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Manganese exposure among smelting workers: Relationship between blood manganese–iron ratio and early onset neurobehavioral alterations

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ABSTRACT

A biomarker for detection of early onset neurobehavioral alterations in manganism remains unknown. The purpose of this study was to use a neurobehavioral test battery to identify subtle changes in Mn-induced motor and memory dysfunction and to relate the quantifiable neurological dysfunction to an established Mn-exposure index such as blood manganese–iron ratio (MIR). A total of 323 subjects were recruited to control ($n = 106$), low-exposure (122), and high-exposure (95) groups. The test battery consisted of standard testing procedures including the nine-hole and groove-type steadiness tester, Benton visual retention test, and Purdue pegboard coordination test. No significant health problems or clinically diagnosed neurological dysfunctions were observed. Benton test did not reveal any abnormal memory deficits among Mn-exposed smelters, nor did the groove and nine-hole tests detect any abnormality in dynamic and static steadiness in tested subjects. Purdue pegboard test showed a remarkable age-related decline in fine movement coordination among all study participants regardless of the Mn-exposure condition. Mn exposure significantly exacerbated this age-related deterioration. Statistical modeling revealed that the plasma and erythrocyte MIR (i.e., pMIR and eMIR, respectively) were associated with Purdue pegboard scores. Among all subjects whose MIR were above the cut-off value (COV), pMIR was significantly correlated with pegboard scores ($r = -0.261$, $p = 0.002$), whereas for those subjects over the age of 40, the eMIR, but not pMIR, was associated with declined pegboard performance ($r = -0.219$, $p = 0.069$). When both factors were taken into account (i.e., age > 40 and MIR $>$ the COV), only pMIR was inversely associated with pegboard scores. Combining their usefulness in Mn-exposure assessment, we recommend that the blood Mn–Fe ratio may serve as a reasonable biomarker not only for assessment of Mn exposure but also for health risk assessment.

1. Introduction

The neurological signs and symptoms of manganism are similar to those of idiopathic Parkinson's disease (IPD). Manganism can be partially differentiated from IPD by differences in pathology, diagnostic imaging and type of motor impairment (Calne et al., 1994; Olanow, 2004; Wang et al., 1989). A hallmark pathological difference between IPD and manganism is the presence of a kinetic tremor (action tremor) associated with extrapyramidal dysfunction, as opposed to a persistent resting tremor with IPD (Cersosimo and Koller, 2006; Olanow, 2004; Roels and Buchet, 1987).

During the early stages of progressive manganism, patients display non-specific symptoms such as weakness, apathy, somnolence, headaches, and minor motor dysfunction, which are generally reversible if the exposure is reduced or eliminated (Cersosimo and Koller, 2006; Deschamps et al., 2001; Lander et al., 1999; Mergler et al., 1999). With chronic exposure, the disease may progress with a combination of motor deficits with more severe psychological disturbances manifesting in memory loss, psychotic episodes, anxiety, and hallucinations (Bouchard et al., 2007b; Calne et al., 1994; Couper, 1837; Levy et al., 2003). In the late stages of clinical manganism, the patients present with established movement disorders comprised of bradykinesia, dystonic postural abnormalities, gait disturbances, cock-walk, cogwheel rigidity, impaired motor coordination and, in worst cases, permanent and complete disability (Calne et al., 1994; Levy et al., 2003; Mergler, 1999; Olanow, 1994). Whether the neuropsychological impairment of manganism is directly caused by Mn exposure or Mn-induced acceleration of IPD progression or a combination of both is unknown (Finley and Santamaria, 2005; Gorell et al., 1997; Racette et al., 2001, 2005).

Symptoms of acute Mn exposure are reported; but more commonly consequences of chronic exposure are observed in

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clinics. Acute Mn exposure, known as "metal fume fever," is relatively less incapacitating. Patients exhibit irritated upper respiratory tract, flu-like symptoms and motor deficits which can be alleviated upon removal of exposure (Barceloux, 1999; Racette et al., 2001; Roels and Buchet, 1987). Chronic, low-level occupational Mn exposure is prevalent among smelters, welders, dry-cell battery production workers, and Mn-oxide and salt production workers, leading to irreversible damage to neurological structures (Crossgrove and Zheng, 2004; Inoue and Makita, 1996; Jiang et al., 2006; Roels et al., 1999; Tanner, 1993; Wang et al., 2008). The unknown period of time prior to the stage where neuropathology becomes permanent is critical for the prevention of permanent damage. Unfortunately, there is no method currently available for diagnosis of early signs and symptoms of manganism. Therefore, identification of early onset manganism through the use of biomarkers is of paramount importance to the prevention of manganism.

Identification and validation of a biomarker necessitates a significant connection between two interrelated components: (1) a dose-related association between exposure and biomarker level and (2) a relationship between the biomarker and associated neurological deficit. Ideally, a useful biomarker should bridge the gap between the exposure and exposure-related functional impairment. We have previously established the manganese–iron ratio in erythrocytes and plasma (eMIR and pMIR, respectively) as novel indicators of Mn exposure. The MIR is a quotient where the numerator reflects external Mn exposure and the denominator indicates biological alteration (Fe) which is significantly associated with external airborne Mn levels (Cowan et al., in press). By using the established cut-off value (COV), the MIR can reasonably distinguish Mn-exposed workers from the controls. The COV was established previously using receiver operator characteristics (ROC) analysis which computes each possible threshold value in order to determine the optimal cut-off value for maximizing the discrimination between two groups of data points (Cowan et al., in press). However, the questions as to whether the blood MIR was associated with neurobehavioral impairment and whether such an association may be affected by other physiological factors were unanswered.

