NeuroToxicology 33 (2012) 687–696

Contents lists available at SciVerse ScienceDirect

NeuroToxicology

Tremor, olfactory and motor changes in Italian adolescents exposed to historical ferro-manganese emission

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ARTICLE INFO

Article history:
Received 9 August 2011
Accepted 11 January 2012
Available online 31 January 2012

Keywords:
Neuromotor changes
Children
Airborne particles
Soil
Metals
Manganese

ABSTRACT

Background and objective: Increased prevalence of Parkinsonism was observed in Valcamonica, Italy, a region impacted by ferroalloy plants emissions containing manganese and other metals for a century until 2001. The aim of this study was to assess neurobehavioral functions in adolescents from the impacted region and the reference area of Garda Lake.

Methods: Adolescents age 11–14 years were recruited through the school system for neuro-behavioral testing. Metals including manganese, lead, iron, zinc, copper were measured in airborne particulate matter collected with 24-h personal samplers, and in soil, tap water, blood, urine and hair. Independent variables included parental education and socio-economic status, children's body mass index, number of siblings, parity order, smoking and drinking habits.

Results: A total of 311 subjects (49.2% females), residing in either the exposed ($n = 154$) or the reference ($n = 157$) area participated. Average airborne and soil manganese were respectively 49.5 ng/m^3 (median 31.4, range 1.24–517) and 958 ppm (median 897, range 465–1729) in the impacted area, and 27.4 ng/m^3 (median 24.7, range 5.3–85.9) ng/m^3 and 427 ppm (median 409 range 160–734) in the reference area. Regression models showed significant impairment of motor coordination (Luria-Nebraska test, $p = 0.0005$), hand dexterity (Aiming Pursuit test, $p = 0.0115$) and odor identification (Sniffin' task, $p = 0.003$) associated with soil manganese. Tremor intensity was positively associated with blood ($p = 0.005$) and hair ($p = 0.01$) manganese.

Conclusion: Historical environmental exposure to manganese from ferroalloy emission reflected by the concentration in soil and the biomarkers was associated with sub-clinical deficits in olfactory and motor function among adolescents.

1. Introduction

Early life exposure to metals such as lead (Pb) and manganese (Mn) has been shown to cause neurotoxic effects of particular concern in susceptible subgroups like children. While pediatric exposure to Pb has been extensively studied for cognitive impairment, the neuro-developmental effect of Mn has been ascertained only recently. Exposure to Mn in water (Wasserman et al., 2006; Bouchard et al., 2011) and airborne dust (Riojas-Rodríguez et al., 2010; Menezes-Filho et al., 2011) has been found to be associated with cognitive impairment measured as reduced IQ. Since Mn neurotoxicity is known for extra-pyramidal effects in adults and has been related to early Parkinsonism (Lucchini et al., 2007), control of motor function may be impaired also in younger individuals after early life exposure. Exposure to Mn can occur from various sources via inhalation and ingestion routes. Recent animal studies have also suggested that the olfactory uptake of airborne particulates may be important in brain Mn uptake, since it would circumvent physiological barriers (e.g., blood–brain barrier) that normally regulate to some extent brain uptake (Aschner and Dorman, 2006). Although studies on the uptake of Mn through the human olfactory system are not available, it is likely that olfactory transport of Mn plays an important role in human neurotoxicity (Lucchini et al.,

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doi:10.1016/j.neuro.2012.01.005

2012; Aschner et al., 2005), especially when carried by ultrafine particles (Elder et al., 2006). Mexican children exposed to severe air pollution have shown deficits of odor identification related to signs of inflammation in the olfactory bulb (Calderón-Garcidueñas et al., 2010). The province of Brescia, where ~1,200,000 inhabitants live in a territory of ~4800 km^2, ranks as the third most industrialized province of Italy. The province has a long history of metallurgic production, with the iron and ferroalloy industry being especially prevalent in this area. Ferroalloy air emissions over the past century have increased environmental levels of various metals in the province, including manganese (Mn), lead (Pb), iron (Fe), copper (Cu), zinc (Zn), chromium (Cr), and nickel (Ni) (Zacco et al., 2009). Cross sectional studies conducted by our group on ferroalloy workers have shown over the past two decades impairment of motor functions related to Mn in blood (MnB) (Lucchini et al., 1995) and to cumulative exposure indices obtained as a time integrated estimate of average exposure to airborne Mn (Lucchini et al., 1999). An environmental epidemiological study of non-worker residents of Brescia also showed an increased prevalence of Parkinsonism (crude rate 296/100,000, age-sex standardized rate 407/100,000) compared to national (157.7/100,000) and international (126–144/100,000) rates. Moreover, the Standardized Morbidity Ratios (SMR) for Parkinsonism increased further around the sites of three ferroalloy plants located in Valcamonica, a valley in the pre-Alps within the province of Brescia (Kruskal–Wallis chi-squared 1 df = 17.55, p-value <0.001). The SMRs were positively associated with Mn levels in outdoor deposited dust (Lucchini et al., 2007).

In light of these findings, we undertook a cross sectional study on behavior, cognitive and motor functions among healthy individuals resident in Valcamonica and in a reference non industrial area of the province of Brescia, located on the west shore of the Garda Lake, where concentrations of metals in deposited dust are significantly lower (Zacco et al., 2009). Different age groups were targeted by this project, including pregnant mothers and infants, adolescents, ferroalloy workers, elderly. Here we report the results on the assessment of motor and neurosensory functions among the adolescents; the investigation of cognitive and behavioral functions and in the other age-groups will be published elsewhere.

2. Methods

2.1. Study sites

The study area includes different sites of the Province of Brescia, Northern Italy (Fig. 1): Valcamonica, a valley of the pre-Alps that runs for about 50 miles in the NE-SW direction with an average width of about 2 miles, and is delimited by mountains of about 10,000 feet. Winds in the valley average 3 miles/h primarily in the SW → NE direction during the day and NE → SW during the night, with no seasonal variation. Here three ferroalloy plants have been operating in the municipalities of Sellero (pop'n 1500) from 1973 to 1987, Breno (pop'n 5000) from 1921 to 2001, and Darfo (pop'n 13,200) from 1902 to 1995. A reference group community with no history of industrial ferroalloy plant activity was identified in the Garda Lake area of the Province. A fourth ferroalloy plant started Mn alloy production in the 1970s and is currently active in the town of Bagnolo Mella, located on the Padana plains. Estimated levels of Mn in settled dust obtained by statistical interpolation of previous measures (Zacco et al., 2009), showed the highest values in the vicinity surrounding the sites of previously and currently operating ferroalloy plants (Fig. 2). Results reported in the present study are based on the sites of Valcamonica and the Garda Lake.

