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Associations of an Industry-Relevant Metal Mixture with Verbal Learning and Memory in Italian Adolescents: The Modifying Role of Iron Status

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The authors declare they have nothing to disclose.

HIGHLIGHTS

- Environmental exposure to individual metals has been associated with neurodevelopmental outcomes in children, and these associations may be modified by iron (Fe) status. However, less is known about metal mixtures.
- A mixture of hair manganese (Mn), blood lead (Pb), hair copper (Cu), hair chromium (Cr), and serum ferritin was jointly associated with better scores on tests of verbal learning and memory, which was driven primarily by Cu.
- A beneficial interaction between Cu and ferritin was estimated, such that Cu was more strongly associated with verbal learning and memory scores at higher percentiles of ferritin.

ABSTRACT

Background: Biomarker concentrations of metals are associated with neurodevelopment, and these associations may be modified by nutritional status (e.g., iron deficiency). No prior study on associations of metal mixtures with neurodevelopment has assessed effect modification by iron status.

Objectives: We aimed to quantify associations of an industry-relevant metal mixture with verbal learning and memory among adolescents, and to investigate the modifying role of iron status on those associations.

Methods: We used cross-sectional data from 383 Italian adolescents (10–14 years) living in proximity to ferroalloy industry. Verbal learning and memory was assessed using the California Verbal Learning Test for Children (CVLT-C), and metals were quantified in hair (manganese, copper, chromium) or blood (lead) using inductively coupled plasma mass spectrometry. Serum ferritin, a proxy for iron status, was measured using immunoassays. Covariate-adjusted associations of the metal mixture with CVLT subtests were estimated using Bayesian Kernel Machine Regression, and modification of the mixture associations by ferritin was examined.

Results: Compared to the 50th percentile of the metal mixture, the 90th percentile was associated with a 0.12 standard deviation [SD] (95% CI= -0.27, 0.50), 0.16 SD (95% CI= -0.11, 0.44), and 0.11 SD (95% CI= -0.20, 0.43) increase in the number of words recalled for trial 5, long delay free, and long delay cued recall, respectively. For an increase from its 25th to 75th percentiles, copper was beneficially associated the recall trials when other metals were fixed at their 50th percentiles (for example, trial 5 recall: β=0.31, 95% CI= 0.14, 0.48). The association between copper and trial 5 recall was stronger at the 75th percentile of ferritin, compared to the 25th or 50th percentiles.

Conclusions: In this metal mixture, copper was beneficially associated with neurodevelopment, which was more apparent at higher ferritin concentrations. These findings suggest that metal associations with neurodevelopment may depend on iron status, which has important public health implications.

1. INTRODUCTION

Environmental exposure to metals is common among children and occurs through several sources, including diet, contaminated drinking water, consumer products, and air emissions (Agency for Toxic Substances and Disease Registry, 2020, 2012a, 2012b, 2004). Living in proximity to certain industries, like ferroalloy plants that manufacture steel, may also lead to increased environmental exposure to metals like manganese (Mn), lead (Pb), chromium (Cr), copper (Cu) and iron (Fe). Residing near ferroalloy industry has been associated with increased body burdens of metals in children in Italy (Butler et al., 2019), the United States (Haynes et al., 2012), Mexico (Riojas-Rodríguez et al., 2010), Canada (Boudissa et al., 2006), and Brazil (Menezes-Filho et al., 2016, 2009). There is ample epidemiologic evidence demonstrating that exposure to individual metals can adversely affect cognition and other neurodevelopmental outcomes in children (Bauer et al., 2020b), but fewer studies have examined the neurodevelopmental impacts of exposure to mixtures of metals, which may interact or act jointly (Ahamed and Siddiqui, 2007; Akinyemi et al., 2019; Amos-Kroohs et al., 2017; Neal and Guilarte, 2013; O'Neal et al., 2014; Wang et al., 2016; Zhao et al., 2018). Because the ferroalloy industry is expected to grow substantially through 2025 (~6% worldwide) ("Ferroalloy Market Share 2018-2025 Industry Growth Outlook Report"), quantifying the impacts of exposure to metal mixtures from ferroalloy industry is an important public health objective, particularly for susceptible populations like children.

Verbal learning and memory are key domains for overall cognitive development and academic achievement in children (Blankenship et al., 2018, 2014). Disruptions in verbal learning and memory may have long term implications for child health, as well as for educational achievement and socioeconomic position in adulthood (Aro et al., 2019). Learning is defined as the ability to acquire new information (Kreutzer et al., 2011), while memory refers to the ability to encode, store and retrieve learned information (Delis et al., 1994); both learning and memory are primarily modulated by the hippocampus and prefrontal cortex (Arnsten, 2009; Hoogman et al., 2017). Metals, such as Pb and Mn, have been detected in these brain tissues in animal models (Neal and Guilarte, 2013; O'Neal and Zheng, 2015; Yamagata et al., 2017), where they may exert toxic effects through various mechanisms, including dopaminergic toxicity, oxidative stress, dendritic degeneration, and disruption of ATP synthesis and neurotransmission (Ahamed and Siddiqui, 2007; Akinyemi et al., 2019; Amos-Kroohs et al., 2017; Neal and Guilarte, 2013; O'Neal et al., 2014; Wang et al., 2016; Zhao et al., 2018). This suggests that verbal learning and memory are domains that may be particularly impacted by these metals, a notion supported by epidemiological studies that have reported adverse associations of individual metals with learning and memory in children (Carvalho et al., 2018; García-Chimalpopoca et al., 2019; Oulhote et al., 2014; Torres-Agustín et al., 2013; Wright et al., 2006).

Furthermore, emerging epidemiological evidence suggests that children with nutritional deficiencies, such as Fe deficiency, may be more susceptible to the neurotoxicity of metals (Amorós et al., 2019; Kupsco et al., 2020; Shah-Kulkarni et al., 2016). Fe is an essential nutrient that plays a role in a multitude of biologic functions, such as cellular oxygen transport and neurotransmitter synthesis, which are critical for normal cognitive function (McCann et al., 2020). Altered Fe status (i.e., deficiency or excess), clinically measured through biomarkers like hemoglobin, ferritin, and transferrin (Gibson, 2005), has been consistently associated with poorer neurodevelopment (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., 2017). Several prior studies of metals and neurodevelopment reported effect modification by Fe status, where the negative associations of Pb, Mn, and Cu with neurodevelopment were stronger in children with lower hemoglobin levels (Amorós et al., 2019; Gunier et al., 2015; Kordas et al., 2007; Kupsco et al., 2020; Shah-Kulkarni et al., 2016). Little is known, however, about how metal neurotoxicity is modified by Fe status during the adolescent period. Metals may be particularly neurotoxic during adolescence because several regions of the brain (e.g., prefrontal cortex, temporal lobe) undergo rapid maturation during this developmental period, including dendritic pruning, maturation of cytoskeletons, myelination, and refinement of synaptic connections and neurotransmission (Arain et al., 2013; Shaw et al., 2020). Adolescents are also particularly vulnerable to Fe deficiency given the increased Fe needed to support rapid physical growth and neural development (Cutler et al., 2009; Das et al., 2017; Leal et al., 2017; Mesías et al., 2013; Movassagh et al., 2017), and given changes in dietary behaviors in this developmental stage, which may alter the toxicokinetics and toxicodynamics of other metals in relation to cognitive development. Females are especially vulnerable to Fe deficiency in the adolescent period due to the onset of menstruation (Zimmermann and Hurrell, 2007). However, few studies have examined effect modification of metal neurotoxicity by Fe status in adolescence, and none to date have assessed effect modification of complex metal mixtures.

The aim of this analysis was to address the current literature gaps on metal mixtures, Fe status, and verbal learning and memory in adolescents. Specifically, we quantified associations of a mixture of metals commonly emitted from ferroalloy industry (Pb, Mn, Cu, Cr) with verbal learning and memory, and investigated whether Fe status modified these associations in a cohort of Italian adolescents. We also aimed to explore sex-specific associations of the metal mixture with neurodevelopment.

