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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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**Samantha Schildroth:** conceptualization, formal analysis, software, writing- original draft, writing-review + editing; **Birgit Claus Henn:** conceptualization, methodology, writing- review + editing, supervision, funding acquisition; **Alexa Friedman:** writing- review + editing, software, validation; **Roberta White:** writing- review + editing, supervision; **Katarzyna Kordas:** writing- review + editing; **Donatella Placidi:** writing- review + editing; methodology, project administration, data curation; **Julia Bauer:** writing- review + editing; **Thomas Webster:** writing- review + editing; **Brent Coull:** writing- review + editing, methodology; **Giuseppa Cagna:** writing- review + editing, project administration, data curation; **Robert Wright:** writing- review + editing, methodology, project administration, funding acquisition; **Donald Smith:** writing- review + editing, methodology, project administration, funding acquisition; **Roberto Lucchini:** writing- review + editing, methodology, project administration, funding acquisition; **Megan Horton:** writing- review + editing, methodology, project administration, funding acquisition.

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

3  
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22  
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24 status, ferritin

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28 **Declaration of conflicts of interest:**

29 *The authors declare they have nothing to disclose.*

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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.

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88 **ABSTRACT**

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

93

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

96

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

104

105 **Results:** Compared to the 50<sup>th</sup> percentile of the metal mixture, the 90<sup>th</sup> percentile was associated with a  
106 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%  
107 CI= -0.20, 0.43) increase in the number of words recalled for trial 5, long delay free, and long delay cued  
108 recall, respectively. For an increase from its 25<sup>th</sup> to 75<sup>th</sup> percentiles, copper was beneficially associated the  
109 recall trials when other metals were fixed at their 50th percentiles (for example, trial 5 recall:  $\beta=0.31$ ,  
110 95% CI= 0.14, 0.48). The association between copper and trial 5 recall was stronger at the 75<sup>th</sup> percentile  
111 of ferritin, compared to the 25<sup>th</sup> or 50<sup>th</sup> percentiles.

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

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## 157 1. INTRODUCTION

158 Environmental exposure to metals is common among children and occurs through several  
159 sources, including diet, contaminated drinking water, consumer products, and air emissions (Agency for  
160 Toxic Substances and Disease Registry, 2020, 2012a, 2012b, 2004). Living in proximity to certain  
161 industries, like ferroalloy plants that manufacture steel, may also lead to increased environmental  
162 exposure to metals like manganese (Mn), lead (Pb), chromium (Cr), copper (Cu) and iron (Fe). Residing  
163 near ferroalloy industry has been associated with increased body burdens of metals in children in Italy  
164 (Butler et al., 2019), the United States (Haynes et al., 2012), Mexico (Riojas-Rodríguez et al., 2010),  
165 Canada (Boudissa et al., 2006), and Brazil (Menezes-Filho et al., 2016, 2009). There is ample  
166 epidemiologic evidence demonstrating that exposure to individual metals can adversely affect cognition  
167 and other neurodevelopmental outcomes in children (Bauer et al., 2020b), but fewer studies have  
168 examined the neurodevelopmental impacts of exposure to mixtures of metals, which may interact or act  
169 jointly (Ahamed and Siddiqui, 2007; Akinyemi et al., 2019; Amos-Kroohs et al., 2017; Neal and Guilarte,  
170 2013; O’Neal et al., 2014; Wang et al., 2016; Zhao et al., 2018). Because the ferroalloy industry is  
171 expected to grow substantially through 2025 (~6% worldwide) (“Ferroalloy Market Share 2018-2025  
172 Industry Growth Outlook Report”), quantifying the impacts of exposure to metal mixtures from ferroalloy  
173 industry is an important public health objective, particularly for susceptible populations like children.

174 Verbal learning and memory are key domains for overall cognitive development and academic  
175 achievement in children (Blankenship et al., 2018, 2014). Disruptions in verbal learning and memory may  
176 have long term implications for child health, as well as for educational achievement and socioeconomic  
177 position in adulthood (Aro et al., 2019). Learning is defined as the ability to acquire new information  
178 (Kreutzer et al., 2011), while memory refers to the ability to encode, store and retrieve learned  
179 information (Delis et al., 1994); both learning and memory are primarily modulated by the hippocampus  
180 and prefrontal cortex (Arnsten, 2009; Hoogman et al., 2017). Metals, such as Pb and Mn, have been  
181 detected in these brain tissues in animal models (Neal and Guilarte, 2013; O’Neal and Zheng, 2015;  
182 Yamagata et al., 2017), where they may exert toxic effects through various mechanisms, including

183 dopaminergic toxicity, oxidative stress, dendritic degeneration, and disruption of ATP synthesis and  
184 neurotransmission (Ahamed and Siddiqui, 2007; Akinyemi et al., 2019; Amos-Kroohs et al., 2017; Neal  
185 and Guilarte, 2013; O’Neal et al., 2014; Wang et al., 2016; Zhao et al., 2018). This suggests that verbal  
186 learning and memory are domains that may be particularly impacted by these metals, a notion supported  
187 by epidemiological studies that have reported adverse associations of individual metals with learning and  
188 memory in children (Carvalho et al., 2018; García-Chimalpopoca et al., 2019; Oulhote et al., 2014;  
189 Torres-Agustín et al., 2013; Wright et al., 2006).

190 Furthermore, emerging epidemiological evidence suggests that children with nutritional  
191 deficiencies, such as Fe deficiency, may be more susceptible to the neurotoxicity of metals (Amorós et al.,  
192 2019; Kupsco et al., 2020; Shah-Kulkarni et al., 2016). Fe is an essential nutrient that plays a role in a  
193 multitude of biologic functions, such as cellular oxygen transport and neurotransmitter synthesis, which  
194 are critical for normal cognitive function (McCann et al., 2020). Altered Fe status (i.e., deficiency or  
195 excess), clinically measured through biomarkers like hemoglobin, ferritin, and transferrin (Gibson, 2005),  
196 has been consistently associated with poorer neurodevelopment (Halterman et al., 2001; Jáuregui-Lobera,  
197 2014; Ji et al., 2017; Lukowski et al., 2010; Parkin et al., 2020; Roy et al., 2011; Tseng et al., 2018; Wang  
198 et al., 2017). Several prior studies of metals and neurodevelopment reported effect modification by Fe  
199 status, where the negative associations of Pb, Mn, and Cu with neurodevelopment were stronger in  
200 children with lower hemoglobin levels (Amorós et al., 2019; Gunier et al., 2015; Kordas et al., 2007;  
201 Kupsco et al., 2020; Shah-Kulkarni et al., 2016). Little is known, however, about how metal neurotoxicity  
202 is modified by Fe status during the adolescent period. Metals may be particularly neurotoxic during  
203 adolescence because several regions of the brain (e.g., prefrontal cortex, temporal lobe) undergo rapid  
204 maturation during this developmental period, including dendritic pruning, maturation of cytoskeletons,  
205 myelination, and refinement of synaptic connections and neurotransmission (Arain et al., 2013; Shaw et  
206 al., 2020). Adolescents are also particularly vulnerable to Fe deficiency given the increased Fe needed to  
207 support rapid physical growth and neural development (Cutler et al., 2009; Das et al., 2017; Leal et al.,  
208 2017; Mesías et al., 2013; Movassagh et al., 2017), and given changes in dietary behaviors in this

209 developmental stage, which may alter the toxicokinetics and toxicodynamics of other metals in relation to  
210 cognitive development. Females are especially vulnerable to Fe deficiency in the adolescent period due to  
211 the onset of menstruation (Zimmermann and Hurrell, 2007). However, few studies have examined effect  
212 modification of metal neurotoxicity by Fe status in adolescence, and none to date have assessed effect  
213 modification of complex metal mixtures.

