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RESEARCH ARTICLE

Manganese exposure among smelting workers: blood manganese–iron ratio as a novel tool for manganese exposure assessment

Dallas M. Cowan[1], Qiyuan Fan[2], Yan Zou[2], Xiujuan Shi[3], Jian Chen[3], Michael Aschner[4], Frank S. Rosenthal[1], and Wei Zheng[1]

[1]School of Health Sciences, Purdue University, West Lafayette, Indiana, USA, [2]Department of Occupational Medicine, Zunyi Medical College, Zunyi, Guizhou, PRC, [3]Guizhou Institute of Occupational Safety and Health, Zunyi, Guizhou, PRC, and [4]Department of Pediatrics, Vanderbilt University, Nashville, Tennessee, USA

Abstract

Unexposed control subjects ($n = 106$), power distributing and office workers ($n = 122$), and manganese (Mn)-exposed ferroalloy smelter workers ($n = 95$) were recruited to the control, low and high groups, respectively. Mn concentrations in saliva, plasma, erythrocytes, urine and hair were significantly higher in both exposure groups than in the controls. The Fe concentration in plasma and erythrocytes, however, was significantly lower in Mn-exposed workers than in controls. The airborne Mn levels were significantly associated with Mn/Fe ratio (MIR) of erythrocytes (eMIR) ($r = 0.77$, $p < 0.01$) and plasma (pMIR) ($r = 0.70$, $p < 0.01$). The results suggest that the MIR may serve as a useful biomarker to distinguish Mn-exposed workers from the unexposed, control population.

Keywords: *Manganese; iron; exposure assessment; Mn-Fe ratio; biomarker; saliva; erythrocyte; smelter*

Introduction

Occupational exposure to manganese (Mn) occurs among workers involved in welding, mining, smelting, ferroalloy steel production and dry-cell battery production (Roels et al. 1992, Bader et al. 1999, Lucchini et al. 1999, Myers et al. 2003, Li et al. 2004, Lu et al. 2005, Bowler et al. 2006, Bouchard et al. 2007a,b, Jiang et al. 2007, Montes et al. 2008). Among the general population, Mn exposure occurs from ingesting contaminated food and drinking water, and from increased environmental levels of Mn resulting from use of the anti-knock gasoline additive methylcyclopentadienyl manganese tricarbonyl (MMT) (Kondakis et al. 1989, Franklin & Solomon 1997, Baldwin et al. 1999, Hudnell, 1999, Woolf et al. 2002, Kaiser, 2003, Bouchard et al. 2007a). Cases of accidental Mn exposure have been reported in the production of homemade illicit drugs and contamination of fruits and vegetables with the Mn-containing fungicide maneb (Ferraz et al. 1988, Sikk et al. 2007). Finally, Mn exposure in young children is known to occur via parenteral nutrition causing neuro- and hepatotoxicity (Reynolds et al. 1994, Fell & Reynolds 1996).

The early onset of Mn intoxication is usually subtle. The initial signs may be categorized as nonspecific neurological manifestations characterized by psychiatric symptoms. Later, the symptoms progress and encompass the extrapyramidal system, resulting in dystonic postural abnormality, bradykinesia, tremor, cog-wheel rigidity, speech difficulty, fixed facial expression and difficulty with fine movements (handwriting). Importantly, the symptoms of Mn intoxication, once established, usually become progressive and irreversible, reflecting permanent damage to neurological structures (Inoue & Makita, 1996, Jiang et al. 2006, Aschner et al. 2007). Thus, early diagnosis is crucial for

Address for Correspondence: Wei Zheng, Professor of Health Sciences and Toxicology, Purdue University School of Health Sciences, 550 Stadium Mall Drive, CIVL-1163D, West Lafayette, Indiana 47907, USA. Tel: 765-496-6447. Fax: 765-496-1377. E-mail: wzheng@purdue.edu

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preventing Mn toxicity in occupational and environmental exposure scenarios.

While methods for clinical diagnosis of established manganism are available, tools for the early detection and diagnosis of Mn intoxication have not yet been developed. Numerous indicators of toxicity have been proposed in the literature. Whole blood Mn concentration has been evaluated for use in differentiating exposed from unexposed subjects on a populational basis, but this method is inadequate on the individual level (Roels et al. 1987, Jarvisalo et al. 1992, Bader et al. 1999, Apostoli et al. 2000, Myers et al. 2003). Similar to whole blood Mn, an elevated serum Mn concentration can be seen among active Mn-exposed welders, yet this increase is not associated with welders' years of employment (Apostoli et al. 2000, Lu et al. 2005). Both Mn concentrations in plasma and serum have been extensively evaluated for use in Mn exposure assessment with limited success. Mn concentrations in urine, hair and nail clippings have also been investigated; the data, however, are inconsistent and not sufficiently refined to distinguish accurately exposed from unexposed workers (Bader et al. 1999, Apostoli et al. 2000, Wongwit et al. 2004, Smith et al. 2007). In a recent study by this group, the concentrations of several metals including Mn in the saliva of career welders were investigated (Wang et al. 2008). Saliva Mn concentrations among 49 welders studied were elevated by 72% compared with soldiers without Mn exposure. Thus, the use of saliva Mn concentrations appears to be promising for the evaluation of Mn exposure. Nonetheless, the utility of this biomarker has not been evaluated in a larger population.

In addition to Mn content in biological fluids, exploration of protein concentrations and catecholamine metabolites, believed to be altered by Mn exposure including transferrin (Tf), transferrin receptor (TfR), ferritin (Ft), prolactin and homovanillic acid (HVA), have not yielded a definitive biomarker for Mn exposure. Serum prolactin levels are reportedly increased in ferromanganese smelter workers (Allessio et al. 1989, Smargiassi & Mutti 1999). However, Roels and colleagues (1992) failed to find any significant alterations in serum prolactin among workers engaged in dry-cell battery production. Ellingsen et al. (2003a,b) further suggested that the Mn-induced elevation of prolactin is generally within the reference limits and therefore is not suitable for use as a biomarker of Mn exposure. HVA, a catcholamine metabolite, is significantly increased in the urine of Mn-exposed welders (Ai et al. 1998, Buchet et al. 1993), yet a large fluctuation within control populations limits the use of HVA as an indicator of Mn exposure (Buchet et al. 1993).

Previous studies by this group indicate that Mn exposure leads to altered blood levels of several iron (Fe) regulatory proteins including Tf, TfR and ferritin (Zheng et al. 1998, Zheng et al. 1999, Zheng & Zhao 2001, Li et al. 2004, Lu et al. 2005). More recently, we have reported that the Mn concentration in the erythrocytes of Mn-exposed smelting workers is increased compared with controls; the increase in the erythrocyte Mn level, however, is significantly correlated with the palladial index as indicated by magnetic resonance imaging analysis (Jiang et al. 2007). As the procedure for separating the plasma and erythrocyte portion is relatively straightforward and because Mn has been shown to accumulate in erythrocytes, it became imperative to evaluate further the utility of erythrocyte Mn content and various Fe-regulatory proteins as markers of Mn exposure in a larger, well-characterized Mn-exposed population.

