Investigation of biomarkers related to manganese exposure in metalworkers

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Abstract

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Manganese (Mn) is found in both environmental and occupational settings, and can cause neurological conditions affecting cognition and mobility. Existing Mn exposure biomarker studies have not reached consensus on a valid and reproducible biomarker when looking cross-sectionally at biological specimens from Mn-exposed individuals. Here, we investigate biomarkers of Mn in three metalworking cohorts using a variety of novel techniques. A mixed model was fit to longitudinal air and blood Mn (MnB measurements to obtain estimates of airborne exposure. Estimated Mn exposures were summed over exposure windows and related to measured MnB. We found that after 30 days of exposure, an increase in air Mn was associated with an increase in MnB. Using the same exposure model, the relationship between modeled air Mn and Mn at the target organ (as determined by magnetic resonance imaging) was investigated. T1-weighted indices of

basal ganglia exhibited increases in signal intensity in relation to increased Mn exposure, demonstrating that T1-weighted changes can be detected in the brain even at low levels of exposure.

Global and targeted urine metabolomics data, generated using mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR), were utilized to investigate biochemical differences between persons exposed and unexposed to Mn. We used univariate analysis to investigate differences between groups defined by exposure. The reproducibility of the global (MS, NMR) and targeted (NMR) metabolomics profiles were investigated by building elastic net models in the primary data set, and testing the models in a second sample collected from the study participants, and in samples from a different cohort. From univariate global analyses, nine ions were found to be related to Mn exposure using the MS data, but no NMR bins were found to be associated with exposure. Mn exposure could be classified fairly well in a second set of samples from the study participants using the elastic net models, however the models were less robust when using samples from a different cohort. This discrepant results could be due to differences in Mn exposure. Work presented here underscores the challenges of using and identifying putative biomarkers for use in occupational settings.

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DEDICATION

To my son Stellan. May you grow up to live in a world free of recognized occupational and environmental health hazards.

Chapter 1. INTRODUCTION

Manganese (Mn) is an established neurotoxicant that is associated with cognitive (Bouchard et al. 2007; Bowler et al. 2007; Mergler et al. 1994) and motor (Lucchini et al. 1999; Roels, Lauwerys, Buchet, et al. 1987; Roels et al. 1985) health outcomes. Mn is common in the human diet and found in foods such as pineapple, whole grains, dark leafy greens, legumes, nuts, seeds, and multivitamins (USDA 2012). The dietary Mn intake for adults is estimated to range between 0.9 to 10 mg/day. The estimated safe and adequate dietary intake established by the U.S. National Research Council is 2.3 mg/day for men and 1.8 mg/day for women (Greger 1998). However, only 1-5% of orally ingested Mn is absorbed into the blood; a level that is tightly regulated based on concentration of Mn in the diet, with less Mn absorbed into the blood when more Mn has been ingested (Aschner, Erikson, and Dorman 2005). Environmental Mn exposure can also occur through inhalation of airborne Mn, although exposure via ambient air sources is estimated to be less than 2 µg Mn/day for an average person (Aschner, Erikson, and Dorman 2005).

While Mn is ubiquitously found in ambient air, drinking water, and foods common to a Western diet, it is in Mn-utilizing work environments, or in communities surrounding Mn-industries, where chronic and elevated exposures are most frequently found. In the United States, it is estimated nearly 700,000 workers are employed in metal-working occupations with direct contact with Mn or Mn-containing fumes (e.g. welders, solderers, brazers, and foundry workers) (Bureau of Labor Statistics 2016), but it can be assumed workers in other manufacturing work environments may also be indirectly or directly exposed to Mn in their workplace. Community exposure to Mn, such as from traffic or industrial sources are also of note, with individuals living in close proximity to Mn-utilizing work environments potentially having elevated airborne

exposure to Mn. Elevated Mn exposure has been shown to increase the prevalence of Parkinsonian disorders (Lucchini et al. 2007; Finkelstein and Jerrett 2007). Potentially elevated community exposures, as well as high and chronic exposures in occupational settings, make Mn a relevant and pressing public health issue.

Historically in occupational settings, Mn exposure is measured using industrial hygiene air monitoring techniques by sampling either the respirable or inhalable Mn fractions with a sampling pump. Particle size is an important determinant of metal fume deposition in the respiratory tract, and therefore of potential health effects and absorption (Antonini 2003; Antonini et al. 2004). Inhaled Mn can be deposited in the nasopharyngeal airways (and may reach the brain through direct olfactory transport), the trachea-bronchial region (and mostly swallowed, entering the gastrointestinal tract), or the alveolar or pulmonary region of the respiratory tract (Antonini, Santamaria, et al. 2006). For those particles deposited in the alveolar or pulmonary region, time and mechanism of uptake into body fluids is further dependent on Mn oxidation state, particle solubility, and particle concentration among other factors (Antonini, Santamaria, et al. 2006; Berlinger et al. 2008).

Consequently, when solely using traditional air monitoring for exposure assessment, the quantitative estimation of workers' exposure and dose of Mn, and subsequent evaluation of exposure-related health effects remains problematic. Another tool for exposure assessment is biomonitoring—as this is often cheaper, easier, individual-specific, and may better estimate body dose as compared to traditional air monitoring techniques. Given the health effects related to Mn exposure, research has focused on finding both exposure and disease biomarkers for Mn and its related neurological outcomes (Apostoli, Lucchini, and Alessio 2000; Zheng et al. 2011; O'Neal and Zheng 2015). A variety of tissues and fluids have been used to evaluate Mn exposure,

including short-term biomarkers of exposure in serum (Chia et al. 1993; Järvisalo et al. 1992; Loranger and Zayed 1995; Myers, Thompson, et al. 2003), plasma (Bowler et al. 2007; Grass et al. 2010), urine (Ellingsen et al. 2006; Järvisalo et al. 1992; Roels, Lauwerys, Genet, et al. 1987), and saliva (Wang, Du, and Zheng 2008), and long-term biomarkers in brain tissue (evaluated via magnetic resonance imaging) (Criswell et al. 2012; Jiang et al. 2007), toenails (Laohaudomchok, Lin, Herrick, Fang, Cavallari, Christiani, et al. 2011), and hair (Bader et al. 1999; Cowan et al. 2009). Mn in whole blood is the most commonly used short-term biomarker, but literature presents mixed results as to its utility. We performed a meta-analysis of existing literature and found that at exposure levels above about 10 $\mu g/m^3$, levels which would be plausible in many work environments and the communities surrounding them, a relationship existed between Mn in blood and Mn in air (Baker et al. 2014). However, these studies had several limitations; the majority of studies included in this meta-analysis were cross-sectional, did not consider baseline blood Mn levels, did not utilize repeat measurements, and did not specify when the blood sample was taken in relation to the air sample.

Another limitation facing occupational Mn biomarker studies is that the pharmacokinetics of Mn in humans exposed to metal fumes is not well established. The current body of Mn toxicokinetic literature is primarily in primates or rodents exposed to manganese chloride, manganese phosphate, or manganese sulfate. These chemicals differ from the Mn compounds in occupational settings (Zheng, Kim, and Zhao 2000; Dorman et al. 2001; Vitarella et al. 2000; Antonini, Santamaria, et al. 2006). To explore the timing between welding fume Mn exposure, uptake, and health effects, Antonini et al. developed a controlled chamber to expose animals to welding fumes (2006). Antonini et al. found that 80% of Mn was cleared from the lungs eight days after exposure to mild steel welding fumes, and nearly all Mn was cleared from the lungs 21

days post-exposure; this elimination time from the lung increased with stainless steel welding fumes (Antonini et al. 2011). Antonini et al. also found that Mn was more likely to leave the respiratory system than Fe, and elevated levels of Mn were found in the kidney, spleen, and specific areas of the brain. Thus, understanding the timing between Mn exposure and uptake into the circulatory system, and incorporating this into Mn biomarker studies, will help to inform the relationship between air Mn and blood Mn. The relationship between time of exposure to Mn and levels of Mn in blood is presented in Chapter 2.

T1-weighted magnetic resonance imaging (MRI) have been utilized as a biomarker of in vivo Mn brain exposure. T1-weighted scans measure the longitudinal proton relaxation time and can be affected by the concentration of paramagnetic metals such as Mn, which lead to a shorter T1 relaxation time and greater MRI signal intensity in the affected regions. Although T1-weighted MRI indices are not a clinical measure of symptoms of chronic manganese poisoning (considered broadly as manganism) Kim et al. found increases in signal intensities on T1-weighted MRI in asymptomatic Mn-exposed workers and increased signal intensity was found to reflect recent Mn exposure (1999). Related to MRI, positron emission tomography (PET) has also been used to detect biomarkers of Mn effects in the brain. Criswell et al. found a 10% reduction in 6-[18F]fluoro-L-DOPA uptake using PET in Mn-exposed welders, suggesting pre-clinical neurotoxicity can be found using highly sensitive imaging methods (2011). However, neither of these studies had longitudinal scans to assess changes over time, as was possible scanning the inception cohort introduced in Chapter 2. An analysis and discussion of the relationship between Mn exposure and longitudinal measures of MRI, UPDRS3, and Grooved Pegboard is presented in Chapter 3. This work represents the first time, to our knowledge, that these measures have been investigated longitudinally in a well characterized inception cohort.

In addition to investigating biomarkers of exposure, such as whole blood and MRI, and biomarkers of effect, such as UPDRS3 and Grooved Pegboard, we undertook global and targeted metabolomics analyses to attempt to determine putative biomarkers of Mn exposure in persons exposed and unexposed to Mn in an occupational setting. Metabolomics is the study of the small molecules (<1000 daltons) in biological systems, often important in metabolic function and signaling, or representing a fingerprint of a specific cellular process (Wishart 2010). Global metabolomics uses mass spectrometry, NMR or other analytical methods in an untargeted approach to detect the small molecules found in a biological sample, often detecting thousands of unique ions in each sample. The typical mass spectrometry approach used for global metabolomics can provide the molecular weight of the ion and its retention time—that is, how long it took to elute chromatographically—but provides no additional information on chemical structure. Global metabolomics is an exploratory analysis method and is a means of hypothesis generation. The selection of putative biomarkers using this approach is dependent on statistical methods to select interesting ions from the thousands of specific ions in the data. Therefore, some ions that are considered statistically significant, may not be related to the exposure in question (i.e., false positive results). Similarly, some ions may not be selected though they truly are related to the exposure in question (i.e., false negative results). Targeted metabolomics quantitates chemically characterized and biologically annotated ions in a biosample—those features that have already been identified by empirical formula or name. Thus, while a targeted metabolomics dataset has fewer compounds than a global metabolomics dataset, the identities of the compounds and their associated biochemical pathways are known. Ideally, results from both global and targeted metabolomics studies should be replicated in independent samples and the chemical nature and biological role of the ions identified (Broadhurst and Kell 2006).