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

219

## 220 **2. METHODS**

### 221 ***2.1 Study Population***

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

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

243 Guardians of potential participants gave informed consent after receiving detailed information on  
244 study protocols. PHIME study protocols were approved by Institutional Review Boards at the Icahn  
245 School of Medicine at Mount Sinai, University of California Santa Cruz, and the Ethical Committee of  
246 Brescia.

## 247 **2.2 Biomarker Collection and Measurement**

248 Blood and hair samples were collected from study participants at enrollment and evaluated for  
249 metals (Pb, Mn, Cu, Cr). For this analysis, we *a priori* selected blood as a biomarker for Pb and hair as a  
250 biomarker for Mn, Cu and Cr. Blood is a commonly used and accepted biomarker of Pb exposure in the  
251 epidemiology literature (Barbosa et al., 2005). There is not a commonly accepted biomarker of exposure  
252 for Mn, Cu, or Cr (Bertinato and Zouzoulas, 2009; Coetzee et al., 2016; Jursa et al., 2018; Lukaski, 1999).  
253 We selected hair to represent exposure to Mn, Cu and Cr in this analysis because: 1) hair had the least  
254 missing data for these metals (<6%); 2) hair metal concentrations have been correlated with  
255 environmental (e.g., dust, soil, air) concentrations in this cohort (Butler et al., 2019; Lucas et al., 2015)  
256 and elsewhere (Coetzee et al., 2016); and 3) hair metal concentrations have been consistently utilized and  
257 associated with neurodevelopmental outcomes in prior epidemiological studies, including studies in the  
258 current study population (Bauer et al., 2020a; Caparros-Gonzalez et al., 2019; Carvalho et al., 2018;  
259 García-Chimalpopoca et al., 2019; Rechtman et al., 2020; Torres-Agustín et al., 2013; Wright et al.,  
260 2006).

261 We have previously described collection methods for each biomarker in depth (Eastman et al.,  
262 2013; Lucas et al., 2015; Lucchini et al., 2012a; Smith et al., 2007). In brief, venous whole blood samples  
263 (4mL) were collected with 19-gauge butterfly catheters and stored in lithium-heparin Sarstedt Monovette  
264 Vacutainers; these tubes contained a clotting factor, and samples were centrifuged within hours of  
265 sampling to separate the serum. Hair samples were collected from the occipital region of the scalp (2-3  
266 cm, or ~20 mg) using stainless steel clippers, reflecting exposure from the past several months (Agency  
267 for Toxic Substances and Disease Registry, 2012a, 2012b, 2004). Because hair may be susceptible to  
268 exogenous contamination, the samples were then extensively cleaned using Triton detergent, nitric acid  
269 and sonication, as has been previously described (Eastman et al., 2013; Lucas et al., 2015). Metals (Mn,  
270 Pb, Cu, and Cr) were quantified in blood and hair using magnetic sector inductively coupled plasma mass  
271 spectrometry (Eastman et al., 2013; Lucchini et al., 2012a; Smith et al., 2007). Biomarker values below  
272 the limit of detection (LOD) were imputed as the LOD/2 (hair Mn: n=1, hair Cr: n=1); LODs were  
273 defined based on repeated measures of procedural blanks across multiple days (n= 4) (Butler et al., 2019).

274 We used serum ferritin to characterize Fe status in this analysis. Ferritin is considered a sensitive  
275 measure of altered Fe status because levels are reduced in the early stages of Fe deficiency (Gibson,  
276 2005). We quantified serum ferritin from blood samples in immunoassays using the Instrument Architect  
277 *i2000SR* – Abbott Laboratories (Abbott Park, IL, USA).

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### 279 ***2.3 Cognitive Assessment***

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

287 participants selected target words on List A from a written list of 44 words that included both target and  
288 distraction words.

289 Available CVLT-C outcomes for analysis included the total number of correct words recalled on  
290 trial 1, trial 5, trials 1-5 (summed), the interference list, short delay trials (free and cued), and long delay  
291 trials (free and cued). We also calculated three additional scores: intrusions, perseverations, and  
292 forgetting. The sum of intrusions is defined as the total number of non-target words reported across all  
293 trials, and perseverations is defined as the total number of target words repeated within a trial summed  
294 across trials. We calculated scores for forgetting by subtracting the number of correct words on the short  
295 delay free recall trial from the number of correct words on the long delay free recall trial (Kreutzer et al.,  
296 2011; Strauss et al., 2006). Positive scores for the recall trials and recognition indicate better learning and  
297 memory, while positive scores for intrusions and perseverations suggest worse performance. Positive  
298 scores for forgetting suggest better memory (i.e., retention). Full descriptions of each CVLT-C outcome  
299 are provided in **Table 1**. For this analysis, we *a priori* selected and analyzed five CVLT-C outcomes that  
300 reflect varying aspects of verbal learning and memory: trial 5, long delay free recall, long delay cued  
301 recall, perseverations, and forgetting.

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

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

316 <sup>a</sup>CVLT-C outcomes included in this analysis.

317

#### 318 *2.4 Collection of Covariate Data*

319 Trained study staff collected information on potential covariates, including sociodemographic  
320 information, using standardized questionnaires that were administered either in-person or via the phone at  
321 time of enrollment. Information was collected on the following covariates: age (continuous, in years),  
322 biological sex (female or male), birth order (first, second, third, or >third born), area of residence (BM,  
323 GL, or VC), self-reported alcohol consumption (yes or no), self-reported smoking status (smoker vs. non-

324 smoker), parental occupation, and parental education level. We categorized each participant's  
325 socioeconomic status (SES) as low, medium or high based on a method developed for Italian populations  
326 that combines information on parental education and occupation (Cesana et al., 1995; Lucchini et al.,  
327 2012b). HOME scores, which reflect cognitive stimulation at home, were calculated (possible range: 0 –  
328 9) for each participant using nine items selected from the HOME Short Form (National Longitudinal  
329 Surveys, 1979).

