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Manganese transporter genetics and sex modify the association between environmental manganese exposure and neurobehavioral outcomes in children

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ABSTRACT

There is increasing evidence that environmental manganese (Mn) exposure early in life can have negative effects on children's neurodevelopment and increase the risk of behavioral problems, including attention deficit hyperactivity disorder (ADHD). Factors that may contribute to differences in sensitivity to Mn exposure are sex and genetic variation of proteins involved in the regulation of Mn concentrations. Here we investigate if sex and polymorphisms in Mn transporter genes *SLC30A10* and *SLC39A8* influence the association between Mn exposure and ADHD-related behavioral problems in children.

The SNPs rs1776029 and rs12064812 in *SLC30A10*, and rs13107325 in *SLC39A8* were genotyped by TaqMan PCR or pyrosequencing in a population of Italian children (aged 11–14 years; n = 645) with a wide range of environmental Mn exposure. Mn in surface soil was measured in situ using XRF technology or modeled by geospatial analysis. Linear regression models or generalized additive models (GAM) were used for analyzing associations between soil Mn and neurobehavioral problems assessed by the Conners' behavior rating scales (self-, and parent-reported). Gene-environment interactions (Mn transporter genotype x soil Mn) were evaluated using a genetic score in which genotypes for the three SNPs were combined based on their association with blood Mn, as an indication of their influence on Mn regulation.

We observed differences in associations between soil Mn and neurobehavior between sexes. For several self-reported Conners' scales, girls showed U-shaped relationships with higher (worse) Conners' scoring at higher soil Mn levels, and several parent-reported scales showed positive linear relationships between increasing soil Mn and higher Conner's scores. For boys, we observed a positive linear relationship with soil Mn for one Conner's outcome only (hyperactivity, parent-reported). We also observed some interactions between soil Mn and the genetic score on Conner's scales in girls and girls with genotypes linked to high blood Mn showed particularly strong positive associations between soil Mn and parent-reported Conners' scales.

Our results indicate that sex and polymorphisms in Mn transporter genes contribute to differences in sensitivity to Mn exposure from the environment and that girls that are genetically less efficient at regulating Mn, may be a particularly vulnerable group.

1. Introduction

Manganese (Mn) is an essential element crucial for brain function and neurodevelopment (Lucchini et al., 2014). There is a narrow range of optimal Mn concentrations in the body and both high and low Mn concentrations have been associated with negative impact on

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neurodevelopment (Claus Henn et al., 2010). Elevated Mn concentrations can be caused by environmental exposure and there is emerging evidence that even modest Mn exposure in early life can disrupt children's neurodevelopment with adverse effects on both cognition and neurobehavior (Beaudin et al., 2017; Bhang et al., 2013; Bouchard et al., 2007; Bouchard et al., 2011; Chung et al., 2015; Mora et al., 2015; Shin et al., 2015; Torres-Agustin et al., 2013). Several studies also show different associations between Mn exposure and neurological effects between girls and boys, suggesting that there could be differences in sensitivity to Mn exposure between sexes (Bauer et al., 2017; Gunier et al., 2015; Menezes-Filho et al., 2014; Mora et al., 2015; Riojas-Rodriguez et al., 2010).

Mn concentrations in the body are regulated by homeostasis which involves the metal transporters proteins SLC30A10 and SLC39A8 (Chen et al., 2015). SLC30A10 is an efflux transporter specific to Mn (Leyva-Illades et al., 2014) and SLC39A8 is an influx transporter with high affinity to Mn, but which also can transport iron (Fe), zinc (Zn) and cadmium (Cd) (He et al., 2006; Wang et al., 2012). Loss-of-function mutations in either of the *SLC30A10* or *SLC39A8* genes (coding for the SLC30A10 and SLC39A8 proteins respectively) cause severe dysregulation of physiological Mn concentrations and neurological symptoms (Boycott et al., 2015; Quadri et al., 2012; Tuschl et al., 2012), which show that SLC30A10 and SLC39A8 are crucial for efficient Mn regulation. Also, common genetic variations in these genes can influence Mn concentrations in different life-stages (Wahlberg et al., 2017; Wahlberg et al., 2016). In a recent study, we showed that single nucleotide polymorphisms (SNPs) in *SLC30A10* and *SLC39A8* strongly contributed to differences in Mn concentrations between children and were associated with neurodevelopmental outcomes, particularly test scores for ADHD-related behavioral problems (Wahlberg et al., 2018). The results indicated that variations in *SLC30A10* and *SLC39A8* may cause differences in the efficiency of Mn regulation between children and thereby influence their neurodevelopment and behavior. We have also demonstrated that the same SNPs influence Mn concentrations in early life differently between developmental life stages (as measured by teeth dentine) and between sexes (Wahlberg et al., 2017).

In this study, we investigate the hypothesis that sensitivity to neurobehavioral problems from environmental Mn exposure may be influenced by both sex and genetics that affect the capacity to regulate Mn concentrations in the body. In a group of children with low to moderate environmental Mn exposure, we evaluate differences in associations of Mn exposure (soil Mn) and ADHD-related behavioral problems (Conners' behavior rating scores) between girls and boys, and between children with different genotypes for SNPs in *SLC30A10* (rs1776029 and rs12064812) and *SLC39A8* (rs13107325).

2. Material and methods

2.1. Study population

In this study, we used a cross-sectional design. The complete study cohort consisted of 719 children, aged 11–14 from the Province of Brescia, Italy. The children come from the three districts Valcamonica valley, Bagnolo Mella and Lake Garda. Valcamonica and Bagnolo Mella both have a history of industrial Mn contamination and environmental Mn exposure (Lucas et al., 2015). In Valcamonica, ferromanganese alloy plants were active during 1902 to 2001, and in Bagnolo Mella, one plant produced ferromanganese since 1973 and was still active during data collection. In contrast, Lake Garda has no history of ferromanganese production. Study areas and recruitment have previously been described (Bauer et al., 2017; Lucchini et al., 2012a).