The current work was carried out on a human population in South western China known to be occupationally exposed to Mn, with the specific aim of identifying a clinical biological indicator (i.e. biomarker) relevant to the health hazards associated with occupational Mn exposure. The hypothesis tested was that occupational exposure to airborne Mn led to an altered level of Mn, Fe or other biological determinants in the biological media of exposed workers in a dose-dependent manner. More specifically, we used a cross-sectional design to: (1) determine the concentrations of Mn, Fe and Fe-metabolic proteins in saliva, plasma, erythrocytes, urine and hair in Mn-exposed smelters, compared with non-exposed control subjects; (2) evaluate the associations between airborne Mn levels and potential biomarkers; and (3) establish a distinct value for a reliable biomarker that accurately distinguishes Mn-exposed subjects from the general, Mn-unexposed healthy population.

Materials and methods

Study population

This cross-sectional study was conducted among Mn-exposed ferroalloy smelting workers. The ferroalloy manufacturer is located in the Guizhou Province, a mountainous region of South western China. The factory has more than 1000 workers and has been in operation for over 20 years. Study subjects were recruited to the high or low Mn-exposure group based on their job classification, day-to-day working proximity to the smelting ovens and on-site monitoring of airborne Mn. As the smelting workers have been exposed to Mn on a daily basis for the years specified in Table 1, the Mn concentrations in their biological matrices are considered to be at the steady state. Subjects assigned to the high Mn-exposure group were those who maintained and operated the smelting process within 2–5 m of the smelting ovens, while those in the low Mn-exposure group were power distribution workers, supporting personnel or supervisory staff whose daily tasks occurred

Table 1. Summary of demographic information.

		Mn exposure groups	
	Control	Low	High
n	106	122	95
Men	86	84	89
Women	20	38	6
Age	37.7 ± 7.37 (18–56)	34.5 ± 6.89** (20–52)	35.4 ± 6.69 (22–53)
Employment years in current job	2.61 ± 1.66 (0.5–6)	5.03 ± 3.21** (0.1–12)	4.11 ± 2.81** (0.1–12)
Distance from home to work (km)	3.97 ± 0.60 (0–25)	1.44 ± 0.20** (0–20)	2.07 ± 2.44** (0–10)
Current smokers	71 (67%)	61 (50%)	66 (69%)
Airborne Mn level (mg m^{-3})	0.003 ± 0.009 (0.00–0.04)	0.026 ± 0.028* (0.01–0.11)	0.177 ± 0.103# (0.098–0.374)

Data represent mean ± SD (range). *$p < 0.05$ compared with control; **$p < 0.01$ compared with control; #$p < 0.05$ compared with control and low exposure. Significance confirmed with Tukey's post-hoc analysis.

in offices where they experienced minimal contact with Mn-containing products or raw materials. The medical records from the manufacturer's clinics suggest a history of occupational cases of Mn intoxication.

The subjects in the control group were recruited from another local factory where Mn is not used as a raw material and is not a contaminant in the working environment. A preliminary study was conducted to ensure the presence of differential Mn concentrations in the working environment among control, low and high exposure groups.

Subjects in all three groups at the time of interview had reported no exposure to other toxic substances, radiation therapy or substance abuse. Subjects who had taken medications which could interfere with Fe metabolism, such as vitamin D, aspirin or herbal supplements, were excluded from the study.

Determination of airborne Mn in work sites

Personal air samples were collected within the workers breathing zone using a sampling train that included an SKC pump (model 224-44XR, calibrated at 2 l min^{-1}), tygon tubing and a sampling cassette with a closed-face MCE filter (37 mm, pore size 0.8 μm). Filters were dried in a desiccator at room temperature before and after sampling. Shifts were divided into two sampling periods, thus reducing the possibility of filter overload. A time-weighted average concentration was calculated following each 8-h work shift. The airborne Mn concentrations were determined by atomic absorption spectrophotometry (AAS) as described below. Collection of biological samples for subjects carrying the personal air monitors occurred within 24 h of exposure measurement.

Subject examination and collection of biological matrices

Participating subjects were invited to the Zunyi Medical College (ZMC) in Zunyi City. A written consent form was obtained from each subject prior to the onset of the study. The study protocol received official approvals from the Office of Clinical Investigation at the ZMC, the human study Institutional Review Board at Purdue University and the Office of Research Protections at the U.S. Army Medical Research and Material Command Center.

A scheduled interview lasting approximately 60 min was conducted by trained clinical researchers. The questionnaire collected detailed information on job classification, occupational history, working years at the current facility, socioeconomic status, smoking habits, family and personal medical history and the presence of behavioural/psychological dysfunctions. A general physical examination was conducted by an occupational physician, followed by a neurological examination by an experienced neurologist. The subjects also received a battery of neurological and behavioural tests (data not shown).

Biological samples (blood, urine, saliva and hair) were collected at the ZMC. Subjects were instructed to collect the first urine sample of the morning and to bring the sample to the appointment. One millilitre of venous blood was drawn from a cubital vein into a non-heparinized tube to prepare serum samples for metal analysis. Another 4 ml of blood was collected in a heparinized tube and maintained at room temperature for 30 min; the sample was then centrifuged at 600 *g* min^{-1} for 5 min. The supernatant was collected as the plasma fraction, and the pellet as the erythrocyte fraction. Hair samples were collected by cutting approximately 2–3 cm of hair from the posterior portion of the head using stainless steel scissors; the cuttings were then stored in Eppendorf tubes. For saliva collection, subjects were asked to gargle three times with distilled, deionized (DDI) water. Each saliva sample was then collected by expectoration into a 2-ml test tube. All samples were stored at −20°C prior to laboratory analyses. All test tubes used in the study were free of metal contamination, as pretested by AAS.

AAS analysis of metal concentrations

To prevent metal contamination, tools and glassware were soaked for 8 h in 10% HNO_3 and washed multiple times with DDI water. For analysis of Mn content in air samples, filter membranes were dried in desiccators at room temperature for 48 h, and the weight was recorded. The filters were then digested with 5 ml of $HClO_4$-HNO_3 mixture (1:9 vol/vol) at 200°C. The dry residues were dissolved in 10 ml of 1% HNO_3. The solutions were diluted by 20–50 fold prior to AAS. Air Mn concentrations were measured by a Model AA240FS Varian flame AAS (Australia Pty Ltd., Clayton, Australia) according to a China National Standard Operation Protocol (GB/T16018-1995) for occupational safety surveillance.

For metals in the plasma, an aliquot (0.5 ml) of plasma was mixed with 5 ml of digestion solution ($HClO_4$:HNO_3; 3:7). The digested samples were heated until dry and stored at room temperature for 4–6 h. The dried samples were then re-dissolved in 5 ml of 1% HNO_3. If necessary this solution was further diluted with 1% HNO_3 prior to AAS analysis. For analysis of metals in erythrocytes, approximately 1.5 g of the pellet blood cell fraction (the upper plasma portion was removed following centrifugation) was mixed with 2 ml of saline; additional saline was added to reach a volume of 4 ml. An aliquot (2 ml) of this fraction was mixed with 10 ml of the digestion solution and heated until dry, followed by the addition of 5 ml of 1% HNO_3. Saliva samples were centrifuged to remove large particles. Both urine and the supernatant of saliva samples were digested by mixing 0.5 ml of the sample with 0.5 ml of 100% HNO_3; the mixtures were allowed to stand at room temperature overnight. All results for urine measurements were normalized with creatinine concentration. Hair samples were soaked three times with 1% HNO_3 and thoroughly washed with DDI water. An amount of hair (0.2 g) was weighed using a precision scale and added to a beaker containing 5 ml of digestion solution ($HClO_4$:HNO_3; 1:9). The solution was diluted appropriately prior to AAS.