## 330 331 **2.5 Data Analysis** 332

333 **2.5.1 Descriptive statistics, confounder selection, and generalized additive models.** Our analytic sample  
334 included all PHIME study participants who completed the CVLT-C (n= 403). Some data were missing  
335 (<6%) for biomarkers and covariates (**Table S1**); therefore, we employed the Markov chain Monte Carlo  
336 method (Zhou et al., 2001) using the *mice* package in R (Buuren and Groothuis-Oudshoorn, 2011) to  
337 impute missing biomarker and covariate data using all available biomarker, outcome, and potential  
338 confounder data. Twenty datasets were imputed under the assumption that data were missing at random.

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

350 outcomes. Spearman correlation coefficients between metals and between CVLT-C outcomes were  
351 estimated across the 20 imputed datasets using the *miceadds* package in R.(Robitzsch and Grund, 2021)

352 Confounders for this analysis were chosen *a priori* using directed acyclic graphs (DAGs) and  
353 prior literature (Bauer et al., 2020b; Carvalho et al., 2018; Kordas, 2010; Torres-Agustín et al., 2013). We  
354 adjusted for age, biological sex, SES, and HOME score as confounders in all analyses. Fe status (i.e.,  
355 ferritin) has been associated with both metal biomarker concentrations and neurodevelopment (Halterman  
356 et al., 2001; Jáuregui-Lobera, 2014; Ji et al., 2017; Lukowski et al., 2010; Parkin et al., 2020; Roy et al.,  
357 2011; Schildroth et al., 2022; Tseng et al., 2018; Wang et al., 2017), suggesting Fe status may be a  
358 confounder of associations between the metal mixture and neurodevelopment. We therefore included  
359 ferritin as a covariate in all regression models. However, effect modification by Fe status was also  
360 considered in both multivariable linear regression and Bayesian Kernel Machine Regression Models  
361 (described below). Ferritin, age, and HOME scores were modeled as continuous covariates, as they were  
362 linearly related to CVLT-C outcomes based on visual inspection of penalized splines (constrained to 4  
363 knots) from generalized additive models (GAMs). Sex and SES were treated as categorical covariates.

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

373

374 **2.5.2 Multivariable Linear Regression.** We first fit fully adjusted multivariable linear regression models  
375 with all four metals (Mn, Pb, Cu, Cr) and ferritin to examine associations of each metal with CVLT-C

376 outcomes. These models initially included all pairwise cross-product terms between metals to identify  
377 potential metal-metal interactions in relation to CVLT-C outcomes. Potential modification by Fe status  
378 was similarly evaluated in a separate model by including all pairwise interaction terms between each  
379 metal and ferritin. We *a priori* selected  $p < 0.10$  as the cutoff for retaining interaction terms in our final  
380 linear regression models. No pairwise metal-metal interaction terms were significant for any CVLT-C  
381 outcome among the full cohort; those interactions were therefore not included in the subsequent linear  
382 regression models. However, there was evidence of metal-ferritin interactions for Cu and Mn in relation  
383 to trial 5 and forgetting, respectively ( $p < 0.10$ ). Final models for trial 5 and forgetting therefore retained  
384 the significant Cu-ferritin and Mn-ferritin interaction terms, respectively, while final models for long  
385 delay free recall, long delay cued recall, and perseverations did not include any metal-ferritin interaction  
386 terms.

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

$$396 \quad [1] \% \text{ difference in perseveration scores} = (e^{(\ln(2) * \beta)} - 1) * 100$$

397

398 **2.5.3 Bayesian Kernel Machine Regression.** Next, we used Bayesian Kernel Machine Regression  
399 (BKMR) to further examine the association of the metal mixture with CVLT outcomes. Although there  
400 was limited evidence of nonlinearity in the GAMs or of pairwise metal interactions in the multivariable  
401 linear regression models, using BKMR allowed for investigation of potential higher-order interactions

402 (i.e., interaction of each exposure with multiple components of the mixture), as well as for the estimation  
 403 of joint effects of the overall mixture with CVLT outcomes (Bobb et al., 2015).

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

$$410 \quad [2] \text{ CVLT score}_i = h(\text{Mn}_i, \text{Pb}_i, \text{Cr}_i, \text{Cu}_i, \text{Ferritin}_i) + \beta_1 * \text{Sex}_i + \beta_2 * \text{Age}_i + \beta_3 * \text{SES}_i + \beta_4 * \\
 411 \quad \text{HOME score}_i + e_i,$$

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

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

$$424 \quad [3] \% \text{ difference in perseveration scores} = (e^\beta - 1) * 100$$

425 To describe the associations of the metal mixture with learning and memory, we estimated the  
 426 following for each CVLT-C outcome: 1) exposure-response profiles for each metal, holding all other  
 427

431 metals at their 50<sup>th</sup> percentiles; 2) exposure-response profiles for each metal estimated at varying (25<sup>th</sup>,  
432 50<sup>th</sup>, 75<sup>th</sup>) percentiles of a second metal, while holding remaining metals at their 50<sup>th</sup> percentiles; 3)  
433 associations of each metal comparing its 75<sup>th</sup> percentile to its 25<sup>th</sup> percentile when all other metals are  
434 held at their 25<sup>th</sup>, 50<sup>th</sup>, or 75<sup>th</sup> percentiles; and 4) the joint association of a percentile change in all metals  
435 simultaneously, compared to the 50<sup>th</sup> percentile of all metals.

436 We ran sensitivity analyses to evaluate the robustness of findings by 1) using the gamma prior  
437 distribution instead of the default inverse uniform distribution; 2) changing the degree of smoothness of  
438 the  $h$  function from the default ( $b=100$ ) to lower and higher degrees of smoothness ( $b=50$  and  $1000$ ,  
439 respectively) (Bauer et al., 2020a; Valeri et al., 2017); and 3) increasing the number of iterations to  
440 50,000 (from 10,000).

441  
442 **2.5.4 Sex-stratified Analyses.** There is evidence in the literature to suggest that 1) associations between  
443 metals and domains of neurodevelopment may be sexually dimorphic, and 2) female adolescents are more  
444 susceptible to Fe deficiency (Bauer et al., 2017; Kounnavong et al., 2020; Llop et al., 2013; Rechtman et  
445 al., 2020; Shaw, 1996; Zhu et al., 2021). Therefore, we assessed potential sex-specific effects in  
446 exploratory analyses. We stratified imputed datasets by sex and re-ran the above multivariable linear  
447 regression and BKMR models to evaluate sex-specific associations.

### 448 3. RESULTS

#### 449 3.1 Study Population Characteristics

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

458 75<sup>th</sup> percentile: 22.0 – 46.0 ng/mL). Summary statistics were similar between the imputed and complete  
 459 data (**Table S2**). Summary statistics were also similar for adolescents included in the analysis and those  
 460 who were excluded due to missing outcome data; however, adolescents who were excluded due to  
 461 missing outcome data (n= 318) had lower ferritin concentrations (median: 21.0 ng/mL; 25<sup>th</sup> – 75<sup>th</sup>  
 462 percentile: 12.0 – 34.0 ng/mL; **Table S2**).