The current study focused on searching for biomarkers associated with neurological deficit. For development of a Mn toxicity biomarker, the term of "early onset" must be taken into consideration. During the early stages of pathogenesis, Mn-induced neuropsychological impairment is subtle, sometime undetectable or unquantifiable. Since Mn neurotoxicity manifests mainly in motor deficit, it is possible to design a standardized neurobehavioral test battery to differentiate exposed from unexposed subjects.

Based on the literature, motor deficit can be quantified by using components of the nine-hole steadiness test for static steadiness or the groove-type steadiness test for dynamic steadiness; the latter was originally developed to measure motor deficit in children and adolescents with disordered brain function (Klonoff and Low, 1974). The nine-hole test alone has been used extensively for evaluation of Mn-related dynamic tremor (Bouchard et al., 2005, 2007a; Mergler et al., 1994). The combination of nine-hole and groove-type tests were used to quantify essential tremor (Louis et al., 2000) and kinetic tremor associated with smoking (Louis, 2007). The Benton visual retention test measures visual perception and nonverbal short-term memory and has been used to evaluate normal memory loss in elderly subjects (Larrabee and Crook, 1994; Seo et al., 2007), pathological memory loss in Alzheimer's and dementia patients (Ferman et al., 2006; Kawas et al., 2003), and neurological function of Mn-exposed welders and ferroalloy plant workers (Bast-Pattersen et al., 2004; Wang et al., 2006). Finally, the Purdue pegboard test, developed by Dr. Joseph Tiffin of Purdue University was originally used for pre-employment screening of workers with exceptional hand–eye coordination (Tiffin and Ascher, 1948), has recently been used for quantifying fine movement disorders and learning disabilities (Bloch et al., 2006; Kane and Gill, 1972; Pitcher et al., 2003; Skinner and Curwin, 2007). We employed these four testing devices in this study for their reliability reported in the literature, readiness in operation and less mandate on the language command.

The hypothesis tested in this cross-sectional study was that one or multiple neurobehavioral testing outcomes reflective of subtle motor deficits may be associated with the changes of proposed MIR biomarkers. The objectives of this study were to (1) conduct medical examination on Mn-exposed subjects previously evaluated, (2) perform neurobehavioral test on each study participants using four aforementioned testing devices, (3) identify the early sign of Mn exposure based on the obtained quantitative scores, and (4) relate the identified measurable behavioral manifestation with the selected internal biomarkers of Mn exposure such as eMIR, pMIR and MnE. Using a neurobehavioral test battery, we hoped to detect subtle neurological impairment and relate it to our established exposure biomarkers. This would enable us to determine Mn exposure and related neurological dysfunction when the exposure is unknown and neuronal impairment is not visible.

2. Materials and methods

2.1. Study population

Both the subjects and ferroalloy manufacturers evaluated in this cross-sectional study are identical to the population previously described (Cowan et al., in press). Mn ferroalloy workers were recruited into high- and low-exposure groups based on their job classification, day-to-day working proximity to the smelting ovens and Mn levels in their work environment. High-exposure subjects included smelting oven-operators directly involved in the smelting process whose daily tasks required a close working proximity to the smelting ovens (approximately 2–5 m). Low-exposure subjects were supervisory and support staff with minimal contact with Mn-related operations and products. Control subjects were recruited from a facility where daily operations were similar to the ferroalloy plant, but without the use of Mn as a raw material and without environment Mn contamination.

Historical medical records indicated occupational cases of Mn intoxication and related neuropsychological dysfunction. Subjects with history of anemia or who were taking medicine containing iron (Fe) were excluded from the study. Subjects who had taken medications which could interfere with Fe metabolism, such as vitamin D, aspirin or herbal supplements, were excluded from the study. All subjects included in this study were right-handed. Smoking status was quantified by calculating pack-years which is the number of packs per day multiplied by the number of years smoking this quantity. Detailed exposure assessment and quantification of Mn content is detailed in the previous paper (Cowan et al., in press).

2.2. Survey and self-reported symptoms

The subjects' clinical examination consisted of four phases: (i) collection of biological samples, (ii) personal and medical survey using questionnaires, (iii) neurological examination, and (iv) neurobehavioral testing. Prior to the study, a written consent form was obtained. Following collection of biological samples (data not shown), a trained research nurse conducted a 60-min interview cataloging each subject's personal, family, health, and occupational history. During this phase, vital statistics including: height, weight, and blood pressure were measured. Self-reported smoking and drinking habits were also recorded.

Subjects were then asked a variety of questions designed to evaluate their general health and possible symptoms related to

manganism. The subject's answers, either positive or negative, to whether he/she had ever experienced these symptoms were tabulated.

2.3. Neurological examination

Neurological examination was performed by an experienced and accredited clinical neurologist. A total of 26 different motor functions were examined according to standard clinical procedures aimed at identifying established clinical signs of manganism. These evaluations included tests such as finger to nose, finger to finger, micrographia, and stability while standing with his/her eyes closed. A positive or negative observation was assigned by the neurologist for each neurological functional test.

2.4. Quantitative neuropsychological test battery

Each subject's motor function, coordination, and memory were examined and quantified using a neuropsychological battery, which included 4 tests with a total of 9 measurements collected. The four tests were the nine-hole steadiness apparatus for static steadiness, the groove-type steadiness platform for dynamic steadiness, the Purdue pegboard (PP) for coordinated fine movement, and the Benton visual retention test for nonverbal learning ability. The justification for these instruments and the outcomes related to visual–motor coordination, static and dynamic steadiness and nonverbal memory have been discussed in published literature (Bouchard et al., 2005; Buddenberg, 2000). During the motor examination, all subjects sat in an identical position to limit variation caused by seating arrangements.

2.5. Assessment of memory by Benton visual retention test

The Benton tester (Harcourt Assessment Inc., San Antonio, TX; 5th edition) is a tool commonly used for measuring nonverbal learning deficits, brain injury, or memory. During the test, the researcher presented 1 of 30 different simple designs from the test collection to the subject who was given 10 s to memorize the drawing before withdrawal. The subject was then asked to reproduce the drawing. The procedure was repeated for each of the 30 designs. For each successfully reproduced picture the subject received a score of 1.

