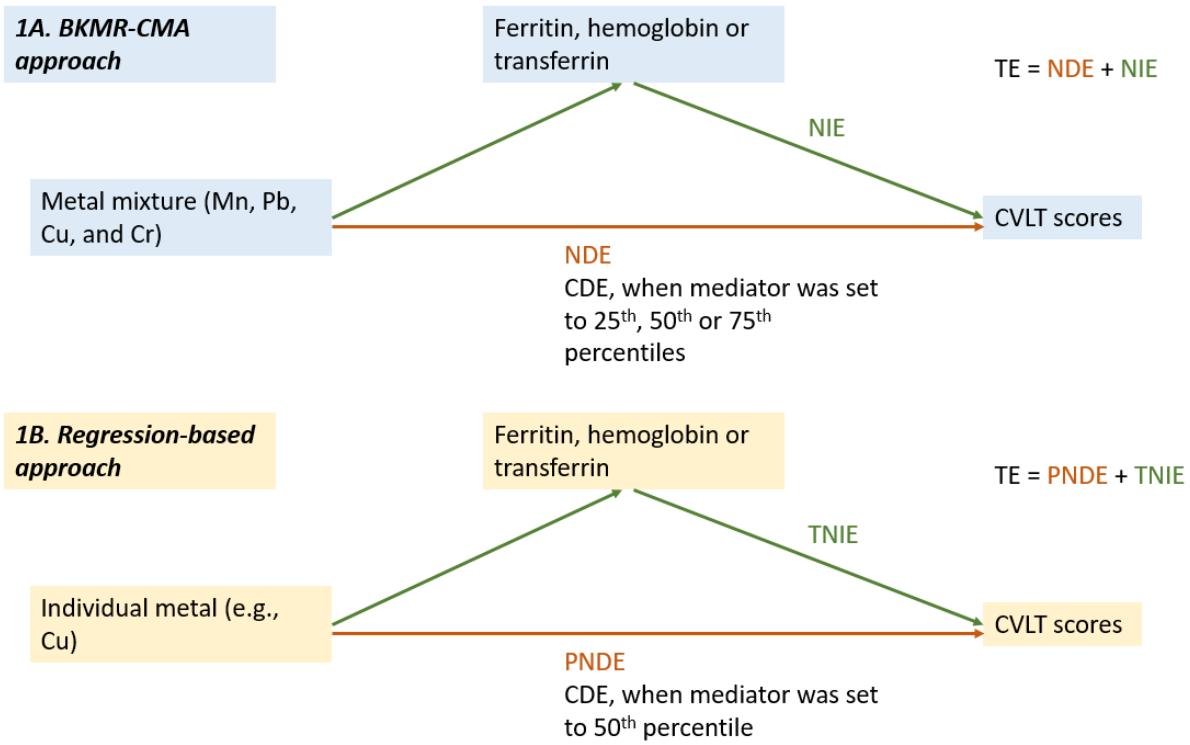
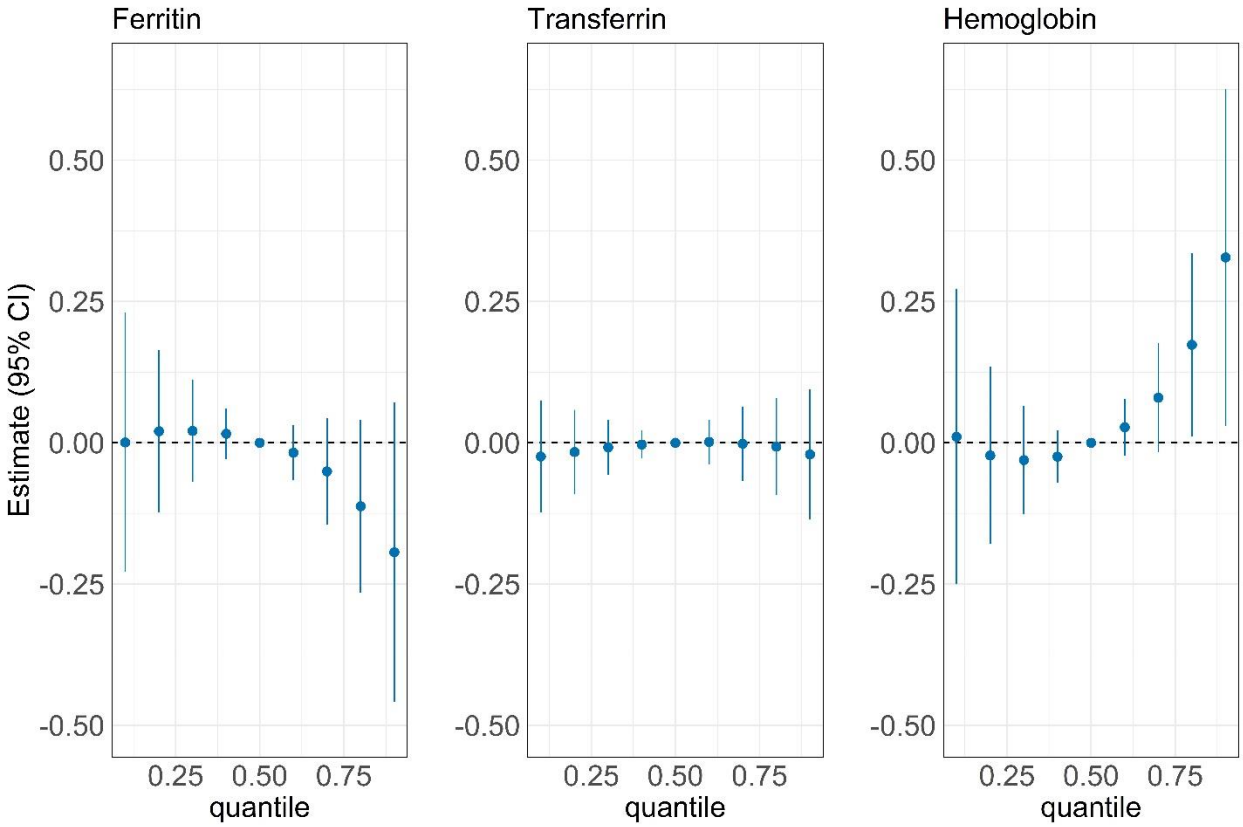
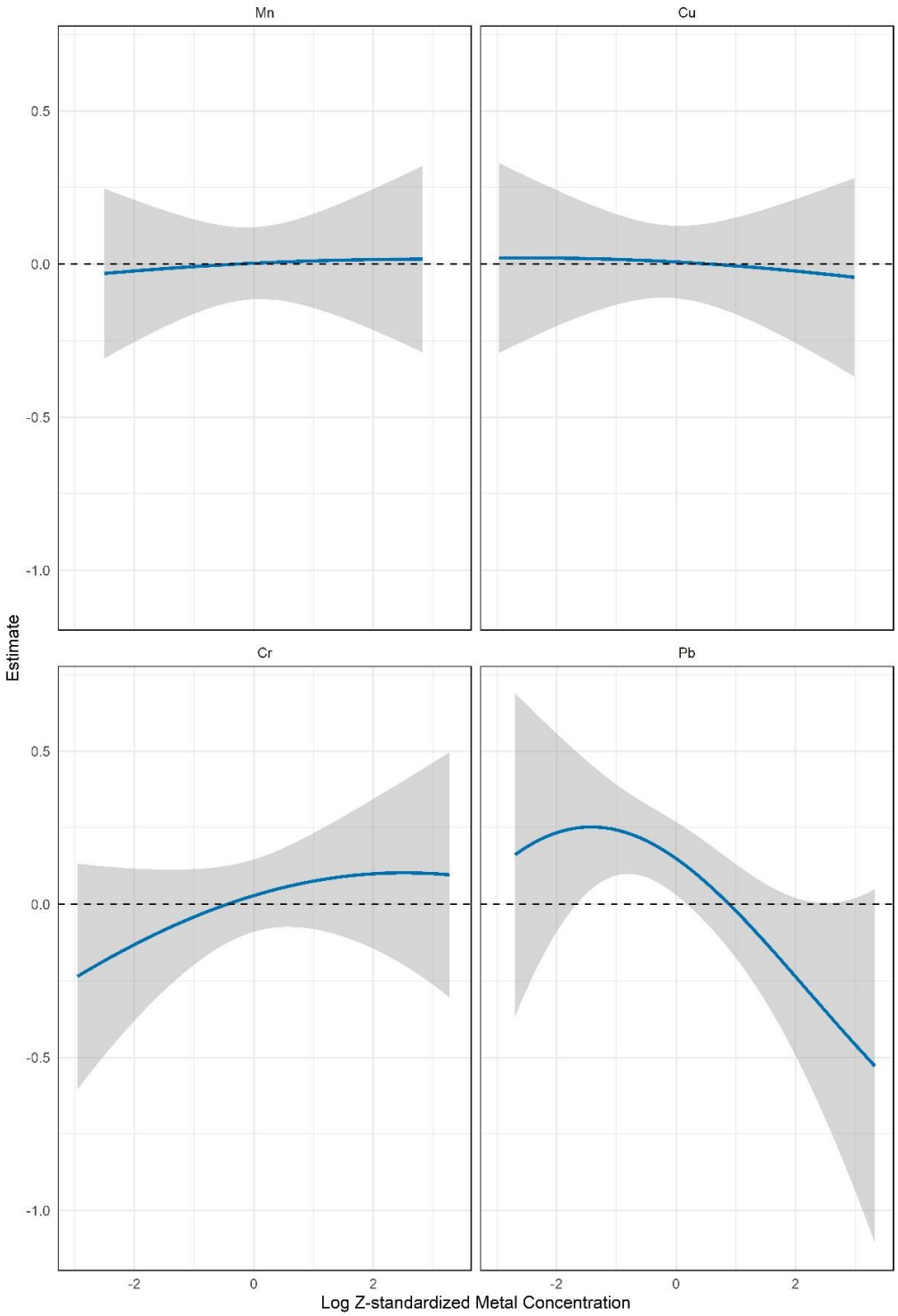

Fe status (as a mediator)
Metals impact Fe status (e.g., hemoglobin) through hematologic toxicity
Fe deficiency and excess are associated with worse neurodevelopment
Environmental metals
Neurodevelopment
Fe status impacts neurodevelopment and alters the uptake, transport and deposition of metals
