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Noninvasive biomarkers of manganese exposure and neuropsychological effects in environmentally exposed adults in Brazil

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HIGHLIGHTS

- Noninvasive biomarkers evaluate Mn exposure to metallurgical emissions.
- An inverse gradient of exposure is observed in those living relatively further.
- Scalp hair, fingernail and axillary hair are useful matrices to assess Mn exposure.
- The levels observed are associated with detrimental neuropsychological effects.

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ABSTRACT

Manganese (Mn), an essential element to humans, in excess can cause neurotoxic damage. So far, Mn exposure assessment has no ideal biomarker. This study aims to investigate the association between Mn exposure, using noninvasive biomarkers, and neuropsychological effects in environmentally exposed adults. The residents of two communities near to a ferromanganese refinery in Bahia, Brazil were evaluated. Volunteers aged 15–55 of both sexes provided scalp hair, axillary hair, fingernail and saliva specimens for Mn determination by electrothermal absorption spectrometry. Several neuropsychological tests were used to evaluate cognitive, attention, memory, motor and executive functions. Significant correlations were observed between Mn in hair (MnH, median 8.95 μg/g), axillary hair (MnAxH,18.49 μg/g) and fingernail (MnFN, 6.91 μg/g) with the performances in several neuropsychological tests. No association was observed between manganese levels in saliva (MnSal, 4.2 μg/L) and any neuropsychological function. Multiple regression analysis detected an inverse association between Log MnH and IQ (β = −4.76 [CI 95% −9.17 to −0.36]) and between Log MnFN and visual working memory (β = −3.33 [CI 95% −6.15 to −0.52]). Direct association was observed between Log MnFN and time of completion in the cognitive flexibility task (β = 56.29 [CI 95% 2.41–110.18]). The Mn biomonitoring using noninvasive biomarkers was able to detect high exposure levels, which were associated with detrimental neuropsychological effects in adults exposed to industrial emissions.

1. Introduction

Manganese (Mn) is an essential mineral (Aschner and Aschner, 2005), that when present in excess in the body, can cause damage to the central nervous system (Aschner et al., 2007). The accumulation of this element in the basal ganglia and cerebral frontal cortex (Reaney et al., 2006; Rivera-Mancía et al., 2011; Sen et al., 2011), as well as its correlation with poor performance on neuropsychological tests (Sen et al., 2011) have been demonstrated in animals and humans.

The action of Mn on neurotransmitters can cause changes in synaptic mechanisms mediated by dopamine, glutamate and γ-aminobutyric acid (GABA) (Burton and Guilarte, 2009), which can trigger poor performance on neuropsychological tests assessing motor, cognitive and behavioral functions. Studies with

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non-human primates exposed to Mn showed behavior similar to Parkinson's disease and impairment of cognitive and motor functions (Burton and Guilarte, 2009; Schneider et al., 2009). Occupational and environmental exposures to Mn have also been correlated with motor impairment (Lucchini et al., 2012; Mergler et al., 1994), working memory (Park et al., 2009), low cognitive performance (Bouchard et al., 2011; Menezes-Filho et al 2011; Riojas-Rodríguez et al 2010; Wasserman et al., 2006), low academic performance (Khan et al., 2012), hyperactivity (Bouchard et al., 2007), externalizing behaviors (Khan et al., 2011), negative effects on attention (Laohaudomchok et al., 2011) and memory(Torres-Agustín et al., 2013).

Communities located nearby mine ore extraction (Riojas-Rodríguez et al., 2010), metal alloys plants (Menezes-Filho et al., 2009a) and near agricultural areas spraying with fungicides such as maneb and mancozeb (Geissen et al., 2010; Mora et al., 2014), among others, are groups at risk of excessive Mn exposure. Epidemiological studies on communities environmentally exposed to this metal have successfully used scalp hair Mn (MnH) and air Mn as biological and environmental markers of exposure (Menezes-Filho et al., 2009a; Riojas-Rodríguez et al., 2010; Rodríguez-Agudelo et al., 2006). In general, the usefulness of a biomarker of exposure should be evaluated by its ability to characterize and differentiate exposed and non-exposed groups, as well as by its ability to predict health disturbances; it should also be able to anticipate neuropsychological impairment as a consequence of short or long term exposure.

Conflicting results in the literature do not contribute to the selection of the ideal biomarker to assess Mn exposure, which would be the one that best correlates biological levels of Mn with environmental Mn concentrations. Blood and hair Mn levels have been widely used in environmental and occupational studies in which neuropsychological outcomes resulting from exposure to this metal were evaluated (Bader et al., 2000; Rodríguez-Agudelo et al., (2006); Bowler et al., 2007; Menezes-Filho et al., 2009b; Zheng et al., 2011). However, Mn blood levels have rarely been associated with performance on neuropsychological tests (Roels et al., 2012; Bhang et al., 2013). Mn levels in saliva, axillary hair and nails, on the other hand, have been used only in exposure assessment studies. None used these biomarkers to investigate their associations with neuropsychological effects (Bader et al., 1999; Cowan et al., 2009; Gil et al., 2011; Laohaudomchok et al., 2011; Mehra and Juneja, 2005; Menezes et al., 2004; Wang et al., 2008; Wongwit et al., 2004).

The authors have been gathering evidences about excessive Mn exposure of children and their mothers who live in communities near a metallurgical plant. It was found negative associations between internal dose of Mn, measured in hair, and intellectual functions, working memory and attention (Menezes-Filho et al., 2011; Carvalho et al., 2013), and direct association with behavioral changes, specially externalizing behavior (Menezes-Filho et al., 2013). In these studies, it was found that only MnH levels were associated with neuropsychological function scores when adjusted for social–economic covariables, like gender, age, height-age z-score and maternal education. Blood Mn levels were not associated