Recruitment was performed through the public-school system. Forms of consent were given to 1232 children of which 870 agreed to participate (participation rate = 70.6%). Out of the 870, 135 were rejected based on inclusion/exclusion criteria and 16 were excluded to maintain balance between age and districts, resulting in 719 children that were enrolled for the study (a participant flow chart is presented in Supplemental Fig. 1). The inclusion criteria were: (i) born in the study area from resident families living in the study area since the 1970s; (ii) living in the study area since birth; (iii) being between 11 and 14 years of age. Exclusion criteria were: (i) pre-existing neurological, hepatic, metabolic, endocrine, and psychiatric conditions affecting neurodevelopment (in order to evaluate genetics influencing normal neurodevelopment); (ii) taking medications with known neuropsychological side-effects; (iii) clinically diagnosed motor impairment of hand and fingers; (iv) pre-existing clinical diagnosis of neurodevelopmental disorders, e.g. attention deficit hyperactivity disorder (ADHD) and autism (v) visual deficits not adequately corrected. Thus, based on these exclusion criteria, the study group represented a neurologically normal group. A first round of recruitment, including 311 children from Valcamonica and Lake Garda, started in 2007 and was completed in 2010; data collection in this round included personal sampling of air, water, and soil, indoor and outdoor dust from participants' homes and schools, and blood. A second round of recruitment started in 2010 and was completed in 2014 and included 408 children from Valcamonica, Lake Garda, and Bagnolo Mella. From the complete cohort of 719 children, DNA samples for genetic analyses were extracted from 686 children. The study cohort for the current project include 645 children for which successful genotyping data was achieved for the 3 SNPs rs1776029, rs12064812 and rs13107325, and which also had data for Mn in soil.

The study design, information about study aims, and forms for informed consent were in accordance with the Helsinki declaration and were reviewed and approved by the ethics committees of the University of Brescia, Icahn School of Medicine at Mount Sinai, and Lund University, Sweden.

2.2. Measurements of manganese in soil

Measurements of Mn concentrations in soil were used to represent environmental Mn exposure in this study. Higher soil Mn compared to the local background levels was found in the exposed regions of Valcamonica and Bagnolo Mella compared to the control region Lake Garda (Borgese et al., 2013; Ferri et al., 2015; Zacco et al., 2009), indicating that soil Mn is a suitable predictor of environmental exposure. Moreover, Mn in soil values were available for the majority of the cohort and previous findings from the same cohort have shown that Mn in soil was associated with other biomarker measures of Mn exposure (Butler et al., 2018; Lucas et al., 2015) and with neuromotor effects in a subset of the cohort (Lucchini et al., 2012a). Measurements on Mn in soil were made in situ on available bare, undisturbed surface soil near each home (e.g., front or back yard area) using a portable X-ray fluorescence instrument (Thermo Scientific Niton, model XL3t). Measurements were made for 37% of subjects, and for the remaining, it was estimated by GIS Kriging models as previously described (Lucchini et al., 2012a).

2.3. Biological sampling and measurements of manganese and lead in blood

Sampling and preparation of blood for analyses of Mn and lead (Pb) concentrations were conducted as previously described (Lucchini et al., 2012a). Blood Mn was used for the generation of a genetic score which is further described in the statistics section. Pb was measured since it is a well-established neurotoxicant which influences neurodevelopment (Lucchini et al., 2012b). Briefly, whole blood samples were collected with butterfly catheters into trace metal free vacutainers, and Mn and Pb levels were measured by magnetic sector inductively coupled plasma mass spectrometry or by Zeeman graphite furnace atomic absorption spectrometry, as described elsewhere (Apostoli et al., 2000; Lucas et al., 2015). All blood samples had Mn concentrations above the limit of detection (LODs), whereas for Pb in blood, a few samples had concentrations below LOD and those were assigned a value of LOD/2.

2.4. Assessment of neurobehavioral problems

2.4.1.

For all children included in the study (N = 645), behavior was assessed using the long version of the Conners' Adolescent Self-Report Scale (CASS), which includes 10 subscales (family problems, emotional problems, conduct problems, cognitive problems/inattention, anger control problems, hyperactivity, attention deficit hyperactivity disorder (ADHD) index, Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) of inattention and DSM-IV of hyperactivity/impulsivity. Also, a total score of the 2 DSM-IV subscales assessing inattention and hyperactivity/impulsivity was calculated (DSM-IV total) (Conners, 2001). The questionnaire was administered to small groups of 4-5 subjects at a time that could answer the questions online on a personal computer directly. A researcher was always present during the compilation to answer the subjects' questions. Children recruited between 2010 and 2014 were also assessed by revised short versions of the Conners' Parents Rating Scales (CPRS) which includes 4 subscales (oppositional problems, cognitive problems/inattention, hyperactivity and ADHD-index) (Conners et al., 1998) which was completed for 396 children. The validated Italian versions of Conners' Rating Scale-Revised (CASS, CPRS-R) were used for the assessment (Conners, 2001).

Raw scores were converted into T-scores, which are standardized scores with a mean of 50 and a standard deviation of 10. Generally, T-scores above 56 are borderline i.e. slightly below the average but not in the clinical range. Otherwise, scores above 60 are significantly pathological and therefore require detailed clinical study (Conners, 2001).

In this study, we have included all subscales from the short version of CPRS. For the long version of CASS, we have limited the analyses to those scales that overlap with the short version of CPRS (i.e. cognitive problems/inattention, hyperactivity and ADHD-index) to allow a comparison between tests. For CASS we also included DSM-IV total due to the DSM-IV being a stringent and widely used test for ADHD diagnosis and thus should provide a good overall assessment of ADHD-related behavior (Wolraich et al., 2011).

2.5. Genotyping

DNA was extracted from whole blood samples using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). Genotyping was performed by TaqMan real-time PCR for rs12064812 and rs13107325 (Thermo Scientific assay IDsC_32155052 and C_1827682 respectively), as previously described (Wahlberg et al., 2017; Wahlberg et al., 2016; Wahlberg et al., 2018). Reactions were analyzed on the ABI 7900HT Fast Real Time PCR System (Applied Biosystems, Thermo Fisher, Waltham, USA), using the manufacturer's recommended standard conditions. Rs1776029, which is situated in a repeat region, was genotyped by pyrosequencing as previously described (Wahlberg et al., 2017) using the PyroMark reagents and PSQ HS96 Pyrosequencing System (Qiagen) according to the manufacturer's protocol. For quality control of genotyping data, > 5% of samples were re-analyzed for all SNPs in a separate round of experiments with a 100% agreement between duplicates. Data quality was also assessed by evaluating Hardy-Weinberg equilibrium using the conventional Chi-Square test.