2. METHODS

2.1 Study Population

The Public Health Impact of Metals Exposure (PHIME) study is an ongoing study of adolescents in northern Italy designed to examine impacts of ferroalloy industry-related metal exposures on neurodevelopmental outcomes. Full details on the study population, including recruitment, have been described previously (Lucchini et al., 2012a). Briefly, 721 adolescents (aged 10 – 14 years) were recruited from three regions of the Brescia province in northern Italy with varying historical ferroalloy industry: Bagnolo Mella (BM), with industrial activity since 1974; Garda Lake (GL), with no historical industrial activity; and Valcamonica (VC), with historical industrial activity that ended in 2001. Enrollment in PHIME occurred in two phases, following two distinct waves of funding for the study. Of the 721 subjects, 311 participants were enrolled during the first phase of the study (2007 – 2010) and 410 participants were enrolled in the second phase of the study (2010 – 2014). All study protocols, including questionnaires, were consistent between the phases. The second phase recruited participants from Bagnolo Mella, collected and measured metals in additional biomarkers (saliva, urine, nails), and administered an abbreviated version of the Home Observation Measurement of the Environment (HOME) Short Form questionnaire to parents (National Longitudinal Surveys, 1979).

Residents in the Brescia province were eligible for enrollment if they 1) were 10 – 14 years of age at enrollment, 2) lived in the study area since birth, and 3) were born into families that lived in the study region since the 1970s. Participants were excluded if they 1) had a clinically diagnosed neurologic, hepatic, metabolic, endocrine, or psychiatric disease, or clinically relevant motor deficits that may have impacted testing, 2) used medication with neurologic side effects, 3) had clinically diagnosed cognitive or behavioral impairment, 4) had visual deficits without corrective measures, or 5) had ever received parenteral nutrition.

Guardians of potential participants gave informed consent after receiving detailed information on study protocols. 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 Biomarker Collection and Measurement

Blood and hair samples were collected from study participants at enrollment and evaluated for metals (Pb, Mn, Cu, Cr). For this analysis, we *a priori* selected blood as a biomarker for Pb and hair as a biomarker for Mn, Cu and Cr. Blood is a commonly used and accepted biomarker of Pb exposure in the epidemiology literature (Barbosa et al., 2005). There is not a commonly accepted biomarker of exposure for Mn, Cu, or Cr (Bertinato and Zouzoulas, 2009; Coetzee et al., 2016; Jursa et al., 2018; Lukaski, 1999). We selected hair to represent exposure to Mn, Cu and Cr in this analysis because: 1) hair had the least missing data for these metals (<6%); 2) hair metal concentrations have been correlated with environmental (e.g., dust, soil, air) concentrations in this cohort (Butler et al., 2019; Lucas et al., 2015) and elsewhere (Coetzee et al., 2016); and 3) hair metal concentrations have been consistently utilized and associated with neurodevelopmental outcomes in prior epidemiological studies, including studies in the current study population (Bauer et al., 2020a; Caparros-Gonzalez et al., 2019; Carvalho et al., 2018; García-Chimalpopoca et al., 2019; Rechtman et al., 2020; Torres-Agustín et al., 2013; Wright et al., 2006).

We have previously described collection methods for each biomarker in depth (Eastman et al., 2013; Lucas et al., 2015; Lucchini et al., 2012a; Smith et al., 2007). In brief, venous whole blood samples (4mL) were collected with 19-gauge butterfly catheters and stored in lithium-heparin Sarstedt Monovette Vacutainers; these tubes contained a clotting factor, and samples were centrifuged within hours of sampling to separate the serum. Hair samples were collected from the occipital region of the scalp (2-3 cm, or ~20 mg) using stainless steel clippers, reflecting exposure from the past several months (Agency for Toxic Substances and Disease Registry, 2012a, 2012b, 2004). Because hair may be susceptible to exogenous contamination, the samples were then extensively cleaned using Triton detergent, nitric acid and sonication, as has been previously described (Eastman et al., 2013; Lucas et al., 2015). Metals (Mn, Pb, Cu, and Cr) were quantified in blood and hair using magnetic sector inductively coupled plasma mass spectrometry (Eastman et al., 2013; Lucchini et al., 2012a; Smith et al., 2007). Biomarker values below the limit of detection (LOD) were imputed as the LOD/2 (hair Mn: n=1, hair Cr: n=1); LODs were defined based on repeated measures of procedural blanks across multiple days (n= 4) (Butler et al., 2019).

We used serum ferritin to characterize Fe status in this analysis. Ferritin is considered a sensitive measure of altered Fe status because levels are reduced in the early stages of Fe deficiency (Gibson, 2005). We quantified serum ferritin from blood samples in immunoassays using the Instrument Architect *i*2000*SR* – Abbott Laboratories (Abbott Park, IL, USA).

2.3 Cognitive Assessment

Concurrent with biological sample collection, trained psychologists administered the California Verbal Learning Test for Children (CVLT-C) to assess verbal learning and memory in the second phase of the study (n= 403) (Delis et al., 1994). The CVLT-C consisted of five recall trials of 15 verbally presented words (List A) that included five words from each of three semantically related categories (e.g., fruits), followed by a recall trial of an interference list (List B). Participants then completed free (i.e., not cued) and semantically cued recall trials following short (immediately following the interference list recall trial) and long (20 minute) delays. Finally, subjects completed a recognition trial, where participants selected target words on List A from a written list of 44 words that included both target and distraction words.

Available CVLT-C outcomes for analysis included the total number of correct words recalled on trial 1, trial 5, trials 1-5 (summed), the interference list, short delay trials (free and cued), and long delay trials (free and cued). We also calculated three additional scores: intrusions, perseverations, and forgetting. The sum of intrusions is defined as the total number of non-target words reported across all trials, and perseverations is defined as the total number of target words repeated within a trial summed across trials. We calculated scores for 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). Positive scores for the recall trials and recognition indicate better learning and memory, while positive scores for intrusions and perseverations suggest worse performance. Positive scores for forgetting suggest better memory (i.e., retention). Full descriptions of each CVLT-C outcome are provided in **Table 1**. For this analysis, we *a priori* selected and analyzed five CVLT-C outcomes that reflect varying aspects of verbal learning and memory: trial 5, long delay free recall, long delay cued recall, perseverations, and forgetting.

CVLT-C outcome	Description	Direction of Beneficial Effect	Memory Processes	Primary brain region(s) subserving function
Trial 1 recall	# of correct target words recalled on trial 1	(+)	Encoding, working memory, attention	Hippocampus Prefrontal cortex
[a]Trial 5 recall	# of correct target words recalled on trial 5	(+)	Encoding, working memory, invoking strategies for learning	Hippocampus Prefrontal cortex
Trials 1-5 recall	# of correct target words recalled on trials 1 through 5	(+)	Encoding, working memory, invoking strategies for learning	Hippocampus Prefrontal cortex
Recall of interference list	# of correct words recalled from the interference list	(+)	Encoding, working memory, invoking strategies for learning, ability to inhibit interference	Hippocampus Prefrontal cortex
Short delay free recall	# of correct target words recalled immediately following the interference list without semantic cue	(+)	Declarative learning, self-structured retrieval	Hippocampus Prefrontal cortex
Short delay cued recall	# of correct target words recalled immediately following the interference list with semantic cue	(+)	Declarative learning, cued retrieval	Hippocampus Prefrontal cortex
[a]Long delay free recall	# of correct target words recalled after long (~20 min) delay without semantic cue	(+)	Declarative learning, self-structured retrieval, retention	Hippocampus Prefrontal cortex
[a]Long delay cued recall	# of correct target words recalled after long (~20 min) delay with semantic cue	(+)	Declarative learning, cued retrieval, retention	Hippocampus Prefrontal cortex
Recognition	# of correct target words identified from a written list of 44 target and non-target words	(+)	Encoding in the absence of forced retrieval	Hippocampus
[a]Forgetting	# of words recalled on long delay free recall minus number of words recalled on short delay free recall	(+)	Loss of information (i.e., interference of consolidation, retrieval or memory stability)	Hippocampus
Intrusions	Total # of responses not from the target list across free and cued recall trials	(-)	Source confusion or response inhibition	Prefrontal cortex
[a]Perseverations	Total # of target words repeated within a trial	(-)	Inhibition from prior responses and source memory impairment	Prefrontal cortex

Table 1. Descriptions of California Verbal Learning Test for Children (CVLT-C) outcomes in PHIME (Davis and Zhong, 2017; Delis et al., 1994; Kreutzer et al., 2011; Nee et al., 2007; Preston and Eichenbaum, 2013; Solesio et al., 2009; Strauss et al., 2006).

[a]CVLT-C outcomes included in this analysis.