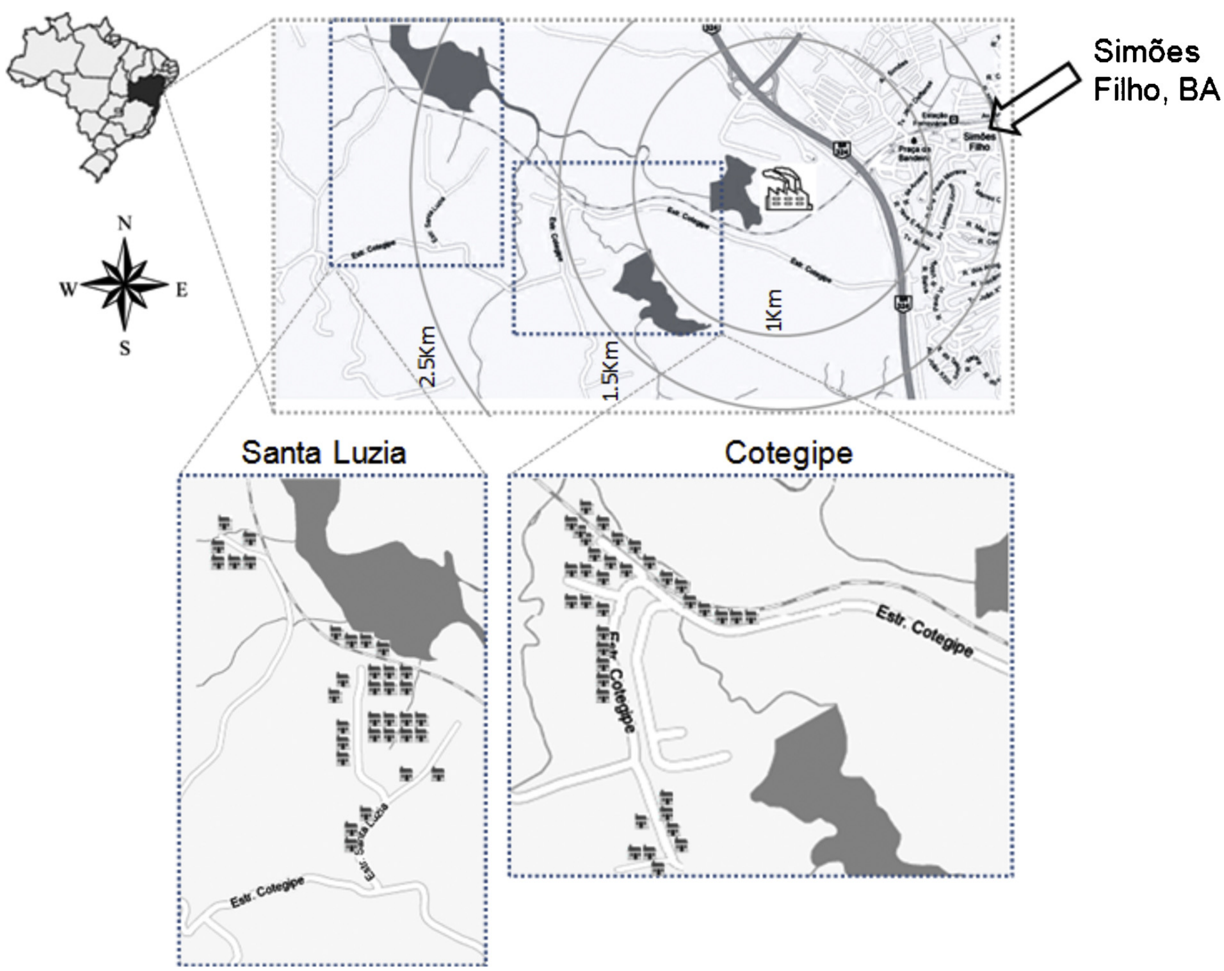

Fig. 1. Schematique map of the study location in the district of Simões Filho, Bahia, Brazil.

with any neurological outcomes evaluated. The authors have demonstrated previously that in these communities, the main exposure route is atmospheric (Menezes-Filho et al., 2009a). The ferromanganese alloy plant emissions are primarily composed of manganese oxides, which are sub-micrometric particles (ranging between 5 and 35 nm Mn m^{-3}) that can reach the pulmonary alveoli (ATSDR, 2012).

In the face of the conflicting findings in the literature regarding the ideal Mn surrogate, this study was carried out on four different noninvasive biomarkers to assess Mn exposure in adults and investigated their associations with performance in several neuropsychological function tests.

2. Materials and methods

2.1. Study design and population

This cross-sectional study was conducted in two communities of the town of Simões Filho, Bahia, Brazil: Cotegipe and Santa Luzia villages (Fig. 1). These communities are situated at an approximate distance of 1.5 and 2.5 km, respectively, from the ferromanganese alloy plant that has been in operation since the early seventies. Thus, it was hypothesized that there might be a gradient of exposure and, possibly, effects associated with the area of residence of the participants. Inhabitants of both villages have reported complaints of fine black dust deposits on their homes, which they link to the industrial atmospheric emissions. Previous reports have documented elevated environmental and human Mn levels in children and their mothers residing in Cotegipe village (Menezes-Filho et al., 2009b, 2011).

According to the last demographic census conducted in 2010 by the Brazilian Institute of Geography and Statistic (IBGE), the town of Simões Filho has a population of 118,047 inhabitants, per capita GNP of US$ 13,594 and HDI 0.730. Approximately, 31% of the households are maintained with a monthly income of 2–5 minimal wages, equivalent to US$ 540-1350 (IBGE, 2013). A census was conducted between March and September 2011 in both communities; Cotegipe had about 900 residents and Santa Luzia village, 750 residents. The socioeconomic classification from the Brazilian Association of Population Studies (ABEP, 2011), based on the possessions of comfort items in the home (washing machine, fridge and vacuum cleaner, car, color TV, bathroom, etc.) was applied. The total ABEP score is ranked into five descending classes (from A to E). The two communities are considered of low socioeconomic status; most families are ranked as class "C" (54%) and "D" (39%); very few families were either "E" or "B" (3%) and none classified as "A". The main income sources are subsistence farming, production of cassava flour and shellfish harvesting, processing and selling in local street markets.

In this study, all inhabitants of both sexes, aged between 15 and 55 years who resided in one of the communities for at least 5 years were intended to be included. The exclusion criteria were: medical diagnosis of neurological problems and being a former labor of the alloy plant. All volunteers who met the inclusion criteria (153 from Cotegipe and 142 from Santa Luzia) were informed about the objectives and procedures of the research. Thus, those who agreed to participate [n = 42 (27.5%) from Cotegipe and n = 47 (33.1%) from Santa Luzia) signed a consent form. The research project was approved by the Research Ethics Committee of the Federal University of Bahia (Registry n° 021/2011).