Standard curves were established each day and run for every 25 samples using freshly prepared standard Mn or Fe solutions in 1% HNO_3. Sample measurements were repeated if the RSD was more than 3% in the flame AAS or 5% in the graphite furnace AAS. To determine the intraday precision of the method, the standard samples were repeatedly measured 10 times; for interday precision the samples were assayed for 10 consecutive days. Both intraday and interday precisions of the AAS were <8%. To estimate the recovery, standard Mn or Fe was added to saliva, plasma, urine, erythrocyte fractions or DDI-water, followed by the same digesting procedure. The values of the sample in the biological matrices were divided by the values in the DDI-water, yielding the recovery. The recovery of both metals from all four body fluids was between 94 and 105%. The wavelength for AA analysis was 279.5 for Mn and 248.3 for Fe. The detection limits of the graphite furnace AAS for Mn and Fe were 0.1 ng ml^{-1} and 0.09 ng ml^{-1}, respectively, and the detection limits of flame AAS for Mn and Fe were 40 μg ml^{-1} and 0.15 μg ml^{-1}, respectively.

Determination of ferritin, transferrin (Tf), transferrin receptor (TfR) and total iron-binding capacity (TIBC) in serum and saliva

Serum ferritin levels were determined by using an ELISA method. The ELISA assay kit was purchased from Ramco Laboratories (Houston, TX, USA; Catalog no. S-22). The assay procedure followed the manufacturer's instructions. Briefly, the sera were diluted 10-fold with the sample diluents supplied by the assay kit. The diluted samples were pipetted to the wells precoated with polyclonal antihuman ferritin antibody. Following addition of horseradish peroxidase (HRP) conjugated secondary antibody, the reaction mixtures were incubated, washed, and the absorbance at 450 nm was recorded. The concentrations of serum ferritin were calculated from a standard curve derived from the same procedure using purified human ferritin. The lowest detection limit for ferritin was 5 ng ml^{-1}.

Serum concentrations of TfR were determined by using a similar ELISA test kit purchased from Ramco Laboratories (Catalog no. TF-94). The experimental procedure followed the manufacturer's instructions. The sera were diluted 100-fold with the sample diluent. The absorbance was determined at 450 nm, and the concentrations of TfR were estimated from a standard curve using human TfR as the standard. The detection limit for transferrin receptor was 10 ng ml^{-1}.

Serum levels of Tf were also determined by an ELISA kit purchased from Bethyl Laboratory (Montgomery, TX, USA; Catalog no. E80-128). The assay procedure followed the manufacturer's instructions. Serum samples were diluted 20 000-fold prior to the assay. The absorbance was read at 490 nm and converted to calculate the serum concentrations using purified human Tf as the standard. The detection limit for transferrin was 3 ng ml^{-1}.

The TIBC was determined using an assay kit purchased from Biosino Biotechnology (Beijing, China; Catalog no. 0380). All reagents (ascorbic acid solution, Fe/UIBC buffer solutions, Fe colour reagent ferrozine) were included in the assay kit. The experimental procedure followed the manufacturer's instructions. Each procedure was calibrated with the Fe standard set by the manufacturer (100 μg dl^{-1}). In short, an aliquot (1 ml) of Fe solution was added to 0.5 ml of each serum sample and allowed to mix for 1 min in order to completely saturate the transferrin. Excess Fe was removed with the addition of 75 mg of $MgCO_3$ powder (base) and allowed to stand for 50 min at 20°C with regular mixing. The

solution was centrifuged at 3000 rpm for 10 min, and the supernatant was collected. An acidic buffer solution was added to the supernatant, the detergent component of the solution, to dissociate Fe(III) from its complex with transferrin, thus reducing all Fe(III) to Fe(II). The ferrozine reagent, which binds with free Fe(II) to produce a red-coloured complex, was then added to the reaction mixture; the concentration of Fe was quantified against a standard curve at a wavelength of 540 nm. The total iron measured was expressed as the TIBC.

Statistical analysis

Demographic information was abstracted from personal and work history survey questionnaires. All data are expressed as mean ± SD. Original data presented as log transformation of data did not alter the statistical findings or change our overall conclusion. Initial comparison of means was accomplished using one-way analysis of variance (ANOVA). Comparison of means using two factor level variables was accomplished by two-way ANOVA. If the ANOVA results indicated significance at $p < 0.05$, a Tukey's pair-wise comparison was used for post-hoc determination of significant differences between groups. For multivariate analyses, a generalized linear model (GLM) was used to determine the significance of all independent variables (exposure group, years of employment, age, sex and income) on response variables. Pearson correlation coefficients were obtained by linear regression. A receiver-operator characteristic (ROC) method was further used to establish the threshold value to maximize the sensitivity and specificity of the selected biomarker. ROC analysis computes each possible threshold value in order to determine the optimal cut-off value maximizing the discrimination between two groups of data points. The area under the curve (AUC) is used to evaluate the effectiveness of diagnostic tests. A value of 1.0 indicates a perfect discrimination and 0.5 indicates discrimination no better than chance. The following grading structure was used to evaluate the AUC: 0.9–1.0 = excellent, 0.7–0.9 = moderate accuracy, 0.5–0.7 = low accuracy, 0.5 = equivalent to chance (Ramanchandran et al. 2002, Fischer et al. 2003). All statistical operations were carried out with the Minitab statistical software (v. 15.1) except for the ROC and AUC, which were carried out with the R statistical software (v. 2.6.1).

Materials

Chemicals and reagents were obtained from the following sources: ultrapure nitric acid (HNO_3, >99.7%) from Tianjin Kemiou Chemical Reagent (Tianjin, PRC), percloric acid ($HClO_4$, >99.5%) from Tianjin Dongfang Chemical Reagent (Tianjin, PRC), and AAS standards of Mn and Fe from Alfa Products (Danvers, MA, USA). DDI water was obtained using a Model SZ-93 Autodouble Pure Water Distillatory from Shanghai Yarong Biochemistry (Shanghai, PRC). All reagents were of analytical grade, HPLC grade or the highest available pharmaceutical grade.

Results

Subjects

A total of 323 subjects were recruited to this study; they were all of the Chinese Han origin. The high-exposure group comprised 95 subjects, of whom six were women, and the low-exposure group consisted of 122 subjects, including 38 women. The control group consisted of 106 non-Mn workers, 20 of whom were women (Table 1). No age difference was observed between control and high-exposure groups. The workers in the low-exposure group were slightly, yet significantly, younger (34.5 ± 6.89) than the controls (37.7 ± 7.37). The control subjects lived approximately twice as far away from the Mn plant compared with the exposed subjects ($p < 0.05$). The average length of employment of workers in their current job was 2.6 years (range 0.5–6.0), 5 years (range 0.1–12) and 4.1 years (range 0.1–12) for control, low- and high- exposure groups, respectively. Smoking status was not significantly different between exposure groups. A summary of demographic data is presented in Table 1.