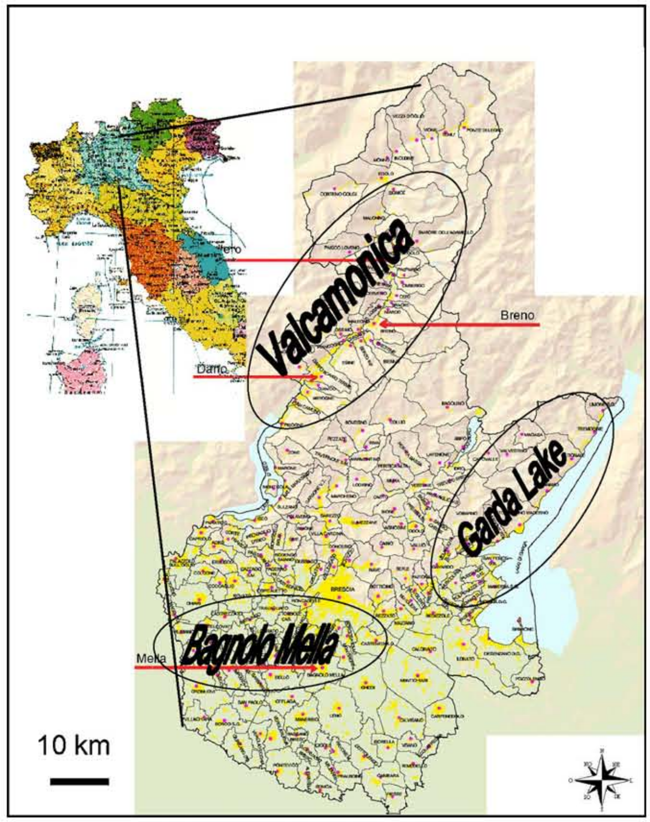

Fig. 1. Location of the study sites.

2.2. Participant enrollment

Children were enrolled through the public school system according to a community-based participatory approach that involved the local communities of Valcamonica and Garda Lake. The aims and methodology of the study were explained through community meetings and conferences and publicized by the local media. Teachers, parents and children were informed with *ad hoc* meetings and brochures. Subjects who agreed to participate filled in a screening questionnaire for the evaluation of inclusion and exclusion criteria. The inclusion criteria included: (i) to be born in the study area from resident families living in the study area since the 1970s; (ii) to live in the study area since birth; (iii) to be aged 11–14 years. The exclusion criteria were represented by: (i) pathological conditions potentially affecting neuro-development, including neurological, hepatic, metabolic, endocrine and psychiatric diseases; (ii) consumption of medications with known neuro-psychological side-effects; (iii) clinically diagnosed motor deficits of hand and fingers; (iv) clinically diagnosed cognitive impairment and behavioral manifestations; (v) visual deficits not adequately corrected. General practitioners physicians and pediatricians of the study areas were informed about the research aims and methods. The study design, the information about the study aims and the forms for informed consent had been reviewed and approved by the ethical committees of the local Public Health agencies of Valcamonica and Brescia.

2.3. Study design

The assessment of participant subjects was divided in three sessions, and carried out on different days over 2 consecutive weeks. Trained medical doctors and neuro-psychologists conducted the first session within dedicated rooms in the local school

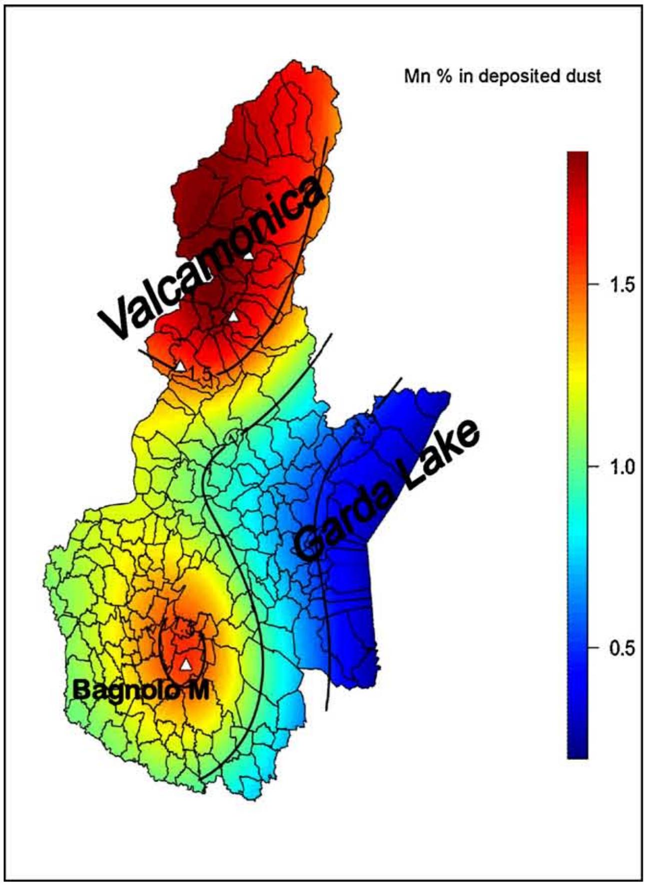

Fig. 2. Mn levels as percentage of total deposited dust in the province of Brescia, Italy. Values estimated with Kriging interpolation obtained with thin-plate spline regression of geo-referenced data (Δ: sites of ferroalloy plant; Valcamonica: previously active; Bagnolo Mella: currently active).

at each site; the session included the collection of socio-demographic data, including socio economic status (SES), family size, parity order, maternal and paternal education level, alcohol drinking and smoking habits, clinical and residential history with *ad hoc* questionnaires, and the administration of a test battery for neuropsychological, neurosensory and behavioral examination. Anthropometric data were measured for the calculation of body mass index (BMI) and a food frequency questionnaire weighted for portion sizes was administered to estimate the daily oral intake of Mn. At the end of the neuropsychological assessment, a portable pump for 24 h collection of PM10 airborne particles was mounted in the child's backpack and collection started. The second session was devoted to the collection of environmental samples including soil and tap water at the residential household of each participant. The third session was dedicated to the collection of biological matrices for the assessment of candidate biomarkers of metal exposure, including blood, urine, and hair. Blood samples were also used to assess cell blood count (CBC), iron status, liver and kidney function. Each individual participant's data was entered with anonymized identification codes accessible only to the research team, and under the responsibility of the study Principal Investigator (RL). According to an ethical procedure on open information about the final study outcomes, individual results on exposure data and hematological parameters were communicated to each participant in written form with comparison to the available exposure protective standards and the normative data for hematological analysis. Since normative data for the neurobehavioral tests were not considered, results were not communicated individually but at group level and discussed in local community meetings.