2.2. Socio-demographic information

A questionnaire to obtain information on socio-demographic characteristics (ethnicity, educational levels, time of residence in the community etc.), lifestyle (smoking, drinking habits, domestic waste destination, etc.) and occupational activity was obtained from volunteer by trained interviewers.

2.3. Mn exposure evaluation

2.3.1. Biological specimen collection

Scalp hair, axillary hair, hand fingernails and saliva samples were collected following the methodologies described by Wright et al. (2006), Menezes-Filho et al. (2009a) and Olmedo et al. (2010). Due to cultural, aesthetic and hygienic habits of the Brazilian women, axillary hair samples were not collected from female volunteers.

For scalp hair sampling, a tuft of approximately 0.5 cm diameter was cut off from the occipital region of the scalp with a stainless steel surgical scissor, tied with a teflon string and stored in plastic sampling bag at room temperature. Hair sampling details and

Table 1
Socio-demographic characteristics of the study population according to the area of residence.

	Cotegipe		Santa Luzia		X^2	p-value
	N	%	N	%		
Gender	42	14.3	47		4.652	0.031
Men	6	85.7	16	34		
Women	36		31	66		
Family income	41		47		1.234	0.267
≤1 minimal wage	28	68.3	37	78.7		
>1 minimal wage	13	31.7	10	21.3		
Schooling	41		47		7.459	0.006
Elementary school	16	39	32	68.1		
Middles school	25	61	15	31.9		
Domestic waste burning	41		47	40.4	0.312	0.576
Yes	19	46.3	19	59.6		
No	22	53.7	28			
ETS	41		47	53.2	10.598	0.001
Yes	8	19.5	25	46.8		
No	33	80.5	22			
	Mean	SD	Mean	SD	T stat.	p-value
Age (years)	32.9	6.35	34.2	10.82	0.691	0.492
Time of residence (years)	22.8	9.52	28.1	12.73	2.229	0.028

cleaning procedures are described elsewhere (Wright et al., 2006; Menezes-Filho et al., 2009a). Sufficient amount of axillary hair was collected from both armpits in male volunteers, with the aid of surgical scissors (Olmedo et al., 2010). As a measure to avoid external contamination, men volunteers were instructed not to use deodorant on the collection day. For hand fingernail collection, participants were asked to wash their hands with the provided liquid soap and rinsing them thoroughly with tap water. After drying, all fingernails of both hands were clipped directly into sampling bags. Women who had painted nails had the enamel removed with acetone-based solution, just before washing their hands (Mehra and Juneja, 2005). Saliva specimen was collected according to the procedures described by Olmedo et al. (2010), Kim et al. (2010), and Watanabe et al. (2009). In summary, the volunteers received deionized water in sufficient amount (approximately 100 mL) to perform three 1 min oral rinses. Afterwards, approximately 5 mL of unstimulated saliva were collected during 10 min directly into the 15 mL screw-capped polypropylene centrifuge tubes (Corning®). Saliva samples were transported in coolers to the laboratory where they were centrifuged for 10 min at about 2318 g. The supernatant was separated into aliquots and kept in the freezer (−22 °C) until analysis.

2.3.2. Mn determination

All biological samples, except saliva, went through a washing procedure to minimize external Mn contamination. For detailed descriptions of hair and fingernail cleaning and analytical procedures, see Menezes-Filho et al. (2009a). Each run included a reagent blank and standard reference material human hair (IAEA-085, International Atomic Energy Agency). Saliva specimens were processed according to the procedures described by Olmedo et al. (2010) and Watanabe et al. (2009). Mn determinations were carried out by electrothermal atomic spectroscopy with Zeeman background correction (GTA-120, Varian Inc.). The intra-batch and batch-to-batch precisions were estimated in 2.4% and 5.9%, respectively. Accuracy in the concentration range of 8.3–9.3 μg/g was 102.6% and the analytical LOD for Mn was 0.1 μg/L.

2.4. Neuropsychological evaluations

Neuropsychological testing was performed individually in a quiet room in the community schools, which assessed intelligence, executive functions, motor function, attention and memory. This evaluation lasted 1.5–2 h. Tests were administered by trained and supervised psychologists.

The Wechsler Intelligence Scale for Adults, third version (WAIS-III) is an intelligence scale, validated in Brazil by Nascimento (2005). The estimated IQ was based on the Vocabulary and Block Design subtests, a reliable and quick method for intelligence assessment (Ringe et al., 2002).

Rey Auditory Verbal Learning Test (RAVLT) evaluates the verbal memory immediate recall, verbal learning and susceptibility to interference and recognition memory (Salgado et al., 2011). This test analyzed recognition memory 30 min after 5 learning trials of 15 words, requiring the identification of these words among 20 distracting words (Malloy-Diniz et al., 2007).

Table 2
Manganese biomarker levels according to the main socio-demographic characteristics. Data expressed as median (range).