Exposure assessment

Because of limited equipment and limited access to the smelting facility, conducting personal air sample collections for all subjects was not possible. Therefore, we collected exposure measurements for representative subpopulations of each group in order to establish job-specific airborne Mn levels. A total of 40 study subjects were asked to carry personal samplers during their normal work shift (control: $n = 20$, low: $n = 10$, high: $n = 10$). The geometric mean of airborne/total dust measured for the control, low- and high- exposure groups was 0.003 mg m^{-3}, 0.03 mg m^{-3} and 0.18 mg m^{-3}, respectively (Table 1). Mn concentrations in the high exposure group ranged from 0.10 mg/m^3 to 0.37 mg/m^3 with the geometric mean slightly below the ACGIH TLV of 0.2 mg m^{-3}. The low-exposure group geometric mean for airborne Mn levels was almost eight times less than the TLV, and the Mn concentration in the control group air samples was virtually undetectable (0.003 ± 0.009 mg m^{-3}).

Mn concentrations in biological matrices

Mn concentrations in saliva (MnS), plasma (MnP), erythrocytes (MnE), urine (MnU) and hair (MnH) were all statistically significantly higher in both exposure groups

than those in controls (Table 2). The saliva, plasma and erythrocyte samples exhibited a significant exposure group-related increase in Mn concentrations compared with the mean levels of control, low- and high- exposure groups (Figure 1A–C). MnS concentrations were nearly identical to those of MnP in all three study groups; this observation corroborated our previous observation in welders (Wang et al. 2008). While MnU concentrations were higher in the exposed groups compared with the control, the level of MnU in the high-exposure group was significantly lower than that of the low-exposure group (Figure 1D). Noticeably, MnH concentrations in both exposure groups were increased by more than 20-fold (Table 2). Although hair samples were thoroughly washed with HNO_3 prior to analyses, these data may reflect, to a certain extent, the external contamination of airborne Mn in the working environment.

Linear regression analyses of the data within each exposure group and the data from all three groups in combination revealed that Mn concentrations in plasma, erythrocytes and hair did not statistically significantly change as a function of workers' employment years on the job; nor were they associated with workers' age (Table 3). MnS concentrations were weakly, yet significantly associated with employment years and age ($r = 0.12$, $p < 0.05$) (Table 3). Mn concentrations in all

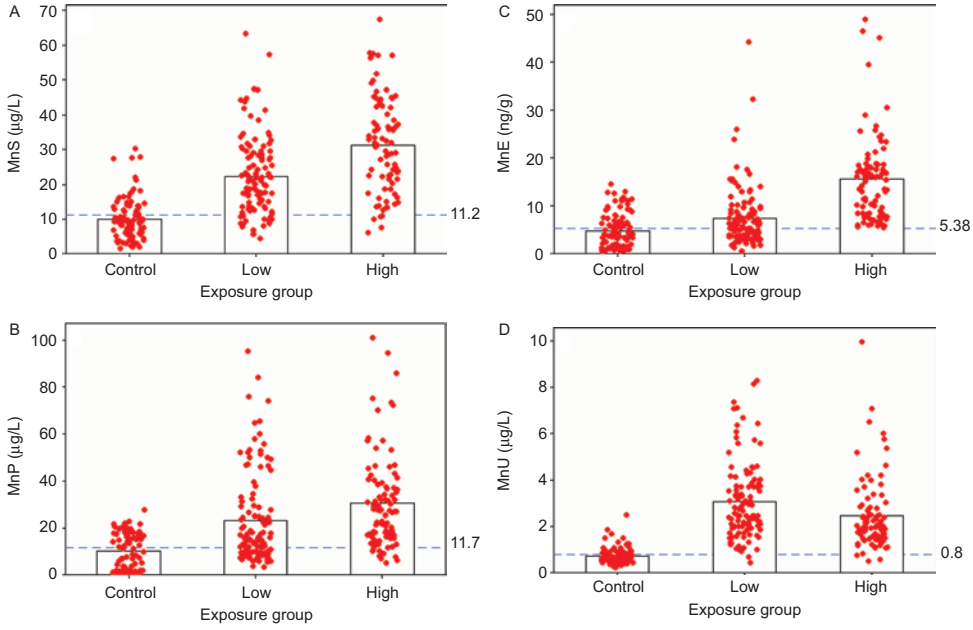

Figure 1. Mn concentrations in biological media of control ($n = 106$), low-exposure ($n = 122$), and high-exposure ($n = 96$) workers. Mn concentrations were determined by atomic absorption spectrophotometry. Bars represent the group mean, dotted lines stand for the upper 95% confidence interval (UCI) of the control mean, and dots are each individual determinant. (A) Mn concentration in saliva (MnS); (B) Mn concentration in plasma (MnP); (C) Mn concentration in erythrocytes (MnE); and (D) Mn concentration in urine (MnU).

Table 2. Metal concentrations in biological matrices.

		Mn exposure groups			
Biological matrices	Control	Low	% Change	High	% Change
Mn concentration					
Saliva ($\mu g\ l^{-1}$)	9.98 ± 6.10	22.3 ± 11.3**	+123	31.3 ± 13.6#	+214
Plasma ($\mu g\ l^{-1}$)	9.97 ± 7.97	23.3 ± 19.2**	+134	30.4 ± 19.5#	+205
Erythrocytes ($ng\ g^{-1}$)	4.68 ± 3.59	7.45 ± 6.15**	+60	15.6 ± 9.01#	+233
Urine ($\mu g\ g^{-1}$ creatinine)	0.64 ± 0.31	2.73 ± 1.46**	+327	2.17 ± 1.35*	+239
Hair ($mg\ kg^{-1}$)	1.51 ± 2.00	32.1 ± 29.2**	+2026	37.6 ± 22.5**	+2390
Fe concentration					
Saliva ($\mu g\ l^{-1}$)	77.8 ± 44.5	139 ± 77.4**	+78	141 ± 82.6**	+81
Plasma ($\mu g\ l^{-1}$)	898 ± 485	533 ± 314**	−41	693 ± 480#	−23
Erythrocytes ($\mu g\ g^{-1}$)	1091 ± 455	750 ± 150**	−31	777 ± 109**	−29
Urine ($mg\ g^{-1}$ creatinine)	0.69 ± 0.27	0.65 ± 0.37	−4	0.53 ± 0.36#	−23
Hair ($mg\ kg^{-1}$)	11.5 ± 15.7	35.9 ± 25.7**	+212	35.0 ± 29.0**	+204

Data represent mean ± SD. **$p < 0.01$ compared with control; #$p < 0.05$ compared with both control and low-exposure groups.Significance confirmed with Tukey's post-hoc analysis.% Change: 100 × (exposed concentration – control concentration)/control concentration.

Table 3. Correlation coefficient comparison among parameters.

	MnS	MnP	MnE	MnU	MnH	sMIR	pMIR	eMIR	Age	Years
MnS	-	0.27**	0.36**	0.31**	0.35**	0.43**	0.22**	0.43**	0.18**	0.12*
MnP	-	-	0.25**	0.08	0.32**	0.17**	0.84**	0.23**	–0.13*	0.01
MnE	-	-	-	0.11	0.26**	0.10	0.19**	0.94**	–0.08	0.00
MnU	-	-	-	-	0.29**	0.12	0.17**	0.16**	–0.08	0.16**
MnH	-	-	-	-	-	0.16**	0.27**	0.24**	–0.06	0.01
sMIR	-	-	-	-	-	-	0.07	0.12	–0.05	–0.06
pMIR	-	-	-	-	-	-	-	0.19**	–0.12	0.01
eMIR	-	-	-	-	-	-	-	-	–0.12	0.00
Age	-	-	-	-	-	-	-	-	-	0.10
Employment years	-	-	-	-	-	-	-	-	-	-

Data represent Pearson correlation coefficient (r) produced by linear regression. *$p < 0.05$; **$p < 0.01$.

biological matrices were further analyzed by stratifying for years of employment as <5, 5–10 and >10 years. Using two-way ANOVA, no employment year-associated increase in Mn levels was found in any of these three groups or in any biological matrices (data not shown). When the personal air sampling data ($n = 40$) were individually paired with potential biomarker levels for linear regression analysis, the airborne Mn level was statistically significantly associated with MnS ($r = 0.77$, $p < 0.01$), MnP ($r = 0.66$, $p < 0.01$) and MnE concentrations ($r = 0.69$, $p < 0.01$) (Figure 2).