Metabolomics analysis techniques have previously most commonly been used to identify biomarkers or physiological alterations related to health effects, such as for various neurologic diseases (Sato et al. 2012; Ibanez et al. 2012; Hassan-Smith et al. 2012; Zhang, Sun, and Wang 2013), various cancers (Wang, Zhang, and Sun 2013; Zhang et al. 2012), and of particular relevance to this work, Parkinson's Disease (Bogdanov et al. 2008; Michell et al. 2008; Ahmed et al. 2009). While using metabolomics to distinguish between patients and controls is a rapidly advancing field, using metabolomics to distinguish between exposed and non-exposed persons is a relatively novel concept, and is a key approach in exploring the exposome—identification of the biological traces of lifelong environmental exposures within individual physiological histories (Wild 2005). Despite this, the few human metabolomics studies investigating biomarkers related to exposure have looked at metabolomics alterations related to dietary exposure to vitamins or minerals, not from chemical exposures in an occupational setting (Johansson-Persson et al. 2013; Astle et al. 2007). Other studies have investigated metabolomics profiles related to exposure using model organisms, but even these rely on dermal exposures, exposures in the water, or exposures through injection as opposed to inhalation exposure (Tyburski et al. 2008; McKelvie et al. 2009; Tuffnail et al. 2009; Santos et al. 2009). Studying the metabolomics profile related to Mn exposure status in humans is highly innovative and could serve as a model for future application of metabolomics in occupational and environmental health studies. This is particularly important given the substantial limitations in available biomarkers of acute and chronic Mn exposure. Chapters 4 and 5 of this dissertation discuss global metabolomics analyses related to the discovery of Mn exposure biomarkers in urine collected from exposed and unexposed workers in the Puget Sound region and Wisconsin. Chapter 6 describes a study using a targeted NMR metabolomics approach to investigate

differences between urine collected from Mn exposed and unexposed persons from the Puget Sound region and Wisconsin.

The work presented herein will help to inform potential biomarkers of Mn exposure, and help to overcome some of the critical barrier to identifying a reliable, reproducible, and easily-accessible biomarker of Mn exposure. This dissertation presents novel biomarker data analysis techniques for blood Mn (Chapter 2), investigates longitudinal imaging biomarkers of Mn exposure in an inception cohort (Chapter 3), and employing global and targeted metabolomics, using both mass spectrometry and nuclear magnetic resonance, as a novel method of attempting to identify urinary biomarkers of Mn exposure (Chapters 4-6).

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Chapter 2. USING EXPOSURE WINDOWS TO EXPLORE BLOOD AS A BIOMARKER OF MANGANESE EXPOSURE*

*Published as: Baker MG, Stover B, Simpson CD, Sheppard L, Seixas NS. *Using exposure windows to explore an elusive biomarker: blood manganese.* Int Arch Occup Environ Health 2016, 89(4); 679-87. Reproduced here per License Agreement with Springer-Verlag Berlin Heidelberg.

2.1 INTRODUCTION

Exposure to manganese (Mn) has been implicated in manganism, a parkinsonian syndrome overlapping clinically with Parkinson's Disease (PD), making Mn exposure assessment of interest to many public health professions (Antonini, Santamaria, et al. 2006; Aschner 2000; Calne et al. 1994). One such tool for exposure assessment is biomonitoring—as this is often cheaper, easier, individual-specific, and may better estimate body dose as compared to traditional air monitoring techniques. Commonly, body fluids such as urine (Järvisalo et al. 1992; Ellingsen et al. 2006; Roels, Lauwerys, Genet, et al. 1987), whole blood (Pesch, Weiss, Kendzia, Henry, Lehnert, Lotz, Heinze, Kafferlein, et al. 2012; Smith et al. 2007; Ellingsen et al. 2006), plasma (Grass et al. 2010; Bowler et al. 2007), or serum (Myers, Thompson, et al. 2003; Chia et al. 1993) are used as biomarkers of exposure to Mn, but hair (Bader et al. 1999; Rodrigues et al. 2008), toenails (Laohaudomchok, Lin, Herrick, Fang, Cavallari, Christiani, et al. 2011; Sriram et al. 2012), and MRI (Baker, Criswell, et al. 2015; Criswell et al. 2012) have also been explored in the literature as exposure biomarkers for Mn.

Ingested Mn is tightly regulated in the body (with the majority being eliminated via feces), and ambient environmental exposures are generally low. Thus, it is inhalation in occupational environments where excessive exposure to Mn usually occurs (Aschner, Erikson,

and Dorman 2005). Welding shops are one such occupational environment, with welders being exposed to eight hour mean personal breathing zone concentrations of Mn ranging from 0.04 mg/m3 to over 2.0 mg/m3 based on the welding type, degree of enclosure, and use of ventilation and respiratory protection (Hobson et al. 2011). While Mn in welding fume deposited in the nasopharyngeal airways can reach the brain through direct olfactory transport, Mn also enters the circulatory system via the lung and crosses the blood brain barrier to reach the target organ (Antonini, Santamaria, et al. 2006; Tjalve and Henriksson 1999). However, timing of uptake from the lung into the blood is dependent on particle size distribution, solubility of the Mn compounds, concentration, and individual factors (Berlinger et al. 2008). Given the neurologic health effects associated with Mn in welding fume, it is reasonable to assume that after uptake, inhaled Mn is circulating in the blood prior to either elimination or crossing the blood brain barrier and reaching the target organ, making blood Mn (MnB) seem an attractive biomarker of Mn exposure (Aschner et al. 2007; Criswell, Perlmutter, Videen, et al. 2011; Racette et al. 2005).

As a result, many studies have explored blood as a biomarker of Mn inhalation exposure, yet there is no clear consensus as to its utility and limitations. In previously published work, MnB levels appear to be fairly tightly regulated within an individual, and there is substantial between-individual variation, which could make interpretation of the relationship between blood Mn and Mn exposure challenging with a common cross-sectional study design (Baker, Simpson, et al. 2015). It has been largely cross-sectional study designs that dominate the literature relating MnB and inhalation exposure. We reviewed the existing body of literature for articles relating airborne Mn and MnB. We weighted the 29 studies included in the review by study size, and fit a segmented regression model. Our results showed that there appeared to be a positive linear relationship between MnB and airborne Mn when Mn exposure exceeded approximately

10µg/m3 (Baker et al. 2014). However, 26 of the 29 studies included in this review were cross-sectional, relating a single MnB and air measurement that might not have been taken at pharmacologically relevant (or even concurrent) time periods.

The pharmacokinetics of Mn in humans exposed to welding fume is not well established, with the current body of toxicology literature focusing on the pharmacokinetics related to primate or rodent exposure to compounds of Mn such as manganese chloride, manganese phosphate, or manganese sulfate. These chemicals differ from the Mn compounds known to predominate in a welding setting (Zheng, Kim, and Zhao 2000; Dorman et al. 2001; Vitarella et al. 2000; Antonini, Santamaria, et al. 2006). To explore the timing between Mn exposure, uptake, and health effects in an occupational setting, Antonini et al. developed a pharmacokinetic model using animals exposed to generated welding fume in a controlled chamber (2006). In contrast, the current manuscript aims to explore the timing between exposure to Mn and possible changes in blood Mn in an occupational setting by utilizing longitudinal blood and air measurements from welder trainees. With repeat exposure measurements in a well-characterized cohort, we are able to determine subject specific estimates of exposure for each day the trainee was enrolled in our study, and then sum these estimates over different time windows of exposure for comparison with longitudinal MnB measurements. With repeat blood measurements for each subject, this manuscript also aims to examine the variance components exhibited in MnB. Understanding the time course between exposure to Mn and uptake into the blood will allow a more meaningful interpretation of exposure-biomarker data, and help to determine the utility of blood as a biomarker for Mn exposure.

2.2 METHODS

Occupational setting and study population

The study setting for this manuscript is a longitudinal inception cohort of 56 student welders with no prior occupational exposure to Mn, who were enrolled in a welding training program at a technical college in Washington state. Subjects were enrolled throughout the duration of the study, with data collection occurring from April 2011 to June 2013. Enrolled welding trainees were monitored for airborne exposure and MnB over the course of their training program. Details of the study design are described elsewhere (Baker et al. 2014). All study protocols were reviewed and approved by the University of Washington Institutional Review Board and subjects provided written informed consent.

The welding training program consisted of five academic quarters, with standard academic breaks in between, where students progressed through different welding processes in the order: oxyacetylene, shielded metal arc welding (SMAW), flux core arc welding dual shield (FCAW-DS), flux core arc welding inner shield (FCAW-IS), gas metal arc welding (GMAW), and gas tungsten arc welding (GTAW). The number of days spent on each process could vary between subjects. Students also intermittently did other metalworking tasks (i.e. cutting and/or grinding) which we characterized as a separate work category if that is what the welder reported doing for the majority of the day. Trainees were in the facility Monday through Friday from about 8 AM to 2 PM, but time spent welding was interspersed with classroom instruction. Each welding booth in the facility had its own adjustable exhaust ventilation hood, and students were given the choice of whether or not to buy and wear respiratory equipment, which was not

provided. There was no formal respiratory protection training or fit testing related to the traineeship.

Each quarter the subject was enrolled in the study a nurse drew 6 mL blood samples at the following times: the morning and afternoon of the first Monday and Friday of the quarter, and morning and afternoon on the last Monday and Friday of the quarter, for a potential eight blood samples each academic quarter. On each day that a subject provided blood samples, they were also fitted with a personal air pump for the duration of activity at the school to assess airborne exposure to Mn. At the end of each sampling day, subjects completed an exposure questionnaire to determine the type of welding done that day, the time spent welding, the use of any respiratory protection, and to assess any metalworking done outside of class time.