	MnH (μg/g)	MnAxH (μg/g)	MnFN (μg/g)	MnSal (μg/g)
Area of residence				
Cotegipe	2.7 (0.6–44,6)[b]	5.8 (3.8–17.2)[b]	4.0 (0.7–16.1)[c]	3.0 (0.4–43.3)
Santa Luzia	10.5 (0.9–42,0)	21.8 (4.4–85.6)	6.5 (1.1–22.2)	3.7 (0.6–81.6)
Sex				
Male	9.7 (1.3–42.0)	17.2 (3.8–85.6)	7.2 (2.9–22.2)	4.1 (0.6–81.6)
Female	4.4 (0.6–44.6)	–	5.3 (0.7–17.0)	3.4 (0.4–43.3)
Age group[a]				
15 to 33	4.5 (1.0–44.6)	7.3 (3.9–37.7)	4.8 (0.7–17.0)	3.5 (0.4–43.3)
34 to 55	7.3 (0.6–42.0)	19.7 (3.8–85.6)	5.8 (1.3–22.2)	3.4 (0.6–81.6)
Family income[b]				
≤1 minimal wage	6.6 (0.6–44.6)	25.8 (5.8–85.6)	5.9 (1.1–22.1)	3.4 (0.4–81.6)
>1 minimal wage	3.3 (0.8–17.5)	7.4 (3.8–21.9)[b]	4.4 (0.7–16.1)	3.5 (0.6–13.5)
Schooling				
Elementary	7.8 (0.6–44.6)	23.7 (4.4–85.6)	6.5 (1.1–22.2)	3.7 (0.9–81.6)
Middle	3.3 (0.7–35.6)[a]	9.0 (3.8–21.9)	4.3 (0.7–16.1)[c]	3.3 (0.4–13.5)
ETS				
Yes	7.2 (1.0–28.3)	7.3 (3.8–44.2)	6.1 (1.3–17.0)	3.5 (0.9–81.6)
No	4.4 (0.6–44.6)	21.6 (3.9–85.6)	5.1 (0.7–22.2)	3.4 (0.4–43.3)
Drinking				
Yes	7.6 (0.6–42.0)	17.2 (5.8–85.6)	5.3 (1.3–22.2)	3.7 (0.4–81.6)
No	4.4 (0.7–44.6)	12.8 (3.8–50.8)	5.8 (0.7–16.1)	3.4 (0.6–43.3)
Domestic waste burning				
Yes	6.6 (0.8–44.6)	21.9 (3.9–85.6)	6.1 (1.3–22.2)	3.1 (0.4–81.6)
No	4.4 (0.6–35.6)	10.9 (3.8–44.2)	5.4 (0.7–16.1)	3.7 (0.9–20.8)
Time of residence[a]				
5 to 26	3.8 (0.6–42.0)	8.7 (3.9–85.6)	4.7 (0.7–22.2)	2.6(0.6–43.3)[b]
26 to 50	7.8 (0.7–44.6)	18.5 (3.8–50.8)	6.4 (1.3–17.0)	4.6 (0.4–81.6)

[a] Age and time of residence in years.
[b] Mann Whitney, $p < 0.05$.
[c] Student t test, $p < 0.05$.

The *Grooved Pegboard Test* (GPT) assesses manual dexterity and fine motor skills, requiring a metal peg to be rotated before inserting as fast as possible. In this study, time to perform the task with the dominant and non-dominant hands are recorded and analyzed. Long execution time indicates low performance (Lafayette Instruments, 2002).

The *Visual Attention Computerized Test third version* (TAVIS-3) was used to assess the selective and sustained attention. In the selective attention task, the volunteers must selectively respond to a target stimulus compared to distracters. The other task evaluates sustained attention through rapid detection response to 64 red dot stimuli that appear on the screen over 10 min. The scores are reaction time, omission errors and commission errors, longer execution time and greater errors indicate lower performance (Duchesne and Mattos, 1997).

The *Trail Making Test* (TMT) has two trials: trail A assesses processing speed in which the participant must connect the numbers in correct sequence (1, 2, 3 etc.); trail B evaluates cognitive flexibility (ability to change symbol), connecting numbers and letters (1A, 2B, 3C etc.) (Strauss et al., 2006; Hamdan and Hamdan 2009). The score is the time to completion.

Digit Span from the WAIS-III and *Corsi Block-Tapping Task* (Kessels et al., 2000; Paula et al., 2010) are two classical tests that assess working memory, responsible for maintenance and manipulation of temporary information. Both tasks have the same logic that consists of recall sequences of numbers (Digits – verbal component) or taps onto cubes (Corsi Blocks – visual component) in forward and backward methods, and the scores is the sum of correct answers.

2.5. Data analysis

Descriptive statistics were used to examine the distribution of socio-demographic data, bioindicators of Mn exposure and neuropsychological function (intelligence, memory, motor function, attention, executive functions and working memory); scores were stratified according to area of residence. For normally distributed variables, the arithmetic mean (AM), median and range are presented. Spearman correlation tests were applied to evaluate bivariate correlations between covariables and exposure bioindicators. Since the distributions of the Mn biomarkers were skewed, data were log 10 transformed for further analyses. Backward stepwise regression models were used to identify variables that were potentially associated with the neuropsychological endpoints (0.100 to enter; >0.05 to exclude). A series of regression models was carried out with (gender, local of residence, time in years of residence in the communities, drinking habits, family income, age, family income) as dependent variables and exposure as independent variable. Each regression was adjusted on variables that could potentially modify the relation between exposure and independent variables. The performance of the regression models was verified with the heteroschedasticity of residuals, outliers detection, the estimate, its standard error and the associated *p*-value. The residual plots clearly showed a linear relation between independent and dependent variables, while accounting for the other variables. All statistical analyses were performed using SPSS version 20 software.

3. Results

3.1. Population characteristics

In this study, 89 volunteers who met the inclusion criteria, representing 30.2% of the 298 inhabitants in the desired age range, who lived in one of the communities for at least 5 years, were evaluated. Women represented 66% of the study population. In Cotegipe village, 36 participants were women (85.7%) and 6 were men (14.3%), while in Santa Luzia, there were 31 women (66.0%) and 16 men (34.0%) (Table 1). On average, the age of the participant populations was similar in the two villages (32.9 years (SD = 6.3) in Cotegipe vs. 34.2 years (SD = 10.8) in Santa Luzia, $p = 0.492$). A statistically significant difference was observed in time of residence in the communities (22.8 years (SD = 9.52) in Cotegipe vs. 28.1 years (SD = 12.7) in Santa Luzia, $p = 0.028$). No difference was observed on the distribution of family income between the two communities ($p = 0.267$). However, the inhabitants of Cotegipe had a higher proportion of people with middle education level than Santa Luzia (61.0 % vs. 31.9%, respectively; $p = 0.006$). It was observed that

Table 3
Performance on neuropsychological functions according to the area of residence.