Fe and Fe regulatory proteins in biological matrices

The Fe concentration was significantly increased in saliva and hair samples when the values of the exposure groups were compared with those of controls (Table 2). The Fe concentration in plasma and erythrocytes, however, was significantly decreased by 23–41% in the plasma and 29–31% in the erythrocytes. Urinary Fe concentrations were significantly reduced by 23% in the high-exposure group (Table 2).

Among Fe regulatory proteins analyzed, ferritin concentrations in both serum and saliva samples did not significantly differ between the three groups. Serum transferrin levels were increased by 19–26% ($p < 0.05$) in Mn exposed smelters compared with controls, while the serum TIBC was decreased among smelters (Table 4). Saliva TIBC showed a similar declining trend in the low-exposure group. The data on the saliva transferrin receptor (TfR) were inconsistent with Mn exposure, as the low-exposure group showed a 34% decrease, and the high-exposure group displayed a 52% increase. Saliva transferrin levels were below the detection limit.

Manganese–iron ratio (MIR)

Since Mn exposure resulted in a general increase in Mn levels in major biological matrices and a general decrease in Fe levels in blood components, it became appealing to investigate the ratio of Mn and Fe concentrations (MIR) within a given media. We thus hypothesized that the decrease in Fe was a biological alteration in a response to Mn exposure and that the MIR would yield a novel and more sensitive measure of Mn exposure. As an example, the MIR in erythrocytes can be calculated as follows:

$$eMIR = \frac{Mne(\mu g/L)}{FeE(mg/L)}.1000$$

$eMIR$: Erythrocyte Maganese-Iron Ratio

MnE : Mn concentration in erythrocytes

FeE : Fe concentration in erythrocytes

The values for all biological matrices were calculated for each study subject, and the statistical data are presented in Table 5.

The MIRs for erythrocytes (eMIR) and plasma (pMIR) exhibited a significant increase in smelters compared with controls ($p < 0.05$, Table 5). The eMIR of the low- and high-exposure groups represents a respective 126% and 349% increase over the eMIR calculated for controls (Figure 3A). A similar exposure group-related increase was also observed for the pMIR, but the pMIR values between the low and high exposure group were not significantly different (Figure 3B). The sMIR was significantly increased in the high-exposure group, but not in the low-exposure group. The MIR for urine, while also greatly increased, did not show an exposure group-related effect. Similar to MnS, MnE and MnP, the MIR values were significantly associated with airborne Mn concentrations for the eMIR ($r = 0.77$, $p < 0.01$) and the pMIR ($r = 0.70$, $p < 0.01$) (Figure 4).

Use of eMIR as an indicator of Mn exposure

To be a reasonable indicator of Mn exposure, ideally the biological measures should display the following: (1) external exposure dose-related changes; (2) large, stepwise percentage increases between adjacent study

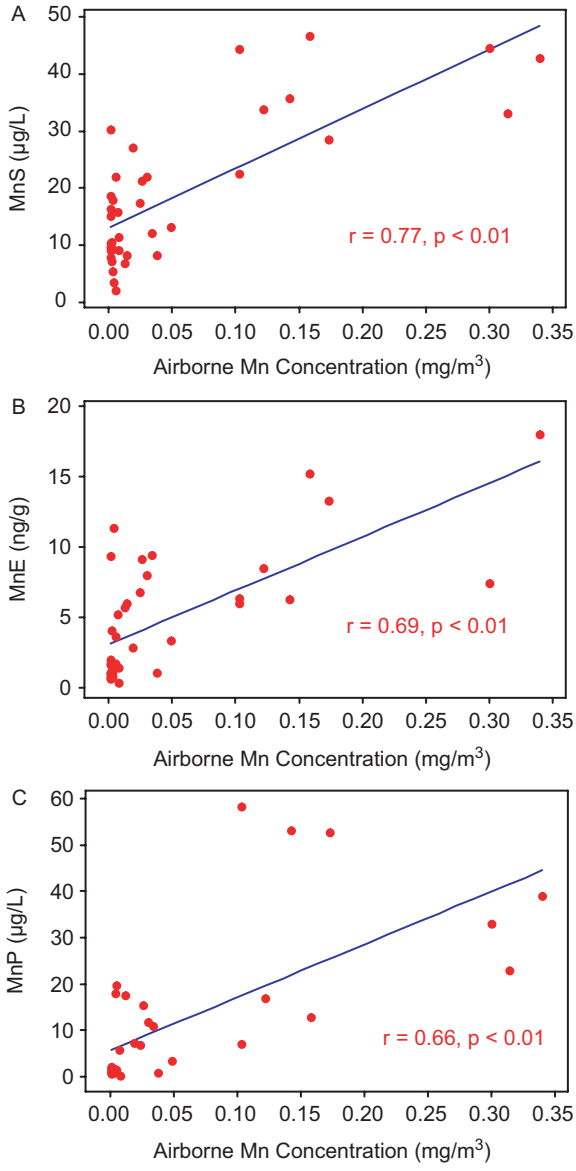

Figure 2. Changes of Mn concentrations in biomatrices as the function of airborne Mn concentrations. A total of 40 study subjects carried the personal air monitor. Mn concentrations were determined by atomic absorption spectrophotometry. Data were analyzed by a linear regression: (A) MnS and air Mn levels ($r=0.77$, $p<0.01$); (B) MnE and air Mn levels ($r=0.69$, $p<0.01$); and (C) MnP and air Mn levels ($r=0.66$, $p<0.01$).

groups; and (3) a reasonable threshold above which it can be predicted with reasonable certainty that an individual has been exposed. Among Mn, Fe and MIR determinants, parameters such as MnS, MnP, MnE, the pMIR and the eMIR showed dose-related increases (Tables 2 and 5). The percentages of the increases between adjacent groups were high for MnS, MnP, MnE, the pMIR and the eMIR. Thus, these five parameters were chosen for further stepwise multiple regression and ROC analysis.

The generalized linear model (GLM) was utilized to determine associations between potential biomarkers and the independent variables including exposure group, years of employment, age, sex and income (Table 6). Model I confirms that a significant group difference existed for all five tested biomarkers. Model II through V were stepwise additions of independent variables to evaluate the effect of other variables on the relationship between exposure and each biomarker. The variance associated with MnS and MnP was affected by independent variables such as age, sex and income. However, both MIRs as well as MnE were only affected by exposure group, although pMIR was somewhat related to age when years of employment was considered ($p=0.051$ in Model III).