Fe status (as a modifier)



Note: BKMR-CMA, Bayesian Kernel Machine Regression Causal Mediation Analysis; TE, total effect; NDE, natural direct effect; NIE, natural indirect effect; CDE, controlled direct effect; Mn, manganese; Pb, lead; Cu, copper; Cr, chromium; CVLT, California Verbal Learning Test; PNDE, pure natural direct effect; TNIE, total natural indirect effect.



Note: Pb, lead; Mn, manganese; Cr, chromium; Cu, copper; BKMR, Bayesian Kernel Machine Regression; SES, socioeconomic status; HOME, Home Observation Measurement of the Environment.



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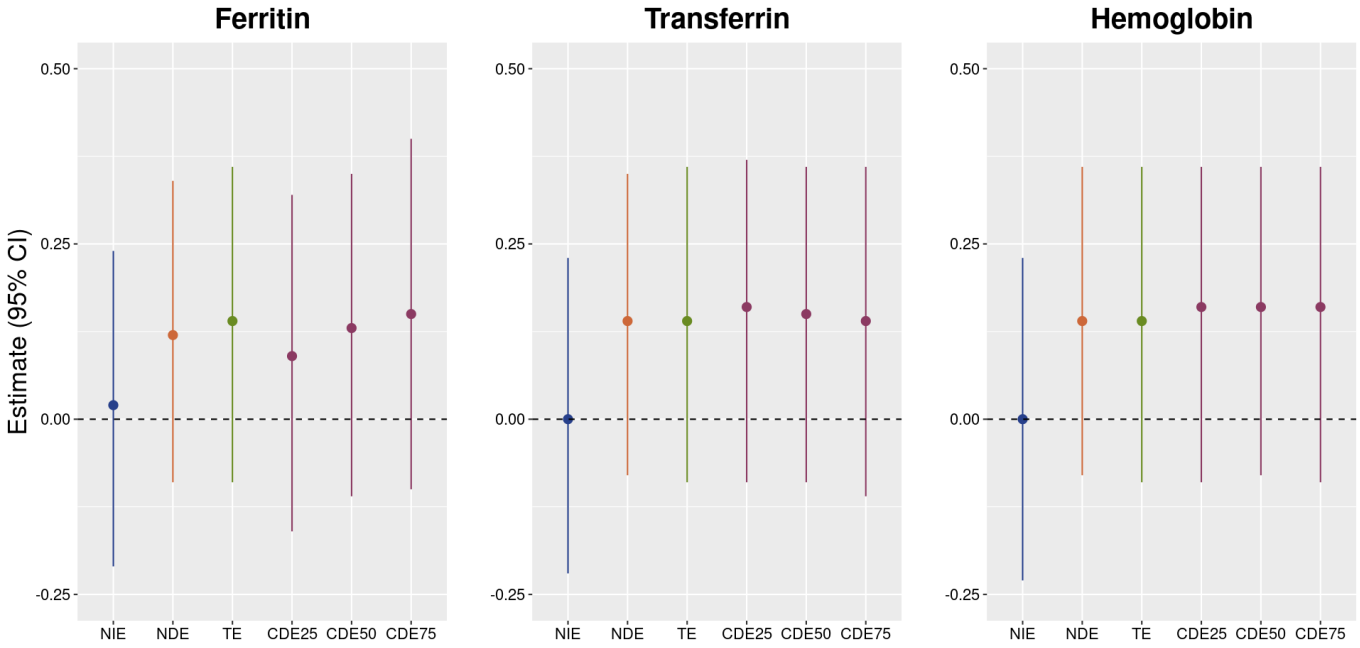
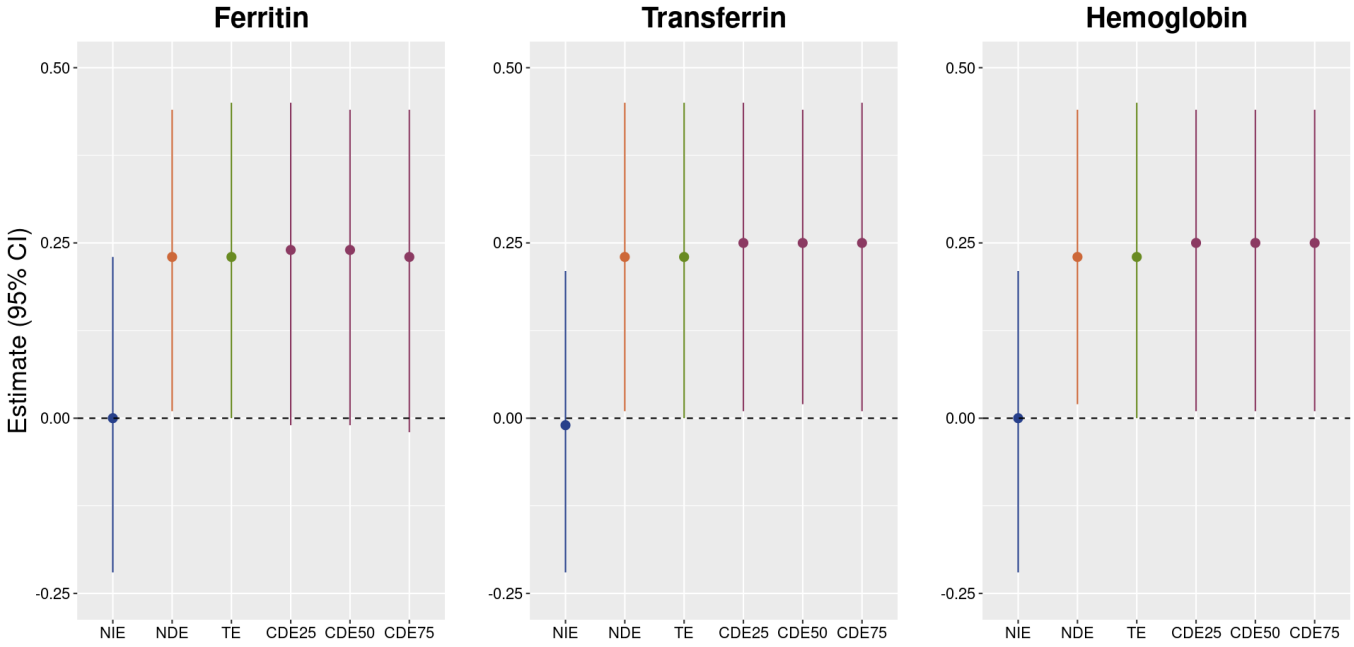
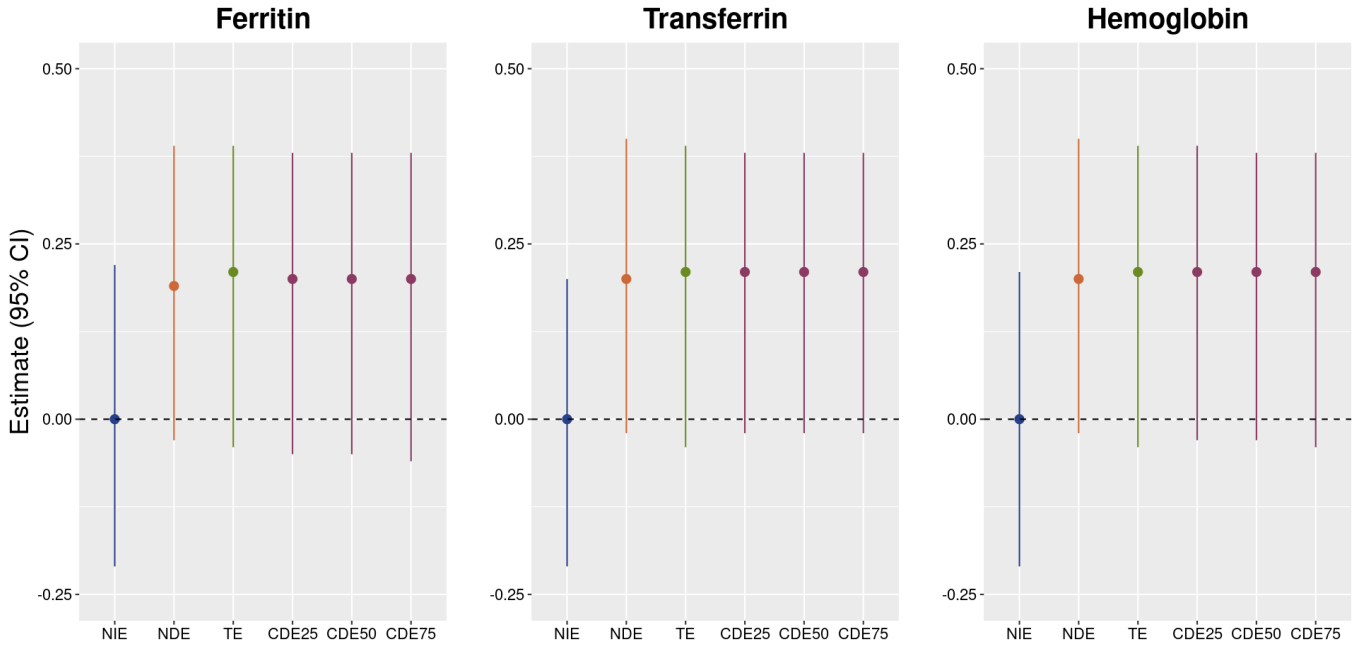
(A) Trial 5 Recall



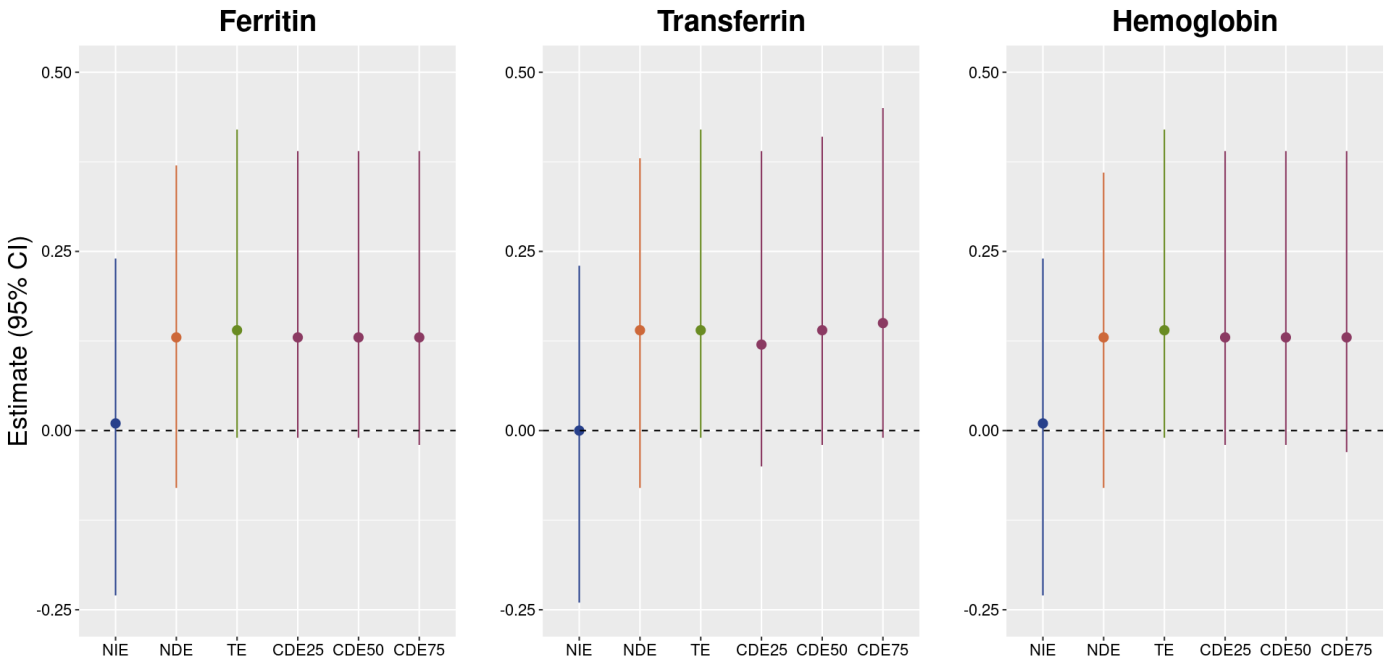