Neuropsychological functions	Cotegipe				Santa Luzia				*p*-value
	n	Median	Min	Max	*n*	Median	Min	Max	
Estimated IQ	39	91*	71	117	43	83	71	112	0.004
Memory	39	12	7	15	45	14	7	15	0.051
Motor (s)									
D.H.[a]	39	79	51	182	45	78	57	237	0.560
N.D.H.[b]	39	85	58	341	45	85	59	377	0.384
Attention (s)									
Selective	38	0.471	0.281	0.536	44	0.471	0.340	0.609	0.593
Sustained	38	0.498	0.345	1.420	44	0.454	0.322	1.256	0.127
Executive functions (s)									
P.S.[c]	37	46	21	113	44	55.5	22	368	0.100
C.F.[d]	37	138	44	138	40	132	53	362	0.415
Working memory									
Visual	39	12	3	19	45	11	1	18	0.074
Verbal	39	9	3	13	45	8	4	16	0.779

[a] Dominant hand.
[b] Non-dominant hand.
[c] Processing speed.
[d] Cognitive flexibility.
* Significant difference at level of $p < 0.05$.

53.2% of the volunteers of Santa Luzia reported exposure to tobacco smoke (ETS) against 19.5% of Cotegipe ($p < 0.001$). There was no difference in the proportion of people reporting burning of domestic waste (46.3% in Cotegipe vs. 40.4% in Santa Luzia; $p = 0.576$).

3.2. Exposure characteristics

Table 2 presents descriptive statistics for Mn biomarker levels (scalp hair, axillary hair, fingernails and saliva), stratified according to the main socio-demographic characteristics. Hair Mn concentrations were significantly different ($p < 0.05$) between area of residence. In Cotegipe, MnH levels were lower than in Santa Luzia (median (range): 2.7 (0.6–44.6) μg/g vs. 10.5 (0.9–42.2) μg/g, $p < 0.05$). The same pattern was also observed with the median levels of MnAxH and MnFN (MnAxH: 5.8 μg/g vs. 21.8 μg/g, $p < 0.05$; MnFN: 4.0 μg/g vs. 6.5 μg/g, $p < 0.05$). MnSal levels were deeply skewed with range between 0.4 to 81.6 μg/L, and no difference in the medians was observed between Cotegipe and Santa Luzia (3.0 vs. 3.7 μg/g, $p > 0.05$).

Male volunteers had a tendency to have higher Mn levels but without reaching statistical significance. Median Mn levels tended to be higher in men than in women (MnH: 9.7 vs. 4.4 μg/g, $p = 0.085$; MnFN: 7.2 vs. 5.3 μg/g, $p = 0.056$; MnSal: 4.1 vs. 3.4 μg/L, $p = 0.302$), but these differences were not significant.

Significantly higher median Mn levels were observed in individuals with lower schooling level. The median MnH levels were 7.8 μg/g vs. 3.3 μg/g ($p = 0.010$) and MnFN levels were 6.5 μg/g vs. 4.3 μg/g ($p = 0.013$), respectively, in those individuals with elementary vs. middle school education level. No statistically significant difference was observed in any of the Mn biomarker levels according to the age group (<34 vs. ≥34 years), ETS, drinking habits and domestic waste burning. Time of residence in the communities was dichotomized in less than 26 years or ≥26 years; those who reported living longer in the communities had a tendency to have higher Mn levels (MnH: 3.8 vs. 7.8 μg/g; MnAxH: 8.7 vs. 18.5 μg/g; MnFN: 4.7 vs. 6.4 μg/g); even though none of these biomarkers reached statistical significance. Only MnSal levels were significantly different (2.6 μg/g vs. 4.6 μg/g, $p = 0.048$).

3.3. Performance on neuropsychological tests and socio-demographic variables

Table 3 presents descriptive statistics of the scores of the neuropsychological tests stratified by area of residence. The median IQ observed in Cotegipe was higher than that observed in Santa Luzia (91 vs. 83, $p = 0.004$). For recognition memory, the median score for the performance of volunteers from Santa Luzia was higher than that from Cotegipe, with borderline statistical significance (14 vs. 12; $p = 0.051$). There was no significant difference between men and women, except in tests assessing memory and verbal working memory. The median score in the RAVLT test was significantly higher for women than for men (14 vs. 12, $p = 0.010$). For the verbal working memory, on the other hand, median score on the Digit Span test was better for men than women (10 vs. 8, $p = 0.043$).

Significant correlations between socio-demographic variables and performance on neuropsychological tests, independently of the area of residence, were detected by Spearman correlation analyses (data not shown). The age of the volunteers was positively correlated with performance in the tests that assess motor function. For the dominant and non-dominant hand time, the coefficients were, respectively, $\rho = 0.301$ and $\rho = 0.346$; ($p < 0.01$, $n = 84$). The coefficient for the processing speed was $\rho = 0.274$, ($p = 0.013$, $n = 81$). Family income was positively correlated with IQ ($\rho = 0.382$; $p < 0.001$, $n = 81$), verbal working memory ($\rho = 0.223$; $p = 0.042$, $n = 83$) and visual working memory ($\rho = 0.318$; $p = 0.003$, $n = 83$), and negatively correlated with motor speed on the Grooved Pegboard test ($\rho = -0.377$ and $\rho = -0.365$; $p < 0.01$, $n = 83$, for the dominant and non-dominant hand, respectively) and processing speed ($\rho = -0.266$; $p = 0.017$, $n = 80$). Moderate positive correlations were observed between years of schooling and IQ ($\rho = 0.560$; $p < 0.001$, $n = 81$), verbal working memory ($\rho = 0.253$; $p = 0.021$, $n = 83$) and visual working memory ($\rho = 0.518$; $p < 0.001$, $n = 83$). This variable was inversely correlated with motor speed ($\rho = -0.321$ and $\rho = -0.274$; $p < 0.05$, $n = 83$, for the dominant and non-dominant hand, respectively), processing speed ($\rho = -0.441$; $p < 0.001$, $n = 80$) and cognitive flexibility ($\rho = -0.393$; $p < 0.001$, $n = 76$).

3.4. Neuropsychological functions and biomarkers of mn exposure

Table 4 presents the Spearman correlation matrix for the associations among Mn biomarker levels and performance on

Table 4
Spearman correlation matrix among Mn biomarkers and scores on neuropsychological tests.