2.4 Collection of Covariate Data

Trained study staff collected information on potential covariates, including sociodemographic information, using standardized questionnaires that were administered either in-person or via the phone at time of enrollment. Information was collected on the following covariates: age (continuous, in years), biological sex (female or male), birth order (first, second, third, or >third born), area of residence (BM, GL, or VC), self-reported alcohol consumption (yes or no), self-reported smoking status (smoker vs. non-smoker), parental occupation, and parental education level. We categorized each participant's socioeconomic status (SES) as low, medium or high based on a method developed for Italian populations that combines information on parental education and occupation (Cesana et al., 1995; Lucchini et al., 2012b). HOME scores, which reflect cognitive stimulation at home, were calculated (possible range: 0 – 9) for each participant using nine items selected from the HOME Short Form (National Longitudinal Surveys, 1979).

2.5 Data Analysis

2.5.1 Descriptive statistics, confounder selection, and generalized additive models. Our analytic sample included all PHIME study participants who completed the CVLT-C (n= 403). Some data were missing (<6%) for biomarkers and covariates (**Table S1**); therefore, we employed the Markov chain Monte Carlo method (Zhou et al., 2001) using the *mice* package in R (Buuren and Groothuis-Oudshoorn, 2011) to impute missing biomarker and covariate data using all available biomarker, outcome, and potential confounder data. Twenty datasets were imputed under the assumption that data were missing at random.

We first examined the distributions of all biomarkers, covariates, and outcomes using one randomly selected imputed dataset. Upon visual inspection of histograms and boxplots, we observed several extreme values for metal concentrations. We excluded participants with concentrations of any metal that were ±3 standard deviations (SD) from the mean across the 20 imputed datasets (n= 20), for a final analytic sample size of 383 adolescents. Summary statistics were calculated for all variables using a randomly selected imputed dataset. Distributions of metal (Mn, Pb, Cu, Cr) concentrations, ferritin levels, and perseverations, one of the CVLT-C endpoints, were right-skewed; therefore, we natural log (ln) - transformed these variables to satisfy modeling assumptions of normality of residuals and to reduce the influence of outlier values. Metal and ferritin concentrations were then z-standardized to account for varying units of measurement in different media (hair, blood, serum). We also z-standardized all CVLT outcomes, which were modeled continuously, to facilitate comparisons of effect estimates across outcomes. Spearman correlation coefficients between metals and between CVLT-C outcomes were estimated across the 20 imputed datasets using the *miceadds* package in R.(Robitzsch and Grund, 2021)

Confounders for this analysis were chosen *a priori* using directed acyclic graphs (DAGs) and prior literature (Bauer et al., 2020b; Carvalho et al., 2018; Kordas, 2010; Torres-Agustín et al., 2013). We adjusted for age, biological sex, SES, and HOME score as confounders in all analyses. Fe status (i.e., ferritin) has been associated with both metal biomarker concentrations and neurodevelopment (Halterman et al., 2001; Jáuregui-Lobera, 2014; Ji et al., 2017; Lukowski et al., 2010; Parkin et al., 2020; Roy et al., 2011; Schildroth et al., 2022; Tseng et al., 2018; Wang et al., 2017), suggesting Fe status may be a confounder of associations between the metal mixture and neurodevelopment. We therefore included ferritin as a covariate in all regression models. However, effect modification by Fe status was also considered in both multivariable linear regression and Bayesian Kernel Machine Regression Models (described below). Ferritin, age, and HOME scores were modeled as continuous covariates, as they were linearly related to CVLT-C outcomes based on visual inspection of penalized splines (constrained to 4 knots) from generalized additive models (GAMs). Sex and SES were treated as categorical covariates.

There is evidence in the literature to suggest that metals, especially nutrients like Mn and Cu, may be nonlinearly associated with neurodevelopment (Bauer et al., 2020a; Claus Henn et al., 2010). Prior to fitting multivariable linear regression models, we utilized GAMs to inspect the shape of the associations between each ln-transformed metal and CVLT-C scores, adjusting for all other metals and selected confounders. We used penalized splines (knots= 4) to allow for non-linear associations between metals and CVLT-C outcomes. We used likelihood ratio tests (LRTs) to compare the fit of models with and without splines; based on the LRTs, there was little evidence that the splines improved the fit compared to linear models (p-values were all >0.05). Therefore, metals were modeled as continuous variables in subsequent linear regression models.

2.5.2 Multivariable Linear Regression. We first fit fully adjusted multivariable linear regression models with all four metals (Mn, Pb, Cu, Cr) and ferritin to examine associations of each metal with CVLT-C outcomes. These models initially included all pairwise cross-product terms between metals to identify potential metal-metal interactions in relation to CVLT-C outcomes. Potential modification by Fe status was similarly evaluated in a separate model by including all pairwise interaction terms between each metal and ferritin. We *a priori* selected $p<0.10$ as the cutoff for retaining interaction terms in our final linear regression models. No pairwise metal-metal interaction terms were significant for any CVLT-C outcome among the full cohort; those interactions were therefore not included in the subsequent linear regression models. However, there was evidence of metal-ferritin interactions for Cu and Mn in relation to trial 5 and forgetting, respectively ($p<0.10$). Final models for trial 5 and forgetting therefore retained the significant Cu-ferritin and Mn-ferritin interaction terms, respectively, while final models for long delay free recall, long delay cued recall, and perseverations did not include any metal-ferritin interaction terms.

Multivariable linear regression models were fit for all 20 imputed datasets using the *miceadds* package (Robitzsch and Grund, 2021). Beta coefficients (β), which estimated the mean difference in the z-standardized CVLT scores for a 1-SD increase in ln-metal concentrations, were pooled across the imputed datasets using standard methods, where standard errors (SEs) were combined using Rubin's rule (Rubin, 2004). For perseveration errors, which were ln-transformed and z-standardized prior to modeling, beta coefficients represent the mean difference in ln-transformed, standardized perseverations per SD increase in ln-metal concentrations. To improve interpretability, we multiplied beta coefficients by the ln-transformed standard deviation for perseverations (**Table 1**) and report findings as the estimated percent difference in perseveration score for a doubling in metal concentrations, calculated as follows:

[1] $\%\ difference\ in\ perseveration\ scores = \left(e^{(\ln(2) * \beta)}\right) - 1 * 100$

2.5.3 Bayesian Kernel Machine Regression. Next, we used Bayesian Kernel Machine Regression (BKMR) to further examine the association of the metal mixture with CVLT outcomes. Although there was limited evidence of nonlinearity in the GAMs or of pairwise metal interactions in the multivariable linear regression models, using BKMR allowed for investigation of potential higher-order interactions (i.e., interaction of each exposure with multiple components of the mixture), as well as for the estimation of joint effects of the overall mixture with CVLT outcomes (Bobb et al., 2015).

BKMR employs a kernel function (*h*) to flexibly model the exposure-response relationship between an outcome and an exposure mixture, where the model assumes that individuals with similar exposure profiles have similar outcomes. Because we aimed to quantify the modifying role of Fe status, we included ferritin in the *h* function to investigate pairwise and higher-order interactions between ferritin and other components of the mixture for each CVLT outcome. The BKMR models took the following form:

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

where *h* is the exposure-response function that accommodates non-linearity and interaction among mixture components, and e_i is the random error term.

We fit a BKMR model for each of the 20 imputed datasets using the default non-informative prior specifications with 10,000 iterations and a 50% burn-in. We used the component-wise variable selection option and estimated posterior inclusion probabilities (PIPs) for each metal and ferritin. PIPs describe the relative importance of each component of the exposure response function in relation to each CVLT outcome while accounting for multiple testing. Findings from each BKMR model across all 20 imputed datasets were pooled using Rubin's rule with previously developed code (Devick, 2019) to obtain an overall estimate and 95% credible interval (CI). As with the linear regression models, we multiplied beta coefficients from models of perseverations by the ln-transformed standard deviation for perseverations (**Table 1**), and report findings as the estimated percent difference in perseveration scores for various percentile changes in metals concentrations, calculated with the following equation:

[3] $\%\ difference\ in\ perseveration\ scores = (e^{\beta} - 1) * 100$

To describe the associations of the metal mixture with learning and memory, we estimated the following for each CVLT-C outcome: 1) exposure-response profiles for each metal, holding all other metals at their 50th percentiles; 2) exposure-response profiles for each metal estimated at varying (25th, 50th, 75th) percentiles of a second metal, while holding remaining metals at their 50th percentiles; 3) associations of each metal comparing its 75th percentile to its 25th percentile when all other metals are held at their 25th, 50th, or 75th percentiles; and 4) the joint association of a percentile change in all metals simultaneously, compared to the 50th percentile of all metals.

We ran sensitivity analyses to evaluate the robustness of findings by 1) using the gamma prior distribution instead of the default inverse uniform distribution; 2) changing the degree of smoothness of the *h* function from the default (b=100) to lower and higher degrees of smoothness (b=50 and 1000, respectively) (Bauer et al., 2020a; Valeri et al., 2017); and 3) increasing the number of iterations to 50,000 (from 10,000).