(B) Long Delay Free Recall



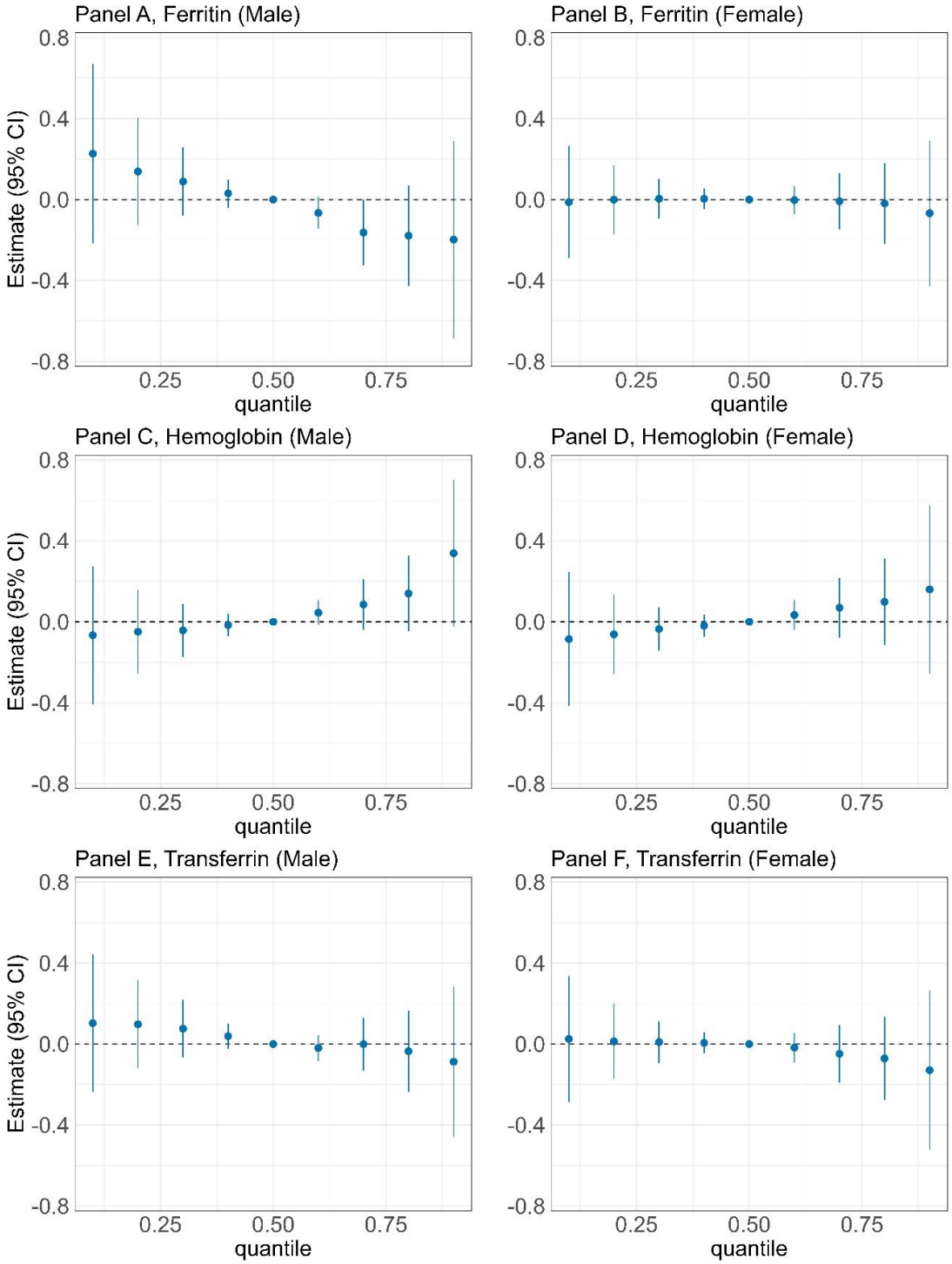
(C) Long Delay Cued Recall



Note: BKMR, Bayesian Kernel Machine Regression; SES, socioeconomic status; HOME, Home Observation Measurement of the Environment; NIE, natural indirect effect; NDE, natural direct effect; TE, total effect; CDE, controlled direct effect.