2.6. Statistics

Several epidemiological studies have suggested that the influence of environmental Mn exposure on neurological function differ between sexes (Bauer et al., 2017; Gunier et al., 2015; Menezes-Filho et al., 2014; Mora et al., 2015; Riojas-Rodriguez et al., 2010). Therefore, analyses of Mn exposure (soil Mn) in relation to neurobehavioral outcomes (Conner's test scores) were performed separately for girls and boys. Differences in continuous variables (e.g. soil Mn, blood Pb, and Conners' scores) between sexes were evaluated using Mann-Whitney test and differences between categorical variables between sexes were evaluated using Chi-square test.

Depending on best fit of the data, linear regression models or generalized additive models (GAM) with smoothing spline function were used for analyses of associations between Mn exposure and neurobehavioral outcomes. The models were adjusted for possible influential factors which included age, socioeconomic status (SES), maternal education and child blood Pb concentrations. Low SES, and particularly low maternal education, may negatively influence neurobehavior (Hosokawa and Katsura, 2018). The SES variable in our data set was calculated as a combination of the highest degree of education and occupation among the parents. To specifically take into account the influence of maternal education, maternal education was included as a separate variable in the models. High blood Pb concentrations are also a risk-factor for ADHD (Ji et al., 2018). Soil Mn and blood Pb were natural log (ln) transformed to fit the model assumptions.

Gene-environment interactions were evaluated by including an interaction term between soil Mn (ln transformed) and genotype in models for analyzing associations between Mn exposure and Conners' scales. Interactions were further evaluated by analyzing associations between Mn in soil and Conners' outcomes with stratification for genotype. Genotype was in the interaction analyses represented by a genetic score based on coding of genotypes for the different SNP in relation to their associations with blood Mn concentrations, as an indication of their influence on Mn regulation. The genotype score has previously been presented in Wahlberg et al., 2018 and is described in Table 1. Briefly, genotypes for each SNP associated with low, medium and high blood Mn concentrations were coded 0, 1 and 2 respectively. The scores were added up to obtain a combined genotype score for each individual ranging from 0 to 5. To increase statistical power in the interaction analyses, the genetic score was further combined into a 2-category genetic score by combining scores 0–2 (final score = 1) and 3–5 (final score = 2). For evaluating a potential dose-response effect in relation to genotype score, we also created a 3-category genetic score by combining scores 0–1 (final score = 1), 2–3 (final score = 2) and 4–5 (final score = 3). The lower scores would thereby represent genotypes associated with low blood Mn, whereas the higher scores represent genotypes associated with high blood Mn. Moreover, for the gene-environment interaction analyses, we also performed sensitivity analyses to evaluate a potential influence of the type of method used to generate Mn in soil values (i.e. measured by X-ray fluorescence or estimated by GIS kriging). For this purpose, we repeated the gene-environment interaction analyses for the CRPS scales with adjustment for the type of method used (categorical variable [measured/estimated] was included in the model) as well as by excluding children with estimated soil Mn values from the analyses.

Statistical analyses were performed using SPSS (Version 25, Chicago, US) and the program R. Graphs were generated using R. *P*-values below 0.05 were considered to indicate statistical significance.

3. Results

3.1. Study population characteristics

Characteristics of the children are presented in Table 2. Mn concentrations in soil showed a large range in the communities as previously reported (Pavilonis et al., 2015), with a minimum value of 124 ppm and maximum value of 17,000 ppm. Soil Mn differed significantly between study sites; it was higher in the districts of Valcamonica and Bagnolo Mella (median = 1009 ppm and 660 ppm respectively) with industrial Mn contamination compared to the control district of Lake Garda (median = 437 ppm). Blood Mn concentrations of children in the cohort have previously been described (Wahlberg et al., 2018) and were in the normal range. In the group included in this study, blood Mn ranged from 4.0 to 34.3 μg/L and there was no significant difference in blood Mn between children from the three different study areas ($p = 0.16$, unadjusted linear regression) or between

Table 1
Information on SNP included in study including description on how genotypes were combined to generate a genetic score representing high and low Mn genotypes.

Gene:Chr	Gene function	Gene expression profile	SNP (alleles[a])	SNP type and functional effect of minor allele/hypothesized effect on Mn regulation	MAF[b] (%)		Genotypes	Mean blood Mn in μg/L (CI[c])	Code[d]
					Eur[e]	Bre[f]			
SLC30A10:1	Coding for the SLC30A10 Mn efflux transporter protein (Leyva-Illades et al., 2014)	High expression in liver, intestine and brain (http://ist.medisapiens.com) including globus pallidus (Quadri et al., 2012)	rs1776029 (G/**A**)	Non-coding upstream variant which may cause reduced *SLC30A10* gene-expression[g] (Wahlberg et al., 2016)/ hypothetically reduced Mn efflux ability in liver, GI tract and globus pallidus neuronal cells.	21	22	GG	10.6 (10.3, 10.9)	0
							GA	12.3 (11.8, 12.8)	1
							AA	14.7 (13.1, 16.4)	2
			rs12064812 (T/**C**)	Non-coding intronic variant which may cause increased *SLC30A10* gene-expression (Wahlberg et al., 2016)/ hypothetically increased Mn excretion ability in liver, GI tract and globus pallidus neuronal cells.	30	30	TT	11.7 (11.3, 12.2)	1
							CT	10.9 (10.5, 11.3)	
							CC	11.2 (10.3, 12.0)	
							CT/CC	11.0 (10.6, 11.3)	0[h]
SLC39A8 (ZIP8):4	Coding for the SLC39A8 Mn influx transporter protein (Boycott et al., 2015)	Liver, kidney, lungs, salivary glands, placenta (http://ist.medisapiens.com), nasal epithelium and olfactory mucosa (Genter et al., 2009)	rs13107325 (C/**T**)	Coding variant causing a substitution of an Ala to Thr which may reduce protein function (Zhang et al., 2016) and *SLC39A8* gene expression in liver (Speliotes et al., 2010) and hypothetically cause reduced Mn uptake in lungs, via nasal epithelium and/or kidney and bile (reabsorption).	8	9	CC	11.7 (11.4, 12.0)	2
							CT	9.9 (9.4, 10.4)	1
							TT	5.5 (2.3, 8.7)	0