Blood Mn sampling and analysis

On mornings and afternoons of sampling days, 6 mL of whole blood was collected in plastic Vacutainer evacuated tubes containing 10.8 mg K2EDTA anticoagulant (BD, Franklin Lakes, NJ) and transported on ice to the American Industrial Hygiene Association (AIHA) Accredited University of Washington Environmental Health Laboratory (UW EHL) within two hours of collection. One mL of whole blood was pipetted into a 15 mL Corning CentriStar polypropylene centrifuge tube (Corning, NY), stored at 4 degrees C until multi-element analysis of whole blood using an Agilent 7500 CE ICP-MS via microwave assisted acid digestion (Bocca et al. 2003). Over the course of the 26 month study, blood was submitted for analysis in 18 batches, but samples were randomly assigned to batches with regard to time of day, time of week, and time of quarter. The limit of detection (LOD) for Mn ranged from 0.1 - 2.0 ng/mL and is based on three times the standard deviation of the blanks. A total of 1170 blood samples were

analyzed. No blood samples fell below the LOD for Mn. The slight variability in LOD is due to changing levels of blank contamination in the UW EHL over the 26 month time period that samples were submitted for analysis. Between batches, the percent recovery for Mn as compared to the control material (ClinChek Level 1 Whole Blood Control, lyophilized for trace elements (Lot 038), Recipe Chemicals, Munich, Germany) ranged between 91% and 134% for all 18 batches with an overall average accuracy of 110% ± 12%. The average within batch variation in the control material was 6.8% ± 6.1%.

Airborne Mn exposure assessment

On each sampling day (four days each academic quarter) the subject was enrolled in the study, they were fitted with a personal air pump (SKC AirChek XR4000, Eighty Four, PA, USA) with an attached pre-weighed 37mm 0.8µm pore mixed cellulose ester filter housed inside a closed face polystyrene cassette and hung on their collar outside the welding garments and helmet. The pumps were calibrated to 2.0 L/min, and this flow rate was checked at the beginning and end of each full-day sampling period. One sample had a ± 10% change in pump flow rate and was excluded. Filters were analyzed gravimetrically for total particulate mass, digested in 10mL of a 1:1 mixture of concentrated nitric acid and deionized water using open vessel microwave assisted digestion, and analyzed at UW EHL for trace metals using an Agilent 7500 CE ICP-MS in He collision mode to eliminate polyatomic interferences. Samples were analyzed using the modified United States Environmental Protection Agency (US EPA) method 6020a Revision 1 (U.S. Environmental Protection Agency 1998). Each day of sampling included two field blanks, which were handled in the same manner as the samples deployed in the field. Over the course of the 26 month study, 622 air samples were submitted for metals analysis in six

batches, and were randomized with regard to time of week and time of quarter. Between batches, assay accuracy based on the spike recovery samples ranged between 88.0% and 110% with an overall average accuracy of 99.4 ± 7.2% for the six batches. The average within batch variation in the spiked samples was 8.2% ± 9.7%. Considering differences in pump flow rate and duration of sampling between the subjects, the maximum LOD for Mn was 0.13 µg/m3 µg, based on three times the standard deviation of the blanks deployed in the field. No air samples fell below the LOD for Mn.

Statistical analysis

Measured Mn air concentrations were normalized to 8-hr TWA Mn concentrations, and as the measured data were determined to be lognormal, the exposure samples were natural log-transformed. A mixed model was fit to obtain estimates of exposure by welding type (fixed effect), adjusted for subject (random effect) on the log-transformed data. While enrolled in the welding program, attendance and progress records were used to determine days each subject was present and welding, and what type of welding the subject was doing each day. Thus, on each day a subject was present and welding, estimated subject-specific exposure based on the type of welding done that day was calculated. Weekends, vacation days, and days the subject was absent were coded as zero-exposure days. Thus, for each subject we generated a personal calendar of estimated Mn airborne exposure for each day they were enrolled in the welding program, based on attendance and type of welding. A total of 18,833 day- and subject-specific exposure estimates were generated, including zero-exposure days. Results of the models on the ln-scale were used to calculate the subject- and day-specific arithmetic mean exposure by using maximum likelihood estimates, incorporating the within-subject variance.

For each blood sampling day, we summed preceding exposures over various exposure windows: one day, seven day, 30 day, 90 day, and cumulative, which was a summation of all exposures from entry into the welding program until the day of the blood sample. All exposure windows are in units of mg/m3-days. The average number of exposed days represented by the cumulative exposure measure is 197 ± 142, ranging from 1 day (the minimum number of days of exposure on a subject's first blood sample) to 805 days. About 25% of cumulative exposure periods included in this mixed model represent time periods of less than 90 days. For afternoon blood samples, the same day estimated exposure counted in the exposure windows; for morning blood samples the previous day was counted as the first day of previous exposure.

The associations between predicted exposures (for all five exposure windows) and measured MnB were assessed on the native scale using a longitudinal linear mixed-effects model including subject as a random effect. Several covariates were considered in the mixed models, including use of respiratory protection (based on self-reported percentage the subject used a respirator during sampling weeks), age at baseline, and smoking status, but none were found to affect the slope parameter of interest. Thus, reported models associating MnB and estimated exposure only include subject as a random effect with no additional fixed effect covariates. All presented models use only afternoon blood values in relation to preceding windows of exposure. Using morning blood values or the change in blood value over the course of a day did not affect the coefficients of association, and we wished to avoid making redundant models by modeling morning blood, afternoon blood, and the change in blood values over the course of a day in relation to preceding windows of exposure.

To directly compare the coefficients of association relating MnB to airborne Mn exposure over the five exposure windows despite each representing different durations of exposure, all

point estimates from the linear mixed-effects models were normalized to a 30-day average exposure value (see equation 1). We standardized the effects to 3 mg/m3-days based on the American Conference of Industrial Hygienists (ACGIH) recommended 8-hr time weighted average threshold limit value (TWA TLV) for inhalable Mn of 0.1 mg/m3 over a 30 day exposure window, by multiplying 0.1 mg/m3 by 30 days in a month. Each estimated coefficient of association between MnB and air exposure for the various exposure windows was normalized to the value of 3 mg Mn/m3 over 30 days (for cumulative exposure we used the mean number of days representing a cumulative exposure, x=197) as:

Equation 1: Calculating 30 day normalized exposure for the exposure windows

$$\left(3\frac{mg}{m^3-days}\right) * \beta_{exp,x} * \left(\frac{x}{30\ days}\right) = standardized\ coefficient$$

x = length of the exposure window

βexp,x = coefficient of association between MnB and air exposure for the exposure window represented by x

2.3 RESULTS

Of the inception cohort, 52 of the 56 subjects were male (93%). The average subject was 28 years of age at baseline, provided 21 blood samples (SD: 5.9, range: 1, 26) while enrolled in our study, and had 11 air samples (SD: 11.4, range: 4, 48). Only 25% of subjects (n=14) used a respirator more than 90% of the time.

Measured and predicted Mn exposures, along with blood values, are summarized by welding type in Table 1. The number of predicted exposure values for each welding type is fewer

than the number of measured exposure values because only one predicted exposure value was generated for each welder for each welding type they completed while enrolled in our study. Thus, welders who did not complete a welding type do not have an estimated exposure value for that welding type. The arithmetic mean predicted values are similar to the measured exposure values, though geometric mean values were more different. The measured exposure mean values are the means of all collected exposure values, whereas the predicted mean values are means of the individual mean levels. Thus, the within-subject variation has already been incorporated into the geometric mean value for the predicted exposure values, so the predicted values have higher geometric mean and lower GSDs relative to the measured exposure values.

Arithmetic mean MnB was highest during GTAW welding (9.6 ± 3.8 ng/mL) which is the last module welders complete in the program, but has some of the lowest measured Mn exposures (arithmetic mean: 5.5 ± 4.4 µg/m3). Arithmetic mean MnB was lowest during oxyacetylene welding (8.0 ± 3.0 µg/m3) which is the first welding module welders complete in the program, and would include most baseline MnB measurements. Oxyacetylene welding also represents the lowest measured airborne Mn exposure (arithmetic mean: 5.2 ± 3.1 µg/m3). The welding type with the highest measured Mn exposure was FCAW-DS, with arithmetic mean exposures of 40.7 ± 32.4 µg/m3. Only 3.4% of samples (n=21) exceed the ACGIH inhalable 8-hr TWA TLV of 0.1 mg/m3, and all measured exposures were below the Occupational Safety and Health Administration permissible exposure limit (OSHA PEL) for Mn in total inhalable dust of 5.0 mg/m3 (ceiling). Table 2 shows the predicted exposure estimates that were used in the longitudinal linear mixed models for all the time points considered in this analysis.

Table 3 shows descriptive statistics for MnB over the various time points, stratified by morning and afternoon samples. For paired samples, MnB tends to decrease over the course of a

day, even when stratifying by time of week and time of quarter. A paired t-test found all but one of these decreases to be significantly different from zero. Despite these apparent decreases, using morning blood values or the change in blood value over the course of a day did not meaningfully affect the coefficients of association in any of the mixed models considered here. A linear mixed model including subject as a random effect was used to estimate differences in MnB over the course of a week and quarter, stratified by time of day. The results are also reported in Table 3. MnB tended to decrease over the course of a week for both morning and afternoon samples (though only significantly different from zero in afternoon samples), and didn't show a change different from zero over the course of a quarter for either morning or afternoon samples.

Table 4A shows the estimated coefficients of association between predicted exposure over the various time windows and MnB measured in the afternoon. Table 4B shows these coefficients standardized to a 30-day exposure period. Afternoon MnB showed no relationship with that day's exposure, or the preceding seven or 30 days of exposure. However, when considering the preceding 90 days exposure, a slight relationship emerges, with a one mg/m3-days increase in air Mn being associated with, on average, a 0.26 ng/mL increase in MnB (SE: 0.13, 95% CI: 0.005, 0.514). Similarly, when considering all preceding exposures (cumulative exposure) a one mg/m3-days increase in air Mn is associated with, on average, a 0.09 ng/mL increase in MnB (SE: 0.04, 95% CI: 0.005, 0.174). Standardized to a 30-day exposure period, a 3 mg/m3 increase in 90 day Mn exposure is associated with a 2.34 ng/mL average increase in MnB (SE: 1.17, 95% CI: 0.049, 4.63), and a 3 mg/m3 increase in cumulative exposure is associated with an average increase in MnB of 1.77 ng/mL (SE: 0.85, 95% CI: 0.125, 3.44). Figure 1 demonstrates the increasing relationship between MnB and exposure over longer time periods or higher levels of cumulative exposure when considering these standardized coefficients.