Note: Pb, lead; Mn, manganese; Cr, chromium; Cu, copper; BKMR, Bayesian Kernel Machine Regression; SES, socioeconomic status; HOME, Home Observation Measurement of the Environment; NIE, natural indirect effect; NDE, natural direct effect; TE, total effect; CDE, controlled direct effect.

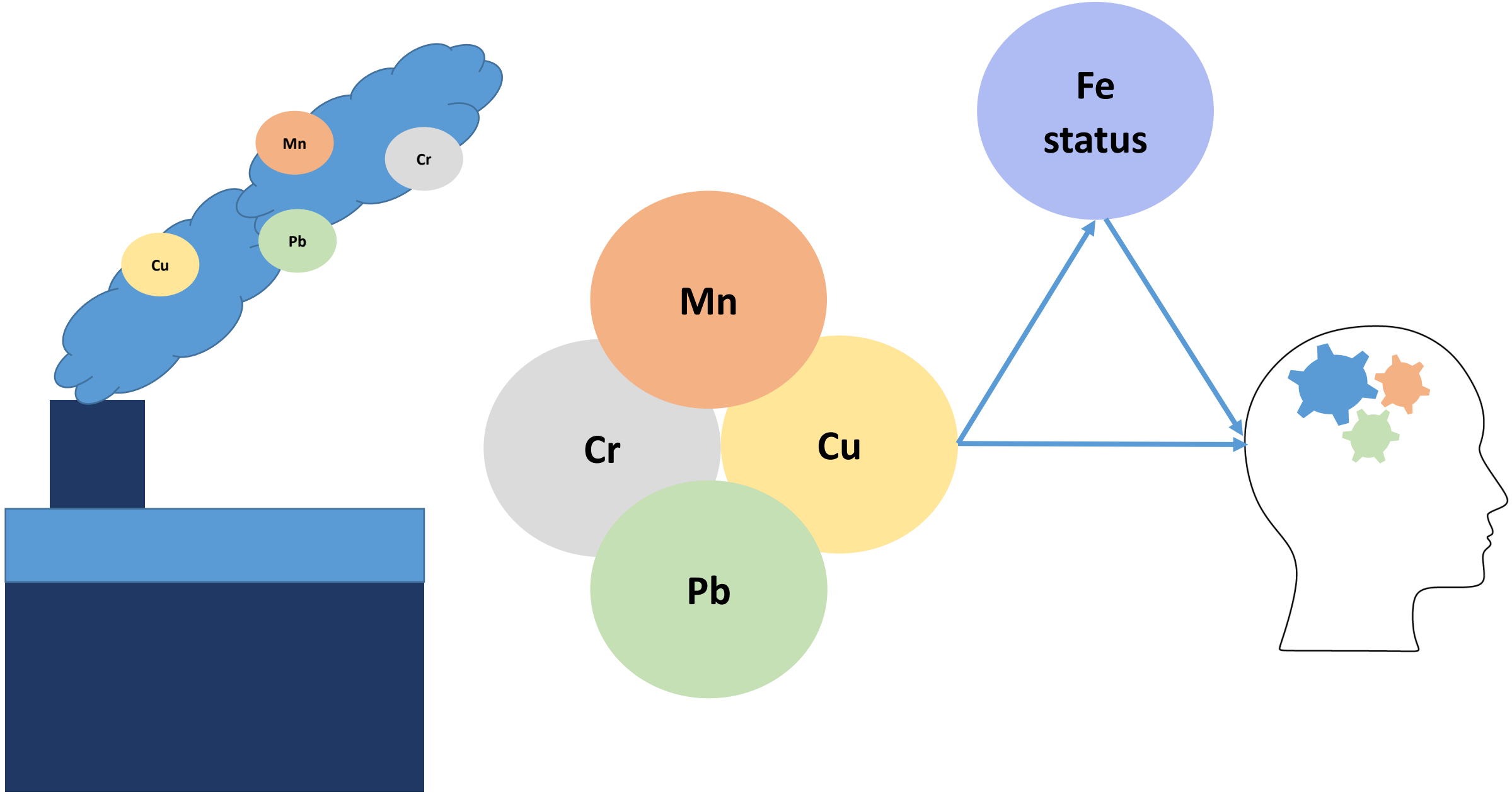


Note: Pb, lead; Mn, manganese; Cr, chromium; Cu, copper; BKMR, Bayesian Kernel Machine Regression; SES, socioeconomic