[a] Minor alleles in bold.
[b] Minor allele frequency (MAF).
[c] 95% confidence intervals.
[d] Code assigned genotype based on the associations with blood Mn concentrations as used to generate a genetic score combining genotypes for the 3 SNPs. 0 refers to a low Mn genotype (hypothetically indicating efficient Mn regulation) for each SNP and 2 refers to a high Mn genotype (hypothetically inefficient Mn regulation) for each SNP. Codes for each SNP was added to generate a genotype score ranging from 0 to 5 which was further combined into a 2-category genetic score by combining scores 0–2 (final score = 1) and 3–5 (final score = 2, as well as a 3-category genetic score by combining scores 0–1 (final score = 1), 2–3 (final score = 2) and 4–5 (final score = 3).
[e] MAF for European populations from Ensembl Genome Browser (http://www.ensembl.org).
[f] MAF for the described study population of children in the regions of Brescia in Italy.
[g] Results demonstrated for *SLC30A10* rs2275707 which is in strong linkage disequilibrium with rs1776029.
[h] Heterozygotes and minor allele homozygotes were combined due to showing similar degree of association with blood Mn concentrations and gene-expression (Wahlberg et al., 2016; Wahlberg et al., 2018), indicating a dominant allele.

Table 2
Study participant characteristics.

Variables	All				Girls				Boys				
	n	mean	median	5, 95 percentiles	n	mean	median	5, 95 percentiles	n	mean	median	5, 95 percentiles	*p* value[a]
Age	645	12	12	11, 14	313	12	12	11, 14	332	12	12	11, 14	0.362
Soil Mn (ppm)	645	966	702	267, 1841	313	910	712	272, 1830	332	1019	700	262, 1854	0.894
Blood Mn (ug/L)	642	11.3	10.9	6.61, 17.0	312	11.5	11.1	6.80, 17.1	330	11.2	10.8	6.40, 16.9	0.229
Blood Pb (ug/L)	642	16.6	13.7	7.20, 32.2	312	14.2	12.1	6.51, 28.0	330	18.9	15.7	8.00, 42.4	< 0.001
CASS cognitive problems/ inattention	645	46	44	36, 64	313	47	44	36, 68	332	45	44	35, 60	0.006
CASS hyperactivity	645	49	46	35, 70	313	47	45	35, 68	332	50	48	37, 72	< 0.001
CASS ADHD-index	645	45	42	36, 63	313	44	40	36, 64	332	45	43	36, 62	0.002
CASS DSM-IV total	645	47	45	36, 63	313	48	45	37, 65	332	46	45	35, 62	0.181
CRPS oppositional problems	356	50	47	36, 73	170	51	49	39, 71	186	49	47	36, 74	0.010
CPRS cognitive problems/ inattention	356	49	44	41, 74	170	51	42	42, 84	186	47	45	41, 61	0.009
CPRS hyperactivity	356	48	45	41, 64	170	49	45	42, 63	186	48	47	40, 65	0.020
CPRS ADHD-index	356	51	47	39, 76	170	53	47	39, 86	186	50	48	39, 63	0.297
Maternal education	633	H = 14%, M = 43%, L = 43%			308	H = 13%, M = 41%, L = 46%			325	H = 15%, M = 46%, L = 39%			0.199
Socioeconomic status (SES)	631	H = 23%, M = 54%, L = 23%			308	H = 23%, M = 50%, L = 26%			323	H = 22%, M = 58%, L = 20%			0.111
2-Category genetic score	645	1 = 45%, 2 = 55%			313	1 = 44%, 2 = 56%			332	1 = 45%, 2 = 55%			0.692
3-Category genetic score	645	1 = 7%, 2 = 72%, 3 = 21%			313	1 = 8%, 2 = 72%, 3 = 20%			332	1 = 6%, 2 = 72%, 3 = 22%			0.742
Participants per study area	645	LG = 219, VM = 243, BM = 183			313	LG = 103, VM = 117, BM = 93			332	LG = 116, VM = 126, BM = 90			0.743

Mn = manganese; Pb = lead; CASS = Conners' adolescent self-report Scale; CPRS = Conners' parents report scale; ADHD = attention deficit hyperactivity disorder; DSM-IV-total = diagnostic and statistical manual of mental disorders, 4th edition (DSM-IV) total test score for inattention and hyperactivity/impulsivity; H = high; M = medium; L = low; LG = Lake Garda; VM = Valcamonica; BM = Bagnolo Mella.

[a] Girls vs boys, Mann-Whitney *U* test or Chi-square.

sexes (Table 2). Blood Pb levels were significantly higher in boys (median = 15.71 μg/L) than in girls (median = 12.11 μg/L), but the concentrations were still relatively low (Lucchini et al., 2012b). The results from the Conners' test differed between girls and boys for several of the subscales; boys scored higher for hyperactivity and ADHD-index by CASS; girls scored higher for cognitive problems/inattention by both CASS and CPRS and oppositional problems by CPRS (Table 2). Comparison between the children included in this study (i.e. children with soil Mn and genetics data, n = 645) and all children in the cohort (n = 719) showed no difference in general characteristics, exposure or neurobehavior between groups (Supplemental Table 1).

3.2. Associations between manganese exposure and scoring for neurobehavioral problems differ between sexes

Mn exposure, represented by Mn concentrations in soil, was analyzed in association with children's scores for Conners' tests (CASS and CPRS) with stratification for sex. Summarized results from stratified linear models for all outcomes are presented in Table 3.

For girls, U-shaped relationships were observed between Mn in soil and some Conners' CASS scales. Therefore, in addition to linear regression, associations for girls CASS scales were analyzed using GAM (Fig. 1; Supplemental Tables 2–5), which provided a better fit than linear models for hyperactivity, DSM-IV-total and cognitive problems/inattention. For hyperactivity and DSM-IV total, GAM showed significant associations between soil Mn and scoring, whereas for cognitive problems/inattention, the association was not significant ($p = 0.057$). For these three scales, both ends of the Mn exposure spectrum (i.e. high and low Mn concentrations in soil) were associated with higher scores (i.e. increased problematic behavior). The breakpoint value for soil Mn, where the associations changed direction, was approximately 1000 ppm (7 on the log scale).