Variance components are also presented in Table 4A, and it can be seen that for all exposure windows considered the majority of variance is between subjects. This was similar to what was found in our previous work on a more restricted sample of subjects from this cohort (Baker, Simpson, et al. 2015).

2.4 DISCUSSION

The longitudinal study design presented here allowed investigation of relationships between measured MnB and modeled inhalation exposures occurring over different time windows, the first time such work has been presented in the literature. With this analysis, we found that when considering the preceding 30 days of exposure, a one mg/m3-days increase in air Mn is associated with, on average, a 0.57 ng/mL increase in MnB (95% CI: -0.04, 1.19), but this estimate is still consistent with a zero slope. However, when considering a 90 day preceding exposure window, and a cumulative exposure window, a one mg/m3-days increase in air Mn is associated with a 0.26 (95% CI: 0.005, 0.51) and 0.09 (95% CI: 0.006, 0.17) ng/mL increase in MnB, respectively, both results that were found to be significantly different from a zero slope.

Point estimates for all the exposure windows (one day, seven day, 30 day, 90 day, cumulative) were normalized to a 3mg/m3-days exposure. This exposure was based on 30 days of exposure at the ACGIH recommended 8-hr TWA TLV for inhalable Mn of 0.1 mg/m3. Upon normalizing the point estimates, an increasing trend emerged when looking at the relationship between afternoon MnB and summed exposures in the preceding seven, 30, and 90 day exposure windows. Though the coefficients for one, seven, and 30 day exposure windows were consistent with a zero slope, when considering the 90 day exposure window a 3 mg/m3-days increase in

exposure over a 30 day period was consistent with a 2.34 ng/mL increase in MnB (SE: 1.17, 95% CI: 0.049, 4.63). Cumulative exposure also maintained a significant increase in MnB, with a 3 mg/m3-days increase in exposure over a 30 day window being consistent with a 1.77 ng/mL increase in MnB (SE: 0.85, 95% CI: 0.13, 3.44).

This relationship between MnB and Mn air exposure at higher accumulated levels of exposure (which would occur after a longer time of exposure) is consistent with what was presented in our previous review relating measures of MnB and Mn exposure with mostly cross-sectional data from the literature. In that meta-analysis, when weighting by study size and fitting a segmented regression, the relationship between MnB and Mn exposure became apparent after a certain threshold of exposure was reached, at about 10 µg/m3 (Baker et al. 2014). Similarly, Pesch et al. (2012) found that measured MnB seemed to relate to measured respirable Mn in air at around 50-100 µg/m3, below which no obvious correlation was present. However, both our review and the work by Pesch et al. found associations between MnB and exposure at lower levels of exposure than we found in this study, as we didn't see an apparent relationship until after 30 days of accumulated exposure, and the mean value used in our model for a 30 day accumulated exposure window was about 420 µg/m3-days. However, models presented in this paper are specific to accumulated exposure and duration of exposure, whereas our review paper and the work by Pesch et al. are specific to daily exposure estimates. As such, neither our review nor the work by Pesch et al. took into account the temporal aspects between air exposure and blood uptake, or used a cumulative measure of exposure for different time periods, which was done in this study, thanks to our ability to quantify temporal resolution with our longitudinal study design. Also, as subjects in our study were welding trainees with no previous occupational exposure to Mn, the delay before seeing a relationship between blood Mn and predicted airborne

Mn could also be due to the time necessary for the blood Mn to reach a steady state in the trainees. With career welders or in persons with long term, consistent exposure to Mn, the relationship between blood Mn and air Mn could manifest sooner, or stronger.

Creating exposure windows based on subject-specific estimates and their personal calendar of exposure is a powerful method to extrapolate the timing between exposure to Mn and its potential uptake into blood. This method could only be utilized because of the longitudinal measurements collected on the subjects, and the detailed information available on their daily tasks and attendance. The mixed model fit allowed creation of unique exposure estimates for each enrolled subject, and for each type of welding they did in the program. The exposure assessment done for this study represents the most robust exposure assessment for any study relating MnB and inhalation exposure to Mn. Using a mixed model including subject as a random effect means the data presented here is essentially the average of all individual trends observed in these data. Therefore, even though the models aren't predicting on an individual basis, they are controlling for inter-subject variation, thus making this substantially different from a repeated-measures design that does not control for within subject trends.

When looking at the variance components related to the relationship between afternoon MnB and seven day, 30 day, 90 day, and cumulative exposure time windows the majority of variance (61%) was found to be between individuals. Even more of the total variance (71%) was between individuals when assessing the relationship between afternoon MnB and the preceding one day of exposure. This supports our previous work, where the majority of variance in MnB levels in a subset of the apprentice welders was between individuals (94%) and only about 6% of the total variance was within an individual (Baker, Simpson, et al. 2015). Thus, in that subcohort, Mn seemed to be fairly tightly regulated in the blood, and the high degree of between-subject

variation in MnB was due to differences in individual biochemistry and baseline MnB, and not due to exposure duration or accumulation. However, this previous work only looked at a subset of the total welding cohort presented in this analysis (n=9 subjects), and analyses were restricted to only their first academic quarter of study in the apprentice training program, a time period of approximately 70 days with relatively low levels of exposure. Thus, it is logical that in this current study, which looks at some longer durations of exposure, there would be more variation within a subject as the tight regulation of Mn in the blood may be overcome due to increased exposure duration. Compared to the previous work, in this study there was an increase in total variance, and in particular, a greater proportion of the total variance was within a subject. This increase in within-subject variance, coupled with the relationship we began to see between MnB and modeled exposure in longer exposure windows, drives the conclusion that MnB may serve as a biomarker of exposure to Mn, but only over longer time periods, such that the tight regulatory control of Mn in the blood is perturbed. As shown in previous studies (Bader et al. 1999; Apostoli, Lucchini, and Alessio 2000), due to its high inter-individual variance, MnB is a biomarker that should be used to differentiate groups by exposure status, rather than for the characterization of the exposure of individuals.

Interestingly, measured MnB exhibited a mean decrease over the course of a day, even when stratifying by time of week and time of quarter. Generally, these decreases were statistically different from zero. This finding was contrary to what was expected to be seen a priori; it was assumed an increase in MnB would be present over the course of a day as exposure increased. Taking urine samples every four hours for a 24 hour period from welders, Jarvisalo et al. found a diurnal variation of Mn in urine, with higher urine Mn values found in the morning compared to in the afternoon or evening (Järvisalo et al. 1992). Given the burden to subjects in

collecting serial blood samples in a similar manner, diurnal variability has not been established for MnB in humans, though seems a plausible hypothesis. In this study, of the 557 paired blood samples taken on a given day, 37% (n=204) exhibited an increase in MnB over the course of a day, wheras 63% (n=353) exhibited a decrease in MnB over the course of a day. Diurnal variability could explain the apparent decrease observed in MnB over the course of a day, and changes in the type of welding being done over the course of a week or quarter (for example, moving from a high exposure welding practice to a low exposure welding practice) could explain some of the decreases observed over long time periods in these data. However, other pharmacokinetic reasons may explain the changes seen over the course of a week and academic quarter (even when controlling for time of day) which would require further investigation.

2.5 Conclusion

The existing body of literature that has explored blood as a biomarker of Mn exposure has employed largely cross-sectional study designs, often trying to relate a single blood and exposure measurement collected on the same day (Baker et al. 2014). The results presented here show that a cross-sectional approach to assess the utility of blood as a biomarker of Mn would not be ideal, since a single blood sample is likely representing exposures accumulated over a longer preceding time period (at least 30 days) not over the sampled work shift. The longitudinal study design and subject-specific exposure modeling allowed the exploration of different time windows of exposure and changes in individual MnB levels over time, which is an improvement over the existing body of literature and can provide more insight into the biochemical properties (and time course) of inhaled Mn in welding fume.

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CHAPTER 2: FIGURES AND TABLES

Table 1. Measured and predicted Mn exposure values (µg/m^3) and blood values (ng/mL) by welding type

	Measured (µg/m3)				Predicted (µg/m^3)***				All Blood (ng/mL)			
Welding Type	**n***	**AM**	**GM**	**GSD**	**n****	**AM**	**GM**	**GSD**	**n***	**AM**	**GM**	**GSD**
Oxyacetylene	80	5.2	4.2	2.0	56	6.4	6.4	1.2	147	8.0	7.4	1.4
SMAW	315	34.7	22.8	3.0	55	36.8	36.0	1.2	547	8.2	7.5	1.5
FCAW (Inner shield)	32	34.5	23.6	3.0	37	35.7	34.9	1.2	92	9.1	8.4	1.5
FCAW (Dual shield)	75	40.7	25.5	3.6	43	46.1	44.9	1.3	141	9.3	8.8	1.4
GMAW	62	28.6	21.0	2.3	34	33.7	33.0	1.2	117	9.4	8.8	1.4
GTAW	36	5.5	4.0	2.3	29	6.4	6.3	1.2	104	9.6	8.8	1.5
Cut/Grind	22	13.1	5.6	3.7	21	8.3	8.0	1.3	22	8.6	8.1	1.4
Total	622	28.6	15.9	3.5	275	26.2	19.2	2.4	1170	8.6	8.0	1.5

*number of collected samples

**number of subjects who did this type of welding

***A mixed model was fit using ln-transformed exposure data to obtain predicted levels of airborne exposure by welding type (fixed effect), adjusted for subject (random effect). Arithmetic mean exposures were then calculated by using maximum likelihood estimates, incorporating the within-subject variance.