2.4. Exposure assessment

Each participant's house was geo-referenced for spatial analysis. Inhalation exposure to PM10 (i.e., 50% collection efficiency for 10 μm aerodynamic diameter particles) airborne particulate matter was determined using 24 h personal air monitoring. Airborne particles were collected on commercial filters (37 mm diameter, PTFE-Teflon) using Personal Environmental Monitors (PEM) connected to a Leland Legacy pump (SKC, Inc., Eighty-Four, PA, USA). The PEM air sampler was mounted onto the student's backpack front strap, in or near the breathing zone, while the pump was carried in the backpack. Pumps were pre-calibrated to a flow rate of 10 L/min, using a soapless piston primary calibrator (Defender, BIOS, Butler, NJ, USA), with post-sampling flow rate confirmation. The pumps were run for 24 consecutive hours with the child's school backpack either carried by the child or placed near the child during school or in the room while they were sleeping. Each child filled a personal diary with complete records of their activities and time spent in indoor/outdoor locations during the air-sampling period. Data on atmospheric conditions during the sampling period were obtained by the online meteorological system of the local Environmental Protection Office (ARPA Lombardia). Total PM10 particulate load on the filter was determined gravimetrically, as well as chemically. Particulate metal content was determined using total reflection X-ray fluorescence (TXRF) spectroscopy, according to a methodology published elsewhere (Borgese et al., 2011, 2012).

Water was sampled from the primary use home tap after a 2 min run at a medium flow rate and examined with TXRF multi-elemental analysis, that has a lowest detection limit for Mn in water of 1 μg/L.

The metal content in the soil was analyzed in situ with a portable X-ray fluorescence (XRF) instrument (Thermo Scientific Niton, model XL3t) equipped with GPS geo-referencing capability. The Niton portable XRF analyzes soil for metals by the generation of an X-ray signal and the instrument interpretation of the energies of the returning X-ray fluorescence signal from the excitation of the metal elements in the soil. Sample collection times ranged from 60 to 100 s. Surface soil measurements were collected in both the impacted and the reference areas as well as in the yards of a select number of subjects' homes. At the homes, two to four randomly spaced readings were taken and averaged. The instrument was internally calibrated prior to each usage. In addition, a series of soil standards reference materials (NIST 2780, 2709a) produced by the U.S. National Institute of Standards and Technology (NIST) were measured at the beginning of each sampling session with the Niton XRF. The collected filters for airborne particles were analyzed in accordance with the patented method PCT/IT2008/000458 (Depero et al., 2009) which allows the filter to be analyzed directly and non-destructively without any chemical treatment using total X-ray fluorescence (TXRF) techniques. The TXRF measurements were performed with the Bruker TXRF system S2 Picofox, air cooled, Mo tube, Silicon-Drift Detector, with operating values of 50 kV and 1000 μA, using an acquisition time of 600 s. The absolute concentration of the elements in the filters was evaluated through calibration with an air particulate standard filter (NIST SRM 2783).

A Food Frequency Questionnaire (FFQ) was used to estimate the daily dietary intake of Mn. The questionnaire included specific food items such as cereals, whole grain products, vegetable (chard, spinach), beans, lentils, chickpeas, fruit (pineapple, hazelnut), tea, cacao, coffee, of known higher Mn content based on the US nutrient database (USDA, 2010). The intake was portion-adjusted based on Italian reference data on food consumption (Leclercq et al., 2009).

2.5. Collection and analysis of exposure biomarkers

Exposure to Mn and Pb was also measured in total blood and urine. Venous whole blood samples (4 mL) were collected using a 19-gauge butterfly catheter into a Li-Heparin Sarstedt Monovette Vacutainer. A spot urine sample (50–200 mL) was collected into a clean, sterile polyethylene container. All samples were stored at 4 °C until analyzed at the laboratory facility of the University of Brescia. Hematological tests were performed to assess iron metabolism and liver function. Hair samples were collected from each subject using stainless steel scissors. Briefly, a 2–3 cm section of hair proximal to the scalp (~100–150 strands, or ~20 mg) was collected from the nape of the neck and stored in polyethylene bags at room temperature until analyses.

Manganese and Pb measurements in blood and Mn in urine (MnU) were performed by Zeeman graphite furnace atomic absorption spectrometry (Varian SpectrAA) in the Industrial Hygiene laboratory at the University of Brescia, Italy, using methods previously reported (Apostoli et al., 2000). Levels of Mn in hair (MnH) were determined by magnetic sector inductively coupled plasma mass spectrometry (Thermo XR-Thermo Element XR ICP-MS). Briefly, hair samples (~10–50 mg) were cleaned of exogenous metal contamination as follows: sonication (20 min) in 0.5% Triton, rinsing 5× with Milli-Q ultrapure water, sonication (10 min) in 1 N trace metal grade nitric acid, rinse with 1 N nitric acid, and rinsing 3× with Milli-Q water. Clean hair samples were dried overnight at 60 °C in a HEPA filtered-air clean room. Subsequently, hair was transferred to pre-weighed 6 mL poly tubes and digested in 0.5 mL 15.7 N double quartz-distilled nitric acid at 80 °C for 6 h in a Class-100 HEPA filtered-air hood. Following digestion, 5 mL Milli-Q water was added to each tube, and tubes were capped and vortexed. Rhodium and thalium were added as internal standards, and samples were analyzed for Pb-208 and Tl-205 (low resolution), and Cr-52, Mn-55, Cu-63, and Rh-103 (medium resolution). Metal concentrations were determined by comparison with certified multi-element standards (Spex Industries). The analytical detection limits for Mn, Pb, Cu, and Cr were 0.0054, 0.0027, 0.027, and 0.0019 ng/mL, respectively, based on repeated measurements of procedural blanks on four different analysis days.

2.6. Neuro-psychological testing

The neurobehavioral test battery was designed based on a review of the tests reported in the Mn literature (Zoni et al., 2007). Since pre-clinical early signs of neurotoxicity were considered in the study design, sensitive neurobehavioral tests were selected to capture exposure-related changes also in the normality range. Based on the study design and the exclusion criteria adopted, normative data were not used in the analysis.