2.5.4 Sex-stratified Analyses. There is evidence in the literature to suggest that 1) associations between metals and domains of neurodevelopment may be sexually dimorphic, and 2) female adolescents are more susceptible to Fe deficiency (Bauer et al., 2017; Kounnavong et al., 2020; Llop et al., 2013; Rechtman et al., 2020; Shaw, 1996; Zhu et al., 2021). Therefore, we assessed potential sex-specific effects in exploratory analyses. We stratified imputed datasets by sex and re-ran the above multivariable linear regression and BKMR models to evaluate sex-specific associations.

3. RESULTS

3.1 Study Population Characteristics

Fifty-three percent of participants were male, and the mean age of participants was 12.3 years (SD: 1.0) (**Table 2**). About half of participants lived near the Bagnolo Mella region (53.3%) and came from families that were classified as medium socioeconomic status (52.5%). The mean abbreviated HOME score, based on 9 items from the Home Observation of the Environment, was 6.0 (SD: 1.7). Ferritin concentrations in this population were within the clinically normal range (University of Rochester, 2021) (median: 32.0 ng/mL; 25th – 75th percentile: 21.0 – 44.0 ng/mL), and tended to be lower in females (median: 30.0 ng/mL; 25th – 75th percentile: 20.0 – 41.0 ng/mL) than in males (median: 33.0 ng/mL; 25th –

75th percentile: 22.0 – 46.0 ng/mL). Summary statistics were similar between the imputed and complete data (**Table S2**). Summary statistics were also similar for adolescents included in the analysis and those who were excluded due to missing outcome data; however, adolescents who were excluded due to missing outcome data (n= 318) had lower ferritin concentrations (median: 21.0 ng/mL; 25th – 75th percentile: 12.0 – 34.0 ng/mL; **Table S2**).

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%)
Self-reported smoking	
Smoker	1 (0.3%)
Non-smoker	382 (99.7%)
CVLT-C outcomes	
Long delay free recall	11.5 ± 2.1
Long delay cued recall	11.8 ± 2.1
Trial 5	12.3 ± 1.9
Perseverations	7.2 ± 6.1
Forgetting	0.3 ± 1.6
Metal biomarkers (median, 25th, 75th 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 biomarker (median, 25th, 75th percentile)	
Ferritin (ng/mL)	32.0 (21.0, 44.0)

Table 2. Characteristics of PHIME study participants included in present analysis (n= 383).
[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.

Median hair metal concentrations were highest for Cu (median: 9.4 µg/g; 25th – 75th percentile: 6.6 – 14.8 µg/g), followed by Mn (median: 0.07 µg/g; 25th – 75th percentile: 0.04 – 0.12 µg/g) and Cr (median: 0.04 µg/g; 25th – 75th percentile: 0.03 – 0.06 µg/g) (**Table 2**). The median blood Pb concentration in study participants (1.3 µg/dL; 25^{th} – 75^{th} percentile: 1.0 – 1.7 µg/dL) was lower than the current Centers for Disease Control and Prevention reference value of 3.5 µg/dL (Centers for Disease Control and Prevention, 2022). Males had higher blood Pb concentrations (median: 1.4 µg/dL; 25^{th} – 75^{th} percentile: 1.1 – 2.0 µg/dL) compared to females (median: 1.1 µg/dL; 25^{th} – 75^{th} percentile: 0.9 – 1.4 µg/dL), while females had higher hair Cu concentrations (median: 10.3 µg/g; 25^{th} – 75^{th} percentile: 7.6 – 16.5 µg/g) compared to males (median: 8.6 µg/g; 25^{th} – 75^{th} percentile: 6.3 – 13.7 µg/g) (**Table S3**). Concentrations of hair Mn and Cr were similar between males and females.

Metal concentrations were not highly correlated; Spearman correlation coefficients ranged from 0.04 (Pb-Cr) to 0.36 (Mn-Cu), and were strongest among the three metals measured in hair (Mn, Cu, Cr) (**Figure S1**). The strongest correlations between the five CVLT-C outcomes were observed among the recall trials, including trial 5, long delay free, and long delay cued recall (r_s = 0.51 - 0.79).

3.2 Multivariable Linear Regression

In fully adjusted linear regression models, a 1-SD increase in ln-hair Cu was associated with better performance (i.e., more words recalled) on trial 5 (β= 0.20, 95% CI= 0.10, 0.31), long delay free (β= 0.28, 95% CI= 0.17, 0.39), and long delay cued (β= 0.22, 95% CI= 0.11, 0.33) recall (**Figure 1, Table S4**). There was evidence of a positive interaction between Cu and ferritin (β-interaction= 0.13, 95% CI= 0.02, 0.25; p-interaction= 0.02), suggesting that the positive (beneficial) Cu association was stronger with increasing ferritin concentrations.

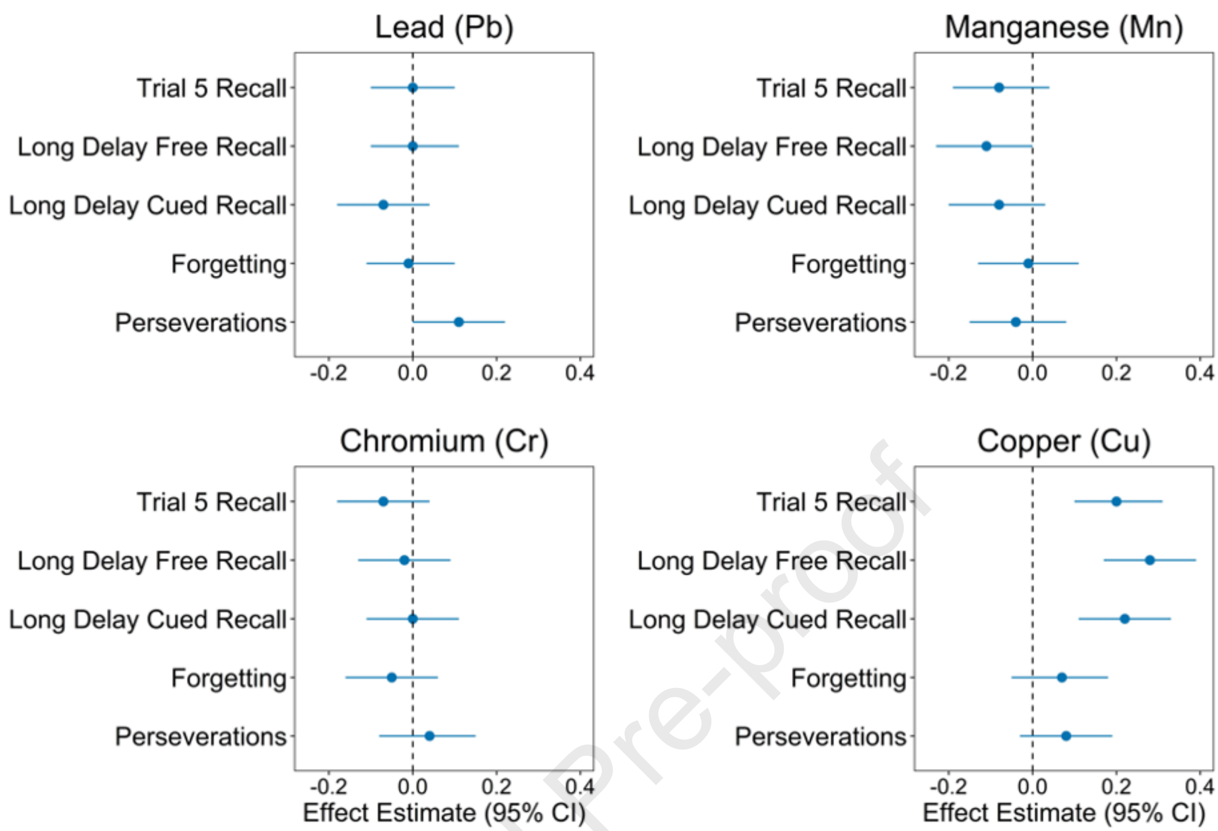

Figure 1. Main effect estimates and 95% confidence intervals from multivariable linear regression models describing associations of ln-transformed Z-standardized metals (Pb, Mn, Cr, and Cu) with Z-standardized CVLT-C outcomes. Perseveration scores were also ln-transformed. Models were mutually adjusted for all metals, ferritin, age, sex, SES, and HOME score. Models for trial 5 and forgetting included Cu-ferritin and Mn-ferritin interaction terms, respectively. Note: Pb, lead; Mn, manganese; Cr, chromium; Cu, copper; CVLT-C, California Verbal Learning Test for Children; SES, socioeconomic status; HOME, Home Observation Measurement of the Environment.