AM=arithmetic mean, GM=geometric mean, GSD=geometric standard deviation

Table 2. Predicted Mn exposure estimates used in the longitudinal linear mixed models, for all time windows considered

			Predicted Mn exposure values (μg/m^3-days)		
Exposure window	**n observations**	**n subjects**	**AM**	**SD**	**Range**
1 day	570	56	30.9	15.4	4.14, 68.9
7 day	570	56	118.4	74.8	4.70, 324.1
30 day	570	56	422.3	296.4	4.85, 1340.9
90 day	570	56	1243.1	772.2	4.85, 3354.3
Cumulative	570	56	2980.0	2339.2	4.85, 10514.1

AM = arithmetic mean

Table 3. Mn Blood descriptives over various time points, stratified by time of day

	Morning				Afternoon				Afternoon - Morning*			
	n	**AM**	**SD**	**95% CI**	**n**	**AM**	**SD**	**95% CI**	**n**	**AM**	**SD**	**95% CI**
Day	600	8.84	3.84		570	8.38	3.19		557	-0.45	0.13	(-0.70, -0.19)
Week												
Begin	315	8.95	3.79		301	8.51	3.30		299	-0.38	0.19	(-0.76, -0.02)
End	285	8.72	3.90		269	8.23	3.06		258	-0.53	0.19	(-0.90, -0.16)
End - Begin**	600	-0.25	0.24	(-0.71, 0.21)	570	-0.33	0.16	(-0.65, -0.01)				
Quarter												
Begin	335	8.91	3.81		313	8.29	3.14		309	-0.64	0.20	(-1.04, -0.24)
End	265	8.75	3.88		257	8.49	3.25		248	-0.22	0.16	(-0.53, 0.08)
End - Begin**	600	-0.18	0.24	(-0.64, 0.29)	570	0.21	0.17	(-0.12, 0.53)				

AM = arithmetic mean

*Paired t-test to assess difference in blood Mn over the course of a day (Afternoon - Morning) stratified by time of week and time of quarter

**Linear mixed model including subject as a random effect to estimate paired difference in blood Mn over the course of a week and quarter, stratified by time of day

Table 4 A, B. Linear mixed model associations between exposure windows and MnB

A.

Fixed Effects (ng/mL)/mg/m^3-days	Coefficient (SE)	CI	Within Var (SE)	%	Between Var (SE)	%	Total Var
1 day exposure window	0.48 (6.37)	-12.02, 12.98	3.7 (0.23)	29%	8.9 (1.2)	71%	12.6
7 day exposure window	-1.36 (1.26)	-3.84, 1.12	3.7 (0.23)	39%	5.9 (1.2)	61%	9.6
30 day exposure window	0.57 (0.31)	-0.04, 1.19	3.7 (0.23)	39%	5.8 (1.2)	61%	9.5
90 day exposure window	0.26 (0.13)	0.005, 0.51	3.7 (0.23)	39%	5.7 (1.2)	61%	9.4
Cumulative exposure	0.09 (0.04)	0.006, 0.17	3.7 (0.23)	39%	5.7 (1.2)	61%	9.4

B.

Adjusted to 30 day exposure period (ng/mL)/(3 mg/m^3-days)	Coefficient (SE)	CI
1 day exposure window	0.05 (0.64)	-1.20, 1.30
7 day exposure window	-0.95 (0.89)	-2.69, 0.79
30 day exposure window	1.71 (0.94)	-0.12, 3.57
90 day exposure window	2.34 (1.17)	0.05, 4.63
Cumulative exposure	1.77 (0.85)	0.13, 3.44

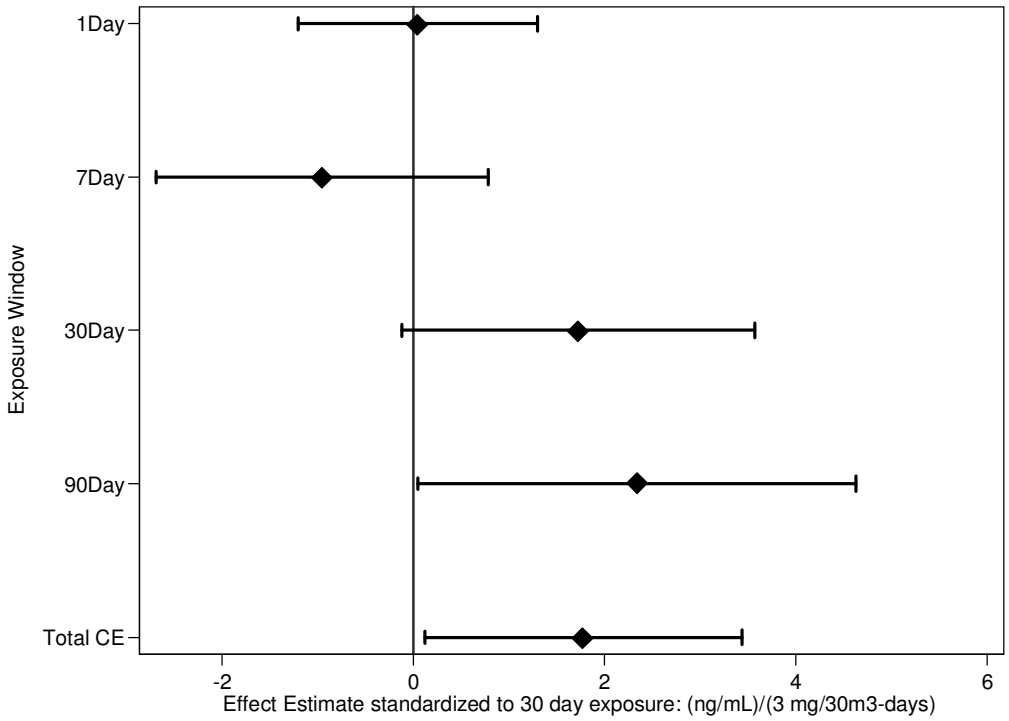

Figure 1. Effect estimate of exposure windows

Chapter 3. NEUROLOGICAL MARKERS ASSOCIATED WITH LOW-LEVEL MANGANESE EXPOSURE**

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3.1 INTRODUCTION

Elevated exposure to manganese (Mn) has long been an occupational health concern; excess Mn in the body crosses the blood-brain barrier, accumulates in the brain, and can cause adverse neurological health effects (Aschner 1999; Antonini, Santamaria, et al. 2006). Manganism is a syndrome characterized by parkinsonism features (bradykinesia, rigidity, postural instability, tremor), dystonia, and cognitive dysfunction, clinical features also common to idiopathic Parkinson's Disease (PD) (Guilarte 2010).

Welders typically experience 8-hour mean personal breathing zone Mn concentrations ranging from less than 0.02 mg/m3 to over 1.0 mg/m3, depending on welding process and control methods (Harris et al. 2005; Ellingsen et al. 2006; Smargiassi et al. 2000; Meeker, Susi, and Flynn 2007). The American Conference of Governmental Industrial Hygienists' (ACGIH) eight-hour time weighted average threshold limit value (8-hr TWA TLV) for Mn in total inhalable dust was recently reduced to 0.1 mg/m3 and the respirable TLV was reduced to 0.02 mg/m3, based on neurological outcomes seen in workers exposed to low levels of Mn (American Conference of Governmental Industrial Hygienists (ACGIH) 2013). Although welding fume is almost entirely respirable, other metalworking tasks commonly done by welders generate larger, inhalable particles. However, the Occupational Safety and Health Administration's Permissible

Exposure Limit (OSHA PEL) for Mn in total inhalable dust of 5.0 mg/m3 (ceiling) is the only enforceable Mn standard in the United States. In Europe, the European Commission Scientific Committee on Occupational Exposure Limits (SCOEL) recommends an 8-hr TWA of 0.2 mg/m3 for Mn in the inhalable fraction and 0.05 mg/m3 for Mn in the respirable fraction, though enforceable standards would vary between European countries.

Manganism has historically been associated with long-term, high levels of occupational exposure to Mn, yet neurological effects have been observed at Mn levels below the OSHA PEL and closer to the ACGIH TLV or SCOEL recommended 8-hr TWA (Mergler 1999; Roels et al. 2012; Zoni, Albini, and Lucchini 2007). Recently, Laohaudomchok et al. reported parkinsonian effects in welders who experience low Mn exposures (median: 0.013 mg/m3) (2011). Ellingsen et al. found welders with a mean Mn exposure of 0.21 mg/m3 to have poorer performance than unexposed referents on Grooved Pegboard, Finger Tapping, and Simple Reaction Time tests (2014). In contrast, in both mineworkers and smelter workers exposed to Mn, Myers et al. found no relationship between Mn exposure and nervous system effects, even at levels above the ACGIH TLV (Myers, Thompson, et al. 2003; Myers, teWaterNaude, et al. 2003).

While neurological function tests such as UPDRS3 and Grooved Pegboard are classified as biomarkers of Mn effect, T1-weighted magnetic resonance image (MRI) have been utilized as a biomarker of Mn in-vivo brain exposure. Mn is a paramagnetic element that strongly influences the magnetic resonance properties of surrounding tissues. Mn shortens tissue T1 relaxation times resulting in increased signal intensity detectible on T1-weighted imaging. Human studies have confirmed the intensity of the T1 pallidal signal correlates with blood Mn levels and cumulative exposure to Mn, but it may not be as accurate at low levels of chronic exposure (Dietz et al. 2001; Chang et al. 2010). A recent paper by Criswell et al. found intensity

changes in the caudate and putamen to be better markers of cumulative exposure (Criswell et al. 2012). In this study we attempt to evaluate caudate, putamen, and pallidal T1 signal as markers of low level Mn exposure in a longitudinal study of welder trainees.

Many tests exist to assess neurotoxicity associated with Mn exposure, both specific (i.e. functional imaging techniques, neuropsychiatric testing for domains of memory) and general (i.e. some tests of mood, dexterity), that have been used either singularly or as part of a battery (Guilarte 2010; Bowler et al. 2006; Levy and Nassetta 2003; Guilarte 2013). Epidemiologic studies assessing neurological outcomes associated with Mn exposure in welders and other occupationally exposed groups are most commonly cross-sectional. Although non-exposed referent groups are frequently included, to our knowledge, no prior studies have included pre-exposure baseline measurements of CNS function on a well-characterized Mn-exposed occupational cohort. While a longitudinal inception cohort study of neurological function allows for individuals to serve as their own controls, the performance-based tests also raise the possibility of learning effects, which can obscure changes over time (Rabbitt et al. 2001).

In this study, we employed three methods to longitudinally assess CNS function in a cohort of previously unexposed welders exposed to low levels of Mn: The Grooved Pegboard examination to assess dexterity and fine motor control (Matthews and Klove 1964), The UPDRS3 for parkinsonian signs such as rest and postural tremor, bradykinesia, and gait disturbance (Racette et al. 2006), and T1-weighted brain MRI, an in-vivo measure of Mn exposure (Criswell et al. 2012; Lin and Koretsky 1997).The purpose of this manuscript is to explore neurological outcomes associated with low-level cumulative exposures to Mn in a longitudinal cohort of asymptomatic welder trainees.