2.6. Evaluation of static and dynamic steadiness

Both the nine-hole steadiness apparatus (Model 32011) and groove-type steadiness platform (Model 32010), obtained from Lafayette Instruments Inc. (Lafayette, IN), require a metal-tipped stylus, with which the subject navigated the testing apparatus, and a battery-powered impulse counter (Lafayette Instruments Inc., Lafayette, IN; Model 58023). When the stylus contacted the metal testing plate, an electrical circuit was completed and the automated counter recorded the number of instances that the stylus contacted the metal plate. The nine cut-out holes in the nine-hole tester progressively decrease in diameter. The subject was asked to place the stylus within each hole, from largest to smallest, for 10 s and the numbers of touches were recorded. The subject was allowed to practice once prior to beginning the formal test. The process was repeated three times for each hand and the average number of touches for each hole was then calculated. A lesser degree of steadiness is usually associated with a greater number of touches for each hole descending order of diameter.

The groove-type steadiness tester was comprised of a grooved compartment, stretching from left to right with width decreasing from 15.9 mm at the opening to 3.2 mm at the end. A metric ruler was positioned parallel to the groove to record the distance where the stylus traveled inside the groove to the point it first touched the metal edge of the groove. The subject was asked to hold the stylus at a 90° angle to the testing plate and move the stylus from left to right, navigating the groove through the entire length regardless of contact. In addition to the distance measured, the number of touches through the entire length of the groove was recorded by an impulse counter. The test was repeated three times for each hand and both the average distance and number of touches were then calculated. The distance from the beginning to the first touch indicates the magnitude of kinetic tremor.

2.7. Determination of fine coordination using Purdue pegboard

The Purdue pegboard (Model 32020), obtained from Lafayette Instruments Inc. (Lafayette, IN), provides information regarding the subject's ability to perform coordinated fine movement tasks with their arms and fingers. The apparatus is a wooden board measuring 30 in. by 14 in. with two rows of peg-holes running parallel down the center of the board. At the top of the board, four shallow compartments hold three types of metal pieces. From left to right, the cups contain (i) 3/4-in. pins, (ii) washers, (iii) collars and (iv) 3/4-in. pins. Both the washers and collars fit tightly over the 3/4-in. pins. The compartments are positioned so the subject can access washers and pins with the left hand and collars and pins with the right hand.

The procedure for the pegboard test is comprised of four operations repeated in triplicate. Each operation is demonstrated by the researcher followed by an opportunity for the subject to practice the procedure. First, the subject has 30 s to place as many pins as possible in the right hole with the right hand, moving down the board toward the subject. This procedure is repeated with the left hand using the left cup of pins. The third operation requires the subject to place pins simultaneously with both the right and the left hand in corresponding right and left holes. The subject has 30 s to complete as many pairs as possible. The average for the three trials is recorded. Finally, the subject is instructed to create an "assembly" using both hands to place pins, washers, and collars in an organized manner. Briefly, the assembly consisted of four operations with alternating hand movements resulting in a four piece "assembly" which was recorded as a score of 4; one point for each step. The subject has one minute to complete as many assemblies as possible. Partial assemblies are counted depending on the number of pieces positioned when time expires. The pegboard test yields two scores: (1) the sum of right, left and both hands (R + L + B), and (2) the number of assemblies (including complete and partial assemblies).

2.8. Manganese–iron ratio (MIR) as a surrogate for Mn exposure

Results of our previous study indicated that Mn exposure was associated with a general increase in Mn concentration and a general decrease in Fe concentration in erythrocytes and plasma leading to the concept of the Mn–Fe ratio (eMIR and pMIR, respectively) as a biomarker for Mn exposure (Cowan et al., in press). The relationships between Mn concentration in erythrocytes (MnE), eMIR, and pMIR values, determined for each subject, and neurological test outcomes were evaluated. The previously established optimal cut-off values, established for distinguishing exposure groups were 8.80, 2.75, and 6.85 for eMIR, pMIR and MnE, respectively, were further used to explore the utility of these biomarkers in assessing the early onset neurobehavioral alternations.

2.9. Statistical analysis

Demographic and personal data were abstracted from the survey questionnaires. Data normality was evaluated and confirmed prior to statistical analysis. To identify differences between

means of each test, a one-way analysis of variance (ANOVA) was used initially to determine significance at the level $p = 0.05$. A Tukey's post hoc pair-wise comparison was used to verify significance between groups. Two-way ANOVA was used to determine differences between means for multiple factor comparison. A Tukey's post hoc test for interaction was further used to determine significance for all combinations of interactions. Statistical data is reported as arithmetic mean ± standard deviation unless stated otherwise. Pearson correlation coefficients (r) were obtained by linear regression analysis. A generalized linear model (GLM) was used to evaluate the influence of each MIR or MnE in a standardized model including age, sex, income, years of exposure, and years of education. Further, because age and education appear to be important independent variables regarding motor function, the cumulative effects of age, education and biomarker level were further evaluated. Corresponding F-statistic and p-values are presented to indicate the relative influence of age and education on each test. The relationship between each MIR and MnE and neuropsychological tests were evaluated by linear regression analysis.

When multiple outcome measurements were collected from same testing device, the scores of each tested task were standardized by computing a z-score using the following formula:

$$z\text{-score} = \frac{x_i - \bar{x}}{\sigma}$$

where x_i is the individual score; $\bar{x}$ is the mean of scores; σ is the standard deviation of the scores.

$$\text{SCI} = \sum z\text{-scores}$$

The z-score represents a common statistical method for standardizing data for comparison purposes. The sum of z-scores yields a parameter called the standardized composite index (SCI), which allows the results of multiple tests to be combined. Statistical operations were accomplished using Minitab statistical software (v15.1).

3. Results

3.1. Subjects

Demographic information is summarized in Table 1 and additional information was previously reported (Cowan et al., in press). In general, a total of 106, 122, and 95 subjects were recruited to the control, low- and high-exposure groups, respectively. Subjects within the Mn-exposed groups smoked significantly less as measured by pack-years ($p < 0.05$). While low-exposure subjects weighed slightly more than control and high-exposure subjects ($p < 0.05$), group-wise comparison of height was virtually identical. Noticeably, blood pressure was significantly higher in both Mn-exposure groups than controls. Both systolic and diastolic blood pressures were increased approximately 10% in both exposure groups compared to control; there was no statistically significant difference between two exposure groups. Interestingly, the control and high-exposure groups had significantly less years of education compared to the low-exposure group, which was comprised mainly of office and supervisory staff ($p < 0.01$).

3.2. Signs and symptoms of Mn-related psychological and motor dysfunction

The occurrences of self-reported symptoms related to manganism were generally low within all groups (Table 2). Overall, few subjects in each group (less than 10%) reported slowed behavior, uncontrollable laughing/crying, decreased libido, and propensity to fall down. Approximately 15% and 14% of low- and high-exposure subjects, respectively, reported decreased memory, compared to less than 3% of control subjects who reported memory loss. The mean age for those reporting decreased memory was 36.9 ± 17.3 and 34.5 ± 5.03 for low- and high-exposure groups, respectively, compared to control subjects which was 38.0 ± 3.0. Regarding self-reported memory loss, subjects in the high- and low-exposure groups were generally younger, although this association did not reach statistical significance. Slightly more subjects in the Mn-exposed groups reported nightly lower leg cramp, nausea, anxiety, and insomnia than control subjects.

Signs of Mn-related dysfunction, as measured by clinical examination by a trained neurologist, were virtually undetectable (<2% testing positive). Examination included more than 25 individual tests designed to identify signs of neurological and behavioral dysfunction related to Mn exposure (Table 3). No more than one subject tested positive for any test and 19/26 of tests resulted in negative findings for all 323 subjects. In addition, no group-wise increases were found. Overall, there were no visible signs of Mn-related dysfunction based on the evaluation by an accredited neurologist.

3.3. Mn exposure and memory impairment

Based on the scores from Benton visual retention test, no significant differences were found between the control and high-

Table 1
Subject information and vital statistics.

	Control	Mn-exposure groups	
		Low	High
n	106	122	95
Age (years)	37.7 ± 7.37 (18–56)	34.5 ± 6.89* (20–52)	35.4 ± 6.69* (22–53)
Current smokers	71 (67%)	61 (50%)	66 (69%)
Smoking: pack-years	17.1 ± 10.7	13.4 ± 11.5	10.6 ± 7.87*
Total education (years)	7.82 ± −2.33 (1–15)	10.5 ± 3.58 (1–15)**	7.75 ± 2.34 (1–15)
Income (RMB)	8000 ± 4780	15013 ± 8742*	9722 ± 10441
Weight (kg)	55.9 ± 8.83	58.4 ± 10.3*	55.9 ± 6.70
Height (cm)	160 ± 5.88	160 ± 6.86	160 ± 5.60
Blood pressure			
Systolic (mmHg)	103 ± 15.7	112 ± 14.2*	115 ± 10.7*
Diastolic (mmHg)	66.8 ± 11.3	75.4 ± 8.88*	77.5 ± 7.49*
Airborne Mn concentration (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 ± S.D. (range).
* $p < 0.05$ compared to control.
# $p < 0.05$ compared to low-exposure group. Airborne Mn concentration reported as geometric mean ± S.D. (range).

Table 2
Self-reported symptoms.

	Control (%)	Mn-exposure groups	
		Low (%)	High (%)
Parkinson's disease	0	0	1 (1.1)[a]
Senile dementia	0	0	1 (1.1)[b]
Anemia	0	0	0
Liver disease	0	4 (3.3)	0
Taking medicine containing Fe	0	0	0
Taking medicine for hypertension	0	3 (2.5)	2 (2.1)
Anxiety	1 (0.9)	8 (6.6)	4 (4.2)
Insomnia	3 (2.8)	10 (8.2)	7
Nausea	4 (3.8)	10 (8.2)	4
Chronic headache	12 (11.3)	9 (7.4)	1 (1.1)
Lethargy	3 (2.8)	6 (4.9)	4 (4.2)
Decreased memory[c]	3 (2.8)	18 (15)	13 (14)
Slowed movement	0	0	0
Hearing loss	0	0	0
Stuttering	0	0	0
Decreased libido	3 (2.8)	5 (6.6)	5 (5.2)
Dribble	2 (1.9)	5 (4.1)	2 (2.1)
Pain in limps	4 (3.8)	4 (3.3)	2 (2.1)
Numbness in limbs	3 (2.8)	4 (3.3)	0
Lower leg cramp at night	1 (0.9)	7 (5.7)	5 (5.2)
Easily fall down or loss of balance	1 (0.9)	2 (1.6)	0
Tired legs	3 (2.8)	2 (1.6)	1 (1.1)
Uncontrollable laugh or cry	0	0	0
Dizziness	4 (3.8)	11 (9.0)	3 (3.2)

Data represent number of subjects reporting various Mn-related symptoms (% occurrence within exposure group).
[a] Age = 53.
[b] Age = 53.
[c] Age: control = 38.0 ± S.D.3.00, low = 36.9 ± 5.04, high = 34.5 ± 5.03.

exposure groups (Table 4). The workers in the low-exposure group appeared to perform better than those in the control group, probably related to the fact that the subjects in this group were supervisory personnel and support staff with the average income at least 60% higher than both the control and high-exposure groups (Table 1). Overall, no significant exposure-related associations were found for the Benton test and all significant differences between groups could be attributed to both education level and age.