status; HOME, Home Observation Measurement of the Environment.

Table 1. Characteristics of PHIME study participants included in present analysis (n= 383).

Characteristic	N (percent) or mean ± SD
Sex	
Female	182 (47.5%)
Male	201 (52.5%)
Age (years)	12.3 ± 1.0
Socioeconomic status index	
Low	89 (23.2%)
Medium	201 (52.5%)
High	93 (24.3%)
HOME score	6.0 ± 1.7
Site	
Bagnolo Mella	204 (53.3%)
Garda Lake	79 (20.6%)
Valcamonica	100 (26.1%)
CVLT-C outcomes (raw scores)	
Long delay free recall	11.5 ± 2.1
Long delay cued recall	11.8 ± 2.1
Trial 5	12.3 ± 1.9
Perseveration errors	7.2 ± 6.1
Forgetting	0.3 ± 1.6
Metal biomarkers (median, 25^{th}, 75^{th} percentile)	
Hair Mn (µg/g)	0.07 (0.04, 0.12)
Hair Cu (µg/g)	9.4 (6.6, 14.8)
Hair Cr (µg/g)	0.04 (0.03, 0.06)
Blood Pb (µg/dL)	1.3 (1.0, 1.7)
Iron biomarkers (median, 25^{th}, 75^{th} percentile)	
Ferritin (ng/mL)	32.0 (21.0, 44.0)
Transferrin (mg/dL)	283.0 (260.0, 308.0)
Hemoglobin (d/dL)	13.8 (13.2, 14.4)

[a]PHIME, Public Health Impact of Metals Exposure Study; HOME, Home Observation Measurement of the Environment; CVLT-C, California Verbal Learning Test for Children; Mn, manganese; Cu, copper; Cr, chromium; Pb, lead.


Mn
Cr
Pb
Cu
Mn
Cr
Cu
Pb
Fe
status