In contrast to Cu, hair Mn was weakly associated with worse performance (i.e., fewer words recalled) on the recall trials: a 1-SD increase in ln-Mn was associated with a 0.08 SD decrease (95% CI= -0.19, 0.04), 0.11 SD decrease (95% CI= -0.23, 0.00), and 0.08 SD decrease (95% CI= -0.20, 0.03) in words recalled on trial 5, long delay free, and long delay cued recall, respectively. Although Mn associations with forgetting were null (β= -0.01, 95% CI= -0.13, 0.11), there was evidence of an interaction between Mn and ferritin (β-interaction= -0.12, 95% CI= -0.23, -0.02; p-interaction= 0.03), such that Mn was adversely associated with forgetting at increasing concentrations of ferritin (**Table S4**).

Pb was also associated with worse performance: a doubling in blood Pb concentrations was associated with a 14.8% increase in perseveration errors (β= 0.11, 95% CI= 0.00, 0.22). Associations for Cr and ferritin were null.

3.3 Bayesian Kernel Machine Regression

Posterior inclusion probabilities for each metal and ferritin across all outcomes are provided in **Table S5**. Cu had the highest PIP for trial 5 (0.98), long delay free (0.94) and long delay cued (0.94) recall, while Pb had the highest PIP for perseverations (0.60). Similar to findings from multivariable linear regression models, Cu was positively associated with each of the recall trials: when all other metals and ferritin were held at their medians, Cu was associated with a 0.31 SD increase (95% CI= 0.14, 0.48), 0.25 SD increase (95% CI= -0.03, 0.53), and 0.27 SD increase (95% CI= 0.07, 0.48) in words recalled on trial 5, long delay free, and long delay cued recall, respectively, when increased from the 25^{th} to the 75^{th} percentile (**Figures S2-S4, panel D**).

A modest interaction between Cu and the other components of the mixture (Pb, Mn, Cr, ferritin) was observed: the association of Cu with trial 5 recall was almost twice as strong when the mixture was fixed at its 75^{th} percentile (for an increase in Cu from the 25th to 75th percentiles, β= 0.37, 95% CI= 0.17, 0.57) compared to when the mixture was fixed at its 25^{th} percentile (β= 0.21, 95% CI= 0.00, 0.43; **Figure 2**). Consistent with linear regression models, this higher-order interaction was driven by the positive interaction between Cu and ferritin, where Cu was more strongly associated with correct responses on trial 5 at higher levels (i.e., 75^{th} percentile) of ferritin (**Figure 2, panels A and B**).

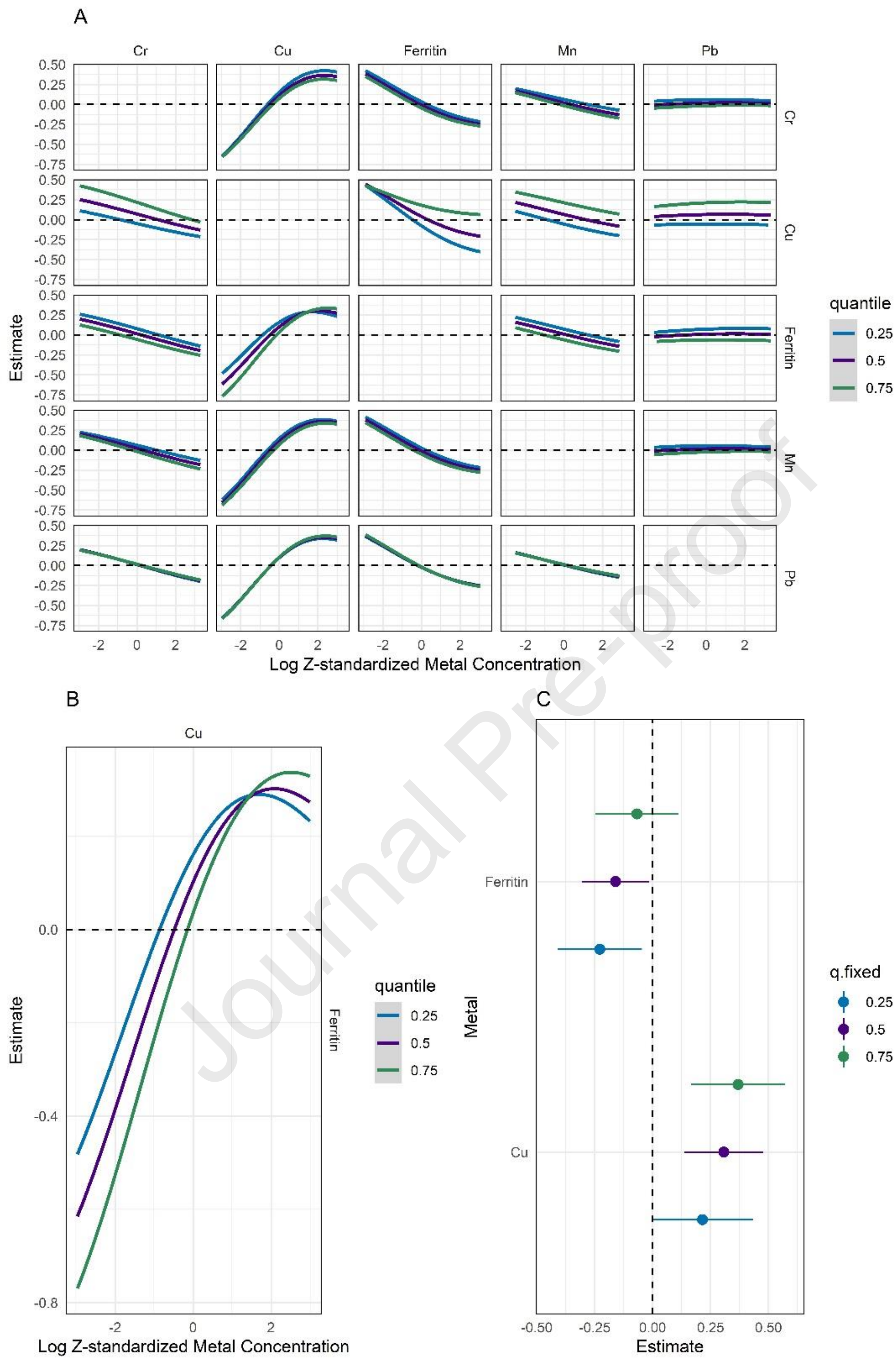

Figure 2. (A) Pairwise exposure-response relationships from BKMR models for each metal and ferritin with trial 5 at varying levels of other metals (25th, 50th, and 75th percentiles), while all other metals were set to their medians. (B) Exposure-response relationships from BKMR models for Cu with trial 5 at varying levels of ferritin (25th, 50th, and 75th percentiles), while all other metals were set to their medians. (C) Estimates and 95% credible intervals for the associations between an increase in Cu and ferritin from the 25th to 75th percentiles and trial 5 recall, when all other metals were set to their 25th, 50th, or 75th percentiles. Metal and ferritin concentrations were ln-transformed and Z-standardized, and CVLT-C outcomes were Z-standardized. Models were adjusted for age, sex, SES, and HOME

score. Note: BKMR, Bayesian Kernel Machine Regression; Cu, copper; CVLT-C, California Verbal Learning Test for Children; SES, socioeconomic status; HOME, Home Observation Measurement of the Environment.

As with multivariable linear regression models, Mn was weakly associated with worse CVLT scores (i.e., fewer words recalled) for the recall trials when all other metals and ferritin were fixed at their median (trial 5: β= -0.08, 95% CI= -0.22, 0.07; long delay free: β= -0.06, 95% CI= -0.23, 0.10; long delay cued: β= -0.07, 95% CI= -0.21, 0.07). The negative interaction between Mn and ferritin estimated in linear regression models was less evident in BKMR models (**Figure S5, panel B**).

Pb was not materially associated with CVLT outcomes (**Figures S2-S6**), with the exception of an association between blood Pb and perseverations: an increase in Pb from the 25th to 75th percentiles was associated with a 15.6% increase in perseveration errors, when all other metals were held at their 50^{th} percentiles (β= 0.08, 95% CI= -0.09, 0.25).