For CRPS, the U-shaped relationship was not observed in girls. Instead, there was a positive and linear relationship between Mn in soil and higher scoring for neurobehavioral problems and the associations were significant for cognitive problems/inattention (B = 4.3,

Table 3
Associations between Mn in soil and Conners' scores by adjusted linear regression models stratified for sex.

Conners' subscales	Girls					Boys				
	n[b]	B[c]	CI[c]		*p*[c]	n[b]	B[c]	CI[c]		*p*[c]
CASS cognitive problems/inattention[a]	305	−1.8	−3.5	−0.13	**0.035**	317	−1.1	−2.5	0.31	0.127
CASS hyperactivity[a]	305	−1.2	−3.2	0.87	0.259	317	−1.1	−2.9	0.72	0.235
CASS ADHD-index	305	0.18	−1.6	1.9	0.844	317	−1.3	−2.6	0.018	0.053
CASS DSM-IV total[a]	305	−1.8	−3.5	−0.13	**0.035**	317	−1.1	−2.5	0.31	0.127
CPRS oppositional problems	166	2.3	−0.66	2.4	0.127	174	0.68	−1.8	3.2	0.596
CPRS cognitive problems/inattention	166	4.3	0.20	8.4	**0.040**	174	0.80	−0.82	2.4	0.333
CPRS hyperactivity	166	0.86	−1.3	3.1	0.441	174	2.0	0.26	3.8	**0.025**
CPRS ADHD-index	166	5.4	0.99	9.7	**0.017**	174	0.41	−1.5	2.4	0.678

Abbreviations: CASS = Conners' adolescent self-report Scale; CPRS = Conners' parents report scale; ADHD = attention deficit hyperactivity disorder; DSM-IV total = diagnostic and statistical manual of mental disorders, 4th edition (DSM-IV) total test score for inattention and hyperactivity/impulsivity; B = unstandardized regression coefficient; CI = 95% confidence intervals; *p* = *p* value.

[a] In girls these subscales showed U-shaped relationship with soil Mn and better fit with generalized additive models (GAM) than linear regression model.

[b] Total number of subjects in adjusted models.

[c] Values from linear regression analyses of associations between Mn in soil and Conner's subscales in models adjusted for age, socioeconomic status (SES), maternal education and child blood lead (Pb) concentrations.

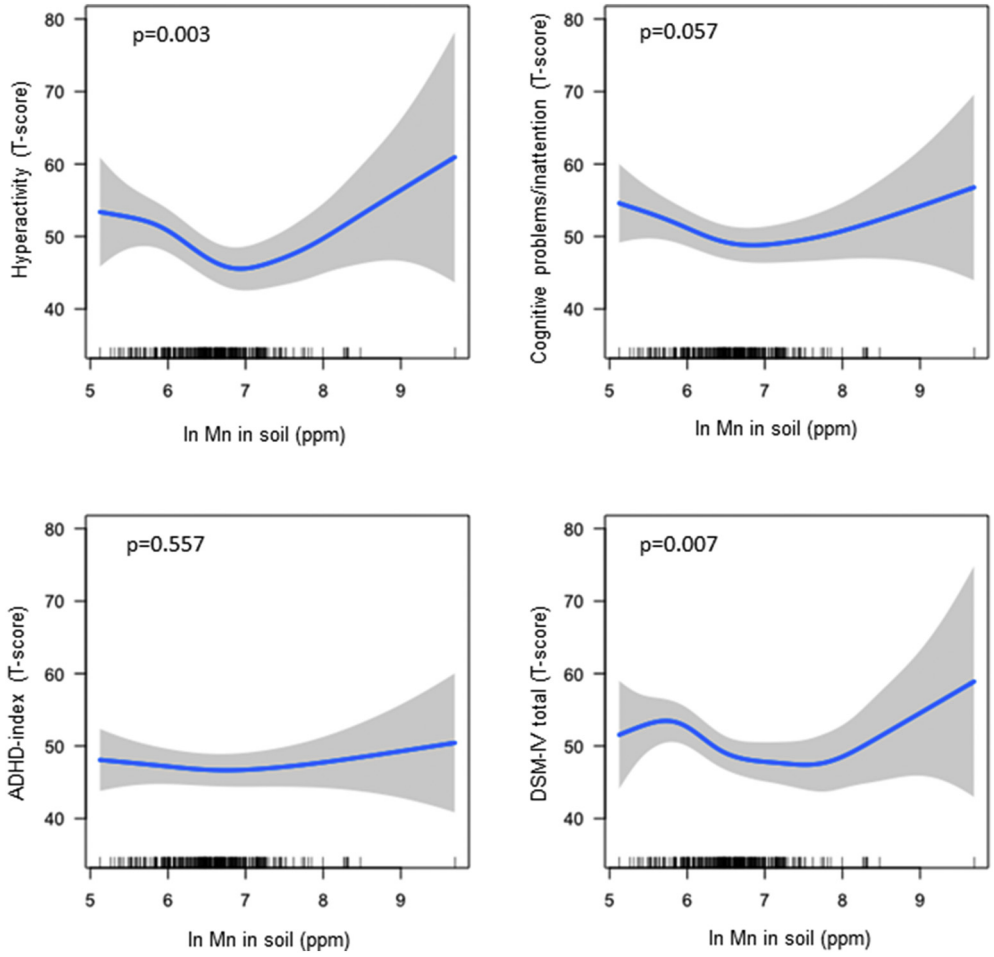

Fig. 1. Associations between Mn in soil and Conners' CASS scales in girls by generalized additive models (GAM; adjusted for age, SES, maternal education and blood Pb concentrations) including 95% confidence intervals (grey). U-shaped associations were observed between Mn in soil and CASS hyperactivity, cognitive problems/inattention (non-significant) and DSM-IV total in girls. At a threshold around 1000 ppm (7 on log-scale), increasing Mn in soil was associated with increasing test scores (i.e. increasing problematic behavior). *P* values represent significance of associations from adjusted GAM.