	MnH (μg/g)	MnAxH (μg/g)	MnFN (μg/g)	MnSal (μg/L)
Estimated (IQ)				
ρ	−0.349**	−0.495*	−0.193	−0.113
p	0.002	0.043	0.104	0.32
n	79	17	72	80
Memory				
ρ	0.159	0.068	−0.029	0.012
p	0.156	0.788	0.805	0.912
n	81	18	73	82
Motor function DH[a]				
ρ	0.223*	0.530*	0.231*	0.125
p	0.045	0.024	0.05	0.264
n	81	18	73	82
Motor function NDH[b]				
ρ	0.127	0.618**	0.1	0.087
p	0.259	0.006	0.398	0.437
n	81	18	73	82
Selective attention				
ρ	−0.147	−0.28	−0.008	0.069
p	0.195	0.261	0.949	0.544
n	79	18	71	80
Sustained attention				
ρ	−0.122	0.047	−0.114	−0.026
p	0.286	0.855	0.344	0.82
n	79	18	71	80
Processing speed				
ρ	0.13	0.697**	0.205	0.125
p	0.258	0.002	0.087	0.274
n	78	17	71	79
Cognitive flexibility				
ρ	0.065	0.479	0.328*	0.057
p	0.58	0.071	0.007	0.627
n	74	15	67	75
Visual working memory				
ρ	−0.251*	−0.717**	−0.303**	−0.135
p	0.024	0.001	0.009	0.226
n	81	18	73	82
Verbal working memory				
ρ	−0.155	−0.523*	−0.112	−0.137
p	0.167	0.026	0.346	0.22
n	81	18	73	82

[a] Dominant hand.
[b] Non-dominant hand.
* Significant correlation at level of $p < 0.05$.
** Significant correlation at level of $p < 0.01$

neuropsychological tests. These bivariate analyses detected significant negative associations between MnH levels and IQ scores ($\rho = -0.349$, $p = 0.002$) and visual working memory ($\rho = -0.251$, $p = 0.024$). This biomarker was also positively correlated with motor function for the dominant hand ($\rho = 0.127$, $p = 0.045$). Mn levels in fingernails (MnFN) were negatively associated with visual working memory ($\rho = -0.303$, $p = 0.009$) and positively correlated with motor function test for dominant hand ($\rho = 0.231$, $p = 0.050$). Mn concentrations in saliva were not significantly associated with any of the neuropsychological functions evaluated. Despite the fact that there were too few data on Mn levels in axillary hair ($n = 18$), very significant correlations were observed between these biomarker levels and several scores of neuropsychological functions: MnAxH was negatively associated with IQ scores ($\rho = -0.495$, $p = 0.043$), visual working memory ($\rho = -0.717$, $p = 0.001$) and verbal working memory ($\rho = -0.523$, $p = 0.026$); but positively correlated with motor speed for dominant hand ($\rho = 0.530$, $p = 0.024$) and non-dominant hand ($\rho = 0.618$, $p = 0.005$). Time to completion on the cognitive flexibility test was also positively correlated ($\rho = 0.697$, $p = 0.002$). These correlations were maintained even after excluding the highest value of MnAxH that seemed to be an outlier.

In multiple regression analyses, the log transformed Mn biomarker levels (MnH and MnFN) were significantly associated with some neuropsychological endpoints, after adjusting for important covariables (gender, area of residence and schooling). LogMnH concentrations were negatively associated with intellectual function: verbal and visual ability [$\beta = -4.76$ (IC 95% –9.17 to –0.36)], after adjusting for gender, area of residence and years of schooling. A summary of the regression models are listed in Table 5. LogMnFN levels were inversely associated with the scores on the Corsi test that assess visual working memory: [$\beta = -3.33$ (IC 95% –6.15 to –0.52)] after adjusting for gender and years of school. This biomarker was also positively associated with time in seconds on TMT-B that measures cognitive flexibility: [$\beta = 56.29$ (IC 95% 2.41–110.18)] after adjusting for years of schooling. Fig. 2a–c depict the scatter plots of log-transformed Mn biomarker levels and the standardized predicted values of the significant neuropsychological functions: visual and verbal ability, visual working memory and cognitive flexibility. The standardized residuals adhered to the linear regression model assumptions (data not shown).

Table 5
Summary of the multivariate linear regression models for Mn biomarkers as predictors of neuropsychological function effects.

	Nonstandardized β coefficients	95% confidence interval
Estimated IQ		
$N = 81$		
Intercept Y	82.82	71.11 to 94.52
Gender[a]	–4.83	–9.23 to –0.43
Area of residence[b]	3.83	–0.29 to 7.96
Schooling[c]	7.65	3.66 to 11.63
Log MnH	–4.76	–9.17 to –0.36
Visual working memory		
$N = 73$		
Intercept Y	13.33	8.44 to 18.23
Gender[a]	–2.18	
Schooling[b]	2.93	–4.00 to –0.36
Log MnFN	–3.33	–6.15 to –0.52
Cognitive flexibility		
$N = 67$		
Intercept Y	172.6	102.53 to 242.67
Schooling[b]	–46.63	–78.23 to –15.04
Log MnFN	56.29	2.41 to 110.12

[a] Men = 0 and women = 1.
[b] Santa Luzia = 0 and Cotegipe = 1.
[c] Elementary school = 0 and middle school = 1.

4. Discussion

The main findings of this study are the high exposure levels evidenced by the measurements of the non-invasive Mn biomarkers (MnH, MnAxH and MnFN) and the association of this excessive exposure with detrimental effects on cognitive function, visual working memory and cognitive flexibility in adults living in two communities close to a ferromanganese alloy plant in Brazil.

In this study, it was found that the median MnH levels are about 8 times higher than the reference value for the Brazilian adult population. Indeed, in a study carried out with about 1500 non-exposed adults in Rio de Janeiro by Miekeley et al. (1998) reported that the normal range of Mn in scalp hair ranges between 0.15 and 1.20 μg/g. Similar results were also observed for Mn levels in fingernails, which are about 4 times higher than the mean observed in healthy and non-exposed young Brazilians in the age range of 12–18 years (mean and SD: 1.6 ± 1.5 μg/g) (Carneiro, 2011). As far as we know, there are no reference values established for Mn in axillary hair or in saliva. The Mn levels observed in this study are superior to those described for occupationally exposed individuals (Bader et al., 1999; Gil et al., 2011; Wang et al., 2008).