3.2 METHODS

Occupational setting and study population

Subjects included 56 welder trainees enrolled in a five quarter welding training program at a technical college in Washington State who joined our cohort prior to occupational exposure to Mn. All 56 enrolled subjects underwent longitudinal UPDRS3 and Grooved Pegboard tests, and 17 underwent repeat MRI measures. Details of the cohort study and the welding traineeship have been previously described (Baker et al. 2014). All study protocols were reviewed and approved by the University of Washington Institutional Review Board and the Washington University Human Research Protection Office, in accordance with the Helsinki Declaration, and subjects provided written informed consent. Data collection for the study began in April 2011 and was completed in June 2013.

Upon entry to the study, and at the end of each academic quarter (approximately 10 weeks) all subjects completed the Grooved Pegboard test and UPDRS3 examination. Similarly, upon entry and at the end of each academic quarter those subjects enrolled in the MRI subcohort were transported to University of Washington Medical Center (UWMC, Seattle, WA, USA) for MRI scans. At the completion of the study, all UPDRS3 examinations and MRI scans were stripped of any time- or subject-identifying information, and interpreted by co-investigators at Washington University (WU).

Grooved Pegboard

Trained research staff administered the Grooved Pegboard (West Lafayette, IN, USA) examination on each subjects' dominant hand and non-dominant hand, following the procedure

outlined by the Grooved Pegboard's user's manual (Matthews and Klove 1964; Lafayette Instrument 2002). A higher score (longer time to complete the test) indicates reduced dexterity and fine motor control.

UPDRS3

Each subject was videotaped performing the Unified Parkinson Disease Rating Scale, motor subsection part 3 (UPDRS3), except for rigidity. Videos were rated by a movement disorders specialist (SRC) blinded to the exposure and clinical status of the subject (Racette et al. 2006; Racette et al. 2005). If the video quality did not allow an individual item to be graded, that subject's total score was not able to be calculated, and thus was not included in analyses. Five UPDRS3 examinations from five different subjects were missing a measure of tremor, likely at random and not affecting our outcome analyses.

Magnetic Resonance Imaging

Subjects interested in participating, and with no contraindications to MRI, were enrolled in the MRI subcohort and transported to UW Diagnostic Imaging Sciences Center at the end of the workday where MRI scans were conducted on a 3T Philips Achieva MR System. Structural anatomic scans included a T1-weighted sagittal, magnetization-prepared rapid gradient echo (MPRAGE; repetition time (TR) = 20.0 ms; inversion time (TI) = 1000 ms, echo time (TE) = 3.14 ms, flip angle = 8°, 0.9x0.9x0.9 mm voxels) and, as is standard, T2-weighted indices were also measured. However, results from T2-weighted imaging are not informative in relation to Mn exposure and are not presented.

A reviewer blinded to the clinical and exposure status of the subject outlined volumes of interest (VOIs) on individual MR images including bilateral caudate, globus pallidus, anterior putamen, posterior putamen, and then calculated a combined basal ganglia as the average signal of all the above VOIs. The intensity of the signal on the T1 weighted image in the VOI was compared to standard frontal white matter control regions by calculating an intensity index (see Equation 2) for each subject. Regional indices were calculated from the T1 MPRAGE images as previously described for the pallidal index (PI) (Criswell et al. 2012).

Equation 2:

$$Intensity\ index = \left[\frac{(Left\ VOI + Right\ VOI)}{(Left\ white\ matter\ control\ region + Right\ white\ matter\ control\ region)}\right] * 100$$

All MRIs were screened for neurological abnormalities at UW prior to interpretation by WU co-investigators.

Estimating Mn Exposure in Air

Four times during each quarter the subject was enrolled in the study, they were fitted with a personal air pump (SKC AirChek XR4000, Eighty Four, PA, USA) with an attached pre-weighed 37mm 0.8µm pore mixed cellulose ester filter hung in their breathing zone, outside the welding helmet. The pumps operated at a flow of 2.0 L/min which was measured at the beginning and end of each full-shift sampling period. Subjects completed an exposure

questionnaire to assess type of welding, use of respiratory protection, and confounding sources of Mn exposure. Air filters were analyzed gravimetrically for total particulate mass, then digested and analyzed for trace metals by ICP-MS at the University of Washington Environmental Health Laboratory (Bocca et al. 2003). Reporting limits for Mn ranged from 0.01 – 0.03 µg depending on analysis-batch-specific field blanks, and were based on three times the standard deviation of the blanks, which were treated the same as the samples in the field. No air samples fell below the reporting limit.

8-hr TWA Mn concentrations were calculated, ln-transformed, and a mixed model was fit to obtain estimates of exposure level by welding type (fixed effect), adjusted for subject (random effect). These estimates were used to predict 8-hr TWA Mn exposure levels, based on the type of welding subjects self-reported to be doing each day of their enrollment. Weekends, vacation days, and days the subjects were absent (as ascertained from school attendance records) were coded as zero exposure. On days when a neurological function test occurred, all preceding predicted daily exposures were summed to create a predicted cumulative exposure from time of entry into the program to the day of the neurological function test, in units of mg/m3-days.

Statistical Analysis

The associations between predicted cumulative exposure at the time of the test and each neurological outcome of interest were assessed using longitudinal linear mixed-effects models allowing for subject as a random effect. For UPDRS3, the neurological outcome of interest was the total UPDSR3 score. As gender is a well-established effect modifier for the Grooved Pegboard (Schmidt et al. 2000; Ruff and Parker 1993), and only four females were enrolled in

our study, pegboard models of dominant hand time are only presented for males (number of subjects = 52).

There was only one female in the MRI subcohort, but removing her n=4 scans from the mixed models did not affect the regression coefficients relating exposure and outcome, thus she is included in all MRI models. While several covariates were considered in the mixed models (including smoking status, alcohol drinker, prior self-reported loss of consciousness, self-reported respirator use, and age at baseline) the only covariate significant in any model was age at baseline, which was found to contribute significantly to the Grooved Pegboard models, and some of the MRI models. Thus, all models were adjusted for subject age at baseline.

Because subjects underwent repeat measures of UPDRS3 and Pegboard over fairly short time periods (mean time between examinations was 66 days), we hypothesized changes in score that appear to be associated with cumulative exposure may in fact be influenced by a learning effect. Therefore, in addition to crude models unadjusted for a learning effect, we also adjusted the UPDRS3 and Pegboard models to control for a potential learning effect. To accomplish this, we corrected the existing models for three predictors, based on guidance from McKnight et al. (1999): number of previous tests (continuous), months since previous test (continuous), and a binary indication of whether or not it was the first test (first test=0, subsequent tests=1). All data analysis was done at University of Washington using Stata 12 (College Station, TX, USA).

3.3 RESULTS

Demographic characteristics for the entire welding cohort and MRI subcohort are summarized in Table 5. The MRI subcohort was similar to the entire cohort. Measured 8-hr TWA Mn concentrations (n=600) ranged from 0.2 to 208 µg/m3 (geometric mean 16.5, GSD

3.4) Only 4.5% of all samples (n=27) exceeded the ACGIH inhalable TLV of 0.1 mg/m3, but assuming Mn in welding fume is primarily in the respirable range (Pesch, Weiss, Kendzia, Henry, Lehnert, Lotz, Heinze, Käfferlein, et al. 2012), 51.8% of all samples (n=211) exceeded the ACGIH respirable TLV of 0.02 mg/m3.

Predicted 8-hr TWA Mn concentrations, which were modeled from the measured 8-hr TWA Mn concentrations, ranged from 4.1 to 68.6 µg/m3 (geometric mean 15.7, GSD 2.2). It was the modeled exposure values which were subsequently used to estimate cumulative exposure.

Table 6 summarizes the neurological outcomes for all time points. None of the pegboard times were outside of the typical reference ranges, based on age and gender (29,34,37). Similarly, none of the UPDRS3 scores were indicative of definite or probable parkinsonism (> 15), though n=14 (5.3%) of the UPDRS3 scores were above 6, a mildly elevated score (26). The average subject underwent 3.8 (SD 1.7) Grooved Pegboard tests (range 1–7) and 3.7 (SD 1.6) UPDRS3 examinations (range 1–7). The average subject in the MRI subcohort underwent 3.7 (SD 1.6) MRI scans (range 1–7).

Table 7A shows coefficients of association between predicted cumulative exposure and time to complete the pegboard test for males using their dominant hand. For males of a similar age completing the pegboard test with their dominant hand, each 1 mg/m3-days increase in cumulative exposure is associated with, on average a -0.60 second (95% CI -1.00 – -0.21) faster completion of the test. When adjusting this model to control for a learning effect, the coefficient of association between cumulative exposure and time to complete the pegboard test was similar (-0.68, 95% CI -1.6–0.27), however the 95% confidence interval indicates that the unadjusted negative association was influenced by a learning effect.

Similar relationships were seen for males using their non-dominant hand (not shown). In both unadjusted and adjusted pegboard models, age at baseline was a significant contributor to the model, and when adjusting for a learning effect, an increase in months between tests resulted in a slight but significant increase in time to complete the test, an average of 1.22 seconds slower (95% CI 0.27–1.83).

Table 7B shows coefficients of association between predicted cumulative exposure and total UPDRS3 score, both adjusted for a learning effect and unadjusted. In the unadjusted model, a 1 mg/ m3-days increase in cumulative exposure was associated with a -0.12 point lower score (95% CI -0.21–-0.04). However, in the adjusted model, the association between UPDRS3 score and cumulative exposure indicates that this test was also influenced by a learning effect.

Table 8 shows the associations between cumulative exposure and MRI outcomes. Among the MRI outcomes, caudate, anterior putamen, posterior putamen, and combined basal ganglia T1 indices were all increased significantly in relation to increased cumulative Mn exposure. The T1 PI did not show any changes associated with low level cumulative exposure. For subjects of a similar age, a 1 mg/m3-days increase in cumulative Mn exposure was associated with, on average, a caudate T1 index with an increased signal intensity of 0.31 (95% CI 0.14–0.48), an anterior putamen T1 index with an average increased signal intensity of 0.26 (95% CI 0.10–0.41), a posterior putamen T1 index with an average increased signal intensity of 0.22 (95% CI 0.08–0.36), and a combined basal ganglia T1 index with an average increased signal intensity of 0.18 (95% CI 0.07–0.30). These average increases correspond to a 0.4% increase from the baseline mean caudate T1 index, a 0.3% increase from the baseline mean anterior putamen T1 index, and a 0.2% increase from both the baseline mean posterior putamen and combined basal ganglia T1 indices.

One subject exhibited a large change between their penultimate and last MRI, the only instance of at least a 10% change between two subsequent scans for any T1 measures among any of the subjects. The findings were not materially changed when sensitivity analyses were performed excluding this subject.

3.4 DISCUSSION

To our knowledge, this is first study to longitudinally explore changes to the CNS associated with Mn exposure among subjects who were asymptomatic at baseline, had no prior occupational exposure to Mn, and known baseline measures. The three methods of assessing neurological outcomes we selected allowed us to evaluate markers of in-vivo Mn exposure (MRI), clinical measures of parkinsonism (UPDRS3), and clinical measures of fine motor skill/dexterity (Grooved Pegboard). As a condition of entry into the study, all subjects were deemed asymptomatic, as assessed by a health screening questionnaire. Baseline UPDRS3 and Grooved Pegboard examinations confirmed subjects to be clinically normal, as UPDRS3 scores were within the normal range (Racette et al. 2006), and Grooved Pegboard completion times were within typical age- and gender-specific reference ranges (Bornstein 1985). Twenty-five percent of the cohort self-reported using respirators. However, field research staff observed very poor respirator practices; for example, no fit testing or respiratory protection training was provided on site, subjects failed to regularly change cartridges, and frequently wore them inconsistently or incorrectly. Thus, even though respirators were used, the degree of protection would not be as much as expected under ideal circumstances. Nevertheless, we tested the impact of respiratory protection on the exposure-response relationship and no effect was observed on either the outcome or the relationship between the exposure and outcome.

Unadjusted findings from the UPDRS3 and Grooved Pegboard mixed models indicated a tendency for pegboard time and UPDRS3 score to be inversely related to cumulative exposure. However, when adjusting for a learning effect, these relationships were no longer apparent. Time since enrolling in the study and cumulative exposure are, unsurprisingly, highly correlated (Pearson's r=0.84). Thus, being able to parse out the effects of increased Mn exposure on Pegboard time or UPDRS3 score from the effects of repeat tests over a fairly short time period in a longitudinal study is challenging. However, it is apparent for both pegboard and UPDRS3 that over the relatively short time periods we repeatedly administered these tests there were no meaningful changes with increased exposure to airborne Mn. In contrast, we did observe hypothesized associations of cumulative exposure with MRI patterns, which are not subject to learning or time effects.

Despite exposure at or near the ACGIH respirable TLV, there was an apparent increase in T1 signal intensities for several parts of the basal ganglia (ranging from a 0.2% to 0.4% increase over the mean) indicative of in-vivo effects associated with Mn exposure. Thus, it appears that even at very low levels of occupational exposure, changes associated with Mn exposure are apparent via MRI techniques, before clinical measures (such as those assessed via UPDRS3 and Grooved Pegboard) manifest, or can overcome influences from time and learning.

Our results corroborate with those reported by Criswell et al. in career professional welders (2011), in which the caudate, putamen, and basal ganglia indices all demonstrated stronger correlations with Mn exposure than the PI, especially at low levels of Mn exposure. Animal and human studies demonstrated Mn deposition throughout all parts of basal ganglia (Larsen et al. 1979; Eriksson et al. 1987). These studies and our results suggest that while earlier studies have focused on the pallidum as the primary target of Mn exposure (as characterized by

the PI), the caudate and putamen may be the better regions to serve as biomarkers of Mn exposure.

Our study design is a primary strength of our study, given it is, to our knowledge, the first inception cohort design to assess CNS related to Mn in a well-characterized, asymptomatic cohort. The controlled nature of the welding training program and repeat full-shift personal air samples allowed us to predict subject-specific cumulative exposures at any time point, and we sampled longitudinally for both biomarkers of exposure and effect related to Mn neurotoxicity. However, we only followed subjects during the time they were in the welding training program. Over this relatively short time period, in our young and otherwise healthy subjects, at the low-levels of exposures we observed, we wouldn't expect clinical indications of parkinsonism to manifest, even with sustained exposure to Mn. Because our cohort consisted of welder apprentices, exposures and work scenarios are not necessarily representative of standard occupational welding settings where exposures would be higher, more variable, and less controlled.

Several studies have reported resolution of T1 hyperintensities in occupationally exposed workers after removal from the source of Mn exposure. Despite improvement in their T1 imaging characteristics, clinical deficits in these workers, if present, frequently persist (Nelson et al. 1993; Yangho 2004; Huang et al. 1998). This suggests while the increased T1 signal intensity is potentially reversible, there may be an exposure threshold at which the MRI changes and neurotoxic effects become permanent.

3.5 CONCLUSION

In conclusion, this study showed that even with 8-hr TWA Mn exposures at or below the ACGIH recommendations, there were increases in T1-weighted indices in the caudate, anterior and posterior putamen, and combined basal ganglia, that were significantly related to increases in cumulative Mn exposure in asymptomatic welder apprentices. However, there were no clinical signs of neurological dysfunction related to increases in cumulative Mn exposure at these low levels and over this relatively short time period as assessed via UPDRS3 and Grooved Pegboard. T1-weighted PI (but not other indices) has been correlated with the Grooved Pegboard (dominant hand) in one cross-sectional study, but other indices have not yet been correlated with clinical measures of parkinsonism, and toxicologically relevant reference values for T1-weighted indices have not yet been established for exposed persons (Chang et al. 2010). As none of our subjects exhibited clinical signs of parkinsonism we cannot address the relationship between MRI outcomes and clinical manifestations of parkinsonism with our cohort. Previously, T1-weighted MRI measures have been positively correlated with Mn exposure, though not at levels as low as in our study or with repeat measurements in a well-characterized inception cohort such as ours. Our study offers additional converging evidence that Mn exposure is associated with T1 signal intensity in various parts of the basal ganglia, making T1-weighted MRI (especially in the caudate/putamen) a biomarker of exposure to Mn, seemingly sensitive across a wide range of exposure levels, and relatively short time periods.

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CHAPTER 3: FIGURES AND TABLES

Table 5. Subject demographics of welder trainees

	UPDRS3/Pegboard Cohort				MRI Subcohort			
	Subjects (N)	**Mean**	**SD**	**Range**	**Subjects (N)**	**Mean**	**SD**	**Range**
Age at baseline (years)	56	28.0	9.6	18–55	17	24.6	7.3	18–41
Smoking pack-years	56	3.7	6.6	0–36	17	1.3	1.8	0–6
Predicted cumulative Mn exp at last test (mg/m^3)	56	4.4	2.6	0.01–10.5	17	3.8	2.8	0.03–8.8
***n* UPDRS3 tests**	56	3.7	1.6	1–7	17	3.8	1.6	1–7
***n* Pegboard Examinations**	52	3.8	1.7	1–7	17	4.0	1.8	1–7
***n* MRI scans**	·	·	·	·	17	3.7	1.6	1–7
Categorical smoking status								
Never	23	41%	·	·	8	47%	·	·
Current	21	38%	·	·	6	35%	·	·
Previous	12	21%	·	·	3	18%	·	·
Drinks alcohol (yes)	39	70%	·	·	12	71%	·	·
Respirator user	14	25%	·	·	4	24%	·	·
Previous head injury (yes)	31	55%	·	·	10	59%	·	·
Male	52	93%	·	·	16	94%	·	·

*Includes all days enrolled in the program, including zero-exposure days

Table 6. Neurological outcome descriptives

Pegboard (sec)	**n (k)***	**Mean**	**Overall SD**	**Within SD***	**Between SD**	**Range**
Males, dominant	199 (52)	62.9	9.5	4.5	7.6	45, 112
Males, non dominant	199 (52)	67.0	11.4	4.8	9.5	47, 112
UPDRS3 score	**n (k)**	**Mean**	**Overall SD**	**Within SD***	**Between SD**	**Range**
Overall total	205 (56)	2.1	2.1	1.1	1.8	0, 9
MRI score	**n (k)**	**Mean**	**Overall SD**	**Within SD***	**Between SD**	**Range**
Caudate T1	63 (17)	86.2	2.9	1.0	2.4	80.2, 96.8
Ant. Putamen T1	63 (17)	90.2	3.0	0.9	2.7	85.2, 98.5
Post. Putamen T1	63 (17)	94.6	3.4	1.0	3.2	87.4, 101.4
Basal Ganglia T1	63 (17)	94.7	2.8	0.8	2.5	89.1, 100.0
Pallidal Index T1	63 (17)	108.0	2.7	1.2	2.3	99.9, 113.8

*Average within-subject standard deviation calculated only using those subjects with at least n=2 tests, which was 47 male pegboard subjects, 51 UPDRS3 subjects, and 15 MRI subjects.

Table 7 A, B. Association of (A) dominant-hand Pegboard times and (B) UPDRS3 scores with cumulative exposure, both unadjusted and adjusted for a learning effect

A. Pegboard	**Unadjusted for learning effect**		**Adjusted for learning effect**	
	β	**95% CI**	**β**	**95% CI**
Cumulative exposure	-0.60	-1.00–-0.21	-0.68	-1.6–0.27
Age at baseline	0.26	0.05–0.47	0.26	0.05–0.47
Number of previous tests	·	·	-0.08	-1.6–1.4
Months since previous test	·	·	1.22	0.27–1.83
Not first test	·	·	-3.1	-6.4–0.20
Within Subject SD*	5.8	5.2–6.5	5.8	5.1–6.5
Between Subject SD*	6.7	5.2–8.5	6.7	5.3–8.6

B. UPDRS3	**Unadjusted for learning effect**		**Adjusted for learning effect**	
	β	**95% CI**	**β**	**95% CI**
Cumulative exposure	-0.12	-0.21–-0.04	-0.01	-0.22–0.19
Age at baseline	0.03	-0.02–0.08	0.03	-0.02–0.08
Number of previous tests	·	·	-0.21	-0.55–0.14
Months since previous test	·	·	0.061	-0.12–0.27
Not first test	·	·	-0.15	-0.92–0.62
Within Subject SD*	1.3	1.2–1.5	1.3	1.2–1.5
Between Subject SD*	1.6	1.3–2.0	1.6	1.3–2.0

Table 8. Association of T1 weighted MRI indices with cumulative exposure, adjusted for age at baseline

MRI score / (mg/m^3-days)	β	95% CI	Within SD*	Between SD*
Caudate T1	0.31	0.14–0.48	1.5	1.8
Ant. Putamen T1	0.25	0.10–0.41	1.3	2.3
Post. Putamen T1	0.22	0.08–0.36	1.2	2.9
Basal Ganglia T1	0.18	0.06–0.29	1.0	2.3
Pallidal Index T1	-0.04	-0.22–0.14	1.6	2.3

Chapter 4. THE USE OF GLOBAL METABOLOMICS TO IDENTIFY BIOLOGICAL SIGNATURES OF MANGANESE EXPOSURE IN URINE***

***Published as: Baker MG, Simpson CD, Lin YS, Shireman LM, Seixas N. *The use of metabolomics to identify biological signatures of manganese exposure.* Ann Work Expo Health. 2017; 61(4): 406-415. Reproduced here per License Agreement with Oxford University Press.