Compared to the 50^{th} percentile, higher percentiles of the mixture (Pb, Mn, Cr, Cu, ferritin) tended to be associated, though imprecisely, with better recall for trial 5, long delay free, and long delay cued recall (**Figure 3**). For example, the 90^{th} percentile of the mixture was associated, though imprecisely, with better scores for trial 5 (β= 0.12, 95% CI= -0.27, 0.50), long delay free (β= 0.16, 95% CI= -0.11, 0.44), and long delay cued (β= 0.11, 95% CI= -0.20, 0.43) recall, compared to the 50th percentile of the mixture. Further, the shape of the association of the overall mixture with trial 5 recall was U-shaped, such that the 10^{th} percentile of the mixture, compared to the 50^{th} percentile, was also positively associated with trial 5 recall (β= 0.14, 95% CI= -0.22, 0.50).

Increasing concentrations of the overall mixture (Pb, Mn, Cr, Cu, ferritin) were also associated with increased perseverations, although associations were imprecise: compared to the 50^{th} percentile, the 90^{th} percentile of the mixture was associated with a 22.0% increase (β= 0.11, 95% CI= -0.16, 0.38) in perseverations (**Figure 3**), which was driven primarily by Pb (**Figure S6, panel D**). The overall mixture was not associated with forgetting.

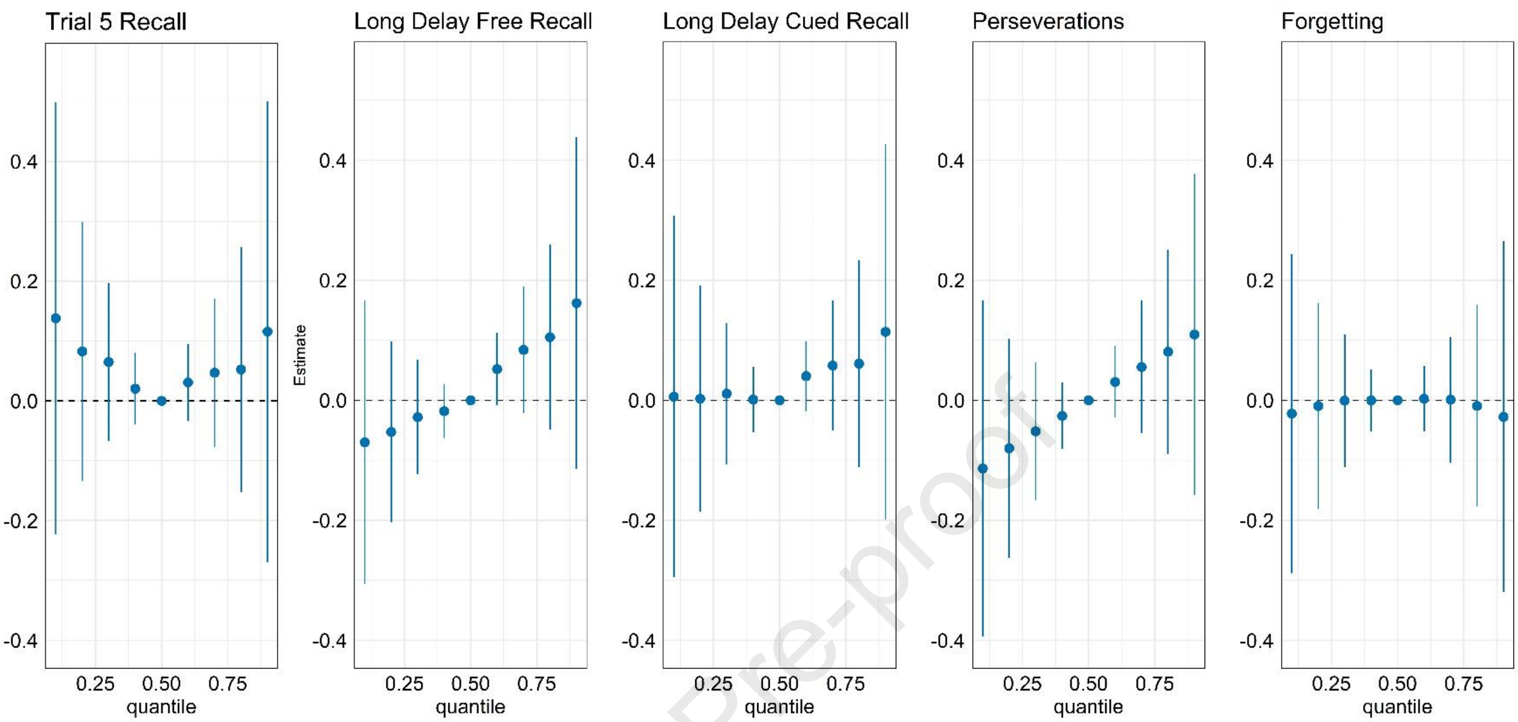

Figure 3. Joint associations of the overall mixture with trial 5 recall, long delay free recall, long delay cued recall, ln-perseverations, and forgetting at increasing percentiles (10th, 20th, 30th, 40th, 60th, 70th, 80th, and 90th) of all metals and ferritin, compared to the medians. Metal and ferritin concentrations were ln-transformed and Z-standardized, and CVLT-C outcomes were Z-standardized. Models were adjusted for age, sex, SES, and HOME score. Note: CVLT-C, California Verbal Learning Test for Children; SES, socioeconomic status; HOME, Home Observation Measurement of the Environment.

3.4 Sex-stratified Analyses

In multivariable regression models exploring sex-specific associations, Cu was positively associated with scores on the recall trials among both females and males, but tended to be stronger in females, suggesting that Cu may be more beneficial among females (**Table S4**). For example, the association between Cu and long delay cued recall was twice as strong in females (β= 0.29, 95% CI= 0.13, 0.44) compared to males (β= 0.14, 95% CI= -0.02, 0.30), though the interaction p-values were >0.10. This is consistent with findings from sex-stratified BKMR models (**Figures S7-S16)**.

Higher concentrations of Mn were negatively associated with trial 5 recall in females (from multivariable regression, β= -0.21, 95% CI= -0.37, -0.04), while this association was null in males (β= 0.04, 95% CI= -0.12, 0.21; p-interaction= 0.09); this was similar in BKMR models (**Figures S7-S8, panel**

D). Among females, Pb was also associated with worse scores for long delay cued recall (from multivariable regression, females: -0.14, 95% CI= -0.29, 0.01; males: β= 0.01, 95% CI= -0.14, 0.17), forgetting (females: β= -0.11, 95% CI= -0.27, 0.04; males: β= 0.11, 95% CI= -0.04, 0.26; p-interaction=0.06), and ln-perseverations (females: β= 0.17, 95% CI= 0.02, 0.32; males: β= 0.02, 95% CI= -0.14, 0.17) compared to males. Associations for Pb were similar across varying percentiles of the mixture in BKMR models (**Figures S11-S16, panel D**).

There was evidence of a positive interaction between Cu and ferritin for trial 5 in multivariable regression models among females (β-interaction= 0.15, 95% CI= -0.01, 0.32; p-interaction= 0.07), whereas the interaction was not evident among males. This is consistent with BKMR models, where a modest interaction between Cu and ferritin for trial 5 was observed only in females (**Figure 4**).