As the eMIR, pMIR and MnE appeared to be a promising indicator for Mn exposure, we further used the ROC analysis to establish the threshold value to maximize the sensitivity and specificity of the eMIR or pMIR to Mn exposure (Table 7). The ROC analysis yielded an eMIR value of 8.8 for comparison between the control and high-exposure groups, which represents the level where sensitivity and specificity were maximized at 88% and 87%, respectively (Figure 5A). The AUC for eMIR was 0.95 which corresponds to excellent discriminating capability. The pMIR and MnE were both below 0.9 indicating low accuracy by our scoring standards. Using the cut-off value (COV) of 8.8, 80 out of 92 control subjects had an eMIR below this COV (87% of controls) (Figure 5B). Of 83 smelters in the high exposure group, 73 had an eMIR above 8.8 (88% of the high exposure smelters). When subjects in the low- and high-exposure groups were combined, the optimal eMIR value with the best sensitivity and specificity (78% each) was 9.68. Using a

Table 4. Iron-regulatory protein concentration within biological matrices.

	Mn exposure groups				
Iron regulatory protein	Control	Low	% Change	High	% Change
Serum ferritin (ng ml^{-1})	31.8 ± 21.6	36.3 ± 24.5	+14	35.5 ± 27.8	+12
Serum transferrin (g l^{-1})	1.65 ± 0.45	1.97 ± 0.36**	+19	2.08 ± 0.33**	+26
Serum TIBC (µmol l^{-1})	75.1 ± 17.9	62.5 ± 9.39**	−17	68.0 ± 8.66$^{\#}$	−9
Saliva ferritin (ng ml^{-1})	24.0 ± 52.7	9.15 ± 36.8*	−62	12.1 ± 29.3	−50
Saliva transferrin receptor (µg ml^{-1})	18 765 ± 19 760	13 432 ± 16 486	−28	29 034 ± 21 452**	+55
Saliva TIBC (µmol l^{-1})	13.8 ± 11.5	6.52 ± 10.6**	−53	12.4 ± 16.8	−10

Data represent mean ± S.D. *$p<0.05$ compared with control; **$p<0.01$ compared with control; $^{\#}p<0.05$ compared with both control and low-exposure groups. Significance confirmed with Tukey's post-hoc analysis.

Table 5. Manganese–iron ratio (MIR) in biological matrices.

		Mn exposure groups			
Biological matrices	Control	Low	% Change	High	High
Saliva, sMIR (x100)	1.92 ± 2.24	2.19 ± 1.98	+14	2.92 ± 2.16**	+52
Plasma, pMIR	1.52 ± 1.38	4.52 ± 3.31**	+197	5.03 ± 3.50**	+231
Erythrocytes, eMIR (x1000)	4.65 ± 3.90	10.5 ± 8.76**	+126	20.9 ± 12.9#	+349
Urine, uMIR	1.04 ± 0.56	7.65 ± 9.33**	+636	5.90 + 5.26**	+467
Hair, hMIR	0.13 ± 0.11	0.87 ± 0.65**	+569	1.37 ± 0.89#	+954

Data represent mean ± SD. **$p < 0.01$ compared with control; #$p < 0.05$ compared with both control and low-exposure group. Significance confirmed with Tukey's post-hoc analysis.

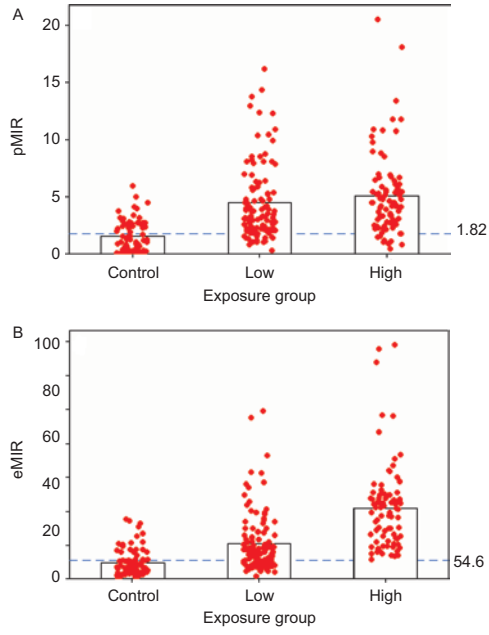

Figure 3. Manganese–iron ratio (MIR) in biomatrices of control, low- and high-exposure groups. The MIR values were calculated by dividing the Mn concentration by the Fe concentration within a given media. Bars represent the group mean, dotted lines stand for the upper 95% confidence interval (UCI) of the control mean, and dots are each individual determinant. (A) MIR of erythrocytes (eMIR); (B) MIR of plasma (pMIR).

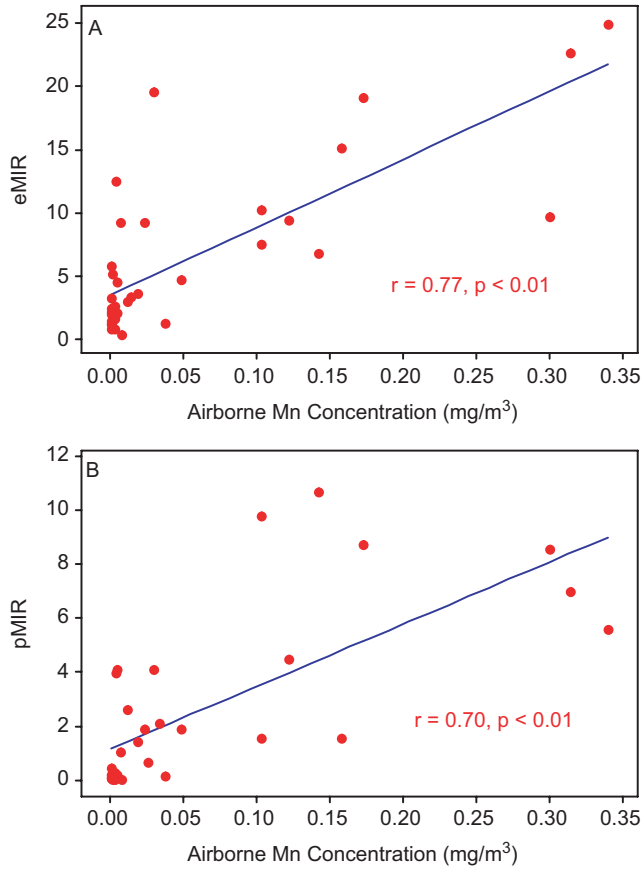

Figure 4. Changes of manganese–iron ratio (MIR) in biomatrices as the function of airborne Mn concentrations ($n = 40$). Data were analyzed by a linear regression: (A) eMIR and air Mn levels ($r = 0.77$, $p < 0.01$); (B) pMIR and air Mn levels ($r = 0.70$, $p < 0.01$).

COV of 9.7, 108 out of 196 total smelters studied (55%) displayed an eMIR significantly higher than the COV, whereas 89% of control eMIR values were below this COV. Similarly, with a pMIR COV of 2.75, 75% of smelters in the high-exposure group were above the COV (Figure 5C), whereas about 84% of controls were below this pMIR COV (Figure 5D, Table 7). Thus, it appeared that both eMIR and pMIR are suitable markers to distinguish Mn-exposed workers from the controls. Using the similar approach, the MnE yielded a poorer percentage of controls below the COV (Table 7).