$p = 0.040$) and ADHD-index (B = 3.9, $p = 0.017$) in adjusted linear regression models.

In boys, we observed an overall negative linear relationship between soil Mn and CASS Conners' scores (i.e. increasing soil Mn was associated with reduced scoring for behavioral problems), but the associations were not significant. For CPRS, there was a positive linear association between Mn in soil and scoring in boys, but the association was weaker compared to the girls and only significant for hyperactivity in adjusted linear regression models (B = 2.0, $p = 0.025$).

3.3. Associations between manganese exposure and neurobehavior are modified by manganese transporter genetics in girls

Results from sex-stratified interaction analyses by linear regression analysis are presented in Table 4.

In boys, there were no significant interactions between soil Mn and the 2-category genotype score for any of the Conners' scales. In girls, we observed that the genetic score 2 showed stronger positive association between soil Mn and Conners' scores compared to genetic score 1. Girls with genetic score 1 showed no association or negative association between soil Mn and Conner's scores (CASS and CPRS). In contrast, girls with genetic score 2 showed strong positive associations between soil Mn and CRPS scales oppositional problems, cognitive problems/inattention and ADHD-index. Significant gene-environment interactions between soil Mn and the genetic score were observed for both CASS and CPRS cognitive problems/inattention.

Adjusting for the type of method used to generate soil Mn values soil (measurement by X-ray fluorescence or estimation by GIS kriging) showed that the method had minor influence on interaction analyses for the CPRS scales (< 10% change in the beta coefficient for the majority of analyses, Supplemental Table 5). There were much fewer children with measured soil Mn values, however, in this group we could still observe more positive associations between Mn in soil and CRPS scores, particularly ADHD-index, for girls carrying genetic score 2 (Supplemental Table 6).

Due to the observed U-shaped relationship between Mn in soil and CASS scales in girls, the CASS scales were also evaluated by GAM subgroup analysis. Here we used the 3-category genetic score in order to better evaluate a potential difference in dose-response relationships for the different genetic scores (Fig. 2). We observed that the U-shaped associations between Mn in soil and CASS in girls were most pronounced for girls carrying genetic score 3 (high Mn genotypes). The difference between genotype scores was particularly evident for CASS hyperactivity where we could observe a marked difference between genetic score 1 and 3, both at the lower and higher ranges of Mn concentrations in soil. At the higher soil Mn concentration (> 1000 ppm), genetic score 3 showed the strongest positive associations between Mn in soil and scoring for behavioral problems.

4. Discussion

In this study, we demonstrate sex- and genotype dependent

Table 4
Interactions between 2-category genetic score and Mn in soil on Conners' scales with stratification for sex by adjusted linear regression.

Conners' subscales	Genetic score[b]	Girls						Boys					
		n[c]	B[d]	CI[d]		p[d]	p int[e]	n[c]	B[d]	CI[d]		p[d]	p inte
CASS cognitive problems/inattention[a]	1	132	−4.0	−7.0	−0.97	**0.010**	**0.045**	144	−1.2	−3.1	0.76	0.233	0.373
	2	172	0.28	−2.0	2.6	0.812		172	−0.18	−2.1	1.7	0.848	
CASS hyperactivity[a]	1	132	−2.5	−6.0	0.88	0.144	0.556	144	−1.6	−4.3	1.2	0.257	0.633
	2	172	−0.81	−3.5	1.8	0.545		172	−0.78	−3.4	1.8	0.557	
CASS ADHD-index[a]	1	132	−0.83	−3.6	1.9	0.553	0.430	144	−1.3	−3.3	0.60	0.175	0.978
	2	172	0.49	−1.9	2.8	0.681		172	−1.5	−3.4	0.42	0.126	
CASS DSM-IV total[a]	1	132	−4.1	−7.0	−1.3	**0.005**	0.134	144	−2.4	−4.4	−0.35	**0.022**	0.087
	2	172	−1.0	−3.2	1.2	0.360		172	0.068	−2.0	2.1	0.947	
CPRS oppositional problems	1	76	−1.0	−6.7	4.6	0.718	0.170	86	0.66	−1.9	3.3	0.614	0.841
	2	89	4.0	0.40	7.6	**0.030**		87	0.14	−4.5	4.8	0.952	
CPRS cognitive problems/inattention	1	76	−4.2	−11	3.3	0.267	**0.014**	86	0.070	−2.2	2.3	0.951	0.498
	2	89	8.2	3.0	13	**0.002**		87	1.6	−0.97	4.1	0.226	
CPRS hyperactivity	1	76	0.46	−2.9	3.8	0.784	0.944	86	2.6	0.78	4.5	**0.006**	0.242
	2	89	0.69	−2.4	3.8	0.663		87	1.4	−1.8	4.6	0.391	
CPRS ADHD-index	1	76	−3.2	−11	4.5	0.408	0.063	86	0.14	−2.3	2.6	0.913	0.936
	2	89	9.3	3.6	15	**0.002**		87	0.64	−2.7	4.0	0.702	

Abbreviations: CASS = Conners' adolescent self-report Scale; CPRS = Conners' parents report scale; ADHD = attention deficit hyperactivity disorder; DSM-IV total = diagnostic and statistical manual of mental disorders, 4th edition (DSM-IV) total test score for inattention and hyperactivity/impulsivity; B = unstandardized regression coefficient; CI = 95% confidence intervals; $p = p$ value.

[a] In girls several of CASS subscales showed U-shaped relationship with soil Mn and CASS scales were therefore also analyzed by generalized additive models (GAM) with stratification for genetic score (Fig. 2).

[b] Combined genotypes for SNPs rs12064812, rs1776029 and rs13107325. 1 = genotypes associated with low blood Mn. 2 = genotypes associated with high blood Mn.

[c] Total number of subjects in adjusted models.

[d] Values from linear regression analyses of associations between Mn in soil and Conner's subscales in models adjusted for age, socioeconomic status (SES), maternal education and child blood lead (Pb) concentrations.