Characteristic	N (percent) or mean $\pm$ SD
Sex	
Female	182 (47.5%)
Male	201 (52.5%)
Age (years)	12.3 $\pm$ 1.0
Socioeconomic status index	
Low	89 (23.2%)
Medium	201 (52.5%)
High	93 (24.3%)
HOME score	6.0 $\pm$ 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 $\pm$ 2.1
Long delay cued recall	11.8 $\pm$ 2.1
Trial 5	12.3 $\pm$ 1.9
Perseverations	7.2 $\pm$ 6.1
Forgetting	0.3 $\pm$ 1.6
Metal biomarkers (median, 25 <sup>th</sup> , 75 <sup>th</sup> percentile)	
Hair Mn ( $\mu$ g/g)	0.07 (0.04, 0.12)
Hair Cu ( $\mu$ g/g)	9.4 (6.6, 14.8)
Hair Cr ( $\mu$ g/g)	0.04 (0.03, 0.06)
Blood Pb ( $\mu$ g/dL)	1.3 (1.0, 1.7)
Iron biomarker (median, 25 <sup>th</sup> , 75 <sup>th</sup> percentile)	
Ferritin (ng/mL)	32.0 (21.0, 44.0)

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

464 <sup>a</sup>PHIME, Public Health Impact of Metals Exposure Study; HOME, Home Observation Measurement of  
 465 the Environment; CVLT-C, California Verbal Learning Test for Children; Mn, manganese; Cu, copper;  
 466 Cr, chromium; Pb, lead.

467 Median hair metal concentrations were highest for Cu (median: 9.4  $\mu$ g/g; 25<sup>th</sup> – 75<sup>th</sup> percentile:  
 468 6.6 – 14.8  $\mu$ g/g), followed by Mn (median: 0.07  $\mu$ g/g; 25<sup>th</sup> – 75<sup>th</sup> percentile: 0.04 – 0.12  $\mu$ g/g) and Cr  
 469 (median: 0.04  $\mu$ g/g; 25<sup>th</sup> – 75<sup>th</sup> percentile: 0.03 – 0.06  $\mu$ g/g) (**Table 2**). The median blood Pb

470 concentration in study participants (1.3  $\mu\text{g/dL}$ ; 25<sup>th</sup> – 75<sup>th</sup> percentile: 1.0 – 1.7  $\mu\text{g/dL}$ ) was lower than the  
471 current Centers for Disease Control and Prevention reference value of 3.5  $\mu\text{g/dL}$  (Centers for Disease  
472 Control and Prevention, 2022). Males had higher blood Pb concentrations (median: 1.4  $\mu\text{g/dL}$ ; 25<sup>th</sup> – 75<sup>th</sup>  
473 percentile: 1.1 – 2.0  $\mu\text{g/dL}$ ) compared to females (median: 1.1  $\mu\text{g/dL}$ ; 25<sup>th</sup> – 75<sup>th</sup> percentile: 0.9 – 1.4  
474  $\mu\text{g/dL}$ ), while females had higher hair Cu concentrations (median: 10.3  $\mu\text{g/g}$ ; 25<sup>th</sup> – 75<sup>th</sup> percentile: 7.6 –  
475 16.5  $\mu\text{g/g}$ ) compared to males (median: 8.6  $\mu\text{g/g}$ ; 25<sup>th</sup> – 75<sup>th</sup> percentile: 6.3 – 13.7  $\mu\text{g/g}$ ) (**Table S3**).  
476 Concentrations of hair Mn and Cr were similar between males and females.

477 Metal concentrations were not highly correlated; Spearman correlation coefficients ranged from  
478 0.04 (Pb-Cr) to 0.36 (Mn-Cu), and were strongest among the three metals measured in hair (Mn, Cu, Cr)  
479 (**Figure S1**). The strongest correlations between the five CVLT-C outcomes were observed among the  
480 recall trials, including trial 5, long delay free, and long delay cued recall ( $r_s = 0.51 - 0.79$ ).

481

### 482 **3.2 Multivariable Linear Regression**

483 In fully adjusted linear regression models, a 1-SD increase in ln-hair Cu was associated with  
484 better performance (i.e., more words recalled) on trial 5 ( $\beta = 0.20$ , 95% CI= 0.10, 0.31), long delay free  
485 ( $\beta = 0.28$ , 95% CI= 0.17, 0.39), and long delay cued ( $\beta = 0.22$ , 95% CI= 0.11, 0.33) recall (**Figure 1**,  
486 **Table S4**). There was evidence of a positive interaction between Cu and ferritin ( $\beta$ -interaction= 0.13,  
487 95% CI= 0.02, 0.25; p-interaction= 0.02), suggesting that the positive (beneficial) Cu association was  
488 stronger with increasing ferritin concentrations.

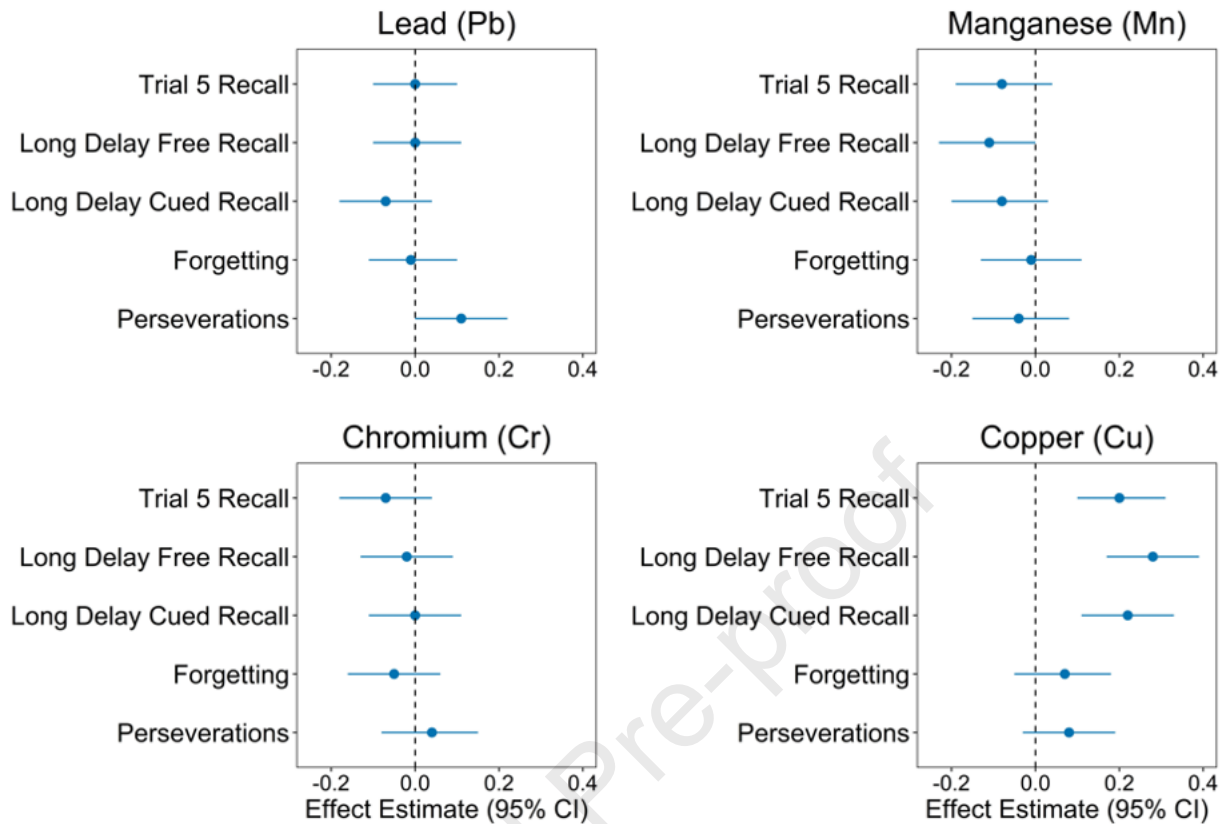
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495 **Figure 1.** Main effect estimates and 95% confidence intervals from multivariable linear regression  
 496 models describing associations of ln-transformed Z-standardized metals (Pb, Mn, Cr, and Cu) with Z-  
 497 standardized CVLT-C outcomes. Perseveration scores were also ln-transformed. Models were mutually  
 498 adjusted for all metals, ferritin, age, sex, SES, and HOME score. Models for trial 5 and forgetting  
 499 included Cu-ferritin and Mn-ferritin interaction terms, respectively. Note: Pb, lead; Mn, manganese; Cr,  
 500 chromium; Cu, copper; CVLT-C, California Verbal Learning Test for Children; SES, socioeconomic  
 501 status; HOME, Home Observation Measurement of the Environment.  
 502

503 In contrast to Cu, hair Mn was weakly associated with worse performance (i.e., fewer words  
 504 recalled) on the recall trials: a 1-SD increase in ln-Mn was associated with a 0.08 SD decrease (95% CI= -  
 505 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  
 506 words recalled on trial 5, long delay free, and long delay cued recall, respectively. Although Mn  
 507 associations with forgetting were null ( $\beta = -0.01$ , 95% CI= -0.13, 0.11), there was evidence of an  
 508 interaction between Mn and ferritin ( $\beta$ -interaction= -0.12, 95% CI= -0.23, -0.02; p-interaction= 0.03),  
 509 such that Mn was adversely associated with forgetting at increasing concentrations of ferritin (**Table S4**).

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

513

### 514 **3.3 Bayesian Kernel Machine Regression**

515 Posterior inclusion probabilities for each metal and ferritin across all outcomes are provided in  
516 **Table S5**. Cu had the highest PIP for trial 5 (0.98), long delay free (0.94) and long delay cued (0.94)  
517 recall, while Pb had the highest PIP for perseverations (0.60). Similar to findings from multivariable  
518 linear regression models, Cu was positively associated with each of the recall trials: when all other metals  
519 and ferritin were held at their medians, Cu was associated with a 0.31 SD increase (95% CI= 0.14, 0.48),  
520 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  
521 trial 5, long delay free, and long delay cued recall, respectively, when increased from the 25<sup>th</sup> to the 75<sup>th</sup>  
522 percentile (**Figures S2-S4, panel D**).

523 A modest interaction between Cu and the other components of the mixture (Pb, Mn, Cr, ferritin)  
524 was observed: the association of Cu with trial 5 recall was almost twice as strong when the mixture was  
525 fixed at its 75<sup>th</sup> percentile (for an increase in Cu from the 25<sup>th</sup> to 75<sup>th</sup> percentiles,  $\beta= 0.37$ , 95% CI= 0.17,  
526 0.57) compared to when the mixture was fixed at its 25<sup>th</sup> percentile ( $\beta= 0.21$ , 95% CI= 0.00, 0.43; **Figure**  
527 **2**). Consistent with linear regression models, this higher-order interaction was driven by the positive  
528 interaction between Cu and ferritin, where Cu was more strongly associated with correct responses on  
529 trial 5 at higher levels (i.e., 75<sup>th</sup> percentile) of ferritin (**Figure 2, panels A and B**).

530

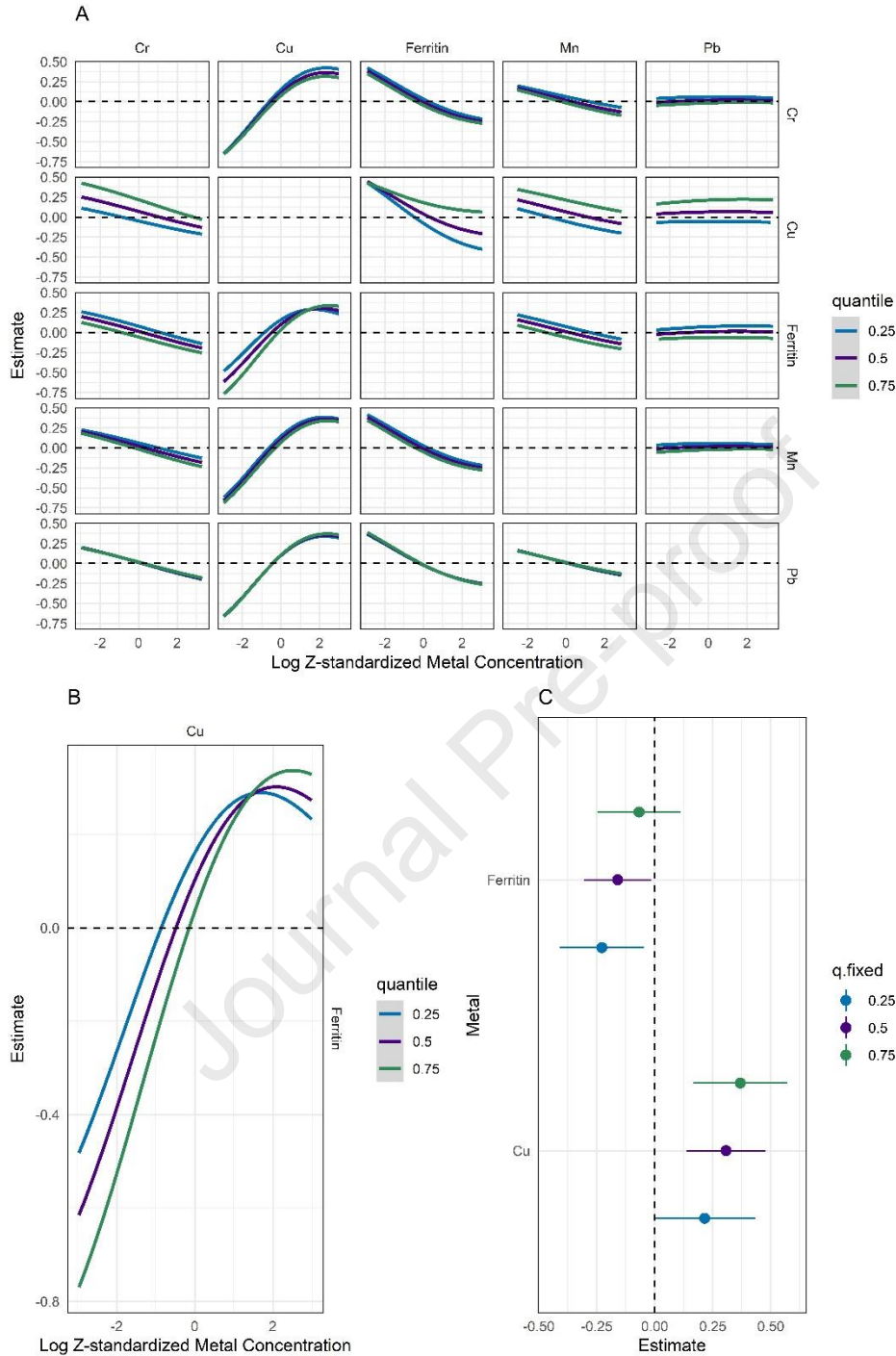
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537 **Figure 2.** (A) Pairwise exposure-response relationships from BKMR models for each metal and ferritin with trial 5  
 538 at varying levels of other metals (25<sup>th</sup>, 50<sup>th</sup>, and 75<sup>th</sup> percentiles), while all other metals were set to their medians. (B)  
 539 Exposure-response relationships from BKMR models for Cu with trial 5 at varying levels of ferritin (25<sup>th</sup>, 50<sup>th</sup>, and  
 540 75<sup>th</sup> percentiles), while all other metals were set to their medians. (C) Estimates and 95% credible intervals for the  
 541 associations between an increase in Cu and ferritin from the 25<sup>th</sup> to 75<sup>th</sup> percentiles and trial 5 recall, when all other  
 542 metals were set to their 25<sup>th</sup>, 50<sup>th</sup>, or 75<sup>th</sup> percentiles. Metal and ferritin concentrations were ln-transformed and Z-  
 543 standardized, and CVLT-C outcomes were Z-standardized. Models were adjusted for age, sex, SES, and HOME

544 score. Note: BKMR, Bayesian Kernel Machine Regression; Cu, copper; CVLT-C, California Verbal Learning Test  
545 for Children; SES, socioeconomic status; HOME, Home Observation Measurement of the Environment.  
546

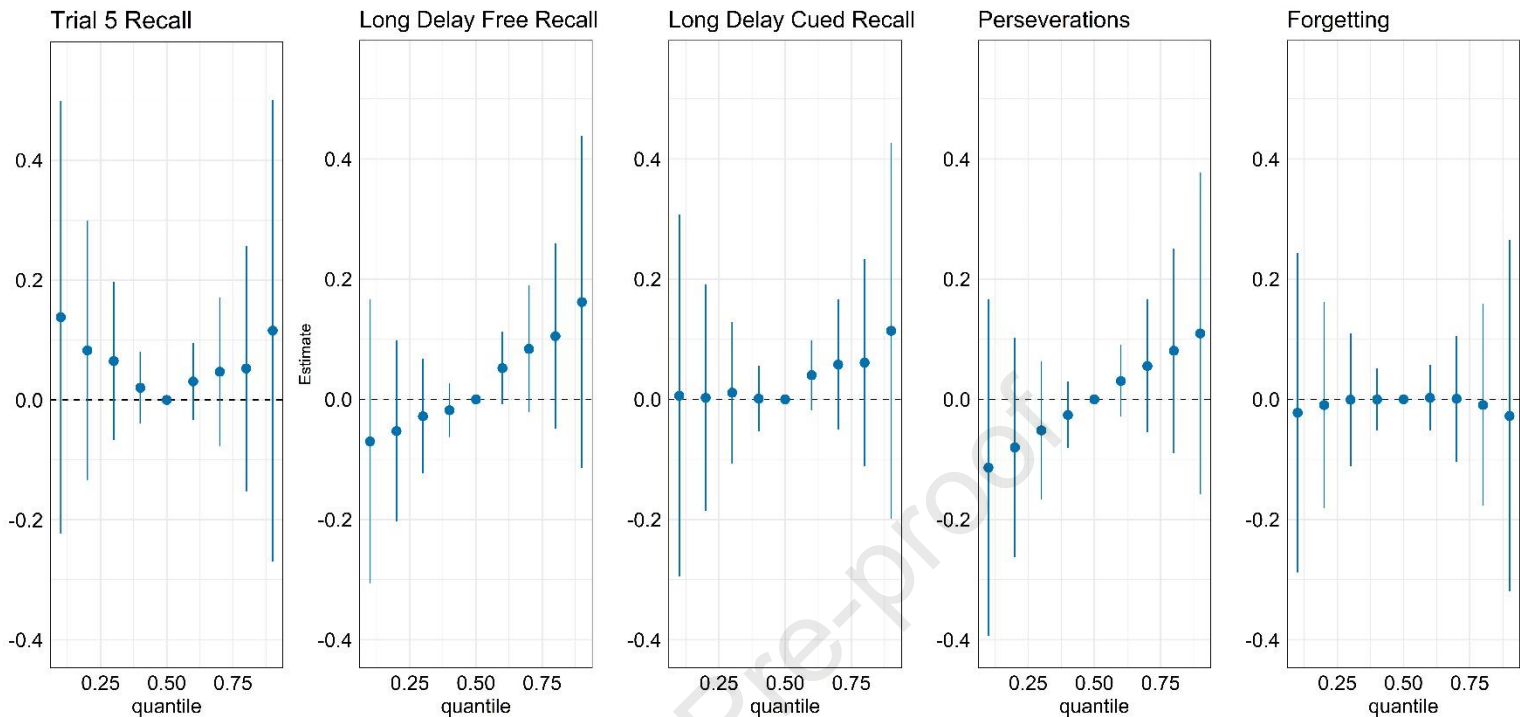
547 As with multivariable linear regression models, Mn was weakly associated with worse CVLT  
548 scores (i.e., fewer words recalled) for the recall trials when all other metals and ferritin were fixed at their  
549 median (trial 5:  $\beta = -0.08$ , 95% CI= -0.22, 0.07; long delay free:  $\beta =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

552 Pb was not materially associated with CVLT outcomes (**Figures S2-S6**), with the exception of an  
553 association between blood Pb and perseverations: an increase in Pb from the 25th to 75th percentiles was  
554 associated with a 15.6% increase in perseveration errors, when all other metals were held at their 50<sup>th</sup>  
555 percentiles ( $\beta = 0.08$ , 95% CI= -0.09, 0.25).