Similarly to other studies on manganese neurotoxicity (Zoni et al., 2007) motor coordination was explored with 5 subtests of the Luria Nebraska Motor Battery (Golden et al., 1980), a standardized test battery composed by a total of 11 clinical scales. The test we administered, each lasting 10 s, were: dominant hand clench, non dominant hand clench, alternative hand clench, finger-thumb touching with dominant hand, finger-thumb touching with non dominant hand. The sum of the scores of these 5 subtests yielded a final score that represents, therefore, a partial Luria motor score. For motor coordination we used also a computerized version of Finger Tapping (Iregren et al., 1996). To assess psychomotor speed we used the Finger Tapping test, computerized version from the SPES (Iregren et al., 1996): task of participant is to tap a push-button alternatively with the dominant and non-dominant hand within 5 min. From the same computerized system we selected also the Visual Simple Reaction time test for vigilance and psychomotor response: the reaction time is the time required to respond to the presence of a visual stimulus represented by a red rectangle appearing on the computer screen. The subject is asked to press a button as soon as a stimulus appears on the screen. Hand dexterity and perceptual speed were assessed with the Pursuit Aiming test (Fleischman, 1954). For this test, the subject must place a dot with a pencil inside 2 mm diameter circles as quickly as possible for two series of 60 s each. Tremor was assessed by the Tremor 7.0 of Danish Products Developments-DPD (Després et al., 2000). During the test, the subject holds a stylus for 10 s and the hand vibrations are recorded and displayed in a time axis plot. The accelerations are analysed by methods drawn from vibrations measurements. Body Sway was recorded by a balance plate (SWAY system by DPD), which produces signals from three sensors to provide a map of the position of the force center during the test period. This centre is defined as the center of equilibrium of the three vertical forces, recorded at the three supports of the sway plate. During the test the subject stands erect on the sway plate and the change in position of the force center can be observed in a X–Y coordinate system. The test was repeated in two conditions of open and closed eyes. To assess odor identification we used Sniffin' Sticks-Olfactory Screening 12 test (Burghart Medical Technology). The subject smells the tips of 12 fiber sticks filled with different common scents and must identify the odors from a list of 4 concepts each. The result of the test is the sum of the correctly identified odors. We selected this test because the smells are more familiar to the Italian subjects compared to other produced elsewhere, and also because this test was already successfully used in children (Hummel et al., 2007).

2.7. Statistical analysis

Preliminary non-parametric statistics (Mann–Whitney and Kruskal-Wallis) were used to test the null hypothesis that the observed values were drawn from the same population in the impacted and reference area. Since air samples and soil measurements were not collected for the entire population we used GIS Kriging interpolations to obtain values for the missing points and gain power in the subsequent regression analyses. The median distance between points with and without direct soil measures was 780 m (20 m–5.37 km), while for the air measures the median distance was 119 m (range 20 m–1.9 km). Ordinary Kriging is a geo-statistical technique used to estimate data at unobserved locations from the observations at nearby locations and is based on spatial correlation of the data. Therefore we used a Stratified (by area) Ordinary Kriging model based on log-transformed Mn levels to estimate the unobserved individual soil Mn values. Since spatial correlation of airborne measurements was weak, we used Inverse Weighted Distance interpolation (Bivand et al., 2008) to estimate the unobserved air Mn values. Cross-validation was used to assess the reliability of the Kriging models. To test the hypothesis that Mn exposure affects motor and sensory functions in children and to obtain an adjusted estimate of the effect we used multiple (linear and logistic) regression analysis. Deviations from linearity in the relationship between exposure measures and covariate and dependent variables was evaluated comparing the ordinary least square model (OLS) or the logistic model with a semi-parametric generalized additive model (GAM) where the choice of the smoothness of the non-parametric part of the model was driven by generalized cross validation (Wood, 2006). When linearity could not be rejected we reduced the model to the simplest OLS (or logistic) model or we entered the continuous variable parametrically in the GAM model. We used soil Mn levels as the surrogate of Mn exposure to the subjects because of the collinearity (concurvity) between the air and soil Mn levels. Using only the soil Mn data resulted in more stable models and no gain in information was obtained when retaining the air Mn data in the models, as indicated by Akaike's information criterion (Venables and Ripley, 2002). We retained in the regression models all the variables that could have been related both to the exposure and to the outcome, including age, gender, SES, family size, parity order, maternal and paternal education, alcohol and smoking habits, BMI. These variable were included on an "*a priori*" basis, even when they showed to be statistically non significant, in order to obtain an unbiased estimate of the effect of Mn exposure (Harrell, 2001). All the analyses and graphics were made with R (R Development Core Team, 2011).

3. Results

3.1. Social-demographics

The study included 311 adolescents from different families and households, selected from a total of 414 originally enrolled. Exclusion was due to: not meeting the residence criteria ($n = 61$); refusal to participate ($n = 22$), neurological and psychiatric diseases ($n = 12$); incomplete informed consent ($n = 5$); endocrine diseases ($n = 1$); metabolic disease ($n = 1$); total parenteral nutrition ($n = 1$). Participants resided in Valcamonica ($n = 154$) and in the Garda Lake area ($n = 157$). According to the three sites of previous ferroalloy operations, the subjects from Valcamonica were further sub-classified in three areas including upper- ($n = 23$), mid- ($n = 85$), lower- ($n = 46$) Valcamonica.

Gender was equally distributed between females (49.2%) and males (50.8%), and median age was 155 months, with 18.3% of 11 years, 33.8% of 12 years, 40.5% of 13 years, 7.4% of 14 years old subjects. SES was low in 24.8%, medium in 41.2% and high in 34% of the families, and maternal education low in 38%, medium in 52.3% and high in 9.7% of the cases. Participants with no siblings were 15.3%, one sibling 61.7%, two siblings 19.5%, three or more 3.6%. Parity order was first in 52%, second in 38.3% and third or more in 9.7% of the cases. Alcohol consumption was declared by 9 subjects (3%), and cigarette smoking by 3 subjects (1%). Median BMI was 20.0, similar to the results from other Italian surveys (Toselli et al., 2010) and the reference Italian standards (Cacciari et al., 2006). Clinical values for CBC, liver and kidney function, and physiological iron status for the 311 included subjects were all within the normal clinical range. No significant differences occurred among the study sites for all socio-demographic covariates except for the SES, that resulted higher among the Garda Lake subjects (chi-squared = 7.4783, df = 2, p-value = 0.02377).