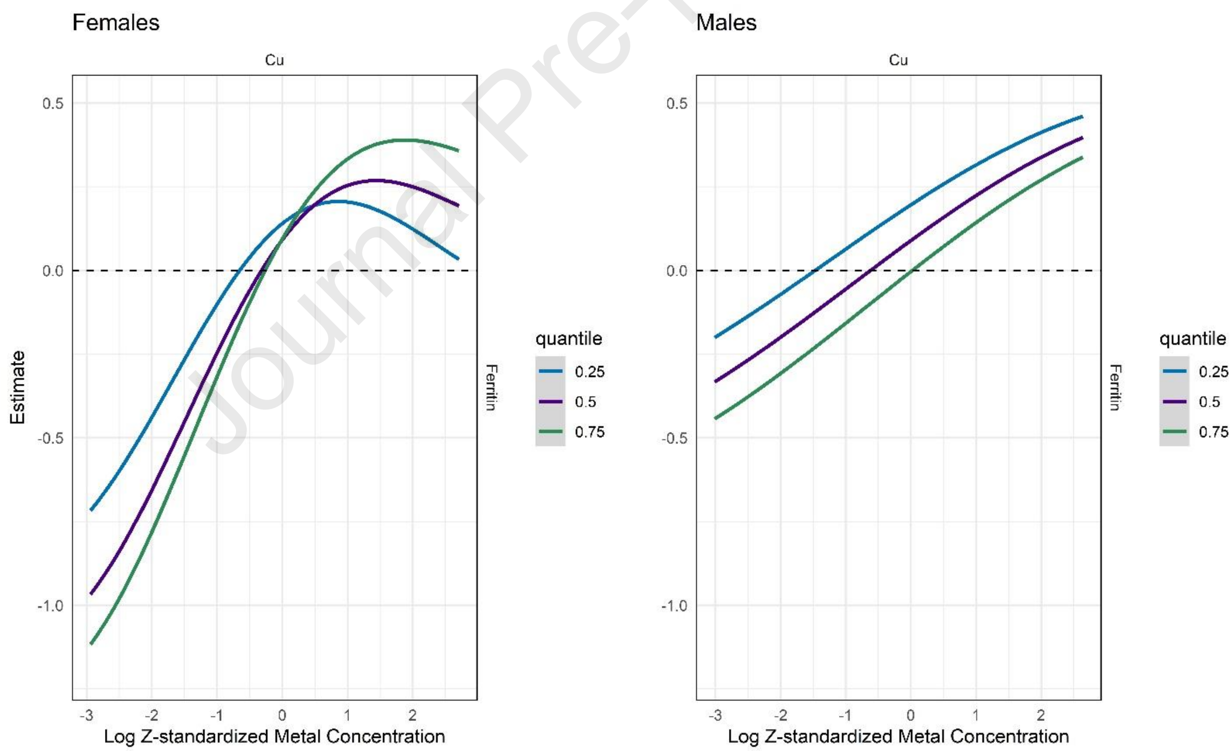

Figure 4. Pairwise exposure-response relationship from sex-stratified BKMR models for Cu with trial 5 at varying levels of ferritin (25th, 50th, and 75th percentiles), while all other metals were set to their medians. Metal and ferritin concentrations were ln-transformed and Z-standardized, and CVLT-C outcomes were Z-standardized. Models were adjusted for age, SES, and HOME score. Note: BKMR, Bayesian Kernel Machine Regression; Cu, copper; CVLT-C, California Verbal Learning Test for Children; SES, socioeconomic status; HOME, Home Observation Measurement of the Environment.

3.5 Sensitivity Analyses

We examined the robustness of our BKMR findings by 1) changing the default uniform distribution to a gamma distribution; 2) changing the smoothness of the kernel function from the default (100) to 50 and 1000; and 3) increasing the iterations from 10,000 to 50,000. Findings from sensitivity analyses, shown in **Figures S17-S20**, were similar to main analyses. However, the ferritin-Cu interaction for trial 5 was attenuated when setting the prior to a gamma distribution (**Figure S17**) and changing the smoothness of the kernel to r=1000 (**Figure S19**). Our overall conclusions were otherwise unchanged in the sensitivity analyses.

4. DISCUSSION

In this cross-sectional analysis of metal exposure and cognition in Italian adolescents, higher hair Cu was consistently associated with better learning and memory, while increasing hair Mn and blood Pb concentrations were associated with worse cognitive function measured by the recall trials and the number of perseveration errors, respectively. Further, we found evidence that Fe status modified associations of transition elements (i.e., Cu, Mn) in the metal mixture and neurodevelopment: a positive interaction between Cu and ferritin was observed for trial 5, suggesting the beneficial association of Cu with recall was stronger at higher concentrations of serum ferritin. Conversely, the negative interaction between Mn and ferritin with forgetting suggests that negative (adverse) associations of higher Mn with cognitive performance may be worse at higher ferritin concentrations.

Consistent with our findings, hair Mn and blood Pb have been associated with worse learning and memory in prior studies, including on the subtests of the CVLT-C, Children's Auditory Verbal Learning Test (CAVLT), and the Developmental Neuropsychological Assessment Battery (NEPSY) (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). These findings are supported by mechanistic and animal evidence: Pb and Mn are transported into the brain via metal transporters, such as the divalent metal transporter 1 (DMT1) or transferrin-mediated mechanisms, and may exert several mechanisms of toxicity (e.g., dopaminergic dysregulation, disruption of neurotransmission, dendritic degeneration, and cytotoxicity) (Ahamed and

Siddiqui, 2007; Akinyemi et al., 2019; Neal and Guilarte, 2013; O'Neal et al., 2014; O'Neal and Zheng, 2015; Yamagata et al., 2017). In animal models, Pb exposure has been shown to alter protein kinase C activity, which plays a key role in regulating membrane structure, transcription, cell growth, neurotransmitter release, neuronal plasticity, and ion channels (Sanders et al., 2009). Protein kinase C specifically modulates learning and memory, and altered activity following Pb exposure has been shown to lead to prefrontal cortex toxicity (Sanders et al., 2009). Mn has been shown to induce toxicity in hippocampal neurons by attenuating long-term potentiation (Amos-Kroohs et al., 2017; Wang et al., 2016; Zhao et al., 2018), which may explain the negative associations we observed between Mn and the recall trials that reflect various aspects of learning and memory modulated in part by the hippocampus (**Table 1**). These findings may also reflect alterations in intrinsic functional connectivity in brain regions, including the frontal and temporal lobes, following early life Mn exposure (Rechtman et al., 2022). However, additional studies in humans using brain imaging methods (e.g., structural and functional magnetic resonance imaging) would provide further insight into the association of metal mixtures with the underlying neuroanatomy and functional connectivity to support epidemiological findings.

Cu is an essential nutrient needed for the formation and maintenance of myelin, cellular respiration, catecholamine synthesis, and long term potentiation (Gaetke et al., 2014; Opazo et al., 2014), and enhances neurotransmission in the hippocampus (Opazo et al., 2014). In animal models, Cu exposure in rats has led to better learning and memory, which supports its essentiality and is consistent with our findings among adolescents (Zhang et al., 2018). However, findings in humans are equivocal. Hair and serum Cu concentrations measured in adolescents were associated with verbal IQ and working memory in two prior studies; both of these studies reported inverted U-shaped associations whereby only the mid-levels of Cu were beneficial for cognitive function (Bauer et al., 2020a; Zhou et al., 2015). Furthermore, one of these studies, conducted in the same PHIME cohort as our analysis, found that the highest hair Cu tertile was associated with worse verbal IQ, suggesting that exposure to higher levels of Cu may be neurotoxic (Bauer et al., 2020a). We did not observe a negative association at high Cu levels in the current analysis, but hair Cu levels were lower in this subset of the PHIME cohort (range: 1.7 – 60.1

μg/g) than in the prior study that utilized the full cohort (range: 1.7 – 191.0 μg/g). It is possible that the highest Cu concentrations in the current analysis were not elevated enough to induce neurotoxicity.

We found that the metal mixture was jointly associated with better scores on the recall trials at higher percentiles of all metals and ferritin, which likely reflects the beneficial association of Cu with recall. This finding is consistent with a prior study in the PHIME cohort that similarly found the overall mixture measured in multiple biomarkers (hair, blood, nails, saliva, urine) was beneficially associated with visuospatial learning and memory (Rechtman et al., 2020). However, in the prior study, the beneficial association of the mixture was observed only in males, and was driven primarily by Cr. In females, the mixture was adversely associated with visuospatial learning and memory (Rechtman et al., 2020), suggesting the neurotoxicity of metals in adolescence may be sex- and domain- (e.g., verbal vs. visuospatial) specific. It should be noted that the associations of the overall mixture with the CVLT-C scores were modest in magnitude: for example, the association with trial 5 recall when all metals are at their 90^{th} percentile (compared to the 50^{th} percentile) is equivalent to an increase of 0.23 words recalled (β*SD, β= 0.12, see **Table 1**). These associations therefore reflect subclinical impacts on learning and memory. Further, we did not observe pairwise or higher order interactions between any of the metals (Mn, Pb, Cu, and Cr) in our study, contrary to previous work in the PHIME cohort (Bauer et al., 2020a). Specifically, Bauer et al. observed interactions between hair Cu- hair Mn and hair Cu- blood Pb, such that the neurotoxic associations of Mn and Pb with verbal IQ were stronger at lower Cu concentrations (Bauer et al., 2020a). These metal-metal interactions are supported by other epidemiological and animal data (Fu et al., 2015; Guilarte and Chen, 2007; Liu et al., 2018; Robison et al., 2013; Zheng et al., 2009). It is possible that we did not observe similar interactions between metals in the current analysis due to our small sample size.