Table 3
Neurological signs of Mn-related dysfunction.

	Control	Mn-exposure groups	
		Low	High
Tremor of the jaw/lip/tongue	1 (0.9%)	0	0
Micrographia	0	0	0
Increased muscular tension in limbs	0	1 (0.8%)	0
Movement	0	0	0
Loss of abdominal reflex	0	0	0
Accentuation in knee	0	1 (0.8%)	0
Accentuation in Achilles tendon	0	0	0
Achilles and knee tendon test	0	1 (0.8%)	0
Ankle cramp	0	0	0
Pathological reflex	1 (0.9%)	0	0
Mismatch in upper limbs movement	0	0	0
Propensity to suddenly sit down	0	0	0
Finger to nose test	0	0	0
Finger to finger test	0	0	0
Difficulty standing with eyes closed	0	0	0
Stuttering	0	0	0
Unclear tongue	0	0	0
Increased muscular tension in face	0	1 (0.8%)	1 (1.1%)
Trembling fingers	0	1 (0.8%)	0
Easily excitable	0	0	0
Trembling in four limbs while standing still	0	0	0
Continuous tension in all four limbs	0	0	0
Resting tremor while standing	0	0	0
Tremor after repeated action	0	0	0
Difficulty walking backward	0	0	0
Cock-walk	0	0	0

Data represents number of subjects tested positive.

Table 4
Benton test and groove test.

	Control	Mn-exposure groups	
		Low	High
Benton test	15.9 ± 5.10	18.9 ± 5.83*	15.4 ± 6.00
Groove test—right hand			
Drawing length (cm)	20.1 ± 1.99	20.7 ± 1.71	20.9 ± 1.57**
Number of touches	4.53 ± 3.46	3.23 ± 3.82	3.06 ± 3.94*
Groove test—left hand			
Drawing length (cm)	20.4 ± 5.42	20.1 ± 1.96	20.0 ± 2.06
Number of touches	4.51 ± 4.41	4.08 ± 3.47	4.05 ± 4.17

Data represent mean ± S.D. Control $n = 106$, low $n = 122$, high $n = 95$.
* $p < 0.05$ compared to corresponding control.
** $p < 0.01$ compared to control.

3.4. Mn exposure and steadiness

In theory, a longer distance traveled by the metal stylus in the groove tester without contact indicates a steadier performance. Additionally, a higher number of touches suggest less steadiness. In contrast to our hypothesized Mn effect, the data for the dominant right hand showed that the smelters in the high-exposure group apparently performed better than control workers for their increased distance and decreased number of touches ($p < 0.05$), although there was no difference observed between the low-exposure group and controls (Table 4). At present we do not have any satisfactory explanations for this observation, however, it should be noted that the average differences in distances and touches were less than one centimeter and 1.5 touches, respectively. No significant differences existed for the left hand.

For static steadiness, a higher score on the nine-hole tester would suggest a reduced steadiness of the subject's hand. Generally, the left hand (non-dominant) produced significantly more touches for each group compared to the right hands (Table 5). However, between the control and high-exposure subjects, the differences were not statistically significant for both hands. The smelters in the low-exposure group, in fact, performed even better than the control workers with less touches ($p < 0.01$) (Table 5). These data suggested that the smelters in this study cohort have not yet developed the hand tremor, either dynamic or static, at the time of evaluation.

3.5. Mn exposure and coordination of fine movement

The results of the Purdue pegboard test provide two types of scores: the assembly score and the summed score of the right, left and both hands. For both outcomes, a higher score indicates a better command of manual dexterity and more steadiness. When the average scores were compared between groups, the smelters in the high-exposure group had lower assembly scores than did control and low-exposure workers; yet, the difference only became significant between the low and high groups (Table 6). However, when the pegboard scores were stratified for age, a general age-related decline in performance became evident for both outcomes, regardless of Mn exposure. Scores in Table 6 indicated that from the young to older age (from the top to the bottom in each column), there was an age-related decline in each group, whereas a visible decrease in both score categories can be seen horizontally from the control to the low and to the high-exposure groups (from the left to the right in each row). Thus, the data suggested that subjects' fine

Table 5
Nine-hole steadiness tester for left and right hands.

Non-dominant	Control	Mn-exposure groups		Dominant	Control	Mn-exposure groups	
		Low	High			Low	High
Hole 1	0	0	0	Hole 1	0	0	0
Hole 2	0	0.16 ± 1.02	0.18 ± 1.44	Hole 2	0	0.11 ± 0.51	0.01 ± 0.01
Hole 3	0.38 ± 1.32	0.42 ± 2.37	0.73 ± 1.65	Hole 3	0.22 ± 0.75	0.22 ± 0.75	0.61 ± 2.48
Hole 4	6.07 ± 4.83	6.07 ± 4.83	3.51 ± 4.50	Hole 4	3.74 ± 3.08	1.36 ± 2.51**	2.58 ± 4.09
Hole 5	11.6 ± 6.86	6.53 ± 6.86	8.37 ± 8.04	Hole 5	7.39 ± 5.69	3.23 ± 3.73**	6.76 ± 7.32
Hole 6	22.8 ± 11.7	15.7 ± 12.4**	20.7 ± 15.8	Hole 6	18.0 ± 9.44	9.76 ± 8.30**	15.9 ± 12.8
Sum of six holes	40.8 ± 20.6	25.5 ± 25.3**	33.54 ± 25.7	Sum of six holes	29.3 ± 16.3	14.6 ± 13.2**	25.8 ± 23.2

Data represent mean ± S.D.
** $p < 0.01$ compared to controls.

Table 6
Purdue pegboard test stratified by age.

Total score		Control	Mn-exposure groups	
			Low	High
Right + Left + Both		52.3 ± 5.87	55.5 ± 6.06**	51.8 ± 6.96
Assembly score		31.4 ± 5.67	33.5 ± 7.52	29.5 ± 6.93#
Age stratified	**Age group**	**Control**	**Mn-exposure groups**	
			Low	High
Right + Left + Both	18–29	56.9 ± 5.74^{a} (11)	58.1 ± 7.14^{ax} (31)	56.3 ± 8.56^{a,c} (21)
	30–39	52.7 ± 5.77^{a,y} (51)	55.2 ± 5.50^{a} (62)	51.7 ± 6.18^{y} (44)
	≥40	50.3 ± 5.27^{y,d} (36)	53.3 ± 5.03^{a,y} (28)	48.2 ± 4.44^{b,y} (26)
Assembly score	18–29	35.9 ± 5.99^{a} (11)	37.1 ± 7.00^{a,x} (31)	33.6 ± 5.65^{a} (21)
	30–39	31.2 ± 5.29^{a,y} (51)	33.2 ± 7.19^{a,y} (62)	30.3 ± 6.80^{a} (44)
	≥40	30.2 ± 5.56^{a,y} (36)	30.0 ± 7.26^{y} (28)	25.0 ± 5.59^{b,y} (26)

Data represent mean ± S.D. (n). Data labeled with the following letter pairs are significantly different from each other ($p < 0.05$): "a" vs. "b", "c" vs. "d", and "x" vs. "y". Tukey's post hoc analyses were used to verify pair-wise comparisons.
** $p < 0.01$ compared to control.
# $p < 0.01$ compared to low-exposure group.

movement coordination was affected not only directly by age but also by Mn exposure.

The standardized composite or cumulative index (SCI) provides the advantage of combining multiple outcomes into a single parameter regardless of the magnitude of individual test outcomes. By plotting SCI of Purdue pegboard scores as a function of age and exposure in Fig. 1, it was apparent that the SCI-PP significantly decreased as the age advanced. Mn exposure exacerbated the age-related decline in SCI-PP. For example, the older high-exposure subjects (age > 40) produced SCI-PP significantly lower scores than the control subjects in the same age group (Fig. 1). The SCI-PP was also positively correlated to subject's education and income ($p < 0.01$), but inversely related to smoking ($p < 0.05$) (Table 7). No statistical difference, however, was observed in the results of the SCI-PP when stratified for years of employment.

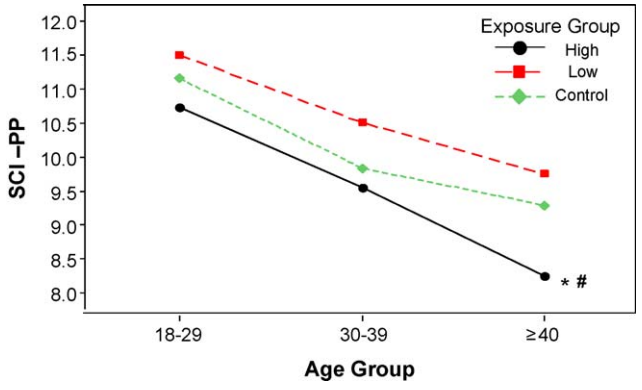

Fig. 1. Effect of age and Mn exposure on standardized composite index of Purdue pegboard scores (SCI-PP). Workers in control ($n = 106$), low-exposure (122), and high-exposure (95) groups were stratified by age (18–29, 20–39, and ≥40). Significance of the comparison was determined by Tukey's post hoc pair-wise comparison for interaction. * $p < 0.05$ compared to high-exposure subjects age 18–29; # $p < 0.05$ compared to both low and control subjects 40 and older.

3.6. Relationship between blood biomarkers and Purdue pegboard scores

Since Mn exposure did not affect the outcomes of groove steadiness, nine-hole steadiness, and Benton memory tests, we chose Purdue pegboard scores to investigate, among three candidate biomarkers (i.e., eMIR, pMIR and MnE), which biomarker was more closely related to the changes of pegboard scores. In the model that included all available independent variables such as age, years of education, sex, income, and years of employment, only the pMIR values were significantly associated with individual PP scores as well as SCI-PP (Table 8).

The cut-off value of a given biomarker suggests a blood parameter above which the subject could be defined as "Mn-exposed" (Cowan et al., in press). For those subjects who had eMIR, pMIR and MnE above the respective COVs, a significant correlation was found between SCI-PP and pMIR (Fig. 2, Table 9). Since age is a main factor in SCI-PP (Fig. 1), all subjects with the age ≥ 40 were analyzed. Only eMIR was inversely associated with SCI-PP ($r = -0.22$); this relationship, however, approached but did not

Table 7
Correlation coefficients among analyzed parameters and SCI-PP.

	SCI-PP	Age	Employment	Education	Smoking	Income
SCI-PP	–	−0.412	0.052	0.318**	−0.186*	0.301**
Age (years)	–	–	0.100	−0.251**	0.513**	−0.164**
Employment (years)	–	–	–	0.164**	0.059	0.223**
Education (years)	–	–	–	–	−0.204*	0.348**
Smoking (pack-years)	–	–	–	–	–	−0.093
Income (RMB)	–	–	–	–	–	–

Data represent Pearson correlation coefficient (r).
* $p < 0.05$.
** $p < 0.01$. SCI: standardized composite index.

Table 8
Relationship between Purdue pegboard scores and biomarkers in presence of independent variables.

Purdue pegboard scores	eMIR				pMIR				MnE			
	Age		eMIR		Age		pMIR		Age		MnE	
	F-Statistic	*P*	*F*-Statistic	*P*	*F*-Statistic	*P*	*F*-Statistic	*P*	*F*-Statistic	*P*	*F*-Statistic	*P*
Assembly	19.32	0.000	0.25	0.619	26.66	0.000	13.38	0.000	21.74	0.000	0.18	0.671
R + L + B	19.33	0.000	0.98	0.322	17.85	0.000	4.19	0.042	21.45	0.000	1.16	0.283
SCI	28.48	0.000	0.15	0.703	33.37	0.000	12.31	0.001	31.98	0.000	0.21	0.644

Each neuropsychological examination was modeled with an identical set of independent variables including: age, sex, income, years of employment and years of education. SCI: standardized composite index; eMIR: erythrocyte Mn–Fe ratio; pMIR: plasma Mn–Fe ratio; MnE: erythrocyte Mn concentration.

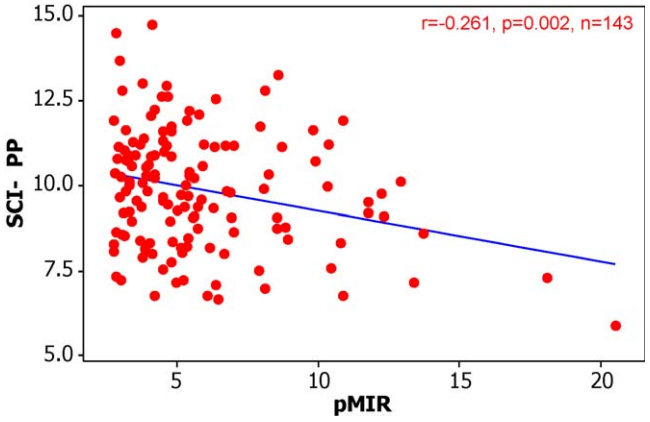

Fig. 2. Changes in SCI-PP as a function of plasma Mn–Fe ratio (pMIR) for subjects with a pMIR greater than the cut-off value (COV) of 2.75 ($n = 143$). Data were analyzed by linear regression ($r = -0.261$, $p = 0.002$).

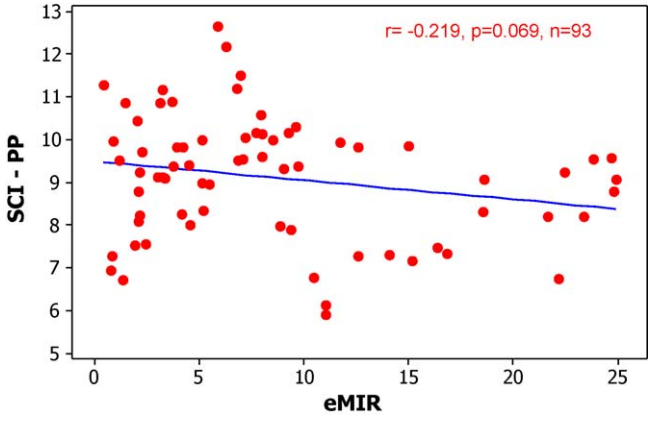

Fig. 3. Changes in SCI-PP as a function of erythrocyte Mn–Fe ratio (eMIR) for subjects of 40 years of age and older ($n = 93$). Data were analyzed by linear regression ($r = -0.219$, $p = 0.069$).

reached the statistical significance ($p = 0.069$) (Fig. 3). When both the COV and age (≥40) were taken into consideration, the pMIR once again showed a significant correlation with SCI-PP ($r = -0.42$, $p = 0.009$) (Fig. 4). MnE was not associated with SCI-PP in any circumstances (Table 9). Thus, both eMIR and pMIR appeared to reflect, to some degree, changes in fine coordinated movement following Mn exposure.

Table 9
Correlation coefficients of biomarkers with Purdue pegboard scores.

	eMIR	pMIR	MnE
Subjects > COV	$n = 136$	$n = 143$	$n = 144$
PP-SCI	0.015	−0.261 ($p = 0.002$)	−0.019
Subjects age ≥ 40	$n = 93$	$n = 93$	$n = 93$
PP-SCI	−0.219 ($p = 0.069$)	−0.103	−0.186
Subjects > COV, ≥40	$n = 31$	$n = 40$	$n = 36$
PP-SCI	−0.256	−0.419 ($p = 0.009$)	0.158

Data represent Pearson correlation coefficient (r). Data analyzed by linear regression. Note: eMIR COV: 8.80; pMIR COV: 2.75; MnE COV: 6.85.

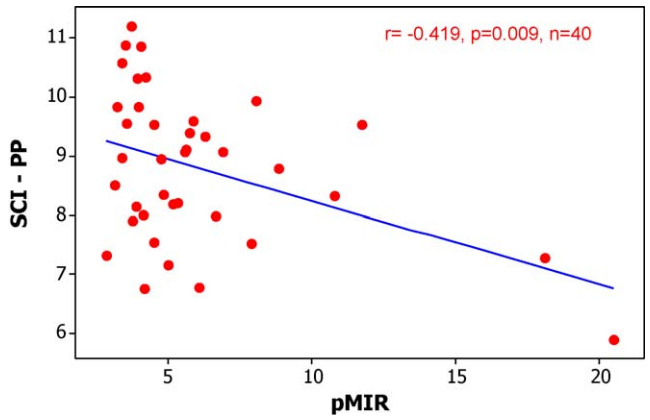

Fig. 4. Changes in SCI-PP as a function of pMIR for subjects with their pMIR values greater than the pMIR COV (2.75) and their age greater than 40 years ($n = 40$). Data were analyzed by linear regression ($r = -0.419$, $p = 0.009$).

4. Discussion

This cross-sectional study utilized four neurobehavioral tests and collected nine different individual measurements in order to characterize the subtle deficits associated with Mn-related neurological impairment. Self-reported symptoms and clinically evaluated signs were minimal and virtually undetectable. Of the 323 subjects examined, less than 2% of subjects in any group tested positive for neurological impairment and less than 10% reported experiencing symptoms of Mn-related deficit. Overall, Mn-related dysfunction was rarely visible to the individual or the clinical practitioner.