3.2. Exposure assessment

Data on Mn exposure assessment are summarized in Table 1a with a comparison between the Valcamonica and Garda Lake areas, and in Table 1b with sub-grouping of Valcamonica according to the former location of the three ferroalloy plants. The average values for the entire Valcamonica were: MnH 0.16 μg/g (median 0.11), MnB 10.99 μg/L (median 10.8), MnU 0.22 μg/L (median 0.10), Mn Air 49.47 ng/m^3 (median 31.39), Mn Soil 958.16 ppm (median 897.49). No significant differences were noted for the biomarkers MnH, MnB, and MnU between Valcamonica and Garda Lake, although a tendency toward increased levels from the lower to mid-upper valley was noticed for all biomarkers. The mean levels of Pb in blood were extremely low in subjects in both study areas: Valcamonica mean 1.72 μg/dL (median 1.60), Garda Lake 1.60 μg/dL (median 1.40). The concentration of Mn in airborne particles was higher in Valcamonica, especially in the mid valley (median 51.73 ng/m^3), compared to the Garda Lake reference area (median 24.72 ng/m^3). Concentrations of the other metals indicated a similar trend for Pb, Cr, Fe, Ni, whereas Cu and Zn were higher in the Garda Lake, possibly due to the agricultural use of chemical products (data not shown). Airborne PM10 particles were also slightly higher in mid Valcamonica (mean 71.64, median 62.22 μg/m^3) compared to the Garda Lake (mean 63.76, median 59.93 μg/m^3). The subjects' diaries kept over the 24 h air sampling period showed an average of 2 h outdoor and 22 h indoor, with slight seasonal variation (1 h outdoor in winter time and 3–5 h in spring time), and no variation across the different study areas. Data on rain precipitation showed that 46% of air particles were collected over 24 h periods that contained some rain, while 54% were collected with no rain during the sampling time, with slight differences between the Valcamonica (40% rain and 60% no rain) and Garda Lake (58% rain and 42% no rain) areas.

Soil values showed a significant increase from the lowest concentration in Garda Lake to the lower-, mid-, upper-Valcamonica sub-areas for Mn and for Pb, Fe, Zn, As, Ni (data not shown).

The concentrations of heavy metals in tap water, reflecting the public water supply from either study area, were not elevated. In fact, water Mn levels were below the LDL of 1 μg/L in all water samples. Only two subjects from Valcamonica (1.30% of participants) and two subjects from the Garda Lake (1.27% of participants) reported drinking water from private wells. The estimated daily oral intake of Mn based on dietary questionnaire was 2.91 ± 1.5 mg/day (median 2.66, range 0.72–9.46) for all participants, with no area differences.

Table 1a
Manganese exposure assessment in biomarkers and environmental media (VC, ValCamonica; GL, GardaLake).

Variable	Levels	n	Min	1st q.le	Mean	Median	3rd q.le	Max	IQR	#NA
Mn hair (ppm)	VC	125	0.02	0.07	0.16	0.11	0.19	1.27	0.12	29
	GL	133	0.02	0.06	0.18	0.12	0.20	3.45	0.14	24
$p = 0.74287$	All	258	0.02	0.06	0.17	0.11	0.19	3.45	0.13	53
Mn blood (μg/L)	VC	151	4.00	8.70	10.99	10.80	13.15	21.60	4.45	3
	GL	148	6.00	8.90	11.24	10.95	12.80	24.10	3.90	9
$p = 0.54844$	All	299	4.00	8.80	11.11	10.90	12.90	24.10	4.10	12
Mn urine (μg/L)	VC	151	0.10	0.10	0.22	0.10	0.10	7.60	0.00	3
	GL	150	0.10	0.10	0.16	0.10	0.10	1.50	0.00	7
$p = 0.74242$	All	301	0.10	0.10	0.19	0.10	0.10	7.60	0.00	10
Mn air (ng/m^3)	VC	125	1.24	15.12	49.47	31.39	60.21	516.70	45.09	29
	GL	64	5.30	16.92	27.37	24.72	35.99	85.93	19.06	93
$p = 0.03461$	All	189	1.24	15.38	41.99	29.37	47.20	516.70	31.82	122
Mn soil (ppm)	VC	31	464.88	658.93	958.16	897.49	1281.06	1729.10	622.13	123
	GL	27	159.76	352.95	426.58	408.58	484.14	734.02	131.19	130
$p < 0.0001$	All	58	159.76	441.66	710.70	579.13	924.80	1729.10	483.14	253

Table 1b
Manganese exposure assessment in biomarkers and environmental media (VC, ValCamonica; GL, GardaLake). The Valcamonica area is subdivided in the locations of the three exposure sources from ferroalloy plants.

Variable	Levels	n	Min	1st q.le	Mean	Median	3r q.le	Max	IQR	#NA
Mn hair (ppm)	Upper VC	21	0.03	0.08	0.23	0.13	0.26	1.13	0.18	2
	Mid VC	54	0.02	0.07	0.14	0.11	0.19	0.65	0.11	31
	Lower VC	23	0.03	0.05	0.11	0.07	0.14	0.49	0.09	23
	GL	104	0.02	0.06	0.13	0.10	0.17	0.72	0.11	53
$p = 0.1434$[a]	All	202	0.02	0.06	0.14	0.10	0.18	1.13	0.12	109
Mn blood (μg/L)	Upper VC	22	7.10	9.03	12.54	12.60	14.78	21.60	5.75	1
	Mid VC	83	4.00	8.70	10.80	10.90	12.85	17.20	4.15	2
	Lower VC	46	5.30	8.55	10.59	10.20	12.07	18.20	3.52	0
	GL	148	6.00	8.90	11.24	10.95	12.80	24.10	3.90	9
$p = 0.1755$	All	299	4.00	8.80	11.11	10.90	12.90	24.10	4.10	12
Mn urine (μg/L)	Upper VC	22	0.10	0.10	0.13	0.10	0.10	0.40	0.00	1
	Mid VC	83	0.10	0.10	0.28	0.10	0.10	7.60	0.00	2
	Lower VC	46	0.10	0.10	0.15	0.10	0.10	0.50	0.00	0
	GL	150	0.10	0.10	0.16	0.10	0.10	1.50	0.00	7
$p = 0.7577$	All	301	0.10	0.10	0.19	0.10	0.10	7.60	0.00	10
Mn air (ng/m^3)	Upper VC	22	8.22	16.86	31.34	23.97	34.91	115.32	18.05	1
	Mid VC	62	7.79	29.78	71.53	51.73	89.57	516.70	59.80	23
	Lower VC	41	1.24	5.25	25.86	15.11	35.51	243.10	30.26	5
	GL	64	5.30	16.92	27.37	24.72	35.99	85.93	19.06	93
$p < 0.0001$	All	189	1.24	15.38	41.99	29.37	47.20	516.70	31.82	122
Mn soil (ppm)	Upper VC	7	1261.56	1340.09	1474.41	1433.72	1608.16	1729.10	268.07	16
	Mid VC	6	650.73	860.40	1060.77	1081.26	1283.33	1413.88	422.93	79
	Lower VC	18	464.88	570.69	723.19	713.20	872.77	1008.85	302.08	28
	GL	27	159.76	352.95	426.58	408.58	484.14	734.02	131.19	130
$p < 0.0001$	All	58	159.76	441.66	710.70	579.13	924.80	1729.10	483.14	253

[a] Kruskal–Wallis test.