Discussion

The present study examined more than 15 biological parameters from five major biological matrices (i.e. saliva, plasma, erythrocytes, urine and hair). Our data suggest that active smelters indeed had elevated Mn concentrations in all biological matrices assayed. Remarkably, however, none of these parameters were associated with workers' years of employment or their age. Unlike our previous reports, where stratifying years of employment as <5, between 5 and 10 and >10 years in many cases revealed such a relationship (Lu et al. 2005, Jiang et al. 2007, Wang et al. 2008), this study did not find any employment year-associated increase in Mn levels in any of the three groups or in any of the biological media.

Table 6. Effect of independent variables on potential biomarkers by generalized linear model analysis.

		eMIR			pMIR			MnE			MnP			MnS		
Models	Variables	F-Statistic	p	Adjusted R^2	F-Statistic	p	Adjusted R^2	F-Statistic	p	Adjusted R^2	F-Statistic	p	Adjusted R^2	F-Statistic	p	Adjusted R^2
Model I	Exp. Group	70.98	0.000	71.00	35.42	0.000	10	70.58	0.000	31.62	34.43	0.000	18.40	89.20	0.000	38.20
Model II	Years Empl	0.00	0.979	0.00	0.03	0.857	0.00	0.00	0.999	0.00	0.02	0.890	0.00	4.09	0.044	1.12
Model III	Years Empl	0.02	0.889	0.48	0.16	0.688	0.72	0.00	0.954	0.00	0.19	0.664	1.24	5.63	0.018	4.15
	Age	3.32	0.070		3.85	0.051		1.58	0.210		5.53	0.019		9.49	0.020	
Model IV	Years Empl.	0.14	0.707	0.16	1.76	0.186	1.01	0.05	0.816	0.00	0.03	0.860	0.90	0.06	0.805	3.82
	Age	0.01	0.937		0.03	0.857		2.07	0.152		0.21	0.649		5.65	0.180	
	sex	2.94	0.088		2.69	0.102		1.38	0.241		5.41	0.021		9.38	0.020	
Model V	Years Empl.	0.14	0.708	0.00	0.90	0.343	0.68	0.00	0.984	0.27	0.08	0.784	0.29	0.11	0.743	3.31
	Age	0.03	0.873		0.01	0.924		2.72	0.100		0.26	0.614		4.64	0.032	
	Sex	3.15	0.077		2.76	0.098		1.46	0.228		4.66	0.032		8.45	0.004	
	Income	0.44	0.509		0.30	0.582		1.13	0.288		0.15	0.697		0.00	0.955	

MIR, manganese–iron ratio.

Table 7. Comparison of cut-off values (COV) of the manganese–iron ratio (MIR).

MIR	Group comparison	COV	AUC	# above COV/ total(high)	# above COV/total (low and high)	# below COV/ total (control)
eMIR	Control vs high	8.8	0.95	73/83 (88%)	124/196 (63%)	80/92 (87%)
	Control vs low and high	9.68	0.87	69/83 (83%)	108/196 (55%)	82/92 (89%)
pMIR	Control vs high	2.74	0.87	67/89 (75%)	133/193 (69%)	69/82 (84%)
	Control vs low and high	3.27	0.70	60/89 (67%)	114/193 (59%)	75/82 (91%)
MnE	Control vs high	6.85	0.89	75/85(88%)	121/200 (61%)	79/102 (77%)
	Control vs low and high	7.7	0.87	70/85 (82%)	102/200 (53%)	82/102 (83%)

AUC, area under the curve.

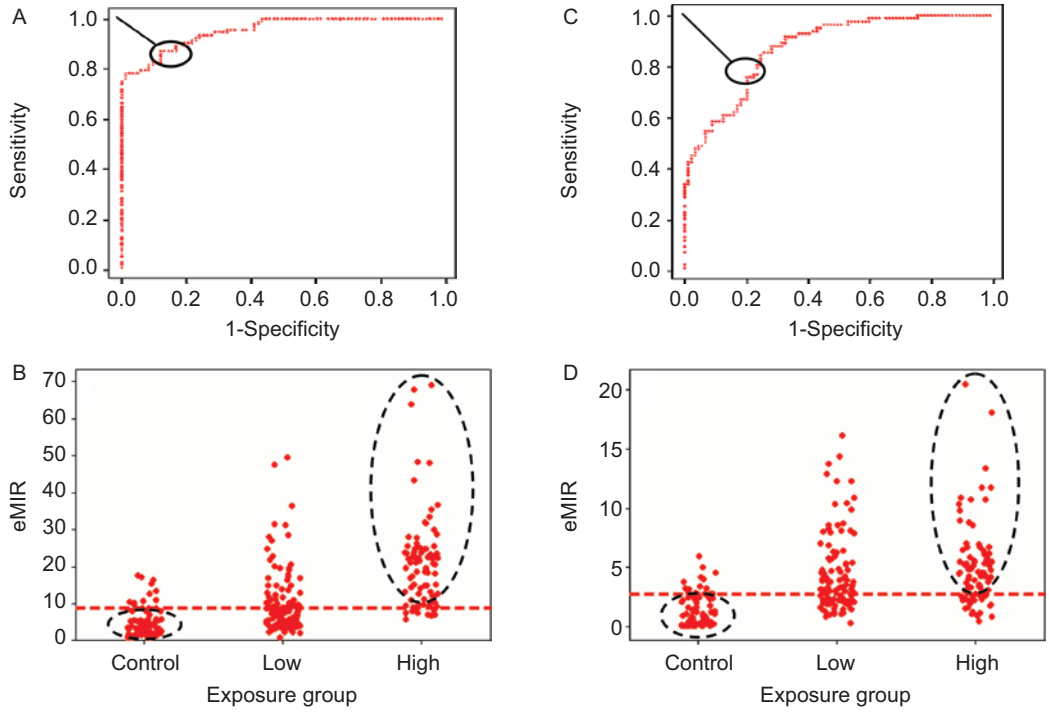

Figure 5. Manganese–iron ratio (MIR) cut-off value (COV) reflective of Mn exposure. The COV was calculated by receiver-operator characteristic (ROC) analysis, which decides the threshold value balancing the optimal (closest to 100%) sensitivity and specificity. (A) The solid circle indicates the COV of eMIR with 87% sensitivity and 88% specificity. (B) Distribution of eMIR in each study group. The dotted oval lines embrace the numbers of worker's eMIR either above or below the eMIR COV (8.8). (C) The solid circle indicates the COV of pMIR with 84% sensitivity and 75% specificity. (D) Distribution of pMIR in each study group. The dotted oval lines embrace the numbers of worker's pMIR either above or below the eMIR COV (2.8).

Therefore, the data from the current study may suggest recent exposure. It is worth noting that the increased Mn levels in the body were observed when the airborne Mn levels were at or near the ACGIH TLV (0.2 mg m^{-3}). Thus, it is plausible and even likely that Mn accumulation and biological alteration of Fe homeostasis could occur at a relatively low environmental air concentration.

The primary goal of this study was to identify a biological marker that accurately distinguishes Mn-exposed individuals from the unexposed, general population. Among 15 biological parameters evaluated, the proteins associated with Fe metabolism, such as transferrin, ferritin and transferrin receptor, displayed varying degrees of change in serum and saliva. For example, serum transferrin was found to be significantly increased by 19% and 26% in the low- and high-exposure groups, respectively, compared with the controls. This observation is consistent with our previous studies of Mn-exposed welders (Lu et al. 2005). The TIBC in both serum and saliva showed a parallel decrease among smelters in comparison to controls. Further, Fe concentrations in plasma and erythrocytes were significantly decreased. These data provide additional evidence to support the theory that Mn exposure distorts Fe homeostasis (Zheng et al. 1999, Zheng & Zhao 2001, Ellingsen et al. 2003, Li et al. 2004, 2005, Lu et al. 2005). However, a relatively small percentage of change, an obvious discrepancy in Fe and ferritin levels between serum and saliva, and a quantitatively large variation among these determinants limit the direct

application and extrapolation of these measurements to Mn-exposure assessment.