The Mn biomarker levels reported here are the highest compared with other environmentally exposed populations. In a mining district in Mexico, Rodríguez-Agudelo et al. (2006) observed that air Mn concentrations ranged between 0.003 and 5.86 μg/m^3, which were significantly associated with several

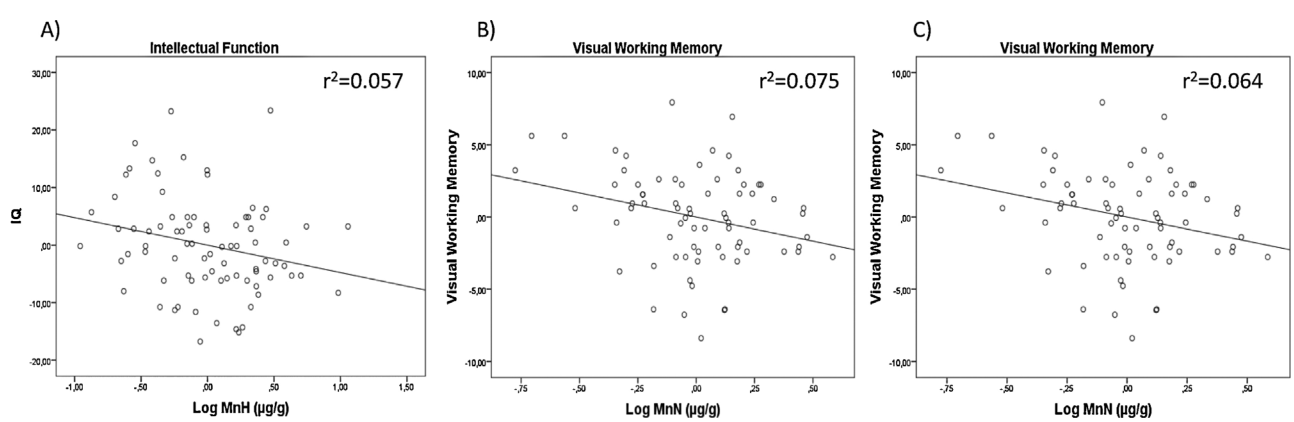

Fig. 2. Partial diagrams of residuals of biomarkers versus residuals of estimated IQ (A), visual working memory (B) and cognitive flexibility (C) and their respective coefficients of determination (r^2).

motor tests; contrary to Mn blood levels that had no association at all. School-aged children from the same region had MnH levels in average 21 times higher than non-exposed children and those levels were significantly and inversely associated with Full-Scale IQ scores (Riojas-Rodríguez et al., 2010). In the USA, Haynes et al. (2010) evaluated a total of 141 residents of Marietta town (OH), living near a similar ferromanganese alloy plant. The annual average ambient air Mn concentrations were estimated by modeling, and air levels ranged 0.02–2.61 μg Mn/m^3. It was observed that hair Mn levels were moderately high (5.80 ± 6.40 μg/g) and significantly associated with Mn air levels after adjusting for iron metabolism. In Costa Rica, air spraying of mancozeb, a Mn based fungicide, on banana plantations, was associated with high hair Mn levels of 449 pregnant women enrolled in a birth cohort study. Hair Mn levels (GM: 1.8 μg/g; range 0.05–53.3 μg/g) were positively associated with living near the banana plantations and Mn levels in drinking water, and inversely associated with gestational age (Mora et al., 2014).

Contrary to what was hypothesized, higher Mn concentrations were observed in those living relatively further from the alloy plant (St Luzia > Cotegipe). This could be explained by the fact that the very fine Mn oxide particles travel further with wind, not settling down immediately after air emission as do the coarse iron oxide particles. As pointed out by Haynes et al., (2012) the coarse fraction ($PM_{2.5-10}$) is often dominated by crustal materials (e.g., windblown dust, resuspension of road dust, demolition of buildings), whereas the particles in fine ($PM_{2.5}$) and ultrafine ($PM_{0.1}$) fractions are mostly formed through combustion processes including metal refining, which is the cases of this exposure source. In order to test this hypothesis, a pilot experiment collecting settled dust using disposable polyethylene Petri-dishes during 30 days inside the houses (n = 10) of both communities was carried out. Higher dust Mn loads were observed in Santa Luzia's residences when compared to those observed in Cotegipe's (260.7 μg/m^2 (range 21.7–482.6) in Santa Luzia vs. 82.3 μg/m^2 (range 25.5–163.7); p = 0.052), corroborating the findings of higher exposure levels in those living relatively further. This finding raises the question that the entire population of the town of Simões Filho may be facing an excessive atmospheric exposure to this neurotoxin. It is noteworthy that this study was based on a sub-sample of the population of two small communities that altogether have less than 1800 inhabitants. The majority of the residents of this town, almost 120,000 people, live just across the highway, separated by an eucalyptus fence but inside a 10 km radius.

It was observed that the male volunteers had a tendency to present relatively higher MnH and MnFN levels, even though with borderline significance. Perhaps, with a larger sample size was achieved, especially of the male volunteers, this difference could reach statistical significance. However, these results are in accordance with the reports of Carneiro et al. (2002), Haynes et al. (2010) and Riojas-Rodríguez et al. (2010) also found no differences in Mn levels between sexes. As far as Mn in nails is concerned, no difference between sexes was observed either.

Education level is an important determinant factor on pollutant exposure and cognitive function. As a matter of fact, education is generally regarded as a confounding variable due to its association with neuropsychological outcomes when the pollutant is a neurotoxin. Significantly higher MnH and MnFN levels were observed in those individuals with lower education level (Table 2). Education level was a significant covariable associated with the three neuropsychological endpoints: intellectual function, visual working memory and cognitive flexibility. Education level is a proxy for social–economic status, which reflects an enriched home quality, better nutrition and more access to education, culture and information. Several studies have reported similar findings. In a previous study, maternal education in years was an important covariable explaining children's IQ score inverse association with log MnH levels (Menezes-Filho et al., 2011). Bouchard et al. (2011) also observed that maternal education was a significant covariable in adjusting the association of IQ scores with Mn levels in drinking water, total Mn consumption and MnH levels in school-aged children in Quebec. Maternal education was also an important covariable in adjusting the association of MnH levels of Mexican children and IQ scores (Riojas-Rodríguez et al., 2010) to name a few.