556 Compared to the 50<sup>th</sup> percentile, higher percentiles of the mixture (Pb, Mn, Cr, Cu, ferritin)  
557 tended to be associated, though imprecisely, with better recall for trial 5, long delay free, and long delay  
558 cued recall (**Figure 3**). For example, the 90<sup>th</sup> percentile of the mixture was associated, though  
559 imprecisely, with better scores for trial 5 ( $\beta = 0.12$ , 95% CI= -0.27, 0.50), long delay free ( $\beta = 0.16$ , 95%  
560 CI= -0.11, 0.44), and long delay cued ( $\beta = 0.11$ , 95% CI= -0.20, 0.43) recall, compared to the 50<sup>th</sup>  
561 percentile of the mixture. Further, the shape of the association of the overall mixture with trial 5 recall  
562 was U-shaped, such that the 10<sup>th</sup> percentile of the mixture, compared to the 50<sup>th</sup> percentile, was also  
563 positively associated with trial 5 recall ( $\beta = 0.14$ , 95% CI= -0.22, 0.50).

564 Increasing concentrations of the overall mixture (Pb, Mn, Cr, Cu, ferritin) were also associated  
565 with increased perseverations, although associations were imprecise: compared to the 50<sup>th</sup> percentile, the  
566 90<sup>th</sup> percentile of the mixture was associated with a 22.0% increase ( $\beta = 0.11$ , 95% CI= -0.16, 0.38) in  
567 perseverations (**Figure 3**), which was driven primarily by Pb (**Figure S6, panel D**). The overall mixture  
568 was not associated with forgetting.

569



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

576

577

### 578 3.4 Sex-stratified Analyses

579 In multivariable regression models exploring sex-specific associations, Cu was positively

580 associated with scores on the recall trials among both females and males, but tended to be stronger in

581 females, suggesting that Cu may be more beneficial among females (**Table S4**). For example, the

582 association between Cu and long delay cued recall was twice as strong in females ( $\beta = 0.29$ , 95% CI=

583 0.13, 0.44) compared to males ( $\beta = 0.14$ , 95% CI= -0.02, 0.30), though the interaction p-values were

584  $>0.10$ . This is consistent with findings from sex-stratified BKMR models (**Figures S7-S16**).

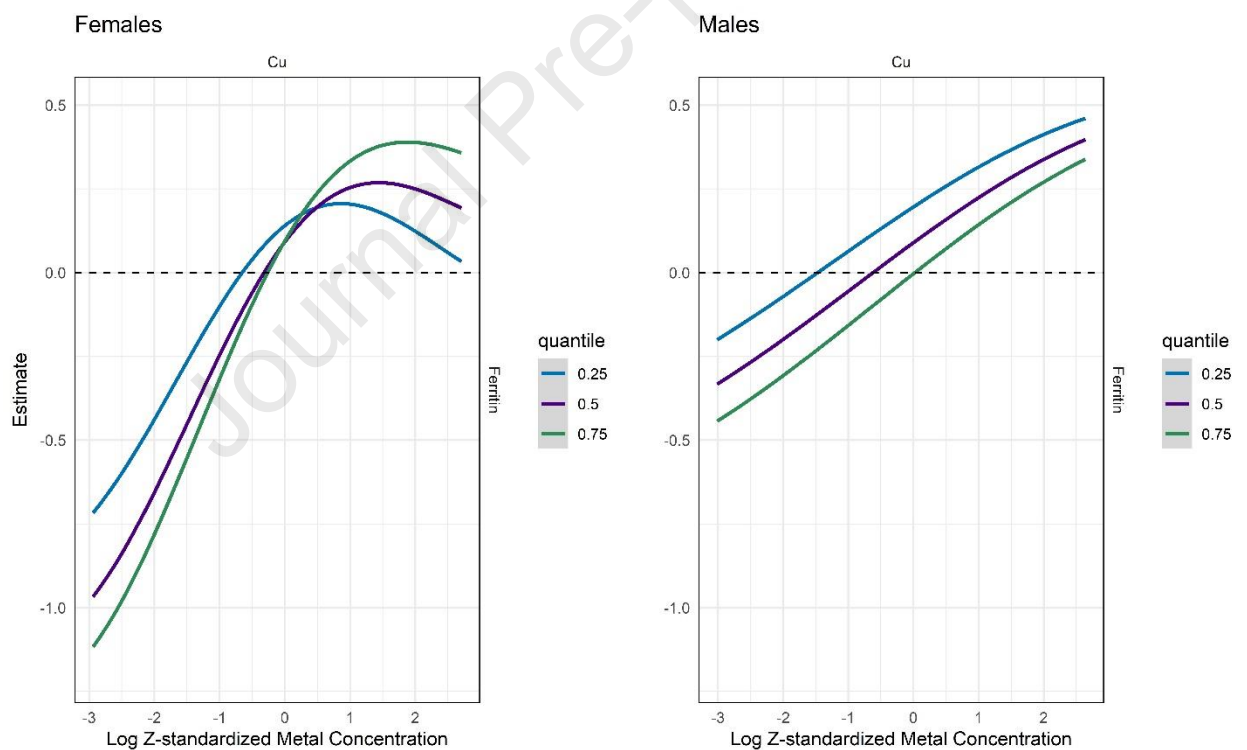
585 Higher concentrations of Mn were negatively associated with trial 5 recall in females (from

586 multivariable regression,  $\beta = -0.21$ , 95% CI= -0.37, -0.04), while this association was null in males ( $\beta =$

587 0.04, 95% CI= -0.12, 0.21; p-interaction= 0.09); this was similar in BKMR models (**Figures S7-S8, panel**

588 **D)**. Among females, Pb was also associated with worse scores for long delay cued recall (from  
 589 multivariable regression, females:  $\beta = -0.14$ , 95% CI=  $-0.29, 0.01$ ; males:  $\beta = 0.01$ , 95% CI=  $-0.14, 0.17$ ),  
 590 forgetting (females:  $\beta = -0.11$ , 95% CI=  $-0.27, 0.04$ ; males:  $\beta = 0.11$ , 95% CI=  $-0.04, 0.26$ ; p-  
 591 interaction=0.06), and In-perseverations (females:  $\beta = 0.17$ , 95% CI=  $0.02, 0.32$ ; males:  $\beta = 0.02$ , 95% CI=  
 592  $-0.14, 0.17$ ) compared to males. Associations for Pb were similar across varying percentiles of the  
 593 mixture in BKMR models (**Figures S11-S16, panel D**).