Table 2
Significant differences for neurobehavioral test at the site comparison (VC, ValCamonica; GL, GardaLake; CE, closed eyes).

Variable	Levels	n	Min	1st q.le	Mean	Median	3rd q.le	Max	IQR	#NA
Sniffin test	GL	156	5.00	9.00	10.08	10.00	11.00	12.00	2.00	1
	VC	152	4.00	9.00	9.61	10.00	11.00	12.00	2.00	2
$p = 0.0045707$	All	308	4.00	9.00	9.85	10.00	11.00	12.00	2.00	3
Aiming Pursuit	GL	156	2.00	23.00	58.63	48.50	85.50	251.00	62.50	1
	VC	152	0.00	21.75	44.39	37.00	61.25	189.00	39.50	2
$p = 0.012798$	All	308	0.00	23.00	51.60	41.00	71.00	251.00	48.00	3
Luria Nebraska	GL	156	31.00	58.00	67.54	66.00	76.25	107.00	18.25	1
	VC	151	37.00	53.00	62.76	61.00	70.00	98.00	17.00	3
$p = 0.0018979$	All	307	31.00	55.00	65.19	64.00	73.00	107.00	18.00	4
Sway intensity CE	GL	156	0.92	4.01	5.10	4.79	6.23	15.19	2.22	1
	VC	152	1.30	4.33	5.50	5.21	6.45	12.06	2.12	2
$p = 0.055633$	All	308	0.92	4.13	5.30	5.03	6.33	15.19	2.19	3

3.3. Neuro-psychological testing

Differences between Valcamonica and Garda Lake subjects were noted for health outcomes including Sniffin' sticks task ($p = 0.005$), Aiming Pursuit test ($p = 0.01$), the Luria-Nebraska partial motor scale ($p = 0.002$), and body sway with closed eyes ($p = 0.05$) (Table 2). The multivariate analysis with GAM and (Generalized Linear Model-GLM) logistic models showed a significant association of soil Mn levels with the Luria-Nebraska motor coordination test (Table 3, Fig. 3), with the Aiming Pursuit hand steadiness test (Table 4, Fig. 4) and with Sniffin' sticks odor identification task (Table 5, Fig. 5). The smooth curves are represented using penalized regression splines with smoothing parameters selected by Generalized Cross Validation (Wood, 2006). A significant association was also observed between MnH and MnB and tremor intensity in the dominant hand, with a border-line relation also between soil Mn and tremor intensity

Table 3
Adjusted effect of soil Mn on the Luria-Nebraska motor coordination test score, ordinary least square model.

	Estimate	SE	*T* value	*Pr*(>|*t*|)
Intercept	51.828	11.495	4.509	0.0000
Gender (M vs F)	−1.690	1.534	−1.101	0.2717
Age	1.594	0.906	1.758	0.0798
SES[a]	2.970	2.091	1.420	0.1566
SES[b]	4.002	2.465	1.623	0.1056
Maternal education[a]	−0.716	1.847	−0.387	0.6987
Maternal education[b]	−3.852	3.269	−1.179	0.2396
Smoking habit	−12.438	7.861	−1.582	0.1147
Alcohol consumption	0.509	4.613	0.110	0.9122
Mn Soil	−0.009	0.002	−3.521	0.0005

[a] Medium vs low.
[b] High vs low.

Table 4
Adjusted effect of soil Mn on the Aiming Pursuit hand dexterity test score, semi-parametric (GAM) model.

	Estimate	SE	z-Value	Pr(>|t|)
Intercept	129.7736	4.9641	26.142	0.0000
Gender (M vs F)	−4.8431	3.0155	−1.606	0.1094
SES[a]	1.1482	4.1185	0.279	0.7806
SES[b]	5.4496	4.9228	1.107	0.2692
Maternal education[a]	−7.2377	3.6392	−1.989	0.0477
Maternal education[b]	−4.1177	6.4537	−0.638	0.5240
Smoking habit	−22.4593	15.4284	−1.456	0.1466
Alcohol consumption	−4.5436	9.1090	−0.499	0.6183
Non parametric	Approximate significance of smooth terms			
	Edf	Ref.df	F	p-Value
Age	2.821	3.441	4.604	0.0023
Mn soil	4.324	5.385	2.922	0.0115

[a] Medium vs low.
[b] High vs low.

(Table 6). No association was observed between the health effects outcomes and the other parameters of internal (MnU) and external (Mn Air, Mn Water, Mn daily oral intake) exposure to Mn in the regression models (data not shown). We checked for the presence of residual confounding with a sensitivity analysis on the inclusion of additional covariates in the models, including parity order, family size, SES, maternal education, BMI, alcohol intake, smoking habits, and concentration of Pb and other metals in air, soil and water. These covariates were generally not statistically significant when entered into the model and in any cases did not change sensibly the regression coefficients associated with Mn exposure. Age was positively associated with the motor and odor tests, but not with tremor, indicating a better performance among the most grown up subjects. A gender difference was observed for odor identification (Table 5), and tremor (Table 6) with lower performance and increased tremor intensity among boys. SES did not generally influence motor and odor testing, whereas maternal education was positively associated to odor identification (Table 5). Although declared by a small number of subjects, alcohol consumption was negatively associated to odor identification (Table 5) and cigarette smoking increased tremor intensity (Table 6).

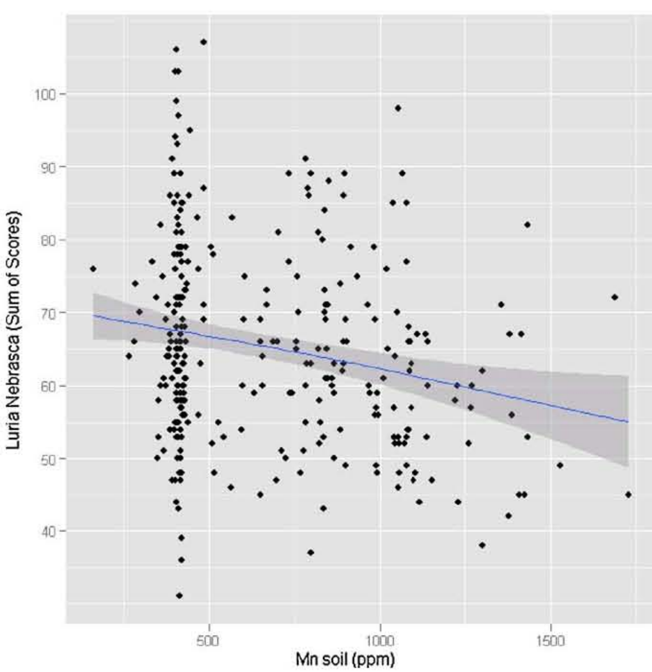

Fig. 3. Soil Mn and motor coordination as measured with the Luria-Nebraska test.