Three of the four biomarkers (MnH, MnFN and MnAxH) used in this work are expected to reflect a longer-term exposure when compared to blood Mn levels. Hair Mn determination, for example, when quantified in the first centimeter, is supposed to reflect an exposure occurred on average 1 month prior to sample collection, given the hair growth speed to be approximately 0.35 mm per day or about 1 cm per month (Tobin, 2005). Nail growth, however, is much slower (3.47 mm/month for fingernails) (Yaemsiri et al., 2010). As it is necessary to wait for its full growth, on average fingernails may reflect an exposure that occurred 5 and 6 months before its collection and use as an analytical matrix (Gupchup and Zatz, 1999). Very few studies have applied Mn in finger or toenails as a biomarker of exposure in environmental exposure settings. Neither has investigated its association with neuropsychological endpoints. In this study, Mn in fingernails varied between 0.7 and 22.0 μg/g, with significant differences observed between area of residence and schooling. Slightly higher Mn levels were observed in those living longer in the communities. A significant correlation (Spearman ρ = 0.226, p = 0.047) was observed between MnFN levels and exposure time (years living in one of the villages). Also, this biomarker was inversely correlated with scores of visual working memory and directly correlated with time in cognitive flexibility task. Even after adjustments for gender and schooling in multivariate analyses, these associations were maintained significant. This biomarker is suggestive to reflect a longer term exposure.

Intellectual performance estimated by the estimated IQ (WAIS-III) was inversely associated with Mn levels in hair. In this current investigation, a similar effect on intellectual function as those reported previously with children and their mothers from Cotegipe village was observed (Menezes-Filho et al., 2011). Maternal intelligence, estimated by Raven Standard Progressive Matrices, was inversely associated with MnH levels (β = −2.69, 95% CI −5.43 to 0.05), after adjusting for age, family income and years of school. This coefficient is quite similar to the current study (β = −4.75), independently if the adult lived in Cotegipe or in Santa Luzia village, gender or years of education. Bouchard et al. (2011) using WASI-III for children 6–13 years-old exposed to Mn through municipal drinking water, found an adjusted coefficient for log MnH levels association with Full-Scale IQ of −3.3 (95%CI −6.1 to −0.5). In 79 children between 7 and 11 years of age from the Molango mining district in central Mexico, a negative association was also demonstrated with MnH levels and verbal IQ, but the coefficient was 10-fold lower (Riojas-Rodríguez et al., 2010). To our knowledge, no studies have evaluated intellectual function in adults environmentally exposed to manganese.

Impairment of motor function deriving from excessive Mn exposure has been widely described in the literature. In recent years, some studies have shown motor deficits in Mn environmentally exposed populations (Hernadéz-Bonilla et al., 2011; Kim et al., 2011; Lucchini et al., 2012; Rodríguez-Agudelo et al., 2006). Similar to Hernández-Bonilla et al. (2011), who evaluated motor performance using grooved pegboard in children exposed to Mn from mining activity, it was also demonstrated a correlation between MnH levels and execution time in grooved pegboard test, but the association was no longer significant after adjustments for covariates.

Biomarkers that reflect recent exposure to Mn, are hardly ever associated to neuropsychological endpoints. Menezes-Filho et al. (2009b) reviewed studies with children exposed to Mn. In 13 studies published up to 2007, only those that assessed Mn levels in hair, in drinking water or in tooth enamel were able to find significant associations with neuropsychological endpoints, in opposition to blood Mn levels that failed as a measure of exposure. In this study, MnSal showed no correlation with performance on any tests performed. The levels of Mn in saliva indicate recent exposure and are a surrogate of the blood concentrations of this metal (Cowan et al., 2009; Wang et al., 2008). Very few studies have detected significant association between Mn blood levels with neuropsychological outcomes in epidemiological studies, with the exception of the San Francisco Bay Bridge welder study (Bowler et al., 2007). Those that did find were studies with infants or young children and Mn concentrations measured in blood or in cord blood (Claus Henn et al., 2010; Bhang et al., 2013; Lin et al., 2013). Thus, it was expected very little utility of MnSal levels for this purpose, which indeed occurred. Comparison of these results with data reported in the literature is limited, considering the scarcity of studies that used axillary hair, nails and saliva as monitoring matrix to assess Mn exposure. There are no studies correlating MnAxH, MnFN and MnSal levels with performance on neuropsychological tests in adults.

The major limitation of this study is the small number of volunteers, particularly from the masculine sex. The scarcity of male participation in health related studies are well known and generally they are not available due to confounding working hours. In order to cope with this kind of situation, evaluation appointments during weekends were set up. In general, this low participation rate is due to the poor awareness of the population, which is related to the very low schooling level. As this investigation had a limited budget, no stipend was paid to any volunteer. Certainly, this aspect compromised the participation rate. The small sample size limited the power of the multivariate regression analyses and thus the identification of other possible exposure determinant factors. This drawback especially limited the interpretation of Mn in axillary hair utility as an exposure biomarker and its ability to detect associations with neuropsychological endpoints .

This study had important strengths. It was possible to measure four Mn biomarkers and apply a somewhat comprehensive battery of neuropsychological tests that assessed cognitive, executive, motor, attention, long-term and working memory functions of the residents of two communities that have been impacted by the atmospheric emissions of the ferromanganese alloy plant.

5. Conclusions

This investigation is the first to measure four noninvasive biomarkers to assess the association between Mn exposure and neuropsychological outcomes. Elevated exposure to Mn in two communities near an ferromanganese refinery was demonstrated, which was higher in the one relatively further. This fact raises the issue of the wide spread of the submicron particles of Mn oxides, that may be contaminating a larger number of people. This controversial findings need to be further investigated using air emission modeling. Above all, it is shown that this high exposure level is associated with detrimental effects on several neuropsychological endpoints, especially those related to intellectual and memory function, despite the limitation of the low participation rate in both communities.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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