When we considered Fe status as a modifier, a modest interaction between Cu and ferritin was estimated for number of words recalled in trial 5, such that the positive association of Cu with recall was stronger at higher concentrations of ferritin. Mechanisms of Cu and Fe cellular uptake and transport are closely interconnected and evidence suggests that these metals may interact at cellular receptors (e.g., DMT1), especially at the blood brain barrier (Skjørringe et al., 2012). Moreover, Fe is required for catecholamine synthesis and plays a role in Cu-dependent dopamine beta-hydroxylase function (Ponting, 2001; Skjørringe et al., 2012), such that increased concentrations of both Fe and Cu may optimize enzymatic processes required for dopaminergic function. Therefore, the modest interaction we observed could be due to the shared necessity of these metals for optimal brain function. It should be noted that our study participants had clinically normal concentrations of ferritin. The positive interaction we observed between Cu and ferritin, whereby the beneficial association of Cu was stronger at higher concentrations of ferritin, may not occur in populations where the concentrations of Fe or Cu are suboptimal or elevated, as both insufficiently low and excess levels of Fe and Cu can induce neurotoxic effects (Skjørringe et al., 2012). Only one prior epidemiological study has assessed modification of Cu-neurodevelopment associations by Fe status in children (Amorós et al., 2019). This study reported that prenatal (maternal blood) Cu was negatively associated with memory and verbal performance on McCarthy Scales in children whose mothers had serum Fe concentrations in the lowest tertile, and this association was null for children whose mothers had greater than the first tertile of serum Fe concentrations (Amorós et al., 2019). The findings of this study, like ours, suggest that impacts of Cu on neurodevelopment may vary by Fe status.

Conversely, we observed a weak interaction between Mn and ferritin, where Mn was more strongly associated with worse neurodevelopment at increasing concentrations of ferritin. This finding is contrary to two prior studies that observed stronger negative associations between prenatal (maternal blood) Mn concentrations and neurodevelopment among children whose mothers had lower hemoglobin or ferritin concentrations in pregnancy (Gunier et al., 2015; Kupsco et al., 2020), as well as animal evidence supporting competitive uptake of Mn and Fe in brain tissues of rats (Ye et al., 2017). We are not aware of any current epidemiological, animal, or mechanistic data to support a negative interaction between Mn and Fe. It is possible that this finding is spurious: the interaction was weak, it was observed only in multivariable linear regression models, and we had a limited sample size.

Based on exploratory analyses of our data, there was suggestive evidence of sexual dimorphism in the associations between metals and CVLT scores. Pb was more strongly associated with worse scores for the long delay cued recall trial, perseverations, and forgetting among females compared to males. These findings are consistent with animal studies: Pb exposure has been found to attenuate hippocampal potentiation only in females (Llop et al., 2013), and poorer hippocampal plasticity in females compared to males has been reported (Barha et al., 2011; Yagi and Galea, 2019). Sex differences in hippocampal plasticity may further explain the stronger negative association of Mn with the recall trials, particularly trial 5 recall, in females. Two prior studies in adolescents (ages 7-12 years) similarly found stronger adverse associations between hair Mn and performance on learning, immediate recall and delay effect, as assessed by the CAVLT, among females (Carvalho et al., 2018; Torres-Agustín et al., 2013). In our study, hair Cu was associated with increased perseveration errors among males only; these findings may reflect sex differences in Cu biomarker concentrations, as males had lower hair Cu compared to females (median: males= 8.6 µg/g; females= 10.3 µg/g). Cu is an essential element, and deficiency or overload can lead to neurotoxic effects, such as mitochondrial disruption, induction of apoptosis and altered neurotransmission (Gaetke et al., 2014; Kalita et al., 2018).

There were several strengths to this analysis. Specifically, we were able to examine modification of a metal mixture by Fe status, making this among the first studies to identify Fe status as a modifier of associations between a metal mixture and neurodevelopment. The use of ferritin as a sensitive metric of Fe status (Gibson, 2005) is also a strength; most previous epidemiological studies of metals and cognition have assessed the role of Fe status using less sensitive metrics, like hemoglobin. This may explain, in part, null findings for metal-Fe status interactions in relation to neurodevelopment reported in several previous studies (Lynch et al., 2011; Wasserman et al., 1992; Wolf et al., 1994). Our analysis also focused on the adolescent period, an understudied but developmentally susceptible period for metal exposure because of the rapid maturation of brain structures at this age (Chulani and Gordon, 2014). Further, we characterized verbal learning and memory function using an objective cognitive assessment commonly used in epidemiological studies. The CVLT-C has numerous outcomes that quantify different aspects of learning and memory; by assessing associations of metals on five of these outcomes, we were able to identify associations of metals with specific aspects of memory and learning, such as encoding, retrieval, and declarative learning. Identifying associations of metal toxicity with specific aspects of memory and learning may be useful for implementing future public health interventions.

The main limitation of this study was its cross-sectional design, which limits any causal interpretation of our findings because we were not able to establish temporality. This is particularly relevant when considering our findings on modification by Fe status, because the uptake of metals, particularly in the duodenum, is closely linked with Fe uptake (McCann et al., 2020). Therefore, our findings could instead be interpreted as the modification of associations of Fe status with neurodevelopment by metal biomarker concentrations. Nonetheless, our study makes an important initial contribution in elucidating the potential modifying role of Fe status on metal mixtures in relation to neurodevelopment. We were additionally not able to account for prior metal exposure (e.g., in the prenatal, postnatal or early childhood periods), where early life exposures may be associated with brain connectivity and cognitive function in adolescence (Bauer et al., 2021; Rechtman et al., 2022). We were also unable to control for second-hand smoke exposure, which has been associated with both increased metal biomarker concentrations and adverse neurodevelopment in children (Gao et al., 2022; Karatela et al., 2019), possibly resulting in residual confounding. Our study population was comprised of healthy adolescents, and generally had normal Fe status with minimal indication of any Fe deficiency or overload, which limits the generalizability of these findings to other study populations. Interaction between Fe status and metals may be more (or less) pronounced in Fe-deficient populations (Kupsco et al., 2020), and warrants further research. Additionally, ferritin concentrations may be altered in the presence of infection or inflammation (Gibson, 2005), but we were not able to measure markers of inflammation (e.g., C-reactive protein) to determine if ferritin concentrations in this population were impacted by inflammatory processes.

Participants in the PHIME study may have higher metal exposures than the general population of Italian adolescents given their residential proximity to ferroalloy industry, which may limit the generalizability of our findings. The CVLT-C was only administered in the second wave of PHIME and our sample was significantly reduced compared to the size of the full cohort, which likely affected the precision of our estimates. Because we used objective measures to quantify both metal concentrations and cognitive function, we would expect any exposure or outcome misclassification to be non-differential. We used hair concentrations of Mn, Cr, and Cu to characterize exposure to these metals. Hair, like other biometrics (e.g., blood, nails), is not currently considered a validated biomarker of exposure for Mn, Cr, or Cu (Bertinato and Zouzoulas, 2009; Coetzee et al., 2016; Jursa et al., 2018; Lukaski, 1999). Therefore, non-differential exposure misclassification of these metals is possible. However, the literature indicates that hair concentrations reflect metal exposure from various environmental sources, including dietary intake, over a period of several months, suggesting hair is a reasonable biomarker of exposure for these metals (Agency for Toxic Substances and Disease Registry, 2012b, 2004; Coetzee et al., 2016; Eastman et al., 2013; Kousa et al., 2021; Ntihabose et al., 2018). Although hair samples may be prone to exogenous contamination,(O'Neal and Zheng, 2015) we employed a validated method to extensively clean samples prior to analysis (Eastman et al., 2013; Lucas et al., 2015).

In conclusion, we identified associations of metals individually and as a mixture with verbal learning and memory in adolescents exposed to varied ferroalloy industry. In these data, Mn and Pb were negatively associated with cognitive function, while Cu was positively associated with encoding, learning, and retrieval. Ferritin modified associations between Cu and recall, suggesting Fe status may be an important factor in the beneficial association of Cu with cognition at the concentrations measured in this study. Further research is needed to better understand the role of Fe status in adolescent populations with a wider range of Fe concentrations, using prospective study designs.

ACKNOWLEDGMENTS
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HIGHLIGHTS

- Environmental exposure to individual metals has been associated with neurodevelopmental outcomes in children, and these associations may be modified by iron (Fe) status. However, less is known about metal mixtures.
- A mixture of hair manganese (Mn), blood lead (Pb), hair copper (Cu), hair chromium (Cr), and serum ferritin was jointly associated with better scores on tests of verbal learning and memory, which was driven primarily by Cu.
- A beneficial interaction between Cu and ferritin was estimated, such that Cu was more strongly associated with verbal learning and memory scores at higher percentiles of ferritin.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: