Final Report

Project ID: R21OH010700

Grants Management Specialist:
Mary Pat Shanahan
Centers for Disease Control and Prevention
Procurement and Grants Office
626 Cochrans Mill Road, Bldg 140
Pittsburgh, PA 15236
Email: mpu0@cdc.gov
Phone: 412-386-4453

Program Official:
Steve Dearwent
Office of Extramural Programs
National Institute for Occupational Safety and Health
Centers for Disease Control and Prevention
1600 Clifton Road N.E., Mailstop E74
Atlanta, GA 30333
Email: sed7@cdc.gov
Phone: 404-498-6382

Title: Bone Manganese as a Biomarker for Early Diagnosis of Manganese Neurotoxicity in Occupationally Exposed Workers

PI: Linda H. Nie, School of Health Sciences, Purdue University; hnie@purdue.edu
550 Stadium Mall Drive, West Lafayette, IN 47906; phone: 765-4942625

Co-investigators: Dr. Ellen M. Wells, School of Health Sciences, Purdue University
Dr. Wei Zheng, School of Health Sciences, Purdue University

Institution: Purdue University, 610 Purdue Mall, West Lafayette, IN 47907

Project starting and ending date: Sep.1, 2014 – Aug.31, 2017, with one year no cost extension

Date of the report: Nov.28, 2017

Table of Contents

List of Terms and Abbreviations:

CEI: cumulative exposure index;
BnMn: bone manganese;
BMn: blood manganese;
FMn: fingernail manganese;
$MnYears_{15:}$ Number of years employed in a job with manganese exposure, summed over the past ~15 years
$MnYears_{TOT:}$ Number of years employed in a job with manganese exposure, summed over the entire work history
$MnCEI_{15:}$ Cumulative exposure index for manganese, summarized over the past ~15 years
$MnYears_{TOT:}$ Cumulative exposure index for manganese, summarized over the entire work history
NAA: neutron activation analysis;
IVNAA: in vivo neutron activation analysis;
HPGe: high-purity germanium detector;
WHO/UCLA AVLT: World Health Organization/ University of California Los Angeles Auditory Verbal Learning Test;
UPSIT-TC: University of Pennsylvania Smell Identification Test – Traditional Chinese Version;
IQR: interquartile range.

Abstract

Occupational exposure to manganese (Mn) occurs among workers involved in welding, smelting, mining, and battery production. In the US alone, millions of workers, including over 300,000 welders, are at high risk of excessive Mn exposure. Mn exposure is associated with many types of diseases, and the most concerned is neurological disorders. In its final stage, Mn toxicity manifests as a neurological disorder termed manganism, which closely resembles Parkinson's disease. The symptoms of chronic Mn toxicity, once established, become progressive and irreversible. Hence, a biomarker for the assessment of cumulative Mn exposure and early diagnosis of Mn neurotoxicity is crucial. In this project, a potential valuable biomarker for long term cumulative Mn exposure was tested among a well-established workers population with a known history of Mn exposure. This biomarker involves measuring Mn in human bone *in vivo*. This technology has been developed and validated in the PI's laboratory for the past seven years. In this study, we recruited 30 workers from ferroalloy factory and 30 workers from manufacturing factory, performed noninvasive bone manganese (BnMn) measurement on these subjects, estimated cumulative exposure index (CEI) of Mn using an existing exposure model, and established the relationship between BnMn and estimated cumulative Mn exposure. We also conducted neurocognitive and neurobehavioral tests on the recruited subjects and determined the relationship between BnMn concentrations and neurological test scores among this population. The results show that BnMn is significantly higher in workers from ferroalloy factory than that in workers from manufacturing factory. We found that there is a significant correlation between BnMn and years of employment as well as 15 years of cumulative Mn exposure index. For the association between BnMn and neurological effects, we found BnMn was significantly associated with decreased cognitive function and decreased motor function. We concluded that BnMn can be used as a good biomarker for cumulative Mn exposure and Mn exposure induced neurological effects. The use of a new biomarker to understand the neurotoxic effects of exposure to Mn is critical to millions of workers who have been exposed to Mn for two reasons. First, neurological disorder is one of the main health issues for these workers; second, neurological impairment reduces the workers' productivity and is a major cause for work related injuries.

Section 1

1.1. Significant or Key Findings

a. Bone manganese (BnMn) is significantly higher in workers from ferroalloy factory than that in workers from manufacturing factory (mean ± error: 10.3±2.7 ppm vs. 3.9±1.5 ppm, $p<0.05$);
b. There is a significant correlation of BnMn with years of employment ($p=0.001$);
c. BnMn was significantly correlated with fingernail manganese (FMn) ($p<0.01$), Number of years working in a Mn-related job in the past ~15 years ($MnYears_{15}$) ($p=0.01$), and a cumulative exposure index for manganese in the past ~15 years ($MnCEI_{15}$) ($p<0.01$); but not with blood Mn (BMn), $MnYears_{TOT}$ (based on lifetime work history), and $MnCEI_{TOT}$ (based on lifetime work history); the association remains after adjusting for age, education, and smoking;
d. BnMn was significantly associated with decreased cognitive function, represented by decreasing AVLT average scores along with decreasing animal naming scores, even after adjusting for confounding factors;
e. BnMn was significantly associated with decreased motor function, represented by decreasing Rhythmic P/S fast, decreasing Rhythmic F-Tap fast, increasing Center Frequency, and increasing tremor intensity, after adjusting for confounding factors.

1.2. Translation of Findings.

Bone Mn measurement can be performed for Mn exposed workers to determine the cumulative Mn exposure for these workers. This is the only method available so far to estimate cumulative Mn exposure for a worker or for a Mn exposed population. With further research, a threshold of BnMn can be determined for removal of the workers from the work environment.

1.3. Research Outcomes/Impact.

The study showed that BnMn is a valuable biomarker for cumulative Mn exposure and for Mn induced neurological effects. Using BnMn as a biomarker, important occupational/ environmental health research can be conducted to study Mn toxicity and neuro-toxicity of exposure to multiple metals. A research proposal was submitted to NIOSH by the PI and the same research team to study neurological effects of combined exposure to Mn, Pb, and Al using bone Mn/Pb/Al as biomarkers.

Section 2

2.1. Background for the project

Manganese (Mn) is an essential micronutrient for human health. It plays a vital role in bone growth, blood sugar regulation, immune cell maintenance and metabolism of lipids, proteins, and carbohydrates (Hurley et al., 1987). Mn can cause adverse health effects when the intake is either too low or too high. Mn deficiency cases are rare, as dietary Mn typically provides sufficient Mn intake. However, Mn overexposure and associated detrimental health effects have been documented, which we will briefly review in the following section. The main source of clinically diagnosed Mn intoxication is from occupational exposure. As the fourth most widely used industrial metal, the inhalation exposure to airborne Mn presents high risks to workers such as miners, ferroalloy smelters, and welder (Bouchard et al., 2011, Couper, 1837, Cowan et al., 2009, Lu et al., 2005, Wang et al., 1989). In addition, there are many environmental sources of Mn which can lead to overexposure in the general population. These sources include pesticides, contaminated water, milk or food (Bouchard et al., 2011, O'Neal and Zheng, 2015), and gasoline additives (Butcher et al., 1999).

Mn intoxication is primarily associated with neurological disorders (Racette, 2014, Rodier, 1955, Sassine et al., 2002, Wennberg et al., 1991). Generally, the appearance of constant pattern of behavioral or cognitive abnormalities only becomes obvious after several years of exposure (Iregren, 1998). The signs and symptoms include poor eye-hand coordination, bradykinesia, reduced cognitive function, as well as tremor with voluntary movements. In severe cases, a devastating neurological impairment called "manganism" occurs, a syndrome which closely resembles but is not identical to Parkinson's disease (PD) (Crossgrove and Zheng, 2004, Emara et al., 1971, Ky et al., 1992, Levy and Nassetta, 2003, Mena et al., 1967, Nelson et al., 1993, Rodier, 1955, Schuler et al., 1957, Wennberg et al., 1991, Whitlock et al., 1966, Williams et al., 2012). Some studies found that the cases of manganism were more likely to happen with patients of prolonged exposure history, which suggested that development of the disease is related to cumulative exposure (Rodier, 1955, Schuler et al., 1957).

Commonly used Mn biomarkers include blood, urine and saliva. Because of the short half-life and large intracellular distribution of Mn, results of Mn concentration in these biometrics are highly variable and are often found to be not significantly associated with Mn-induced neurological effects (Bader et al., 1999, Crossgrove and Zheng, 2004, O'Neal and Zheng, 2015, Santos et al., 2014, Wongwit et al., 2004, Zheng et al., 2000). Thus, using Mn levels in blood, urine or saliva as the biomarkers of Mn deposition are generally not recommended (O'Neal and Zheng, 2015). Hair and nail Mn have also been proposed as Mn biomarkers. However, data show large variations among individuals, although the group-based data appear to be more useful (Bader et al., 1999, Wongwit et al., 2004). In addition, hair and nail are potentially subject to external contamination, and hair and nail Mn only reflect the exposure for the past several months. Magnetic resonance imaging (MRI) technology has provided evidence of Mn deposition in the brain even in the absence of clinical symptoms of Mn toxicity (Dydak et al., 2011, Jiang et al., 2006). While brain Mn accumulation can be reflected by an increased MRI signal, one downside of the technology is that Mn may be removed from the brain after a short period of time (within months) (Arjona et al., 1997, Kim et al., 1999). Hence, brain Mn concentrations obtained using MRI only reflect recent Mn exposure. In addition, the MRI signal of Mn in brain are not specific to Mn, because the signal is not an intrinsic property of the Mn ion alone (Fitsanakis et al., 2006). Overall, to date, the lack of a reliable body Mn burden biomarker limits the capacity for cumulative Mn exposure assessment.

Bone is a promising potential biomarker for Mn exposure assessment. Around 40% of human body Mn is stored in bone (Liu et al., 2014). Derived from the value provided by

International Commission on Radiological Protection (ICRP) 23 and ICRP 70 (Group and Snyder, 1975, Valentin, 2002), there is approximately 1 μg Mn per g dry bone, which is equal to approximately 5 μg Mn per gram of Ca. In a recent rat study, the data revealed that the half-lives of Mn in various rat bones were between 77 and 690 days with an average of 143 days in the whole rat skeleton. Taking into account that every 16.7 days of a rat's life is equal to one human year, the half-life of Mn in human bone can be estimated as from 4.6-41.3 years, with an average of 8.6 years in the whole human skeleton (O'Neal and Zheng, 2015, Sengupta, 2011). Because bone is one of the main long-term storage organs for Mn in humans, it is logical to propose that bone manganese (BnMn) is a relevant and valuable biomarker for assessment of cumulative Mn exposure.

Over the past several years, we have developed the method to measure BnMn using neutron activation analysis (NAA), which is a powerful modality for noninvasive *in vivo* elemental measurement (Liu et al., 2013, Liu et al., 2014, Liu et al., 2017). The general principle of NAA is to convert the stable isotopes of some elements into unstable radioisotopes; the decay information can then be collected for analytical purposes. The stable isotope of Mn, ^{55}Mn, with its natural abundance of 100% and its relatively large thermal neutron capture cross section, can be readily activated by low energy neutrons. The resulting ^{56}Mn nucleus is radioactive and decays to excited state of ^{56}Fe by the emission of a β- particle. This is followed by the internal transition of ^{56}Fe from an excited state to a ground state, which emits an 847 keV γ-ray. The branching ratio of 847 keV γ-ray is 98.8%. By measuring the 847 keV characteristic γ counts, concentration of ^{55}Mn in the sample can be determined. We have developed and characterized a NAA system with Mn detection limit of 0.64 μg Mn per g bone (ppm) (Liu et al., 2017). Another *in vivo* NAA (IVNAA) system has also been developed in other labs (Aslam et al., 2009) that used a large accelerator. In contrast, the neutron generator utilized in our lab is much more compact, and hence it is transportable. In this project, the IVNAA system was shipped to China for use in an epidemiology study.

This study was an interdisciplinary collaboration between different research groups including researchers from medical physics, occupational/environmental health, epidemiology, and metal toxicology. In this report, we describe the project in four aspects: a) the bone Mn technology and BnMn in Mn-exposed workers and controls; b) correlation between Mn biomarkers (bone Mn, blood Mn, fingernail Mn, and cumulative exposure index (CEI)); c) association between Mn biomarkers, especially BnMn, and neurocognitive function; d) association between Mn biomarkers, especially BnMn, and motor function.

2.2. Specific Aims

Specific Aim 1: To test the hypothesis that BnMn is significantly correlated with cumulative Mn exposure. To test this hypothesis, we did the following: a) recruited 31 workers from a ferroalloy factory and 30 controls from a manufacturing factory in Zunyi, China; b) performed noninvasive bone Mn measurement on these subjects and assess the differences in BnMn levels between the ferroalloy worker population and the control population; c) collected airborne Mn concentrations and blood Mn information for these subjects; d) estimated cumulative Mn exposure using work history information; e) established the relationship between Mn biomarkers and the estimated cumulative Mn exposure.

Specific Aim 2: To test the hypothesis that Mn accumulation in bone is positively associated with neurological disorders by using validated neurocognitive and neurobehavioral test batteries. To test this hypothesis, we did the following: a) conducted cognitive and motor performance tests on the subjects described above; b) determined the relationship between BnMn concentrations and neurobehavioral test scores among the exposed and control groups using multivariable statistical models.

2.3. Methodology

2.3.1. *In Vivo* Neutron Activation Analysis System (IVNAA)

Details regarding the NAA system design and setup can be found in our previous publications (Liu et al., 2017, Liu et al., 2014). In short, the system consists of a customized compact deuterium-deuterium (DD) neutron generator, a customized moderator/ reflector/ shielding assembly, and an HPGe detection system with a 100% relative high efficiency detector (GMX100P4-95; Advanced Measurement Technology, Oak Ridge, TN).

The DD neutron generator used in this study produces 2.45 MeV neutrons in an approximately isotropic manner. Because the interaction cross section $\sigma(E)$ (and hence the probability to produce Mn γ-ray signal) increases with the decrease of the energy of the incident neutron, moderator and reflector were used to maximize the number of low energy neutrons presents in the irradiation cave. Additional shielding materials were added to minimize the neutron as well as photon dose.

To minimize the radiation dose to the subject for *in vivo* elemental quantification, Mn was measured in the hand bones. The participant's arm was extended away from the body as their hand was placed in an irradiation cave. Meanwhile, the rest of the body was shielded from the neutron beam. The hand was irradiated for 10 minutes and then the participant was transited to the HPGe detector system for a 60 minutes measurement, with spectra saved at 5 minutes to obtain the calcium (Ca) counts and 60 minutes to obtain the Mn counts. The time between the end of the irradiation and the beginning of measurement was fixed at 5 minutes. Figure 1 (a) shows a participant's hand being irradiated. Figure 1 (b) shows the HPGe measurement system, with the measurement cave clearly seen. Both systems were covered in customized wooden plates to make them more user-friendly.

(a) (b)

Figure 1. The neutron irradiation system (a) and the HPGe measurement system (b).

2.3.2. *Mn-doped Bone Equivalent Phantoms and System Calibration*

We created bone equivalent phantoms with Mn concentrations of 0, 5, 10, 15 and 20 ppm. The matrix of the phantoms contains the same amount of calcium (Ca) and other elements to mimic the real bone, as described previously (Liu et al., 2014, Liu et al., 2013). The phantoms are used to calibrate the system for *in vivo* BnMn measurement. The net counts from both the Mn and Ca 3084 KeV characteristic γ -rays were calculated by an in-house spectral analysis program. The ratio of Mn/Ca were calculated and plotted against the Mn concentration for system calibration. Because the activated Mn and Ca counts came from the same neutron beam (with thermal neutrons as the major component) and Ca concentration is the same in each phantom and presumably in the bone in *in vivo* measurements, the fluctuation of activated Ca counts reflects the variation of neutron flux, hand palm attenuation, and counting geometry factors. Therefore, Mn/Ca ratio was used for system calibration, and normalizing the Mn signal

to Ca signal is expected to correct for the variation of neutron flux, hand palm attenuation, and counting geometry factors.

2.3.3. *Spectrum Analysis*

The γ-rays emitted from irradiated phantoms or human bone were detected by an HPGe detector system. The signals were collected and processed by DSPEC plus digital pulse processing system and Maestro γ-ray spectroscopy. The raw data were imported to MATLAB for Gaussian peak analysis. Levenberg-Marquardt method was used for calculation of net peak area. Least-square algorithm was used to determine the goodness of fit for peak fitting. Multi-peak fitting was applied to differentiate the peaks between 847 keV of Mn and 844 keV of Mg.

2.3.4. *Study Population*

The human study was approved by Purdue Institutional Review Board (IRB) and Zunyi Medical College Ethical Review Board (ERB). Participants signed an informed consent document prior to participation in the study. Sixty-one male workers were recruited as exposed and control groups from a ferroalloy factory (n=31, exposed) and a manufacturing facility (n=30, control) respectively. Exclusion criteria included individuals who had cognitive symptoms, active neurological or psychiatric disease, and movement impairments that had known causes which were not related to manganese exposure. No participant was excluded based on these exclusion criteria. One worker from the ferroalloy factory did not complete BnMn measurements and was excluded from further analyses, leaving a total of 60 participants. The participants completed the BnMn NAA measurement, had a blood sample collected to test for BMn, had fingernails collected to obtain concentration of Mn in fingernails (FMn) and completed a questionnaire. The questionnaire was administered by study staff to obtain data on demographics and work history.

2.3.5. *Bone and Blood Mn Measurement and Analysis*

Before a bone Mn measurement, the participant's right hand and lower arm were thoroughly washed with soap. A trained research assistant then cleaned these areas with 50% alcohol to further reduce or eliminate Mn contamination. The participant was guided to sit on a chair in front of the neutron irradiation system and to place his right hand inside the irradiation cave. A bag filled with water was wrapped around the participant's arm to fix the arm and to further reduce the neutron dose to the rest of the body.

The hand was irradiated for 10 minutes to activate the ^{55}Mn atoms in the hand bone to ^{56}Mn. The hand equivalent dose was measured as less than 50 mSv and whole body effective dose was estimated to be about 17 μSv (Liu *et al*. 2014). The participant moved to another laboratory with a high purity germanium (HPGe) detector to collect the γ ray signals released from the hand bone. A spectrum from a 5 minute measurement was collected to obtain signals from short-lived radionuclides and a spectrum from a 60 minute measurement was collected to obtain Mn and Ca signals. The spectra were analyzed and the BnMn concentrations were calculated using the calibration line obtained from the Mn-doped bone equivalent phantoms.

A trained phlebotomist collected a whole blood sample from the participant using standard protocols and a trace-metal-free vacutainer. The participant's skin was cleaned with an alcohol swab before sampling. All the samples were frozen and kept at -80 °C immediately after the collection. Blood samples were shipped to the Chinese Centers for Disease Control and Prevention in Beijing for assessment of blood BMn using inductively coupled plasma mass spectrometry (ICP-MS) (Ding et al., 2012). Briefly, the collected blood samples were digested in 0.5% ultrapure nitric acid. The samples were then diluted and analyzed using XSERIES 2 ICP-MS (Thermo Fisher, USA) (Zhang et al., 2015).

2.3.6. *Years of Occupational Mn Exposure and Cumulative Exposure Index*

Work history and demographic information were collected using a questionnaire administered by study staff. For current and past jobs, participants reported their job titles, employer, and dates of employment. Work history was used to create two cumulative occupational Mn exposure indices (MnCEI) using methods adapted from prior research (Ramlow et al. 1996, Fayerweather et al. 1997). One index included the entirety of each participant's work history ($MnCEI_{TOT}$). The second incorporated jobs held after January 1, 2000 ($MnCEI_{15}$); this was an average of 16.1 (SD: 0.2) years prior to the study date. This date was chosen based on the $t_{1/2}$ of BnMn (~8-9 years) with the assumption that ~16 years would represent approximately two $t_{1/2}$ for BnMn and reflect a period where a substantial amount of Mn could be expected to remain in bone tissue.

To calculate the MnCEI, past and current jobs were first categorized into 20 occupational groups based on Bureau of Labor Statistic's Occupation Finder (Bureau of Labor Statistics. 2015). These 20 occupational groups were divided into 3 exposure categories (high, medium, and low) based on potential for occupational Mn exposure. Determination of exposure category for each occupational group was based on previously published data on air Mn concentrations (Cowan et al. 2009a; Hedmer et al. 2014; Myers et al. 2003; Sierra et al. 1995; Westberg et al. 2001; Zayed et al. 1996). Both the $MnCEI_{TOT}$ and $MnCEI_{15}$ were calculated with the following equation, where rank = the exposure groups (high = 3, medium = 2, low =1) for each job and Y = years employed in the job.

$$MnCEI = \sum_{i=1}^{n} rank_i Y_i$$

The difference in calculation of the two CEIs is that for $MnCEI_{TOT}$ n=total number of jobs and for $MnCEI_{15}$ n=total number of jobs after January 1, 2000.

A variable for total years of occupational Mn exposure was also created for participants' entire occupational history and for the previous 15 years. This is the sum of years employed in a current or past job with the potential for Mn exposure. The potential of Mn exposure was based on the same methods used for calculating the CEI. Jobs were considered to have Mn exposure if the exposure rank was ≥2.

2.3.7. *Cognitive and Olfactory Testing Procedures*

A group of trained research assistants conducted individual neuropsychological assessments on participants that included the animal and fruit naming tests of semantic verbal fluency, the World Health Organization/University of California Los Angeles Auditory Verbal Learning Test (WHO/UCLA AVLT), and the University of Pennsylvania Smell Identification Test – Traditional Chinese version (UPSIT-TC). In Animal/Fruit Naming tests, participants were asked to name as many 1) animal, and then 2) fruits that they possibly could in 1 minute (Tombaugh et al. 1999; Bowler and Lezak, 2015). The WHO/UCLA AVLT is a test of verbal learning and retention (Bowler et al. 2017; Maj et al. 1993). Participants were given 15 common words and asked to repeat the list of words during five acquisition (learning) trials retention (Bowler et al. 2017). After the five acquisition trials, a new interference list was given and participants were yet again asked to repeat the list of words back to the trained research assistant (trial 6) (Bowler et al. 2017). Finally, participants were asked to repeat as many words from the original acquisition list in a post-interference recall trial (trial 7) (Bowler et al. 2017). The UPSIT-TC test consisted of four booklets each containing 10 odors (Jiang and Liang 2016). Participants release each scent (N=40) using the tip of a pencil and are required to choose from 1 of 4 possible scents given (Jiang and Liang 2016).

2.3.8. *Motor Testing Procedures*

Participants completed the CATSYS battery and the Purdue Pegboard test with the aid of trained research assistants. The CATSYS 2000 system (Snekkersten, Denmark) is a computerized test that assess tremor, postural stability, reaction time and coordination CATSYS

testing procedures are explained in depth elsewhere (DPD 2000; Ellingsen et al. 2015). The standard CATSYS battery was used in this study. A tremor-sensitive stylus (Figure 2: 5) was used to assess tremor in participants' hands. While sitting in a chair, participants held the stylus like a normal pen about a hand-length in front of their waist. Participants were required to hold the stylus as still as possible, without any support, for approximately 10 seconds. This was repeated for both the right and left hand. Four tremor values were produced for each hand: tremor intensity, central frequency, harmonic index, and tremor deviation.

Postural stability was assessed using a force plate (Figure 2: 1) that records vertical forces in 3 points to determine the position of the force center on the plate. Participants were asked to stand on the plate without their shoes and stare ahead to a spot on the wall. Four balance tests, each running for one minute, were performed: SWAY 1) eyes open on plate; SWAY 2) eyes closed on plate; SWAY 3) eyes open on plate with ~ 1cm polystyrene foam placed on top of plate; and SWAY 4) eyes closed with ~ 1cm polystyrene foam placed on top of plate. Scores from the normal conditions (test 1) were reported in this study.

Coordination and controlled movement was assessed using a recording drum (Figure 2: 4) with a microphone inside that records hand movement. To ensure accurate testing, the drum was placed on a computer mousepad on top of a desk. Tests performed using this drum include the hand pronation/supination tests, finger tapping tests, and max frequency tests. For the protonation/supination (P/S) tests, participants were asked to hit the drum palm up and then palm down in tune with the provided beep for 20 seconds. The first test was at a slow pace of ~ 1 hit/second (beep). The second test was at a faster pace where participants were asked to hit the drum in time with the beep. These tests were repeated for both hands. For the finger-tapping (F-Tap) tests, the methods were repeated using participants' index finger. The outcomes produced for each hand were Rhythmic P/S Slow, Rhythmic P/S Slow Standard Deviation, Rhythmic P/S Fast, Rhythmic P/S Fast Standard Deviation, Rhythmic F-Tap Slow, Rhythmic F-Tap Slow Standard Deviation, Rhythmic F-Tap Fast, and Rhythmic F-Tap Fast Standard Deviation.

For the max frequency tests (Max Frequency P/S and May Frequency F-Tap) participants were required to perform the movements for as long as they could in time with the metronome beep as it increased in pace. Outcomes were produced for each hand. Reaction time was assessed using a reaction handle (Figure 2: 3). Participants used their thumb to press the black button on the handle in response to a beep from the system. This test was repeated for both the right and left hand.

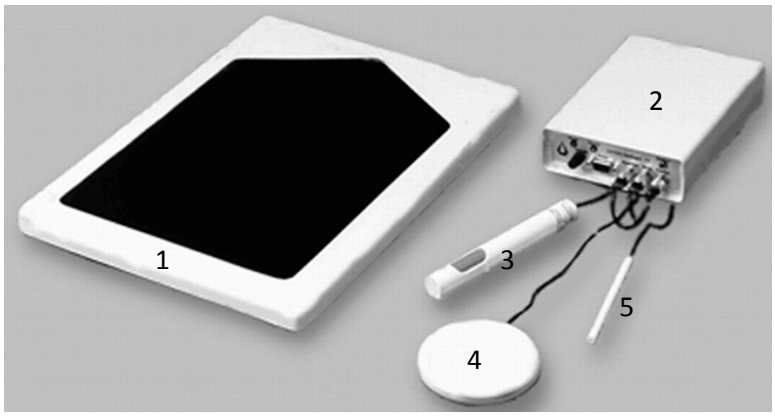

Figure 2. CATSYS motor system (DPD 2000).
1: Force Plate; 2: Data Logger; Reaction Handle; 4: Recording Drum; 5: Tremor-sensitive Stylus

The Purdue Pegboard (Lafayette Instruments, Lafayette, IN, USA) test (Figure 3) is a test of manual dexterity that has been described elsewhere previously (Cowan et al. 2009). The pegboard procedure consists of 4 operations that focus on the coordination of 1) the right hand;

2) the left hand; and 3) both hands. For the first test, participants must place as many pegs down the right side of the board as they can in 30 seconds using just their right hand. This is repeated for the second test using just their left hand down the left side of the board. For the third test, participants use both hands to place as many pegs down both sides as they can in 30 seconds. For the fourth test, participants are required to construct as many assemblies, made from pins, washers, and collars, as they can in 1 minute. This test was repeated twice and an average score was provided.

Figure 3. Purdue Pegboard motor system (LIC 2009).

2.3.9. *Statistical Analysis*

All statistical analyses were completed using Stata 13.1 (College Station, Texas, USA) or MatLab. For all the data analysis in this report, a significant level is defined as a confidence level >95% (or $p<0.05$), and a marginally significant level is defined as a confidence level of >90% and <95% (or $0.05<p<0.1$). One participant from the ferroalloy factory did not complete the BnMn measurement; thus, most analyses are limited to N=60. Study protocols were translated to Mandarin then back-translated to English to check the accuracy of the data prior to use. Age and years of education attained were collected from participants' completed questionnaires. Tertiles of BnMn and total years of occupational Mn exposure were created. BnMn, fingernail Mn (FMn), and blood Mn (BMn) concentrations were lognormally distributed; therefore, summary statistics are presented as medians and interquartile ranges (IQRs) and a natural log transformation of these variables was used in analyses. A constant of 5.99 was added to all BnMn concentrations to ensure all values were positive prior to the log transformation (Atkinson, AC. 1994). This method would affect measures of central tendency for BnMn but not its correlations or associations with other variables.

Mean and standard deviations (or median and IQRs) for demographic and Mn exposure variables were reported for each tertile of BnMn as well as for the entire population. One-way ANOVA was used to assess whether these variables were significantly different between BnMn tertiles. Scatter plots with linear regression fit lines were created to show the relationship between BnMn with other measurements of Mn exposure. Unadjusted and adjusted linear regression models were created to determine the association between lnBnMn (continuous) and BnMn tertiles (categorical) with each alternative measurement of Mn exposure. Covariates included in the adjusted models were age (continuous) and education (continuous).

Animal and fruit naming scores were reported as the number of correct words a participant gave. A difference score, between trials 5 – 1 (AVLT Dif.), as well as a five-trial average score (AVLT Avg.) were created for the WHO/UCLA AVLT to show the number of words learned across the five acquisition trials (Bowler et al. 2017). Number of correct words and number of intrusions from the interference list (trial 6) and recall list (trial 7) were also reported.

Mean and standard deviations (SDs) for cognitive and olfactory tests were reported for each tertile of BnMn as well as for the entire population. A linear regression p-trend was used to

assess any statistical difference between BnMn tertiles for each variable. Due to the translation of BnMn, biomarker concentrations for the total population were summarized using medians and interquartile ranges (IQR) to represent the original distributions. Medians and IQRs of untransformed BMn and FMn were also reported by tertiles of BnMn as well as for the entire population.

Unadjusted scatter plots with linear regression fit lines were created to show a visual representation of the relationship between all 3 naturally log transformed biomarkers and neurological test scores. Spearman correlations were performed to assess the association between potential covariates (age, education, factory of employment, length of time in current position, current smoking status, current drinking status), Mn biomarkers (BnMn, FMn, BMn) and test scores (Animal Naming, Fruit Naming, AVLT Avg. AVLT Dif., AVLT Trial 6, AVLT Trial 6 Intrusions, AVLT Trial 7, AVLT Trial 7 Intrusions, UPSIT). Unadjusted and adjusted regression models were created to assess how well naturally log transformed BnMn (continuous) and tertiles of naturally log transformed BnMn (categorical) predict the cognitive and olfactory test scores. Beta-coefficients (β) and 95% Confidence Intervals (C.I.s) were reported. Covariates included in the adjusted models were age (continuous), education (continuous), and current factory of employment (dichotomous). Olfactory models were also adjusted for current smoking status (dichotomous). These covariates were based on spearman correlations, model coefficients of determination, and previous literature (Bowler et al. 2007).

For the motor function, locally weighted scatter plot smoothing (LOWESS) curves were created to assess the possible non-linear relationship between the biomarkers and the test scores. Based on these graphs, it was shown that for many of the test scores, the linear relationship with Mn changed as the concentration increased. As a result, 3 linear splines of the Mn biomarker were created based on each LOWESS curve. This allowed for a piecewise estimation of the relationship between the Mn biomarkers and the test scores. Outliers were removed from each model based on the LOWESS curve. A data point was considered an outlier if it skewed the curve for all 3 biomarkers. Removing these outliers did create a smoother LOWESS curve. Spearman correlation coefficients (ρ) and p-values (p) were reported for the Spearman correlations. Due to the number of outcomes, the chances of a significant p-value being solely due to chance increases. Therefore, a more stringent *p*-value < 0.01 was considered statistically significant for the Spearman correlations in this case. Beta-coefficients (β) and 95% Confidence Intervals (C.I.s) were reported for the regression models.

2.4. Results and Discussion

2.4.1. *Phantom Spectrum vs. In Vivo Spectrum*

To verify that the phantom is a good representative of hand bone, the spectra of phantom measurement and *in vivo* measurement have been compared. Figure 4 shows the 60 minute measurement of a 20 ppm Mn phantom and the 60 minute *in vivo* measurement of human hand after 10 minutes of irradiation and 10 minutes of decay. The Mn peak is also shown as an enlarged section in the plot. Most of the large peaks are from natural radioisotopes. The background region at the Mn peak

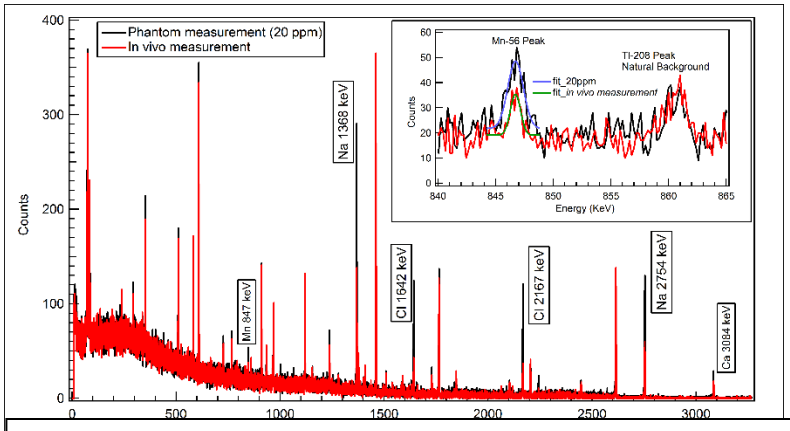

Figure 4. Spectra collected by HPGe detector for a 60 minute measurement of a 20 ppm Mn phantom and a 60 minute *in vivo* measurement of human hand with BnMn concentration of 20.6 ppm

for phantom is similar to that for the *in vivo* measurement. In addition, Ca peaks are comparable to each other. However, the peaks for Na and Cl are significantly different, which indicates that the Na and Cl added to the phantom may not be equivalent to concentrations in real bone.

2.4.2. *Calibration Line*

The calibration line for the system was established from a set of Mn-doped bone equivalent phantoms. Each of the phantoms was placed in the irradiation cave and irradiated for 10 minutes. During the neutron irradiation, Mn along with other elements such as sodium (Na), chlorine (Cl), magnesium (Mg) and calcium (Ca) were activated and characteristic γ-rays were emitted. The activated ^{56}Mn had a half-life of 2.58 hours, which gave us sufficient time to transfer the phantom from irradiation site to measurement site. A 5-minute spectrum was collected using the 100% high efficiency HPGe detector after a 5-minute decay to collect the spectrum for short-lived radioisotopes, such as ^{28}Al, which will not be discussed in this report. After the 5-minute spectrum collection, another 60-minute spectrum was collected to obtain Mn and Ca signals. The activation product for Ca was ^{49}Ca which decays with a half-life of 8.8 minutes. Although 60 minutes is not an ideal time period to collect Ca signal, using the data collected from the same time period will make the Mn/Ca ratio more consistent and does not impact calculation of Ca because of the low background at the energy range of the Ca peak. Table 1 shows the Mn counts under 847 keV and Ca counts under 3084 keV for 60 minute measurements. The uncertainties for the counts were calculated from the peak fitting routine, and the uncertainties for the Mn/Ca ratios were calculated from the uncertainties of the Mn and Ca γ-ray counts.

Table 1. The Mn, Ca counts and Mn/Ca ratio of the Mn-doped bone phantoms from 60 minute measurements.

Phantom	^{56}Mn	^{49}Ca	Mn/Ca Ratio
0 ppm	-6±18	385±7	-0.01±0.07
5 ppm	87±19	295±6	0.29±0.06
10 ppm	137±21	387±6	0.35±0.05
15 ppm	193±23	326±6	0.59±0.07
20 ppm	240±21	324±6	0.74±0.06

1 ppm = 1 μg/g phantom . Values are counts or ratio ± uncertainty

The calibration line of Mn/Ca ratio vs. Mn concentration is illustrated in Figure 5. This was used to calculate the BnMn concentrations from the human study.

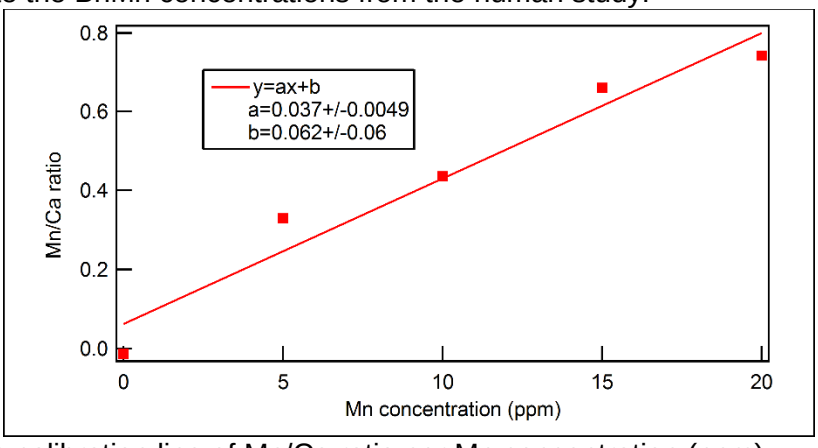

Figure 5. The calibration line of Mn/Ca ratio per Mn concentration (ppm).

2.4.3. *Participant Demographics and Mn Biomarkers*

Data in Table 2 summarize the study population's age, education, years of employment at their current job, as well as BnMn and BMn concentrations. Among all 60 participants with data on BnMn, age was 47.4±7.9 years (mean±standard deviation), education was 10.0±3.9 years, and years of employment in their current job was 9.0±6.8 years. The mean of all three variables was slightly lower in exposed versus control groups, but these differences were not statistically significant.

Table 2. *Population Characteristics.*

	N	Minimum	Maximum	Median	IQR	Mean	SD
(Ferroalloy factory (exposed)							
Age (years)	30	33	58	46	8	46.7	6.1
Education (years)	30	1	15	9	6	9.1	3.6
Years of Employment	30	0.2	18.0	9.0	4.0	8.4	4.1
Blood Mn (μg/L)	30	9.6	39.7	15.2	5.9	15.9	5.6
Bone Mn (μg/g dry bone)	30	-5.0	43.0	3.1	16.3	10.3	14.5
Manufacturing factory (control)							
Age	30	29	62	51	17	48.2	9.4
Education (years)	30	2.5	17	11	5	10.9	4.0
Years of Employment	30	1.0	38.0	5.5	6.3	9.5	8.7
Blood Mn (μg/L)	30	8.4	22.4	13.5	3.1	13.2	3.3
Bone Mn (μg/g dry bone)	30	-4.4	27.0	0.9	4.6	3.9	7.9

SD = Standard deviation; IQR = Interquartile range. Years of employment refers to current position.

Among all participants, the median±interquartile range of Mn biomarkers were 14.1±3.9 μg/L for BMn and 2.6±7.2 μg/g for BnMn. Both median BnMn and BMn were higher in the exposed group versus the control group, although this was of borderline significance for BnMn (BnMn *p*=0.056; BMn *p*=0.033). Both mean BnMn and BMn were significantly higher in the exposed group versus the control group (BnMn p=0.037; BMn p=0.029). The variability in BnMn concentrations is also substantial: the IQR is 16.3 μg/g in the exposed group and 4.6 μg/g in the control group.

2.4.4. *Correlation Between Mn Biomarkers and Years of Employment*

Spearman correlation coefficients between exposed/control status, years of employment, BMn, and BnMn are shown in Table 3. There is a significant correlation between exposed/control status and BMn (p=0.032) and a marginally significant correlation between exposed/control status and BnMn (p=0.055). There is also a significant correlation of BnMn with years of employment (p=0.001), but the correlation of BMn with years of employment is only marginally significant (*p*=0.068). BMn and BnMn were not significantly correlated with each other (p=0.226).

Table 3. Correlations between Factory of Employment, Years of Employment, BMn, and BnMn,

	Factory of Employment	Years of Employment	BMn	BnMn
Factory of employment	--			
Years of employment	0.118 (0.371)	--		
BMn	0.277 (0.032)	0.268 (0.068)	--	
BnMn	0.249 (0.055)	0.409 (0.001)	0.159 (0.226)	--

N=60. Values are Spearman's ρ (*p*-value).

Results from unadjusted regression models and regression models adjusted for age and education are presented in Table 4. Regression model results do not vary substantially after adjustment. Employment at the ferroalloy factory (vs. the manufacturing facility) and years of employment in the current job, but not ln(BMn) are statistically significant predictors of ln(BnMn). Employment in the ferroalloy factory is related to higher ln(BMn), but is of borderline statistical significance following adjustment for age and education (unadjusted p=0.017; adjusted p=0.058). Years of employment in the current job is not a significant predictor of ln(BMn) (unadjusted p=0.413; adjusted p=0.372). To further clarify the result, ln(BnMn) was plotted against year of employment, as shown in Figure 6, with ferroalloy and manufacturing factories presented separately.

Table 4. Results from Regression Models Predicting ln(BnMn)

Independent variable	Unadjusted Model			Adjusted Model		
	β	95% CI	*p*	β	95% CI	*p*
Factory of employment	0.37	-0.05, 0.78	0.080	0.44	0.01, 0.87	0.047
Years of employment	0.05	0.02, 0.08	0.001	0.05	0.02, 0.08	0.002
ln(BMn)	0.46	-0.30,1.22	0.233	0.54	-0.27, 1.36	0.188

CI = Confidence interval. N=60. Adjusted models include age and education as covariates.

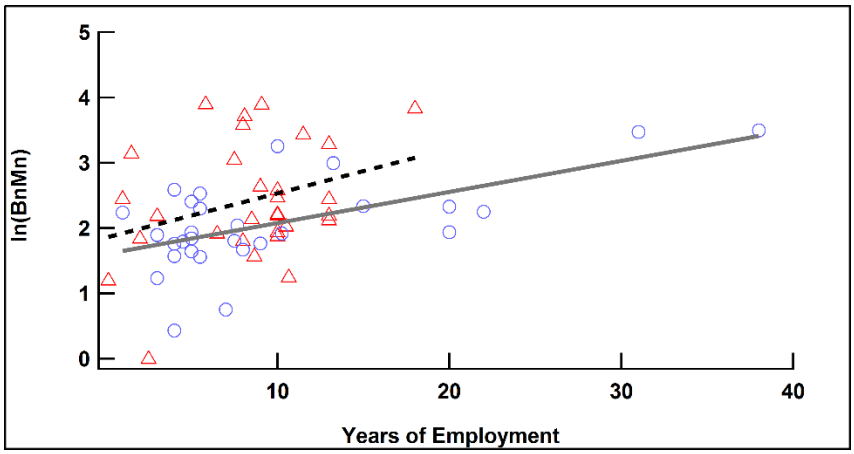

Figure 6. Natural-log transformed bone manganese (ln(BnMn) by year of employment at current job. Circles represent manufacturing factory (control) participants; triangles represent ferroalloy factory (exposed) participants. The solid and dashed lines represent the unadjusted linear association of ln(BnMn) by years of employment among those from the manufacturing factory (control) and ferroalloy factory (exposed), respectively.

2.4.5. *Correlation between BnMn and other Mn biomarkers (blood Mn, fingernail Mn, and Mn CEI)*

Table 5 shows summary measures for age, education, years in current occupation, years of total occupational Mn exposure, years of occupational Mn exposure within the past 15 years, $MnCEI_{TOT}$, and $MnCEI_{15}$ by tertiles of bone manganese. Mean (SD) for age, education, total years of occupational Mn exposure, and $MnCEI_{TOT}$ were not significantly different between BnMn tertiles. The number of years in the participant's current occupation was significantly different between BnMn tertiles (p = 0.006): mean years in current occupation for the highest tertile of BnMn (11.4 years, SD=9.0) was more than double that of the lowest tertile of BnMn

(5.6 years, SD=2.9). Additionally, both $MnYears_{15}$ (p = 0.01) and $MnCEI_{15}$ (p = 0.002) significantly increased as BnMn increased.

The range of Mn biomarkers were 8.43 to 39.70 μg/L for BMn, 0.15 to 935.65 μg/g for FMn, and -5.00 to 43.02 μg/g for BnMn. Median (interquartile range) BMn, FMn and BnMn were 14.1 (4.0) μg/L, 6.1 (39.8) μg/g, and 2.6 (7.2) μg/g, respectively (Table 5). Both FMn (p = 0.003) and BnMn (p <0.001) were significantly different between BnMn tertiles. Median FMn increased by 0.3 μg/g from tertile 1 to 2 and 61.3 μg/g from tertile 2 to 3. Median BnMn increased by 3.36 μg/g from tertile 1 to 2 and 15.9 μg/g from tertile 2 to 3.

Spearman correlation co-efficient for measures of Mn exposure are reported in Table 6. BnMn was significantly correlated with FMn (p=<0.01), $MnYears_{15}$ (p=0.01), and $MnCEI_{15}$ (p=<0.01) but was not correlated with BMn (p=0.22), $MnYears_{TOT}$ (p=0.31), and $MnCEI_{TOT}$ (p=0.22). All 4 measures of cumulative Mn exposure ($MnYears_{15}$, $MnYears_{TOT}$, $MnCEI_{15}$, and $MnCEI_{TOT}$) were significantly correlated with each other.

Figure 7 shows unadjusted scatter plots and linear regressions for measures of Mn exposure compared to ln(BnMn); further details about these unadjusted regression models as well as regression models adjusted for age and education can be found in Table 7. Ln(BnMn) has a positive, but not statistically significant, unadjusted association with ln(BMn) (β = 0.05; 95% confidence interval (CI) = -0.03, 0.14) as well as $MnCEI_{TOT}$ (β = 4.73; 95% C.I. = -2.26, 11.72). However, ln(BnMn) has a statistically significant positive unadjusted association with ln(FMn) (β = 1.35; 95% CI = 0.70, 1.99) and $MnCEI_{15}$ (β = 4.95; 95% CI = 1.54, 8.35). After adjusting for age and education these positive significant trends for ln(BnMn) with ln(FMn) (β = 1.38; 95% CI = 0.75, 2.00) or $MnCEI_{15}$ (β = 5.33; 95% CI = 2.07, 8.59) persisted. Ln(BnMn) also had a statistically significant association with $MnYears_{15}$ (β = 1.73; 95% CI = 0.17, 3.30).

The relationship between measures of Mn exposure with tertiles of BnMn are also displayed in Table 7. The lowest tertile of BnMn is the reference group for these comparisons. After adjustment for age and education, the highest tertile of BnMn was significantly associated with higher ln(FMn) (β = 2.74; 95% CI= 1.51, 3.99) and $MnCEI_{15}$ (β = 12.60; 95% CI = 6,38, 18.81). $MnYears_{15}$ was associated with both the middle (β = 4.10; 95% CI = 1.14, 7.05) and highest (β = 4.84; 95% CI = 1.92, 7.78) tertile of BnMn.

Table 5. Population Characteristics by Tertiles of Bone Manganese.

Characteristic	BnMn Tertiles			Total Population
Range of BnMn (μg/g)	-5.0 to 0.7	0.7 to 5.1	5.5 to 43.0	-5.0 to 43.0
N	20	20	20	60
Age (years)[a]	46.3 (8.3)	48.2 (8.4)	47.8 (7.3)	47.3 (7.9)
Education (years)[a]	9.5 (4.6)	10.6 (3.3)	10.0 (3.7)	10.0 (3.9)
Years in Current Occupation[a, c]	5.6 (2.9)	9.9 (5.9)	11.4 (9.0)	9.0 (6.8)
Mn $Years_{TOT}$[a]	10.8 (9.3)	16.4 (10.7)	13.9 (8.3)	13.7 (9.6)
Mn $Years_{15}$[a, c]	6.2 (4.8)	9.8 (5.4)	10.8 (3.6)	8.9 (5.0)
$MnCEI_{TOT}$[a]	31.1 (17.6)	40.0 (23.2)	42.1 (24.2)	37.5 (22.0)
$MnCEI_{15}$[a, c]	19.5 (9.1)	24.2 (11.4)	31.2 (10.6)	25.0 (11.3)
Blood (μg/L)[b]	13.2 (2.9)	14.3 (4.1)	14.4 (6.4)	14.1 (4.0)
Fingernail (μg/g)[b, c]	3.1 (13.7)	3.4 (29.7)	64.7 (286.32)	6.1 (39.8)
Bone (μg/g)[b, c]	-0.76 (2.6)	2.6 (2.5)	18.5 (21.1)	2.6 (7.2)

Mn= Manganese; BnMn = Bone Manganese; MnYears = Years of Occupational Mn Exposure; MnCEI = Manganese Cumulative Exposure Index; TOT = Lifetime occupational history; 15 = Occupational History since 2000.

[a]Mean (Standard Deviation); [b]Median (Interquartile Range)
[c]ANOVA p≤ 0.05 for differences across BnMn tertiles

Table 6. Spearman's ρ (p-value) for Correlations between Manganese Exposure Measurements, N=60.

Variable	BMn (μg/L)	FMn (μg/g)	BnMn (μg/g)	$MnYears_{TOT}$	$MnYears_{15}$	$MnCEI_{TOT}$
BMn (μg/L)	-	-	-	-	-	-
FMn (μg/g)	0.25 (0.06)	-	-	-	-	-
BnMn (μg/g)	0.16 (0.22)	0.44 (<0.01) [a]	-	-	-	-
$MnYears_{TOT}$	0.06 (0.62)	-0.08 (0.53)	0.13 (0.31)	-	-	-
$MnYears_{15}$	0.28 (0.03) [a]	0.23 (0.07)	0.32 (0.01) [a]	0.77 (<0.01) [a]	-	-
$MnCEI_{TOT}$	-0.008 (0.05) [a]	-0.10 (0.45)	0.16 (0.22)	0.85 (<0.01) [a]	0.59 (<0.01) [a]	-
$MnCEI_{15}$	0.22 (0.09)	-0.43 (<0.01) [a]	0.43 (<0.01) [a]	0.58 (<0.01) [a]	0.81 (<0.01) [a]	0.66 (<0.01) [a]

Mn= Manganese; BMn = Blood Mn; FMn = Fingernail Mn; BnMn = Bone Manganese; MnYears = Years of Occupational Mn Exposure; MnCEI = Manganese Cumulative Exposure Index; TOT = Lifetime occupational history; 15 = Occupational History since 2000.
[a] = p≤ 0.05

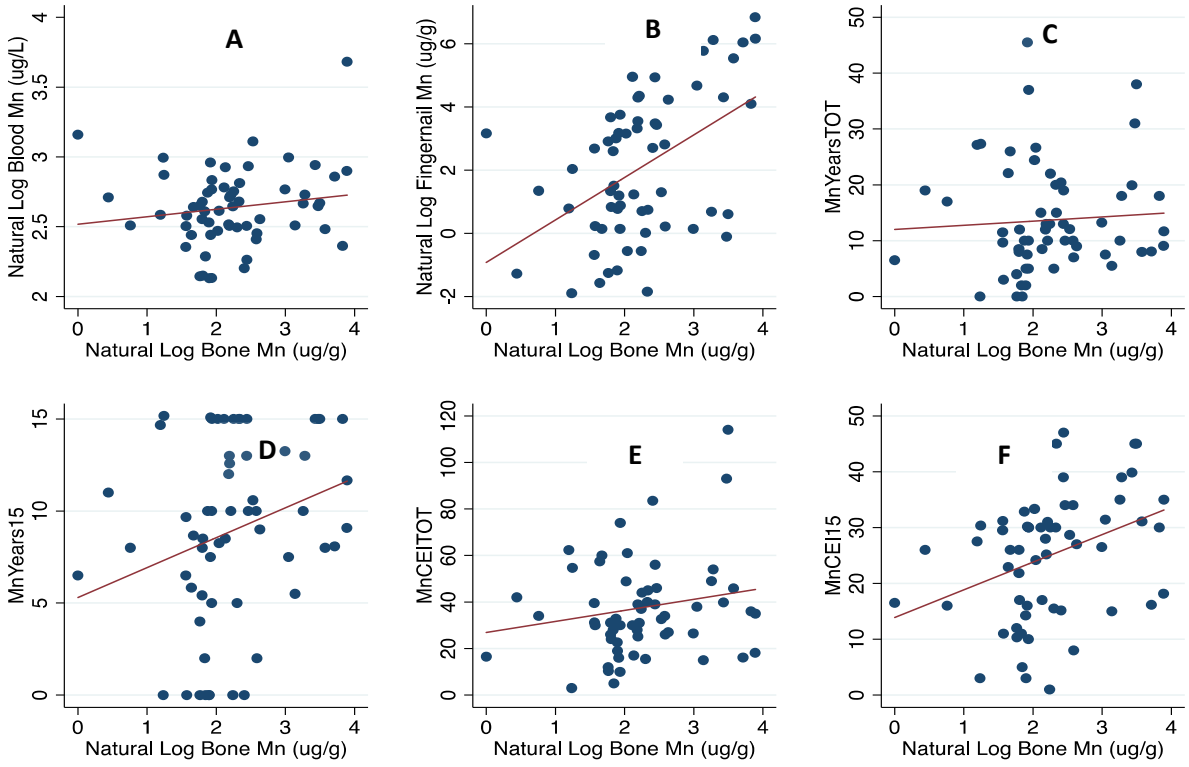

Figure 7. Scatterplots and unadjusted regression lines of natural log BnMn vs. A: ln(BMn); B: ln(FMn); C: $MnYears_{TOT}$; D: $MnYears_{15}$; D: $MnCEI_{TOT}$; D: $MnCEI_{15}$ (n=60). Mn= Manganese; ln(BMn) = Natural Logarithm of Blood Mn; ln(FMn) = Natural Logarithm of Fingernail Mn; MnYears = Years of Occupational Mn Exposure; MnCEI = Manganese Cumulative Exposure Index; TOT = Lifetime occupational history; 15 = Occupational History since 2000.

Table 7. β (95% Confidence Interval) from Regression Models Comparing each Mn Exposure Measure to BnMn (N=60)

Mn Exposure Measure/Model	BnMn Tertiles [a]		ln(BnMn)
	Tertile 2	Tertile 3	
ln(BMn)			
Unadjusted	0.02 (-0.15, 0.19)	0.14 (-0.04, 0.31)	0.05 (-0.03, 0.14)
Adjusted[b]	0.05 (-0.12, 0.22)	0.15 (-0.02, 0.32)	0.06 (-0.03, 0.14)
ln(FMn)			
Unadjusted	0.88 (-0.41, 2.16)	2.65 (1.36, 3.93) [c]	1.35 (0.70, 1.99) [c]
Adjusted[b]	1.08 (-0.17, 2.33)	2.74 (1.51, 3.99) [c]	1.38 (0.75, 2.00) [c]
$MnCEI_{TOT}$			
Unadjusted	8.27 (-5.58, 22.12)	10.93 (-2.92, 24.78)	4.73 (-2.26, 11.72)
Adjusted[b]	6.35 (-6.80, 19.50)	9.38 (-3.67, 22.42)	3.42 (-3.19, 10.03)
$MnCEI_{15}$ (years)			
Unadjusted	4.67 (-1.91, 11.24)	11.73 (5.16, 18.31) [c]	4.95 (1.54, 8.35) [c]
Adjusted[b]	6.19 (-0.08, 12.45)	12.60 (6.38, 18.81) [c]	5.33 (2.07, 8.59) [c]
$MnYears_{TOT}$			
Unadjusted	5.59 (-0.41, 11.60)	3.12 (-2.89, 9.12)	0.75 (-2.34, 3.85)
Adjusted[b]	4.82 (-1.08, 10.73)	2.53 (-3.33, 8.39)	0.26 (-2.75, 3.27)
$MnYears_{15}$			
Unadjusted	3.65 (0.71, 6.60) [c]	4.59 (1.64, 7.54) [c]	1.63 (0.07, 3.18) [c]
Adjusted[b]	4.10 (1.14, 7.05) [c]	4.84 (1.92, 7.78) [c]	1.73 (0.17, 3.30) [c]

Mn= Manganese; BnMn = Bone Mn; BMn = Blood Mn; FMn = Fingernail Mn; ln = natural logarithm; MnYears = Years of Occupational Mn Exposure; MnCEI = Manganese Cumulative Exposure Index; TOT = Lifetime occupational history; 15 = Occupational History since 2000.
[a] Tertile 1 (referent) includes BnMn values from -5.0 to 0.7; tertile 2 includes 0.7 to 5.1; tertile 3 includes 5.5 to 43.0; [b] Adjusted for age (continuous) and education (continuous); [c] = p≤ 0.05

2.4.6. *Association between Mn biomarkers and cognitive function*

Population characteristics are reported in Table 8. Mean (SD) age and years of education for were 47.3 (7.9) and 10.0 (3.9) years respectively for the entire population. On average, participants have been in their current position for 9.0 years (SD = 6.8). Years in current occupation was significantly different between the BnMn tertiles (p=0.05). More than half of the total population were current drinkers (73.3%) and current smokers (76.7%).

Summary statistics for the cognitive and olfactory tests as well as the biomarkers are reported in Table 9. Both the AVLT 5-trial average scores (p=0.02) and olfactory scores (p=-0.05) were significantly different across BnMn tertiles, with decreasing scores as BnMn increases. Both FMn (p=<0.01) and BnMn (P<0.01) are also significantly different across BnMn tertiles with increasing concentrations as BnMn tertiles increase.

Scatter plots with unadjusted linear fit lines were created for BnMn, FMn, and BMn vs. AVLT average scores (Figure 8) and olfactory scores (Figure 9). There was a significant decrease in AVLT average scores [β (95% CI) = -0.9 (01.5, -0.2)] and olfactory scores [β (95% CI) = -1.7 (-3.7, -0.03)] as BnMn increased. AVLT average scores [β (95% CI) = -0.5 (-0.7, -0.3)] and olfactory scores [β (95% CI) = -1.1 (-1.7, -0.05)] also significantly decreased with increasing FMn. There was a significant decrease in AVLT average scores [β (95% CI) = -2.8 (-4.7, -0.9)] with increasing BMn but not with olfactory scores [β (95% CI) = -5.2 (-10.5, 0.2)].

Spearman correlations between Mn biomakrers, test scores, and potential covariates were tested. Current years in occupation, current factory of employment, age, and education were all significantly associated with either a Mn biomarker or test score. After a Bonferroni correction, the association between current factory and ln(FMn) (ρ=0.82, p = <0.01), education and Animal Naming (ρ=0.46, p = <0.01), and ln(FMn) and AVLT Avg. (ρ=0.53, p = <0.01) remained significant.

Adjusted regression models were used to assess the relationship between the Mn biomarkers and test scores. Increasing ln(BMn) was significantly associated with decreasing Animal Naming scores [β (95% CI) = -1.5 (-3.0, -0.7)] and decreasing AVLT Avg. scores [β (95% CI) = -0.6 (-1.2, -0.09)]. There was a decrease in AVLT Avg. scores [β (95% CI) = -1.2 (-2.3, -0.04)] when comparing the highest tertile of BnMn to the lowest. Decreasing scores for Trial 6 were associated with the middle tertile of BnMn [β (95% CI) = -1.3 (-2.4, -0.08)] Trial 6 Intrusions increased for both the middle tertile [β (95% CI) = 0.5 (0.04, 0.9)] and highest tertile of BnMn [β (95% CI) = 0.5 (0.07, 1.0)] when compared to the lowest tertile of BnMn. Increasing ln(FMn) was also associated with decreasing AVLT Avg. scores [β (95% CI) = -0.4 (-0.7, -0.03)] as well as decreasing AVLT Dif. Scores [β (95% CI) = -0.4 (-0.7, -0.02)]. When compared to the lowest tertile, the highest tertile of FMn was significantly associated with decreasing AVLT Avg. scores [β (95% CI) = -2.1 (-3.2, -1.0)]. BMn was not significantly associated with any of the cognitive or olfactory test scores. A sensitivity analysis was conducted to account for FMn samples with the strongest likelihood of contamination. There was still a significant association between increasing FMn and decreasing AVLT Avg. scores [β (95% CI) = -0.5 (-0.8, -0.1)]. The middle tertile of BnMn was still associated with decreasing AVLT Trial 6 scores [β (95% CI) = -1.2 (-2.5, -0.04)].

Table 8. Population Characteristics by BnMn tertile and total population.

Characteristic	**Total**	**BnMn T1**	**BnMn T2**	**BnMn T1**
	N (%)			
Total	60 (100)	20 (100)	20 (100)	20 (100)
Age (years)				
29 - 43	17 (28.3)	7 (35.0)	4 (20.0)	6 (30.0)
44 - 52	28 (46.7)	10 (50.0)	10 (50.0)	8 (40.0)
>52	15 (25.0)	3 (15.0)	3 (15.0)	6 (30.0)
Education (years)				
0 - 8	18 (30.0)	9 (45.0)	3 (15.0)	6 (30.0)
9 - 13	30 (50.0)	7 (35.0)	13 (65.0)	10 (50.0)
>13	12 (20.0)	4 (20.)	4 (20.0)	4 (20.0)
Years in Current Occupation[a]				
0 - 5	19 (31.7)	11 (55.0)	5 (25.0)	3 (15.0)
6 – 10	25 (41.7)	8 (40.0)	7 (35.0)	10 (50.0)
11 – 15	10 (16.6)	1 (5.0)	5 (25.0)	4 (20.0)
>15	6 (10.0)	-	3 (15.0)	3 (15.0)
Current Smoker				
No	14 (23.3)	5 (25.0)	5 (25.0)	4 (20.0)
Yes	46 (76.7)	15 (75.0)	15 (75.0)	16 (80.0)
Current Drinker				
No	16 (26.7)	6 (30.0)	6 (30.0)	4 (20.0)
Yes	44 (73.3)	14 (70.0)	14 (70.0)	16 (80.0)

Bone Mn Tertiles (BnMn T): Tertile 1 = -5.0 – 0.7 μg/g; Tertile 2 = 0.7 – 5.1μg/g; Tertile 3 = 5.5 – 43.0 μg/g; [a] = significantly different between tertiles at ≤0.05 level

Table 9. Mean (Standard deviation) of neuropsychological tests and Median (IQR) of FMn and BMn by BnMn tertile.

Variable	Total	BnMn Tertiles			
		BnMn T1	BnMn T2	BnMn T3	p-value
Animal Naming [a]	15.8 (5.0)	16.5 (5.1)	16.2 (4.7)	14.5 (5.1)	0.2
Fruit Naming [a]	11.3 (3.2)	11.4 (3.2)	11.9 (3.6)	10.5 (2.8)	0.4
AVLT Avg. [a, c]	8.5 (2.2)	9.1 (2.2)	8.9 (1.6)	7.5 (2.4)	0.02
AVLT Dif. [a]	4.5 (2.0)	4.8 (2.0)	4.6 (2.1)	4.0 (2.0)	0.2
AVLT Trial 6 [a]	4.6 (1.9)	5.4 (2.2)	4.1 (1.4)	4.4 (1.9)	0.1
AVLT Trial 6 Int. [a]	0.5 (0.7)	0.2 (0.4)	0.6 (0.7)	0.6 (0.9)	0.1
AVLT Trial 7 [a]	8.8 (3.4)	9.5 (3.7)	8.7 (2.4)	8.1 (3.8)	0.2
AVLT Trial 7 Int. [a]	0.4 (0.7)	0.5 (0.8)	0.4 (0.6)	0.3 (0.8)	0.4
UPSIT [a]	20.2 (5.8)	21.7 (7.1)	21.0 (4.7)	18.0 (5.2)	0.05
$MnCEI_{TOT}$ [a]	37.5 (22.0)	31.1 (17.6)	39.4 (23.2)	42.0 (24.2)	0.1
$MnCEI_{15}$ [a, c]	25.0 (11.3)	19.5 (9.1)	24.2 (11.4)	31.2 (10.5)	<0.01
BMn [b]	14.1 (4.0)	13.2 (2.9)	14.3 (4.0)	14.4 (6.4)	0.1
FMn [b, c]	6.1 (39.8)	3.1 (13.7)	3.4 (29.7)	64.6 (286.3)	<0.01
BnMn [b, c]	2.6 (7.2)	-0.8 (2.6)	2.6 (2.5)	18.5 (21.1)	<0.01

Overall N = 60 (UPSIT N=58)
Bone Mn Tertiles (BnMn T): Tertile 1 = -5.0 – 0.7 μg/g; Tertile 2 = 0.7 – 5.1μg/g; Tertile 3 = 5.5 – 43.0 μg/g; Fingernail Mn (FMn); Blood Mn (BMn)
AVLT Avg. = average words correct over trials 1 – 5
AVLT Dif. = difference in words correct between trials 5 and 1;
AVLT Trial 6/7 Int. = number of intrusions during trials 6 and trial 7
[a] = Unadjusted means (standard deviations); [b] = Unadjusted median (interquartile range)
[c] = significantly different at ≤0.05 level

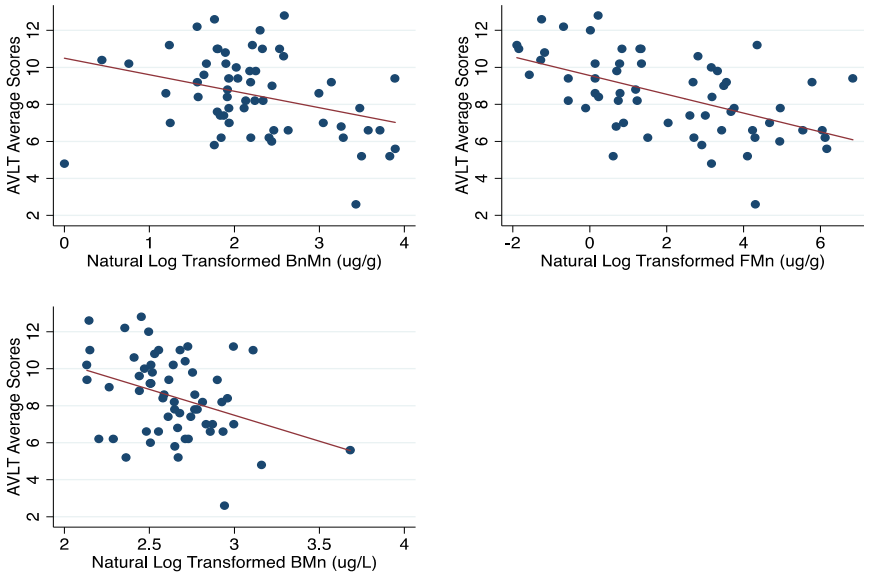

Figure 8. Scatterplots and unadjusted regression lines of natural log transformed Mn Biomarkers vs. AVLT Avg. Scores Bone Mn (BnMn); Fingernail Mn (FMn); Blood Mn (BMn); AVLT Avg. = average words correct over trials 1 – 5.

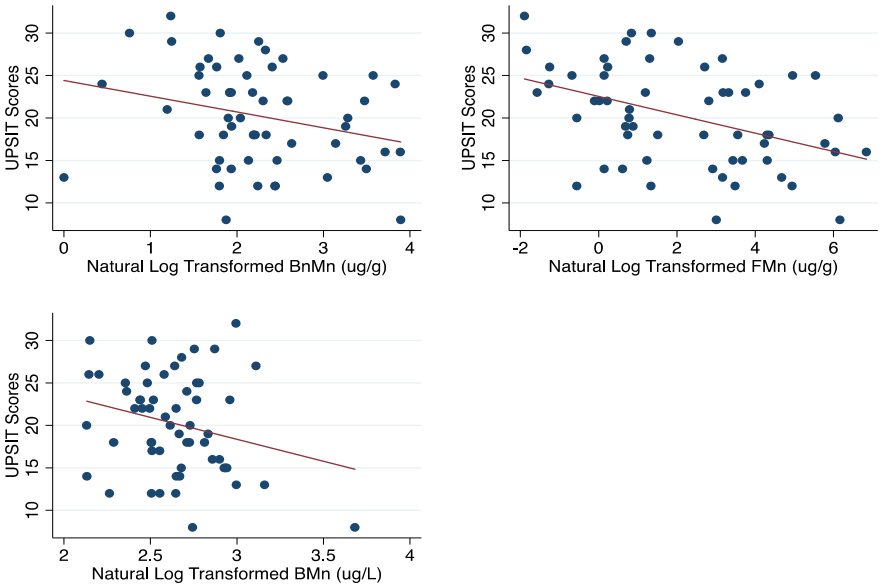

Figure 9. Scatterplots and unadjusted regression lines of natural log transformed Mn Biomarkers vs. UPSIT Scores *Bone Mn (BnMn); Fingernail Mn (FMn); Blood Mn (BMn)*

2.4.7. *Association between Mn biomarkers and motor function*

Results show a significant decrease in precision for the Rhythmic P/S test at the slow speed (non-dominant hand) as BnMn tertiles increase (p = 0.03). Tremor deviation significantly decreased as BnMn increased for both the dominant (p=0.04) and non-dominant hands (p=0.04). The Spearman correlation co-efficient for the associations between covariates, Mn biomarkers and motor test scores were tested. Age was significantly associated with decreases in Max Frequency P/S (NonDom) (ρ = -0.33; p = 0.01), Max Frequency F-Tap (Dom) (ρ = -0.39; p = <0.01), and Purdue Pegboard scores for both hands (ρ = -0.38; p = <0.01). Age was also significantly associated with increased Sway Velocity for both Sway test 3 (ρ = 0.33; p = 0.01) and Sway 4 (ρ = 0.32; p = 0.01). Education was associated with decreasing precision in the

Rhythmic P/S test slow (NonDom) (ρ = -0.38; p = <0.01) and increasing Purdue Pegboard Assembly scores (ρ = 0.33; p = <0.01).

Current factory of employment was associated with decreasing Tremor Standard Deviation (Dom) (ρ = -0.33; p = 0.01), decreasing Mean Sway (Test 1) (ρ = -0.39; p = <0.01), decreasing Transversal Sway (Test 1) (ρ = -0.38; p = <0.01), and decreasing Purdue Pegboard Assembly scores (ρ = -0.33; p = 0.01). Drinking status was associated with decreasing Max Frequency P/S (Dom) (ρ = -0.37; p = <0.01), increasing Center Frequency (Dom) (ρ = 0.33; p = 0.01), and increasing Sagittal Sway (Test 2) (ρ = 0.32; p = 0.01). BMn was associated with increasing Sway Velocity (Test 2) (ρ = 0.32; p = 0.01). FMn was associated with decreasing Max Frequency P/S (Dom) (ρ = -0.32; p = 0.01), decreasing Transversal Sway (Test 1) (ρ = -0.40; p = <0.01), and decreasing Sway Area (Test 1) (ρ = -0.35; p = <0.01). FMn was also significantly associated with current factory of employment (ρ = 0.82; p = <0.01) and BnMn (ρ = 0.41; p = <0.01). BnMn was not significantly associated with any of the motor test outcomes at the 0.01 level.

An example of the lowess curves created for Mn biomarkers vs. motor tests is shown in Figure 10 (Mn biomarkers vs. Reaction Time Dominant Hand). For all 3 graphs, the relationship between Mn biomarker and Reaction Time does not look completely linear. Especially for ln(BnMn) and ln(BMn), there are areas of decreasing and increasing reaction time as Mn increases. After adjusting for age, education, current factory of employment, and current drinking status, increasing ln(BnMn) was associated with decreasing Rhythmic P/S fast (NonDom) (β = -0.019; 95% C.I. = -0.036, -0.002), decreasing Rhythmic F-Tap fast (NonDom) (β = -0.027; 95% C.I. = -0.045, -0.008), increasing Center frequency (Dom) (β = 0.443; 95% C.I. = 0.029, 0.858), and increasing tremor intensity (β = 0.011; 95% C.I. = 0.0001, 0.022).

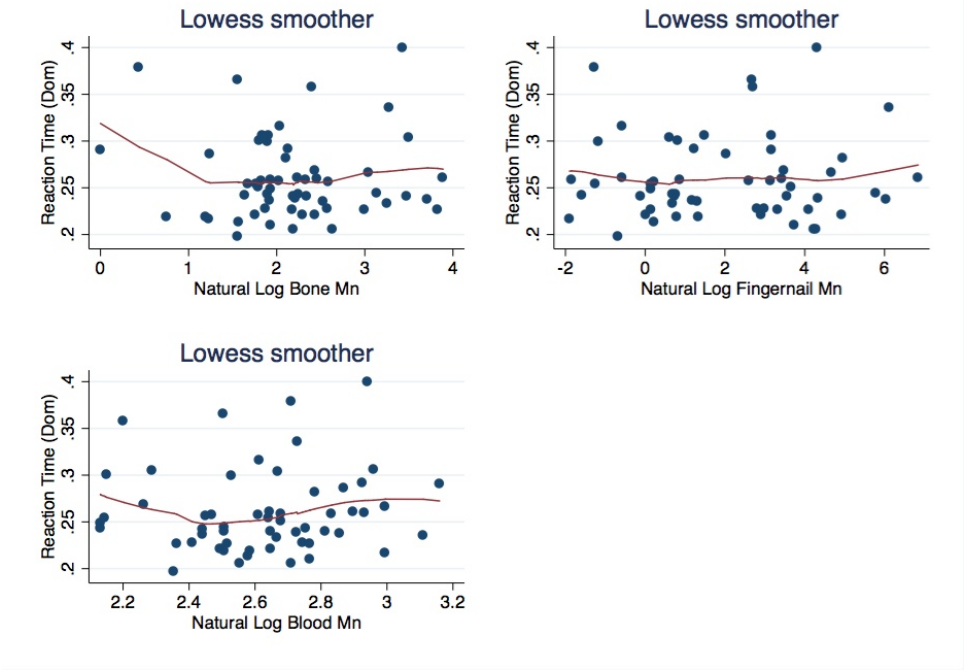

Figure 10. Spearman's ρ (p-value) for correlations between Covariates, Mn Biomarkers and motor test outcomes

When ln(BnMn) was divided into splines, the relationship between the biomarker and motor tests changed as Mn increased. For example, for the Rhythmic F-Tap slow SD test, the lowest (β = 0.057; 95% C.I. = 0.004, 0.109) and highest (β = 0.052; 95% C.I. = 0.014, 0.091) spline were associated with increases in the SD whereas the middle spline was associated with decreasing (β = -0.069; 95% C.I. = -0.115, -0.023) SD. The highest spline of ln(BnMn) was

associated with decreasing scores for the Purdue Pegboard test for both hands (β = -1.212; 95% C.I. = --2.350, -0.074).

Continuous ln(FMn) was associated with decreasing Max Frequency P/S (Dom) (β = -0.380; 95% C.I. = -0.652, -0.109) and decreasing Max Frequency F-Tap (Dom) (β = -0.316; 95% C.I. = -0.604, -0.028). The highest spline of lnFMn was associated with increasing sway for several sway outcomes under the Sway 2 conditions. Continuous ln(BMn) was associated with decreasing Rhythmic P/S slow (NonDom) (β = -0.092; 95% C.I. = -0.178, -0.005) , decreasing Rhythmic P/S Fast (Dom) (β = -0.061; 95% C.I. = -0.118, -0.005), decreasing Rhythmic F-Tap slow (NonDom) (β = -0.103; 95% C.I. = -0.203, -0.004), increasing sway velocity (Sway 2) (β = 4.82; 95% C.I. = 1.00, 8.65), and increasing sway intensity (β = 2.07; 95% C.I. = 0.71, 3.43).

2.4.8. *Discussion*

A DD neutron generator based IVNAA system was transported to China for the purpose of measuring BnMn in human body and determination of the relationship between BnMn with BMn as well as years of employment. This is the first time a transportable IVNAA system was used to quantify Mn in bone in an occupationally exposed population, and the second time it was used in a human population. In an initial pilot study using the system among U.S. adults, we found a mean BnMn of 0.66 μg/g (Wells et al., 2017). *In vivo* BnMn levels have also been reported by Pejovic-Milic *et al*. with the BnMn obtained by a larger accelerator-based NAA system (Pejovic-Milic et al., 2009). They found mean BnMn levels of 2.9±0.4 and 0.1±0.7 μg Mn/g Ca among the exposed and control groups, which correspond to 0.58±0.08 and 0.02±0.14 μg Mn/g dry bone respectively. Reported means from both studies are substantially lower than the mean (7.1 μg/g) within this population, although relatively similar to our median concentration (2.7 μg/g).

Based on the current blood analysis, the median BMn was 15.9 μg/L in the exposed group and 13.2 μg/L in the control group. This is notably higher than BMn reported among several community based studies: geometric mean BMn was reported to be 9.3 μg/L within a study of Ohio adults (Kim et al., 2015), the arithmetic mean BnMn from United States adult males was estimated at 9.2 μg/L (Oulhote et al., 2014), and median BMn among Chinese males living in a Beijing suburb was 9.6 μg/L (Zhang et al., 2015). However, it is within the range of BMn values reported in studies of occupationally-exposed populations, many of which fall between 5 and 20 μg/L (Baker et al., 2014).

On average, both BMn and BnMn were higher among the ferroalloy workers (exposed) than the manufacturing workers (controls). The unadjusted association of BMn with exposed/control was statistically significant, but for BnMn it was of borderline statistical significance. After adjustment for age and education, we did observe a statistically significant association of BnMn with exposed/control groups. We originally hypothesized that there would be a clearer distinction of BnMn between these groups; however, a closer look at job tasks and occupational history could explain these results. Current jobs the workers reported at both factories could reasonably result in either high or low Mn exposure. For example, current jobs among ferroalloy workers include Mn powder processing, ore exaction, ore grinding, Mn electrolysis, and Mn filtration, all of which are likely to have high Mn exposure; however, some reported working in sewage treatment and factory management, which may not result in substantial occupational Mn exposure. Similarly, some participants from the manufacturing facility reported job tasks would likely have little occupational Mn exposure, such as a managers, drivers, and marketing; however, several also reported at least some welding-related tasks, which could result in substantial Mn exposure.

It is important to remember that while we are comparing two different biomarkers of Mn exposure, these measures are expected to represent different time periods of exposure. BnMn, with a half-life of >8 years, is more of a cumulative Mn exposure biomarker, while BMn reflects

exposures over the last day or few days before, and is noted to be highly variable (O'Neal et al., 2014, Zheng et al., 2011). Thus, it is not surprising that BMn and BnMn would not be significantly correlated with each other in our analysis, as our participants' occupational Mn exposure may have changed over time. Further support from this comes from reports from our participants that several of the workers from manufacturing facility had previously been employed at the ferroalloy facility or a similar facility involved with Mn ore processing, which would likely influence the BnMn measurements. This is consistent with our observations that BnMn, but not BMn was associated with years of employment in the current position.

We hypothesized that BnMn would be associated with MnCEI and MnYears, as these are both estimates of long-term exposure, but not necessarily BMn or FMn. BnMn was significantly associated with FMn, but not BMn; this is consistent with our hypothesis that BnMn would be associated with exposure measures representing longer exposure periods. Additionally, there was a significant relationship seen between BnMn and cumulative exposure variables representing the past 15-16 years ($MnYears_{15}$, $MnCEI_{15}$), which persisted after adjustment for age and education. However, BnMn was not significantly associated with lifetime Mn exposure variables ($MnYears_{TOT}$, $MnCEI_{TOT}$). Pejović-Milić et al. found that BnMn was significantly associated with a lifetime MnCEI among 30 welders and 10 controls (Pejović-Milić et al. 2009). Both studies suggest that BnMn is associated with long-term exposure; but results for the upper length of the long-term exposure differ. Our results suggest that BnMn is associated with exposure over the past 15 years, but not necessarily over worker's full occupational career is consistent with an estimate of the BnMn half-life of 8.5 years in humans (O'Neal et al. 2014); this suggests BnMn concentrations may reflect a period of approximately 2 half-lives which could be substantially shorter than a lifetime occupational history.

In contrast to other publications (Bouchard et al. 2008; Bowler et al. 2007; Lucchini et al. 1999)), we were unable to use current air Mn data to create a Mn CEI. However, we observed that many participants from the ferroalloy factory primarily worked with wet ore.
This suggests that inhalation may not be the primary route of Mn exposure for many of the study participants. Thus, if air measurements had been available, they may not have been fully reflective of the total worker Mn exposure. Instead of using air concentrations, we adapted existing methods to rank exposure by job title to approximate Mn exposure for our MnCEI (Ramlow et al. 1996, Fayerweather et al. 1997). There are several potential sources of bias for this method including exposure misclassification and recall bias. Participants were required to recall employment history that for some spanned decades. This could have affected the cumulative exposure variables due to recall bias. Additionally, exposure ranks were assigned based on the job titles thus misclassification could occur due to jobs being incorrectly ranked due to a variation in expected exposures from the job title versus the worker's actual exposure.

We assessed BnMn's usefulness in reflecting decreases in cognitive and olfactory test scores. In the study, we used spearman correlations as well as regression models to assess the relationship between Mn exposure variables and test scores. Based upon Spearman correlations, BnMn was significantly associated with decreasing AVLT Avg. scores (ρ= -0.34; p=<0.01) and UPSIT scores (ρ= -0.27; p=0.04). After adjusting for age, education and current factory of employment in linear regression models, BnMn was still significantly associated with decreasing AVLT Avg. scores [β (95% CI) = -0.6 (-1.2, -0.09)] along with decreasing Animal Naming scores [β (95% CI) = -1.5 (-3.0, -0.7)].

Using tertiles of BnMn to assess non-linear relationships between test scores and BnMn, the middle [β (95% CI) = 0.5 (0.04, 0.9)] and highest [β (95% CI)= 0.5 (0.07, .1.0)] tertiles of BnMn both had significant associations with increasing AVLT Trial 6 intrusions when compared to the lowest tertile. When compared to the lowest tertile, the highest tertile of BnMn was associated with decreases in AVLT Avg. scores [β (95% CI)= -1.2 (-2.3, -0.04)] whereas the middle tertile was associated with decreases in AVLT Trial 6 scores [β (95% CI)= -1.3 (-2.4, -

0.08)]. BnMn also appeared to be a stronger predictor of cognitive and olfactory decline than the more acute biomarkers (BMn = days; FMn = months). After adjusting for covariates, BnMn was the stronger predictor of decreasing function in 5 out of 9 of the test scores reported. These relationships however were not significant.

FMn was also significantly associated with decreasing AVLT Avg. scores (ρ= -0.53; p= <0.01) and UPSIT scores (ρ= -0.40; p= <0.01) along with decreasing AVLT Trial 6 (ρ= -0.30; p= 0.02) and 7 scores (ρ= -0.35; p= <0.01). However, after adjustment for covariates, FMn was only significantly associated with decreases in AVLT Avg. scores [β (95% CI) = -0.4 (-0.7, -0.03))] and AVLT Dif. Scores [β (95% CI) = -0.4 (-0.7, -0.02)]. The highest tertile of FMn was significantly associated with decreases in AVLT Avg. scores [β (95% CI)= --2.1 (-3.2, -1.0)]. BMn was not associated with any decreases in cognitive or olfactory test scores.

Our results between BMn and UPSIT scores is similar to findings by Antunes et al. (2007) who saw no significant correlation between BMn and UPSIT scores in a group of bridge welders (ρ= 0.20; p=<0.20). However, our findings for BMn are in contradictin to previous research. Both blood and toenail Mn have previously been associated with decline in several cognitive tests assessing visual and spatial working memory (Hassani et al. 2016). Another study assessing Mn exposure in a group of Italian ferralloy workers saw significant association.

BnMn in rats has been associated with Mn concentrations in both the hippocampus and striatum, two parts of the brain that play an important role in cognition (O'Neal et al. 2014). In our study, BnMn saw significant associations with decreases in both AVLT and Animal Naming two well-establish tests of verbal memory and fluency function. Verbal memory tests like the AVLT have been associated with hippocampal impairment (Vyhnalek et al. 2014) whereas verbal fluency tests like the Animal Naming test have been associated with decreased striatal matter (Ellfolk et al. 2014). Our results suggest that BnMn may be reflective of decreasing verbal memory and fluency and possibly decreased striatal and hippocampal function.

Bone Mn has been suggested as a possible indicator of decreased motor function in exposed individuals. Results from this study suggest a non-linear relationship between the Mn biomarkers and many of the motor outcomes. In a previous study assessing BnMn vs. manual dexterity using the Purdue Pegboard test, BnMn was significantly associated with decreasing test scores (Wells et al. 2017). In our study, continuous ln(BnMn) was not significantly associated with decreasing Pegboard scores. However, once lnBnMn was separated into splines, the highest spline of lnBnMn was significantly associated with decreasing Purdue pegboard scores for both hands. Similar non-linearity was seen in FMn as well as BMn.

The non-linear relationship between Mn and neurological outcomes has been an area of debate due to the theory that there is a threshold of where no adverse effects are seen due to the homeostatic nature of Mn (Finley and Santamaria 2005; Santamaria 2008). Using both the continuous and spline regressions in this study is important because it allows us to fit a linear and non-linear model to the data. If we just applied a linear regression model to the data, we could have misrepresented the results and obscured the possibility of a threshold for Mn in this population (Santamaria 2008). Non-linear relationships between biomarkers and Mn are usually seen in environmental populations (Henn et al. 2010), so this study could provide evidence of the relationship in an occupational population. Another strength of the study is that we used well established motor tests. These tests have been used before and have proven their utility in non-English speaking populations (Cowan 2009; Iwata et al. 2007).

Some limitations of this study were identified. First, some bone Mn measurements may be affected by external Mn contamination. Some of these workers' responsibilities require hands-on work with solutions containing Mn, and many of them did not use gloves or other personal protective equipment. This was identified during the data collection period, after noting that fingernails of some of the participants were discolored. All participants had their fingernails cut and thoroughly washed their hands and lower arms using soap before the BnMn measurement. Alcohol was also used to clean skin surface of the hand and the lower arm. For

several exposed participants, the research assistant further brushed their hand and fingernails using a soft brush to reduce the contamination. While these protocols reduced the potential for external contamination, it is possible that this still may have influenced our results.

The second limitation is that the IVNAA system did not perform at the expected efficiency. This was due to the impaired performance of the HPGe detector induced by the change of the utility frequency from 60 Hz in the U.S. to that of 50 Hz in China. The frequency change downgraded the detector efficiency and elevated background counts. The sensitivity of the system may have been compromised because of the degradation of the HPGe detector.

The third limitation of this study was related to the population and the study design. A small population size could have resulted in limited study power. However, as individuals with high Mn exposure were recruited we were still able to observe statistically significant results with fewer study participants. The cross-sectional design of the study limits our ability to draw any conclusions about causality. In addition, although this study controlled for age, education and exposure status through current factory of employment, there may have been other potential covariates that could have explained the relationship between Mn biomarkers and test scores.

The use of fingernails in our study could also be a potential limitation. We took precautions to limit the external contamination present on the fingernail samples, however our results could have still been affected. We performed a sensitivity analysis that excluded the samples that are most-likely at risk for contamination. We still saw significant associations between FMn and decreasing AVLT. Avg. scores [β (95% CI) = -0.5 (-0.8, -0.1)] as well as the middle tertile of BnMn and decreasing AVLT Trial 6 scores [β (95% CI) = -1.2 (-2.5, -0.04)] suggesting that some of the variance in the models could not be explained by potential contamination.

Despite these limitations, the study does demonstrate that it is feasible to apply the IVNAA technology to quantify Mn in bone and to use BnMn as a biomarker for cumulative Mn exposure assessment. More importantly, we were able to obtain significant results for the association between BnMn and neurological test scores, which suggests that BnMn is also a valuable biomarker for Mn-induced health effects.

2.5. Conclusions

In conclusion, our data show that BnMn is a promising biomarker for cumulative Mn exposure assessment and for Mn exposure induced health effects especially neurological effects. Future work is needed to confirm the results in a larger population and to use BnMn as a biomarker in metal related epidemiology studies.

Publications – published or in press (* indicates corresponding author(s); trainees are underlined)

1. Liu Y, Rolle-McFarland D, Mostafaei F, Zhou Y, Li Y, Zheng W, Wells EM*, Nie LH*. *In Vivo* Neutron Activation Analysis (IVNAA) of Bone Manganese (Mn) in Workers, Physiological Measurement, In press
2. Wells EM*, Liu Y, Rolle-McFarland D, Mostafaei F, Zheng W, Nie LH*. In Vivo Measurement of Bone Manganese and Association with Manual Dexterity: a Pilot Study. Environmental Research, 160(2017)35-38
3. Liu Y, Mostafaei F, Sowers D, Hsieh M, Zheng W, Nie LH*. Customized Compact Neutron Activation Analysis System to Quantify Manganese (Mn) in Bone In Vivo. Physiological Measurement, 38(2017)452-465
4. Byrne P, Mostafaei F, Liu Y, Koltick D, Zheng W, Nie LH*. The Study of In Vivo Quantification of Aluminum (Al) in Human Bone with a Compact DD Generator-based Neutron Activation Analysis (NAA) System. Physiological Measurement, 37(2016)649-660
5. Sowers D, Liu Y, Mostafaei F, Blake S, Nie LH*. A Dosimetry Study of Deuterium-Deuterium Neutron Generator-Based *in vivo* Neutron Activation Analysis. Health Physics, 109(2015)566-572
6. Mostafaei F, Blake SP, Liu Y, Sowers DA, Nie LH*. Compact DD Generator-based Neutron Activation Analysis (NAA) System to Determine Fluorine in Human Bone In Vivo: A Feasibility Study. Physiological Measurement, 36(2015)2057-2067

Publications – submitted or in preparation (* indicates corresponding author(s); trainees are underlined)

7. Rolle-McFarland D, Liu Y, Zhou J, Mostafaei F, Zhou Y, Li Y, Fan Q, Zheng W, Nie LH*, Wells EM*. The Association between Bone, Fingernail, and Blood Manganese with Motor Function. Environmental Epidemiology. In preparation (drafted and being reviewed by co-authors)
8. Rolle-McFarland D, Liu Y, Zhou J, Mostafaei F, Zhou Y, Li Y, Fan Q, Zheng W, Nie LH*, Wells EM*. The Association between Bone, Fingernail, and Blood Manganese with Cognitive and Olfactory Function. Environmental Health Perspectives. In preparation (drafted and being reviewed by co-authors)
9. Coyne M, Neumann C, Zhang X, Byrne P, Liu Y, Weaver CM, Nie LH*. Compact DD generator based In Vivo Neutron Activation Analysis (IVNAA) System to Determine Sodium Concentrations in Human Bone. Physiological Measurement. Submitted
10. Rolle-McFarland D, Liu Y, Zhou J, Mostafaei F, Zhou Y, Li Y, Fan Q, Zheng W, Nie LH*, Wells EM*. In Vivo Neutron Activation Analysis of Bone Manganese: A Potential Biomarker of Cumulative Manganese Exposure. International Journal of Hygiene and Environmental Health, (to be submitted on the week of Dec.4, 2017)

References

ARJONA, A., MATA, M. & BONET, M. 1997. Diagnosis of chronic manganese intoxication by magnetic resonance imaging. *N Engl J Med,* 336**,** 964-5.

ASLAM, CHETTLE, D. R., PEJOVIC-MILIC, A. & WAKER, A. J. 2009. Opportunities to improve the in vivo measurement of manganese in human hands. *Phys Med Biol,* 54**,** 17-28.

BADER, M., DIETZ, M. C., IHRIG, A. & TRIEBIG, G. 1999. Biomonitoring of manganese in blood, urine and axillary hair following low-dose exposure during the manufacture of dry cell batteries. *Int Arch Occup Environ Health,* 72**,** 521-7.

BAKER, M. G., SIMPSON, C. D., STOVER, B., SHEPPARD, L., CHECKOWAY, H., RACETTE, B. A. & SEIXAS, N. S. 2014. Blood manganese as an exposure biomarker: state of the evidence. *J Occup Environ Hyg,* 11**,** 210-7.

BOUCHARD, M. F., SAUVE, S., BARBEAU, B., LEGRAND, M., BRODEUR, M. E., BOUFFARD, T., LIMOGES, E., BELLINGER, D. C. & MERGLER, D. 2011. Intellectual impairment in school-age children exposed to manganese from drinking water. *Environ Health Perspect,* 119**,** 138-43.

BUTCHER, D. J., ZYBIN, A., BOLSHOV, M. A. & NIEMAX, K. 1999. Speciation of methylcyclopentadienyl manganese tricarbonyl by high-performance liquid chromatography-diode laser atomic absorption spectrometry. *Anal Chem,* 71**,** 5379-85.

COUPER, J. 1837. On the effects of black oxide of manganese when inhaled into the lungs. *Br Ann Med Pharmacol,* 1**,** 41-2.

COWAN, D. M., FAN, Q., ZOU, Y., SHI, X., CHEN, J., ASCHNER, M., ROSENTHAL, F. S. & ZHENG, W. 2009. Manganese exposure among smelting workers: blood manganese–iron ratio as a novel tool for manganese exposure assessment. *Biomarkers,* 14**,** 3-16.

CROSSGROVE, J. & ZHENG, W. 2004. Manganese toxicity upon overexposure. *NMR Biomed,* 17**,** 544-53.

DING, C. G., ZHU, C., LIU, D. Y., DONG, M., ZHANG, A. H., PAN, Y. J. & YAN, H. F. 2012. [Inductively coupled plasma mass spectrometry for the simultaneous determination of thirty metals and metalloids elements in blood samples]. *Zhonghua Yu Fang Yi Xue Za Zhi,* 46**,** 745-9.

DYDAK, U., JIANG, Y. M., LONG, L. T., ZHU, H., CHEN, J., LI, W. M., EDDEN, R. A., HU, S., FU, X., LONG, Z., MO, X. A., MEIER, D., HERAZLAK, J., ASCHNER, M., MURDOCH, J. B. & ZHENG, W. 2011. In vivo measurement of brain GABA concentrations by magnetic resonance spectroscopy in smelters occupationally exposed to manganese. *Environmental health perspectives,* 119**,** 219.

EMARA, A. M., EL-GHAWABI, S. H., MADKOUR, O. I. & EL-SAMRA, G. H. 1971. Chronic manganese poisoning in the dry battery industry. *Br J Ind Med,* 28**,** 78-82.

FITSANAKIS, V. A., ZHANG, N., AVISON, M. J., GORE, J. C., ASCHNER, J. L. & ASCHNER, M. 2006. The use of magnetic resonance imaging (MRI) in the study of manganese neurotoxicity. *Neurotoxicology,* 27**,** 798-806.

GROUP, I. C. O. R. P. T. & SNYDER, W. S. 1975. *Report of the task group on reference man*, Pergamon Oxford:.

HURLEY, L. S., KEEN, C. L., MANGANESE, E. U. & MERTZ, W. 1987. Trace elements in human health and animal nutrition. Academic Press, New York.

IREGREN, A. 1998. Manganese neurotoxicity in industrial exposures: proof of effects, critical exposure level, and sensitive tests. *Neurotoxicology,* 20**,** 315-323.

JIANG, Y. M., MO, X. A., DU, F. Q., FU, X., ZHU, X. Y., GAO, H. Y., XIE, J. L., LIAO, F. L., PIRA, E. & ZHENG, W. 2006. Effective treatment of manganese-induced occupational Parkinsonism with p-aminosalicylic acid: a case of 17-year follow-up study. *Journal of occupational and environmental medicine/American College of Occupational and Environmental Medicine,* 48**,** 644.

KIM, S. H., CHANG, K. H., CHI, J. G., CHEONG, H. K., KIM, J. Y., KIM, Y. M. & HAN, M. H. 1999. Sequential change of MR signal intensity of the brain after manganese administration in rabbits. Correlation with manganese concentration and histopathologic findings. *Invest Radiol,* 34**,** 383-93.

KIM, Y., LOBDELL, D. T., WRIGHT, C. W., GOCHEVA, V. V., HUDGENS, E. & BOWLER, R. M. 2015. Blood metal concentrations of manganese, lead, and cadmium in relation to serum ferritin levels in Ohio residents. *Biol Trace Elem Res,* 165**,** 1-9.

KY, S., DENG, H., XIE, P. & HU, W. 1992. A report of two cases of chronic serious manganese poisoning treated with sodium para-aminosalicylic acid. *British journal of industrial medicine,* 49**,** 66-69.

LEVY, B. S. & NASSETTA, W. J. 2003. Neurologic effects of manganese in humans: a review. *Int J Occup Environ Health,* 9**,** 153-63.

LIU, Y., BYRNE, P., WANG, H., KOLTICK, D., ZHENG, W. & NIE, L. H. 2014. A compact DD neutron generator-based NAA system to quantify manganese (Mn) in bone in vivo. *Physiol Meas,* 35**,** 1899-911.

LIU, Y., KOLTICK, D., BYRNE, P., WANG, H., ZHENG, W. & NIE, L. H. 2013. Development of a transportable neutron activation analysis system to quantify manganese in bone in vivo: feasibility and methodology. *Physiol Meas,* 34**,** 1593-609.

LIU, Y., MOSTAFAEI, F., SOWERS, D., HSIEH, M., ZHENG, W. & NIE, L. H. 2017. Customized compact neutron activation analysis system to quantify manganese (Mn) in bone in vivo. *Physiological Measurement,* 38**,** 452-465.

LU, L., ZHANG, L., LI, G., GUO, W., LIANG, W. & ZHENG, W. 2005. Serum concentrations of manganese and iron as the potential biomarkers for manganese exposure in welders. *Neurotoxicology,* 26**,** 257-265.

MENA, I., MARIN, O., FUENZALIDA, S. & COTZIAS, G. C. 1967. Chronic manganese poisoning. Clinical picture and manganese turnover. *Neurology,* 17**,** 128-36.

NELSON, K., GOLNICK, J., KORN, T. & ANGLE, C. 1993. Manganese encephalopathy: utility of early magnetic resonance imaging. *Br J Ind Med,* 50**,** 510-3.

O'NEAL, S. L., HONG, L., FU, S., JIANG, W., JONES, A., NIE, L. H. & ZHENG, W. 2014. Manganese accumulation in bone following chronic exposure in rats: steady-state concentration and half-life in bone. *Toxicol Lett,* 229**,** 93-100.

O'NEAL, S. L. & ZHENG, W. 2015. Manganese toxicity upon overexposure: a decade in review. *Current environmental health reports,* 2**,** 315-328.

OULHOTE, Y., MERGLER, D. & BOUCHARD, M. F. 2014. Sex- and age-differences in blood manganese levels in the U.S. general population: national health and nutrition examination survey 2011-2012. *Environ Health,* 13**,** 87.

PEJOVIC-MILIC, A., CHETTLE, D. R., OUDYK, J., PYSKLYWEC, M. W. & HAINES, T. 2009. Bone manganese as a biomarker of manganese exposure: a feasibility study. *Am J Ind Med,* 52**,** 742-50.

RACETTE, B. A. 2014. Manganism in the 21st century: the Hanninen lecture. *Neurotoxicology,* 45**,** 201-207.

RODIER, J. 1955. Manganese poisoning in Moroccan miners. *Br J Ind Med,* 12**,** 21-35.

SANTOS, D., BATOREU, C., MATEUS, L., DOS SANTOS, A. M. & ASCHNER, M. 2014. Manganese in human parenteral nutrition: considerations for toxicity and biomonitoring. *Neurotoxicology,* 43**,** 36-45.

SASSINE, M. P., MERGLER, D., BOWLER, R. & HUDNELL, H. K. 2002. Manganese accentuates adverse mental health effects associated with alcohol use disorders. *Biol Psychiatry,* 51**,** 909-21.

SCHULER, P., OYANGUREN, H., MATURANA, V., VALENZUELA, A., CRUZ, E., PLAZA, V., SCHMIDT, E. & HADDAD, R. 1957. [Manganese poisoning; clinical & environmental study in a manganese mine]. *Rev Med Chil,* 85**,** 623-30.

SENGUPTA, P. 2011. A scientific review of age determination for a laboratory rat: how old is it in comparison with human age. *Biomed Int,* 2**,** 81-89.

VALENTIN, J. 2002. Basic anatomical and physiological data for use in radiological protection: reference values: ICRP Publication 89. *Annals of the ICRP,* 32**,** 1-277.

WANG, J. D., HUANG, C. C., HWANG, Y. H., CHIANG, J. R., LIN, J. M. & CHEN, J. S. 1989. Manganese induced parkinsonism: an outbreak due to an unrepaired ventilation control system in a ferromanganese smelter. *Br J Ind Med,* 46**,** 856-9.

WELLS, E. M., LIU, Y., ROLLE-MCFARLAND, D., MOSTAFAEI, F., ZHENG, W. & NIE, L. H. 2017. Measurement of in vivo bone manganese and association with manual dexterity: a pilot study. *Environmental Research, submitted (will update the citation later)*.

WENNBERG, A., IREGREN, A., STRUWE, G., CIZINSKY, G., HAGMAN, M. & JOHANSSON, L. 1991. Manganese exposure in steel smelters a health hazard to the nervous system. *Scand J Work Environ Health,* 17**,** 255-62.

WHITLOCK, C. M., JR., AMUSO, S. J. & BITTENBENDER, J. B. 1966. Chronic neurological disease in two manganese steel workers. *Am Ind Hyg Assoc J,* 27**,** 454-9.

WILLIAMS, M., TODD, G. D., RONEY, N., CRAWFORD, J., COLES, C., MCCLURE, P. R., GAREY, J. D., ZACCARIA, K. & CITRA, M. 2012. *Toxicological Profile for Manganese*, Agency for Toxic Substances and Disease Registry (ATSDR) Toxicological Profiles, Atlanta, Ga, USA.

WONGWIT, W., KAEWKUNGWAL, J., CHANTACHUM, Y. & VISESMANEE, V. 2004. Comparison of biological specimens for manganese determination among highly exposed welders. *Southeast Asian J Trop Med Public Health,* 35**,** 764-9.

ZHANG, L. L., LU, L., PAN, Y. J., DING, C. G., XU, D. Y., HUANG, C. F., PAN, X. F. & ZHENG, W. 2015. Baseline blood levels of manganese, lead, cadmium, copper, and zinc in residents of Beijing suburb. *Environ Res,* 140**,** 10-7.

ZHENG, W., FU, S. X., DYDAK, U. & COWAN, D. M. 2011. Biomarkers of manganese intoxication. *Neurotoxicology,* 32**,** 1-8.

ZHENG, W., KIM, H. & ZHAO, Q. 2000. Comparative toxicokinetics of manganese chloride and methylcyclopentadienyl manganese tricarbonyl (MMT) in Sprague-Dawley rats. *Toxicological Sciences,* 54**,** 295-301.