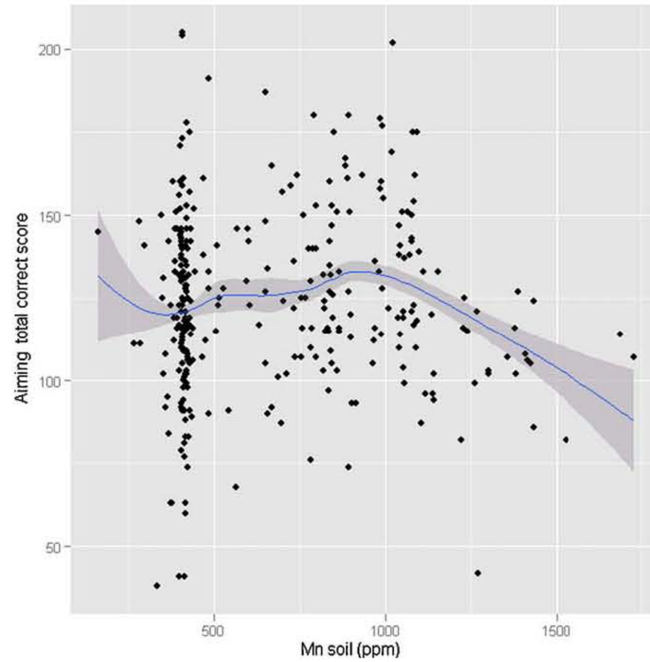

Fig. 4. Soil Mn and hand steadiness as measured by the Aiming Pursuit test.

Table 5
Adjusted effect of soil Mn on the olfactory test, semi-parametric (GAM) logistic model.

	Estimate	SE	z-Value	Pr(>|t|)
Intercept	2.6179	0.2363	11.079	0.0000
Gender (M vs F)	−0.2865	0.0897	−3.196	0.0014
SES[a]	−0.1117	0.1208	−0.925	0.3551
SES[b]	−0.3401	0.1402	−2.427	0.0152
Maternal education[a]	0.2203	0.1050	2.098	0.0359
Maternal education[b]	0.6436	0.1995	3.226	0.0013
Smoking habit	−0.0624	0.4490	−0.139	0.8894
Alcohol consumption	−0.5104	0.2442	−2.090	0.0366
Mn soil	−0.0004	0.0001	−2.937	0.0033
Non parametric	Approximate significance of smooth terms			
	Edf	Ref.df	F	p-Value
Age	4.076	4.623	22.354	0.0003

[a] Medium vs low.
[b] High vs low.

Table 6
Adjusted effect of various Mn sources on (log-transformed) tremor intensity, semiparametric (GAM) model.

	Estimate	SE	z-Value	Pr(>|t|)
Intercept	−2.2765	0.0346	−65.817	0.0000
Gender (M vs F)	0.2564	0.0413	6.213	<0.0001
Smoking habit	0.4172	0.1886	2.213	0.0279
Alcohol consumption	−0.0153	0.1130	−0.135	0.8924
Mn air	0.0004	0.0005	0.928	0.3541
Mn hair	0.2089	0.0813	2.569	0.0108
Non-parametric	Approximate significance of smooth terms			
	Edf	Ref.df	F	p-Value
MN soil	2.584	3.264	2.140	0.0903
Mn blood	7.625	8.529	2.749	0.0053

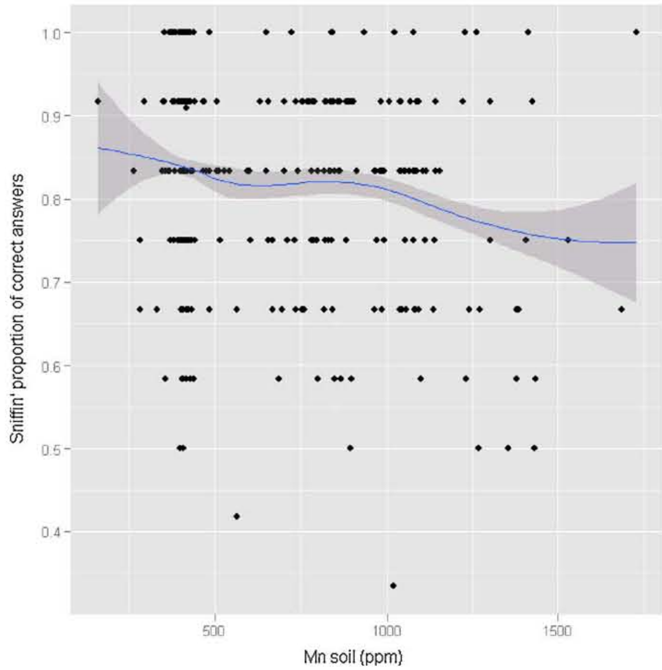

Fig. 5. Soil Mn and odor identification as measured with the Sniffing test.

4. Discussion

The present study shows an association between Mn exposure reflected by Mn levels in surface soil, hair and blood and impairment of motor coordination, hand dexterity, tremor intensity and odor identification in adolescents living in Valcamonica, Italy. Although Mn is a recognized extrapyramidal neurotoxicants in adults since the past century, the most recent studies on exposed children have focused only on cognitive and behavioral implications. Our work is the first showing clear Mn-related motor dysfunction in children, that are very similar to those already observed by our group in chronically exposed ferroalloy workers (Lucchini et al., 1995, 1999). Furthermore, this observation is of particular interest given that in the same region we observed a significantly increased prevalence of Parkinsonism in adults, compared to national and international rates, as possibly linked to increased environmental Mn levels in the Valcamonica dust (Lucchini et al., 2007). It is also noteworthy that olfactory impairment is considered an early sign of Parkinson's disease with a prevalence of ~75% in PD patients. Our observed association of early effects on motor and odor functions with the levels of Mn in soil and not in airborne particles, or with biomarkers of internal Mn exposure (MnH, MnB, MnU) may suggest that these effects are related to past or cumulative environmental exposures rather than current exposure. In fact, the adolescents from Valcamonica were likely exposed to higher levels of Mn in their early life; they were on average 5 years old, when the last ferroalloy plant ceased production in 2001. Metals in soil serve as good indicators of general environmental insult because they are stable and long-lived in the environment, and accumulate in soils over time (Aelion et al., 2009). As such, Mn levels in surface soil measured here would be expected to reflect background soil Mn in addition to cumulative inputs from atmospheric deposition of ferroalloy plant emissions, and thus appear to be a reasonable proxy of past cumulative Mn exposure to the children in this study.