[e] p value for interaction (soil Mn x 2 category genetic score) in linear regression model adjusted for age, socioeconomic status (SES), maternal education and child blood Pb concentrations.

associations between soil Mn levels and neurobehavioral outcomes in children. Several studies have implicated a link between elevated Mn concentrations in early life and neurodevelopmental outcomes in children (Zoni and Lucchini, 2013), and both high and low body Mn levels have been associated with increased behavioral problems (Bhang et al., 2013; Farias et al., 2010; Menezes-Filho et al., 2011; Shin et al., 2015). In this study, we analyzed associations between environmental Mn exposures measured as Mn concentrations in soil with neurobehavioral outcomes. The study population comprises children from areas with and without industrial Mn contamination of their environment, and thus represents a relatively wide spectrum of Mn exposure levels in the low to moderate range. Our data show associations between Mn exposure and neurobehavior in children assessed by the Conners' scales with distinct differences in association strengths and directions between girls and boys and between children with different genotypes for SNPs in Mn transporter genes.

For the girls, we observed a U-shaped relationship between exposure and CASS scores, similar to associations previously demonstrated for blood Mn on neurodevelopment (Claus Henn et al., 2010). At low soil Mn levels, the associations between Mn exposure and CASS scores were significantly negative, suggesting a potentially beneficial influence of higher Mn exposure on neurobehavior. However, at the higher ranges of soil Mn levels, the relationships between Mn exposure and CASS scores became significantly positive, suggesting that the higher Mn exposures may have an adverse influence on girls' neurobehavior.

For boys, we observed linear and overall weakly negative association between Mn in soil and scoring for CASS scales, indicating that soil Mn levels did not have an adverse impact on neurobehavior in the boys. The differences in the association profiles of soil Mn vs neurobehavior between girls and boys are in line with previous findings by Bauer et al. (Bauer et al., 2017), who demonstrated a U-shaped relationship between prenatal Mn exposure (measured as Mn in teeth dentine) and visuospatial learning and memory in girls but not in boys. We hypothesize that a potential threshold of Mn exposure inducing a negative effect on neurobehavior may exist also for the boys, but possibly at higher levels than for girls, and not within the exposure range of this study population. The CRPS scales supported the findings of a higher sensitivity to Mn exposure among girls, however, the U-shaped relationship between exposure and scores was not observed.

Consistent with our results, stronger associations between environmental Mn exposure and neurodevelopmental outcomes in girls compared to boys have been observed in several recent studies (Bauer et al., 2017; Chiu et al., 2017; Gunier et al., 2015), but the reason behind these differences are not known. It may be due to differences in activity patterns between girls and boys which can affect exposure levels and routes, differences in Mn metabolisms and regulation between sexes or differences in the response to Mn induced effects in the brain (e.g. oxidative stress). Sex-differences in neurotoxicity of metals and other toxicants are becoming increasingly evident and potential underlying mechanisms include differences in hormonal levels, mitochondrial activity, gene-expression and epigenetic patterns, as well as nutritional differences between sexes (Dickerson et al., 2017; Llop et al., 2013; Torres-Rojas & Jones, 2018).

We further demonstrate, by gene-environment interaction analyses, that sensitivity to Mn exposure on neurobehavioral outcomes on children is likely influenced by polymorphisms in genes coding for Mn efflux transporter SCL30A10 and influx transporter SLC39A8, and that the genetic influence is particularly strong in girls. To reduce the number of models and enable the evaluation of the combined influence of SNPs, we generated genetic scores, where genotypes were combined based on their previously observed associations with blood Mn concentrations (Wahlberg et al., 2016; Wahlberg et al., 2018), as an indication of their influence on Mn regulation in the body. The highest category of the genetic score represented children which carried genotypes associated with high blood Mn. These children would hypothetically be less efficient in regulating increased Mn concentrations from environmental exposure and accordingly show stronger associations