Among the four neurobehavioral testing devices used in this study, the Benton memory test did not show any signs of visual retention or short-term memory deficit between the control and high-exposure workers; the workers in the low-exposure group even performed slightly better than those in the control and high-exposure group. Noticeably, decreased memory was self-reported in approximately 15% of the exposure workers, compared to 3% of the control group; this phenomenon, however, was not objectively reflected in the Benton test scores. Thus, the data suggest that Mn exposure among this study population appeared unlikely to result in a detectable short-term memory loss at the time of examination.

Two steadiness platforms were employed in this study to examine static steadiness (nine-hole test) and dynamic steadiness (groove test). Based on the literature, Mn exposure causes action tremor (Cersosimo and Koller, 2006; Olanow, 2004; Roels and Buchet, 1987). Both tests in the current study, however, failed to reveal any impaired steadiness in either static or dynamic state among the smelters. It is quite possible that the stage of Mn exposure-related manifestation in this study population remain too early to develop detectable unsteadiness.

Performing a Purdue pegboard test requires the subject not only have good coordinated fine movement with their arms and fingers, but also possess a good working memory for repeated tasks. The combination of manual dexterity, kinetic steadiness, visual–motor coordination and learning ability for repeated actions makes the PP more complicated and therefore more sensitive than the other three testing devices. As demonstrated in this study, the Purdue pegboard test revealed a remarkable age-related decline in tested subjects regardless of Mn-exposure conditions. Since humans naturally lose their fine movement coordinating ability and their vigor in action as they age, the decline in pegboard performance is not completely unexpected. Within the same age group, Mn exposure evidently worsened the performance. Analysis of the composite pegboard scores (SCI-PP) reinforced the inverse relationship between performance and age as well as smoking (Table 7). Interestingly, the composite scores suggested that the performance was positively associated with workers' education level and income. The latter two factors understandably reflect the socio-economic status of the tested subjects. The smelters working in front of furnace were usually less educated, more labor engaged, and having the less income.

One of the primary goals of this study was to identify a biomarker which reflects subtle functional changes among Mn-exposed workers. Our previous results established that the Mn–Fe ratio in plasma and erythrocytes (pMIR and eMIR) are reasonably effective in differentiating Mn-exposed individuals from unexposed controls. By a generalized linear modeling including all potentially confounding independent variables collected, pMIR stood out as the only parameter that was significantly associated with Purdue pegboard performance when all study participants were taken into account ($p < 0.001$, Table 8). Both pMIR and eMIR have cut-off values above which the tested worker is considered to be Mn-exposed. At the eMIR COV of 8.8, about 88% of the high-exposure smelters had an eMIR above the COV, while 87% of controls had an eMIR below the COV. Similarly, with a pMIR COV of 2.75, 75% of smelters in the high-exposure group were above the COV and 84% of controls were below this value (Cowan et al., in press). The results from this study suggested that both eMIR and pMIR were associated with Purdue pegboard scores under different analytical conditions. Among the subjects whose MIR were above the corresponding COV, pMIR was significantly correlated with pegboard scores, whereas for those whose age were above 40 years old the eMIR, but not pMIR, was associated with the declined pegboard performance. When both factors were taken into account (i.e., age $>$ 40 and MIR $>$ the COV), only pMIR was inversely associated with pegboard scores. Thus, it appeared likely that the eMIR was more sensitive to workers' age than the pMIR, while the pMIR was more generally reflective of functional changes than the eMIR.

From the exposure assessment point of view, the eMIR has advantages over the pMIR for its better correlation to airborne Mn levels and a better accuracy (%) in distinguishing the exposed from the control (Cowan et al., in press). Yet the current results from neurobehavioral functional assessment indeed suggest that the pMIR is a more appropriate biomarker of subtle changes in neurobehavioral function. The eMIR appears to be more related to exposure and the pMIR more related to effect, but why? The pMIR may be reflective of unsequestered Mn and iron capable of crossing the brain-barriers, whereas eMIR is a measurement of sequestered Mn and Fe within the cell. As blood cells break down, Mn levels may increase in the plasma and Fe levels decrease as a function of Mn overload or decreased intestinal absorption of Fe need to meet metabolic demand. Either way, the eMIR and pMIR must be further evaluated, and overall we would recommend that both be used for Mn exposure and health risk assessment.

A main hindrance in manganism diagnosis, therapy and prevention is that at the point where Mn-related dysfunction is visible, the impairment may become so progressive that treatment to reverse the effects is not possible regardless of whether the exposure is eliminated. Thus, a biomarker that can identify exposure at an individual level, prior to the onset of clinical manifestation, can prevent progressive manganism and allow workers to lead healthy and unimpeded lives. Our research with the MIR that combines exposure, biological effect and functional changes appear to shed light on the development of useful biomarker for Mn-induced toxicity. The limitation of this study pertains to our sample size, relatively small number of exposure monitoring data points, and homogenous ethnic study population group. Thus, we strongly recommend that the pMIR and eMIR be further evaluated in other populations for its validity as a biomarker of Mn exposure.

In conclusion, among 323 subjects examined in this study, no significant health issues or neurological dysfunction were identified except for the increased blood pressure in Mn-exposed smelters. Benton visual retention test did not reveal any abnormal memory deficits among Mn-exposed smelters, nor did the groove and nine-hole tests detect any abnormality in dynamic and static steadiness in tested subjects. Purdue pegboard test scores showed a remarkable age-related decline in fine movement coordination among all study participants regardless the Mn-exposure situation. Mn exposure evidently exacerbated this age-related deterioration. Both eMIR and pMIR appeared to reflect, to some degree, changes in fine movement coordination following Mn exposure. Our data suggest that the blood Mn–Fe ratio may serve as a reasonable biomarker for assessment of Mn exposure and health risk.

Conflict of interest

We have nothing to declare in any of the categories for any conflict of interest.

Acknowledgments

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