Although still preliminary, the analysis of hair data (that will be fully reported elsewhere) has showed an association with tremor intensity. Hair Mn levels may serve as a better indicator of integrated exposure and acquired body burden over the prolonged period of hair growth, differently from MnB and MnU, which are under rapid homeostatic control. The rigorous cleaning procedure developed for hair samples in this study were designed to minimize risk of external Mn contamination without compromising endogenous (metabolic) Mn, and thereby may have improved the sensitivity of this exposure biomarker to predict the internal dose in storage organs and tissues. Since tremor intensity is related to MnB, this health outcome may be related to current rather than to cumulative exposure.

The detailed exposure assessment, exploring all relevant sources and potential exposure biomarkers, was a major strength of this study. Subject-based measurement of airborne particles over 24 h still shows higher levels of metals in the mid-upper valley compared to the reference area about 7 years after the cessation of active ferroalloy plant emissions. This may be best explained by the weathering and re-suspension of surface particles due heavy traffic, unpaved roads, road/work construction in the Valcamonica. In addition, residual atmospheric emission from other industries located in the mid valley cannot be excluded. Very few air exposure data obtained with personal sampling are available for comparison. Pellizzari et al. (2001) reported lower Mn concentration in PM10 and PM2.5 (median 8 ng/m^3) from Toronto and Indianapolis. Background average levels of 10 ng/m^3 have also been recently surveyed in urban areas in Spain, rising to 25 ng/m^3 in industrial sites (Moreno et al., 2011). The current reference concentration for airborne Mn of USEPA and Health Canada in Canada is 50 ng/m^3 (Health Canada, 2010). The soil Mn levels were relatively consistent in showing an increasing gradient from the lower to the upper Valcamonica, which may be explained by the day time prevailing wind direction (South to North) and also by an overlap of anthropogenic emission on naturally higher soil Mn levels in the upper Valcamonica. This was shown by previous fingerprint analysis of metals in the deposited dust sampled in the entire province of Brescia (Zacco et al., 2009). Further, differences regarding the ferroalloy plants previously operating in the three sub-areas of Valcamonica can partially explain the differences of metal concentrations in airborne particles and surface soil in these sub-areas. The lower Valcamonica shows air levels of metals and PM10 that are similar to the Garda Lake, probably because the plant in this area was closed in 1995, which is more than 10 years before this study. The mid-valley shows the highest air levels probably because the plant in this area was closed most recently in 2001 and because particle re-suspension and residual industrial emissions are still present in this area. The ferroalloy plant in the upper valley was mainly a holding/deposit site and for Mn-rich ores shows the highest soil Mn levels.

In this study, the very low levels of Mn in tap water indicate drinking water does not represent a notable source of exposure. The observed levels below 1 μg/L are substantially lower than the current guideline of 400 μg/L set by the World Health Organization and the U.S. health reference level of 300 μg/L (Ljung and Vahter, 2007). They are also lower than drinking water Mn levels associated with cognitive deficits in children from Quêbec, where water Mn levels ranged from 0.1 to 2700 μg/L (median 30.8) (Bouchard et al., 2011), and Bangladesh, where water Mn levels averaged 795 ± 755 μg/L (Wasserman et al., 2006). Water from the public supply system in the province of Brescia is treated to decrease the Mn level for esthetic reasons. This treatment is generally lacking in private wells, where Mn concentrations may reach high levels (Ljung and Vahter, 2007), but the use of water from private wells was very limited in the present study. Mn oral intake resulted in 81% (118 girls and 130 boys) above the Adequate Intake (AI) by the Institute of Medicine, for children 9–13 years of age (1.6 mg/d for girls; 1.9 mg/d for boys) and in 5% above the Upper Intake Level (UL) of 6 mg/d, although within the range of data reported in the literature (Ljung and

Vahter, 2007). Levels of Mn in blood, urine, or hair were not measurably different in subjects from the impacted Valcamonica versus Garda Lake reference areas, although MnB, MnU and MnH were slightly higher in the subjects from the mid-upper Valcamonica compared to the other two areas (lower Valcamonica or Garda Lake). Blood levels of Mn were similar to those recently measured in 359 school children aged 8–16 years from Durban, South Africa (10.1 ± 3.4 μg/L, median 9.6), exposed to similar Mn levels in PM10 airborne particles (48.7 ± 44 ng/m^3, median 34.3) (Batterman et al., 2011). This is the first study that shows MnH associated with extra-pyramidal impairment. Hair Mn levels have been associated with cognitive deficits in other studies (Wright et al., 2006; Bouchard et al., 2011; Riojas-Rodríguez et al., 2010; Menezes-Filho et al., 2011), though the extent that hair Mn levels reported in those studies reflects external contamination versus internal body burden is not clear, since the hair cleaning methodologies varied widely in those studies, and all of them were notably less rigorous than the methods used in the present study.

A limitation of this study may be the possible residual confounding by unobserved variables. The study is based on a comparison between two adolescents populations that we assumed to be similar but for the exposure and confounding variables that we have recorded. Another limitation may be the association of the exposure location with the subject's home. Although the majority of the time was spent by each adolescent in the house (17 h on average), another 5 h were spent at school and 2 h outdoors, including road transportation. Different exposure levels, especially for airborne particles, may have characterized the different sites. Nevertheless, even considering these limitations, the study indicates that living in an environment characterized by long-term exposure to metals – particularly Mn, can lead to impairment in the development of motor and olfactory functions that may be potentially considered as an early warning for the onset of late neurodegenerative effects in the older age. Preventive strategies toward sustainable soil should be promoted for urban areas close to metal emission from industries and further study should clarify the soil chemistry to allow better understanding of metal solubility, transport and bio-accessibility to plants and living organisms. Further research is scheduled in the Province of Brescia and will target the area of Bagnolo Mella, in the proximity of a currently active ferroalloy plant. This will provide further comparison with human populations exposed to higher levels of metals typically released in the environment by the ferroalloy production.

In summary, this is the first study showing association of manganese in surface soil and airborne particles with motor coordination, hand dexterity and odor identification and of manganese in blood and hair with tremor, in adolescents residing in areas with previous long term emission of metals – including manganese – from ferroalloy emission. The measurement of manganese in soil may represent an indicator of historical cumulative exposure.

Conflict of interest statement

Zimmerman had paid expert testimony/consulting for plaintiff manganese exposure welder/welding rod lawsuits, and all the remainder of authors had declared no conflict of interest.

Acknowledgements

This study was supported by funding from the European Union through its Sixth Framework Program for RTD (contract no FOOD-CT-2006-016253). It reflects only the authors' views, and the European Commission is not liable for any use that may be made of the information contained therein. The project was supported also by Award Number R01ES019222 from the National Institute of Environmental Health Sciences (NIEHS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIEHS or the National Institutes of Health.

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