From the mechanistic point of view, Mn competes with Fe for the same binding site in the active centre of the iron-regulatory protein IRP-1 (Zheng et al. 1998); the altered binding capacity between IRP1 and the stem-loop containing mRNAs that encode various Fe-transport and storage proteins may cause the compartmental shift of Fe from the blood to the cerebrospinal fluid, resulting in a deficient Fe status in the blood compartment (Li et al. 2005, Zheng et al. 1998, 1999). As the changes of Mn and Fe concentrations in biological media may lean in opposite directions, combining these two critical measurements into one parameter may widen the differences between exposed and control individuals and, therefore, increase the sensitivity of Mn-exposure assessment. Based on this logic, we designed the MIR with Mn as the numerator and Fe as the denominator to calculate the MIR for each biological media. Except for saliva, the differences between control and low-exposure groups and between low- and high-exposure groups did become considerably greater in the MIR values than Mn concentrations alone in both plasma and erythrocytes.

Is the MIR better than Mn concentration alone for the accurate assessment of Mn exposure? The GLM analyses revealed the existence of a significant group difference between control, low- and high-exposure groups in the eMIR, the pMIR, MnP, MnE and MnS values. Income, sex and age are non-Mn-related independent variables which may introduce confounding interferences. Using the GLM procedure to systematically add independent variables, it appears that MnP and MnS are significantly associated with non-Mn-related independent variables such as sex and age. The eMIR, pMIR and MnE appear to be significantly associated with only Mn-exposure grouping but not the age, sex, income or years of employment.

Ideally, the biological outcome should be related to variables reflective of the external exposure. Due to limited equipment and limited access to the work sites, we were restricted in our ability to collect external exposure data for each of the 323 participants, therefore our effort to determine the correlation between biomarkers and personal airborne Mn levels was only partially achieved. An additional limitation of this study is the possibility of subject misclassification because we relied on limited personal sampling data and worker questionnaires to derive our exposure classifications. Even with these limited data, however, the linear regression analyses did indeed reveal a significant correlation between the eMIR/pMIR/MnE and the airborne Mn concentration. Thus, among more than 15 potential biomarkers obtained, the eMIR, pMIR and MnE appear to stand out as the promising biomarkers for Mn exposure.

The balance of sensitivity and specificity of a given biomarker with regard to Mn exposure is essential to Mn exposure assessment. The specificity in our study refers to the ability of a biomarker to identify correctly unexposed subjects based on their biomarker value. The sensitivity, on the other hand, is reflected by the ability of a biomarker to correctly identify Mn-exposed subjects based on an individual's biomarker value. A biomarker meeting these criteria can thus be used as a surrogate for exposure when historical exposure characterization data are unavailable. The ROC approach establishes the optimal threshold COV that maximizes the sensitivity and specificity of the potential biomarker of Mn exposure. Using the ROC analysis, at the maximized sensitivity and specificity, an eMIR COV of 8.8 indicated that more than 88% of the smelters in the high-exposure group were above the COV, whereas a similar percentage (87%) of the control workers were below the COV of the eMIR. Noticeably, the MnE values are correlated with eMIR levels. However, the use of MnE as a biomarker is less effective compared with eMIR and pMIR for two reasons: (1) the MnE showed a disproportional increase between control, low- and high-exposure groups (Table 2); and (2) the MnE had a less overall discriminating power to distinguish the exposed from the control based on the percentages above or below its COV and its AUC diagnostic analysis comparing control and high subjects (Table 7). The efficacy of MnE for Mn exposure assessment should be further evaluated in order to determine its usefulness.

A biomarker, by definition, is the biological indication of exposure, effect or susceptibility in humans in response to environmental toxicants (Fowle & Sexton 1992). A positive correlation between the eMIR/pMIR and airborne Mn levels, while tested on a limited basis in the current study, suggests that both eMIR and pMIR may reflect external Mn exposure. More importantly, however, the MIR is reflective of the biological consequences that Mn exposure may elicit. Unlike traditional determination of Mn concentrations in biological media, the MIR embraces one of the most significant systemic effects of Mn toxicity, i.e. Mn effect on Fe metabolism. Red blood cells have been shown to accumulate Mn in a dose-related fashion (Jiang et al. 2007). Systemic Fe levels were also reduced in Mn-exposed humans and animals (Ellingsen et al. 2003, Zheng et al. 1999). Thus, the ratio of Mn and Fe concentrations in blood cells does not simply reflect the steady-state body burden of Mn or Fe in tested individuals, but rather signals a biological response to Mn exposure. Hence, the utility of the eMIR and pMIR in combination with saliva Mn levels should be further explored in populations with different Mn exposure scenarios and among different ethnic backgrounds.

Between eMIR and pMIR, the pMIR appears to be weaker than the eMIR for assessing Mn exposure, for its inability to distinguish between the low- and high-

exposure groups, and for its generally smaller percentage change than the eMIR in identifying the numbers of subjects above or below a given COV. Nonetheless, the relative ease of daily clinical preparation, a reasonable correlation with airborne Mn levels, and a good association with neurological functional changes (data not shown) render the pMIR an attractive biomarker for further study. Thus, based on the current data, we would also recommend pMIR as another alternative biomarker for Mn exposure assessment.

Consistent with our earlier study (Wang et al. 2008), findings herein suggest that the patterns of increase in both saliva and plasma Mn levels are nearly identical, emphasizing that the Mn content in the saliva of exposed subjects directly reflects the Mn concentration in the plasma (Wang et al. 2008). In contrast to decreased Fe levels in the plasma, saliva samples showed a significant increase of Fe concentration when the Mn-exposed smelters were compared with the control subjects. The reason for this increase is unknown. Nonetheless, the saliva MIR between the low and control groups was not significantly different. Furthermore, MnS appears to be associated with non-Mn-related independent variables such as age and sex when exposure group is not considered. It is also noticeable that the air samples of the smelting environment have relatively stable constituents of MnO (20%), SiO_2 (22%), Fe_2O_3 (4%), CaO (4.5%), MgO (4%) and Al_2O_3 (5%) from our early investigation (Jiang et al. 2007). While the airborne Fe concentration in the smelting environment is relatively low, the possible interference from Fe should be taken into account when MIR is used for risk assessment.

In conclusion, more than 15 parameters in saliva, plasma, erythrocytes, urine and hair were examined for their utility as a biomarker for Mn exposure. Mn concentrations in all biological media were significantly elevated in smelters compared with control workers. The concentrations of Fe and Fe metabolic proteins in these matrices were also significantly altered to various degrees. The concept of Mn/Fe ratio (MIR) in biological matrices was developed and tested for its efficacy in assessing Mn exposure. The erythrocyte MIR (eMIR) and plasma MIR (pMIR) exhibit strong correlations with airborne Mn levels and their variances are not significantly affected by non-Mn-related independent variables. Finally, using the cut-off values for eMIR and pMIR, we are accurately able to distinguish Mn-exposed workers from the unexposed, control population.

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