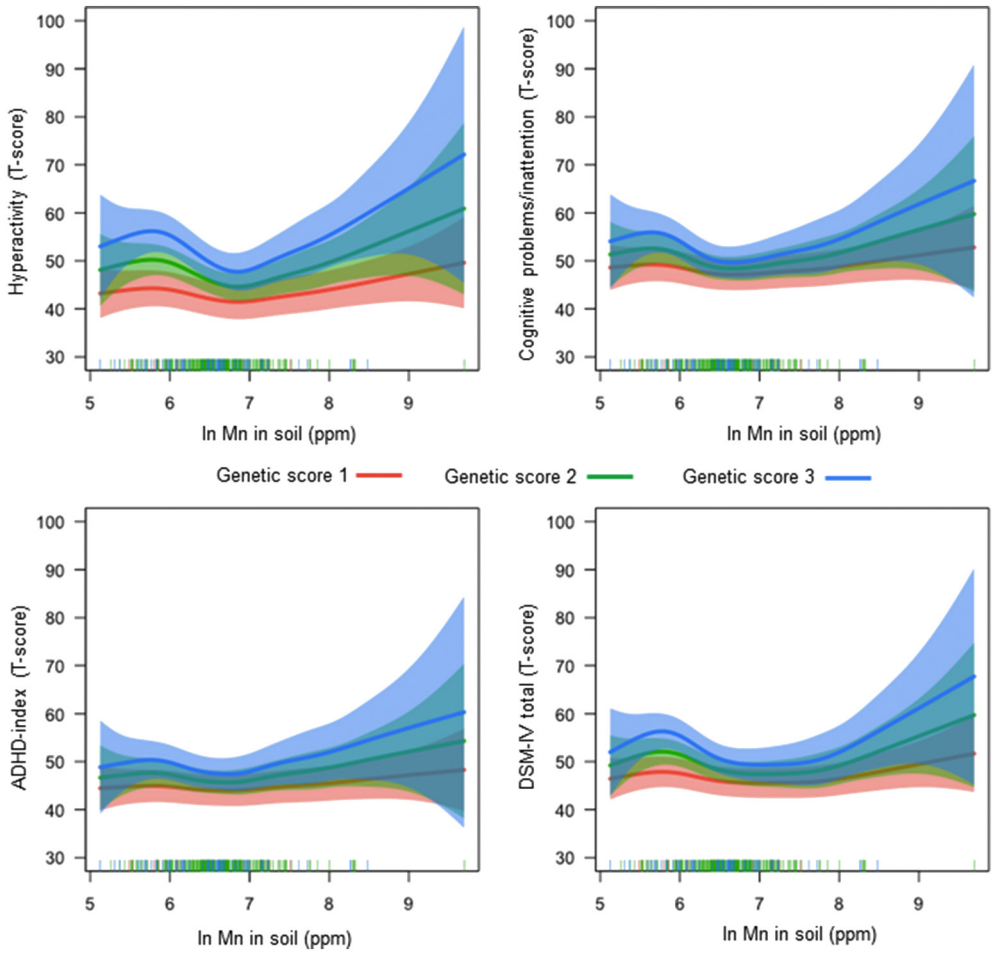

Fig. 2. Analyses of interactions between Mn in soil and genetic score (3 category score) on Conners' CASS scores in girls by GAM (adjusted for age, SES, maternal education and blood Pb concentrations), including 95% confidence intervals. Girls with genetic score 3 (High Mn genotypes) showed the strongest U-shaped relationship between Mn in soil and CASS scoring.

between Mn exposure and neurobehavioral outcomes. In line with this hypothesis, for the majority of CPRS scales, girls with genetic score 2 showed strong positive associations between soil Mn and CPRS scoring, whereas girls with genetic score 1 showed no association. The difference in associations between the genotypes was confirmed by significant interactions between Mn in soil and the genetic score on cognitive problems/inattention. Furthermore, GAM analyses of associations between soil Mn and CASS scores in girls showed a tendency of stronger associations between soil Mn and scoring at the higher exposure range and with increasing genetic score. The differences in associations between soil Mn and Conners' scores were less evident in boys, suggesting that Mn transporter genetics contribute to a higher degree of variation in sensitivity to Mn exposure among girls compared to boys.

The mechanisms behind the genetic influence on neurological outcomes and sensitivity exposure need further investigation. The intake of Mn from environmental contamination in these children may be via the gastrointestinal tract or from air via the lungs. It was previously shown that Mn in soil and air was weakly positively correlated in the same study population of Italian children (Lucas et al., 2015) and Mn in soil as the measurement of environmental Mn exposure in this study, may therefore to some degree also represent exposure to Mn from air. Rs13107325 in *SLC39A8* is a coding SNP and the rare allele (T) causes an amino acid change (alanine/threonine) which has been linked to reduced SLC39A8 protein function and SLC39A8 gene-expression, as well as reduced blood Mn concentrations. SLC39A8 regulate Mn concentrations in the body by Mn reabsorption in kidney and bile (He et al., 2006; Lin et al., 2017), but it has also been implicated in the direct uptake of Mn from air into the brain via the olfactory pathway due to the high expression in the nasal epithelium and olfactory mucosa (Genter et al., 2009). Furthermore, high expression of SLC39A8 in lung tissue (http://ist.medisapiens.com, n.d.) suggests that it may be involved in Mn uptake via the lungs. There is no evidence of SLC39A8 being involved in Mn transfer over the blood-brain barrier, however, reduced Mn concentrations due to dysfunctional SLC39A8 has been linked to impaired glycosylation (Park et al., 2015), which in turn could influence the function of transferrin involved in the transfer of Mn over the blood-brain-barrier (Tuschl et al., 2013). Thus, the rs13107325 rare allele may indirectly cause reduced brain Mn concentrations.

The SNPs rs1776029 and rs12064812 in *SLC30A10* are both non-coding. The rs1776029 rare allele (A) has been associated with reduced *SLC30A10* gene-expression and increased blood Mn concentrations, whereas the rs12064812 rare allele (C) appears to have the opposite effect (Wahlberg et al., 2016; Wahlberg et al., 2018). These SNPs may influence blood Mn concentrations by affecting the degree of SLC30A10 mediated Mn efflux via the liver and GI tract, where *SLC30A10* is highly expressed (http://ist.medisapiens.com, n.d.). In addition to their influence on blood Mn, which could indirectly affect how much Mn that reaches the brain, rs1776029 and rs12064812 may also influence the regulation of Mn in the brain, including in the globus pallidus where *SLC30A10* is highly expressed (http://ist.medisapiens.com, n.d.; Quadri et al., 2012). A recent study in mice showed that SLC30A10 in the brain was particularly important for regulation of brain Mn at elevated Mn exposure, whereas at basal physiological conditions, brain Mn was

mainly regulated via SLC30A10 in the liver and GI-tract (Taylor et al., 2019).

Taken together, SNPs in *SLC39A8* and *SLC30A10* are likely to the influence the regulation of excess Mn from environmental exposure by different pathways and in different directions. Genetic variants associated with high uptake and low efflux activity could cause an increased Mn accumulation in globus pallidus and consequently a stronger influence on neurobehavioral outcomes. The proposed mechanisms linking Mn to neurobehavior and ADHD involve disturbance of dopaminergic neurotransmission by Mn in the brain (Hong et al., 2014).

An apparent strength of our study is the relatively large and well characterised study population of Italian children representing a wide range of Mn exposure. A limitation in the study was low statistical power in stratified interactions analyses, particularly for CPRS. Although the total study group is relatively large, it is not large enough for us to evaluate the associations between exposure and neurological outcomes for all possible genotype combinations which would presumably be more pronounced for some combinations than what is observed for the combined scores with only 2 and 3 categories. This means that we may not capture the full range of genetic sensitivity represented by the selected SNP. Another weakness is the lack of data on maternal Mn concentrations during the fetal period for the same children. *SLC39A8* is expressed in placenta (http://ist.medisapiens.com, n.d.) and may be involved in Mn transfer between the mother and fetus. Thus, some of the associations that we observe here could in fact be the result of exposure via the mother during fetal life.

In conclusion, our results indicate that genetic variation in Mn transporter genes, as well as sex, contribute to differences in sensitivity to environmental Mn exposure on neurobehavioral outcomes in children, and that girls with a genotype associated with high activity of the Mn influx transporter protein SLC39A8 and reduced expression of the SLC30A10 Mn efflux transporter (i.e. high Mn genotype) may be a particularly sensitive group. Our findings highlight the importance in considering sex and genetic susceptibility in risk-assessments of environmental Mn exposure.

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Declaration of Competing Interest

None.

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