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



598

599 **Figure 4.** Pairwise exposure-response relationship from sex-stratified BKMR models for Cu with trial 5  
 600 at varying levels of ferritin (25<sup>th</sup>, 50<sup>th</sup>, and 75<sup>th</sup> percentiles), while all other metals were set to their  
 601 medians. Metal and ferritin concentrations were ln-transformed and Z-standardized, and CVLT-C  
 602 outcomes were Z-standardized. Models were adjusted for age, SES, and HOME score. Note: BKMR,  
 603 Bayesian Kernel Machine Regression; Cu, copper; CVLT-C, California Verbal Learning Test for  
 604 Children; SES, socioeconomic status; HOME, Home Observation Measurement of the Environment.  
 605

### 606 3.5 Sensitivity Analyses

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

## 614 4. DISCUSSION

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

624 Consistent with our findings, hair Mn and blood Pb have been associated with worse learning and  
625 memory in prior studies, including on the subtests of the CVLT-C, Children's Auditory Verbal Learning  
626 Test (CAVLT), and the Developmental Neuropsychological Assessment Battery (NEPSY) (Carvalho et  
627 al., 2018; García-Chimalpopoca et al., 2019; Oulhote et al., 2014; Torres-Agustín et al., 2013; Wright et  
628 al., 2006; Yorifuji et al., 2011). These findings are supported by mechanistic and animal evidence: Pb and  
629 Mn are transported into the brain via metal transporters, such as the divalent metal transporter 1 (DMT1)  
630 or transferrin-mediated mechanisms, and may exert several mechanisms of toxicity (e.g., dopaminergic  
631 dysregulation, disruption of neurotransmission, dendritic degeneration, and cytotoxicity) (Ahamed and  
632

633 Siddiqui, 2007; Akinyemi et al., 2019; Neal and Guilarte, 2013; O’Neal et al., 2014; O’Neal and Zheng,  
634 2015; Yamagata et al., 2017). In animal models, Pb exposure has been shown to alter protein kinase C  
635 activity, which plays a key role in regulating membrane structure, transcription, cell growth,  
636 neurotransmitter release, neuronal plasticity, and ion channels (Sanders et al., 2009). Protein kinase C  
637 specifically modulates learning and memory, and altered activity following Pb exposure has been shown  
638 to lead to prefrontal cortex toxicity (Sanders et al., 2009). Mn has been shown to induce toxicity in  
639 hippocampal neurons by attenuating long-term potentiation (Amos-Kroohs et al., 2017; Wang et al.,  
640 2016; Zhao et al., 2018), which may explain the negative associations we observed between Mn and the  
641 recall trials that reflect various aspects of learning and memory modulated in part by the hippocampus  
642 (**Table 1**). These findings may also reflect alterations in intrinsic functional connectivity in brain regions,  
643 including the frontal and temporal lobes, following early life Mn exposure (Rechtman et al., 2022).  
644 However, additional studies in humans using brain imaging methods (e.g., structural and functional  
645 magnetic resonance imaging) would provide further insight into the association of metal mixtures with the  
646 underlying neuroanatomy and functional connectivity to support epidemiological findings.

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

659  $\mu\text{g/g}$ ) than in the prior study that utilized the full cohort (range: 1.7 – 191.0  $\mu\text{g/g}$ ). It is possible that the  
660 highest Cu concentrations in the current analysis were not elevated enough to induce neurotoxicity.

661 We found that the metal mixture was jointly associated with better scores on the recall trials at  
662 higher percentiles of all metals and ferritin, which likely reflects the beneficial association of Cu with  
663 recall. This finding is consistent with a prior study in the PHIME cohort that similarly found the overall  
664 mixture measured in multiple biomarkers (hair, blood, nails, saliva, urine) was beneficially associated  
665 with visuospatial learning and memory (Rechtman et al., 2020). However, in the prior study, the  
666 beneficial association of the mixture was observed only in males, and was driven primarily by Cr. In  
667 females, the mixture was adversely associated with visuospatial learning and memory (Rechtman et al.,  
668 2020), suggesting the neurotoxicity of metals in adolescence may be sex- and domain- (e.g., verbal vs.  
669 visuospatial) specific. It should be noted that the associations of the overall mixture with the CVLT-C  
670 scores were modest in magnitude: for example, the association with trial 5 recall when all metals are at  
671 their 90<sup>th</sup> percentile (compared to the 50<sup>th</sup> percentile) is equivalent to an increase of 0.23 words recalled  
672 ( $\beta \times \text{SD}$ ,  $\beta = 0.12$ , see **Table 1**). These associations therefore reflect subclinical impacts on learning and  
673 memory. Further, we did not observe pairwise or higher order interactions between any of the metals  
674 (Mn, Pb, Cu, and Cr) in our study, contrary to previous work in the PHIME cohort (Bauer et al., 2020a).  
675 Specifically, Bauer et al. observed interactions between hair Cu- hair Mn and hair Cu- blood Pb, such that  
676 the neurotoxic associations of Mn and Pb with verbal IQ were stronger at lower Cu concentrations (Bauer  
677 et al., 2020a). These metal-metal interactions are supported by other epidemiological and animal data (Fu  
678 et al., 2015; Guilarte and Chen, 2007; Liu et al., 2018; Robison et al., 2013; Zheng et al., 2009). It is  
679 possible that we did not observe similar interactions between metals in the current analysis due to our  
680 small sample size.

681 When we considered Fe status as a modifier, a modest interaction between Cu and ferritin was  
682 estimated for number of words recalled in trial 5, such that the positive association of Cu with recall was  
683 stronger at higher concentrations of ferritin. Mechanisms of Cu and Fe cellular uptake and transport are  
684 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.

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

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

736 learning and memory; by assessing associations of metals on five of these outcomes, we were able to  
737 identify associations of metals with specific aspects of memory and learning, such as encoding, retrieval,  
738 and declarative learning. Identifying associations of metal toxicity with specific aspects of memory and  
739 learning may be useful for implementing future public health interventions.

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

760         Participants in the PHIME study may have higher metal exposures than the general population of  
761 Italian adolescents given their residential proximity to ferroalloy industry, which may limit the

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

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

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**HIGHLIGHTS**

- 1
- 2     • Environmental exposure to individual metals has been associated with neurodevelopmental
- 3         outcomes in children, and these associations may be modified by iron (Fe) status. However, less
- 4         is known about metal mixtures.
- 5     • A mixture of hair manganese (Mn), blood lead (Pb), hair copper (Cu), hair chromium (Cr), and
- 6         serum ferritin was jointly associated with better scores on tests of verbal learning and memory,
- 7         which was driven primarily by Cu.
- 8     • A beneficial interaction between Cu and ferritin was estimated, such that Cu was more strongly
- 9         associated with verbal learning and memory scores at higher percentiles of ferritin.

10

**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:

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