4.1 INTRODUCTION

Manganese (Mn) is an established neurotoxicant associated with deleterious cognitive (Bouchard et al. 2007; Bowler et al. 2007; Mergler et al. 1994) and motor (Lucchini et al. 1999; Roels, Lauwerys, Buchet, et al. 1987; Roels et al. 1985) health outcomes. Environmental Mn exposures, from traffic or industrial sources, have been shown to increase the prevalence of Parkinsonian disorders (Lucchini et al. 2007; Finkelstein and Jerrett 2007). The American Conference of Governmental Industrial Hygienists (ACGIH) has recommended an 8-hour time weighted average threshold limit value (TWA TLV) of 100 µg/m3 for inhalable Mn based on neurological endpoints.

While Mn is ubiquitously found in ambient air, drinking water, and foods common to a Western diet, it is in occupational settings where chronic, elevated exposures are most frequently found. Elevated exposures, encountered in either community or occupational settings, make Mn a public health concern in need of further investigation.

Given the negative health effects related to Mn exposure, research has focused on finding both exposure and disease biomarkers for Mn and its related neurological disorders in readily available biofluids such as blood, plasma, and urine, or by using imaging techniques such as magnetic resonance imaging (MRI) or positron emission tomography (PET) scans (Apostoli,

Lucchini, and Alessio 2000; Zheng et al. 2011; O'Neal and Zheng 2015; Baker, Criswell, et al. 2015; Baker et al. 2016). However, a lack of understanding of the timing between exposure and uptake (Roth 2006; Baker et al. 2014), natural human variability in Mn (Baker, Simpson, et al. 2015; Jarvisalo et al. 1992), and costs or contraindications associated with MRI or PET scans (e.g. metal chips in eyes) make many biomarkers of Mn exposure and effect impractical for use in a field or clinical setting. Thus, the critical barrier to identifying a reliable, reproducible, and easily accessible biomarker of Mn exposure still remains largely unsolved. As such, there is a critical need in the fields of environmental health, neurology, toxicology, and public health to develop informative peripheral biomarkers of Mn exposure and its related diseases, and to better understand the underlying pathophysiology of Mn neurotoxicity.

Global metabolomics is one promising approach to identifying a biomarker of Mn exposure. Metabolomics is the study of the small molecules (<1000 daltons) in biological systems, often important in metabolic function and signaling, or representing a fingerprint of a specific cellular process (Wishart 2010). Global metabolomics is typically an exploratory analysis method and is a means of hypothesis generation because selection of putative biomarkers in biofluids using this approach is inherently statistical, based on comparisons between thousands of ions. Therefore, the false positives in the analysis represent ions that are statistically significant but are not related to the exposure in question. Results from metabolomics studies tend to have limited utility unless the selected putative ions can be replicated in an independent sample and the chemical nature and biological role of the ions identified (Broadhurst and Kell 2006).

Typically, global metabolomics has been used to identify biomarkers or physiological alterations related to health effects, such as for neurologic diseases (Sato et al. 2012; Ibanez et al.

2012; Hassan-Smith et al. 2012; Zhang, Sun, and Wang 2013), cancers (Wang, Zhang, and Sun 2013; Zhang et al. 2012), and, of particular relevance to Mn, Parkinson's Disease (Bogdanov et al. 2008; Michell et al. 2008; Ahmed et al. 2009). While using metabolomics to distinguish between patients and controls is more common, using metabolomics to distinguish between exposed and non-exposed persons is a relatively novel concept (especially in occupational settings) and is a key approach in exploring the exposome—the biological signatures of lifelong environmental exposures within individual physiological histories (Wild 2005). Walker et al. used a metabolome-wide association study (MWAS) framework to investigate metabolic changes in plasma related to trichloroethylene exposure in an occupationally-exposed cohort, and were able to link measured exposure to internal dose and measured endogenous metabolites (2016). However, the other few human metabolomics studies investigating biomarkers of exposure have looked only at dietary exposure to vitamins or minerals, not chemical exposures in an environmental or occupational setting (Johansson-Persson et al. 2013; Astle et al. 2007). Few studies have investigated metabolomics related to Mn exposure, and those that have analyzed biofluids or tissues collected from model organisms, not biofluids collected from humans exposed in an occupational setting (Dorman et al. 2008; Kumar et al. 2015; Fordahl et al. 2012; Neth et al. 2015). Our study of occupational exposed workers is particularly important given the substantial limitations in available biomarkers of acute and chronic exposure to Mn. The objective of this work is to report on the use of a metabolomics analysis approach to investigate the differences between occupational groups classified by Mn exposure status, which could in turn inform a biomarker of exposure to Mn. Work presented here represents a novel method for exposure assessment in environmental and occupational health studies and the first

time, to our knowledge, that metabolomics has been applied in an occupational setting to distinguish between exposed and unexposed workers.

4.2 METHODS

During one week in October 2014, subjects were recruited from two Puget Sound worksites; Mn exposed subjects (n=20) worked at a Mn foundry and Mn unexposed subjects worked as crane operators in a metal recycling facility (n=17). Data were collected on worker demographics including age, race, and ethnicity. No exclusions were made on the basis of race, ethnicity, or gender, though all enrolled subjects identified as male. For each enrolled subject at both worksites, a personal, full-shift, inhalable dust sample was collected using an Institute of Occupational Medicine (IOM, Edinburgh, UK) sampler, and a single spot urine sample was collected at the end of the same day. The study protocols were approved by the University of Washington Institutional Review Board.

To increase the validity of our findings, both the Mn exposed and unexposed groups were divided into a training set (n=12 in the Mn exposed group, n=10 in the Mn unexposed group) and a demographically-similar validation set (n=8 in the exposed group, n=7 in the unexposed group). Urine samples were processed and analyzed without regard to exposure status. After metabolomics data extraction and preprocessing (as described below), statistical analysis of the training data set yielded putative ions related to Mn exposure and a separate analysis was performed to compare the relative abundance of the selected significant ions in the validation data set.

Mn exposure assessment

Personal full shift inhalable dust sampling was conducted at each location using IOM inhalable dust samplers according to the UK Health and Safety Executive's Methods for the Determination of Hazardous Substances 14/4 (Mark and Vincent 1986; Health and Safety Executive 2014). The collected inhalable dust samples were analyzed at the University of Washington Environmental Health Laboratory for total Mn using inductively coupled plasma mass spectrometry (ICP-MS) and adjusted for field blanks handled in the same manner as the filters deployed in the field. Measured inhalable Mn concentration were normalized to 8-hour time weighted average concentrations. For the 37 subjects enrolled, three subjects were missing IOM samples (one from the foundry, and two from the scrap metal recycling yard). Missingness was due to pump failure in the field and is believed to be completely at random.

To explore exposure-response relationships, subjects were divided into three categorical exposure levels based on job title and results from inhalable dust sampling: No Exposure (operators at the scrap metal recycling yard, n=10 in the training set and n=7 in the validation set), Lower Exposure (forklift operators, foundry helpers, and molders at the foundry, n=7 in the training set and n=3 in the validation set), and Higher Exposure (melters or pourers at the foundry, n=5 in the training set and n=5 in the validation set).

Urine preparation and metabolomics analysis

After collection in the field, urine samples were immediately stored on dry ice for transport to a -80 C freezer at the University of Washington. All samples were frozen upon arrival to University of Washington and remained frozen in the -80 C freezer until being thawed on wet ice for sample preparation. All urine samples were prepared and analyzed on the same

day, approximately one month after collection in the field. The procedure is described in full in Tay-Sontheimer et al (Tay-Sontheimer et al. 2014). In brief, 200 µL urine was combined with 800 µL acetonitrile containing deuterated internal standards. After protein precipitation, samples were centrifuged and the supernatant was evaporated under nitrogen gas. Samples were reconstituted in methanol:water with 0.4% acetic acid, vortexed, centrifuged, and transferred to glass autosampler vials. A pooled quality control sample was made from ten randomly selected urine samples and prepared as described above. Samples were analyzed on an Agilent (Santa Clara, CA, USA) 1200 HPLC coupled to Agilent 6520 Accurate Mass quadropole time-of-flight (Q-TOF) mass spectrometer, calibrated for accurate masses between m/z of 118 to 1700. A 3.5 µm, 2.1 x 30 mm Agilent Zorbax SB-C8 guard column and a 1.8 µm, 2.1 x 50 mm Agilent Zorbax SB-Aq analytical column heated to 60 °C were used. The mobile phase consisted of 0.2% acetic acid in water (A) and 0.2% acetic acid in methanol (B) with the gradient starting at 2% B and increasing to 98% B in 13 min, held at 98% B for 6 min, followed by re-equilibration to 2% B for 6.5 min at a flow rate of 0.6 mL/min.

Data were acquired over the 25 minute run in both electrospray ionization positive (ESI+) and negative (ESI-) modes to detect cations and anions, respectively.

Data Preprocessing

Data from the quality control samples were assessed for signal stability and retention time shifting over the course of the run. Preprocessing of the raw data from the Q-TOF was done using the open-source package xcms in R Studio, developed by the Scripps Center for Metabolomics (Smith et al. 2006; Tautenhahn, Böttcher, and Neumann 2008). We used xcms for feature detection, retention time alignment, and recursive filling of missing peaks. The relative

abundance of each ion was normalized by dividing by the sum of the abundances of all ions detected. Signals were multiplied by 10^6 for convenience, were $\log_{10}$ transformed to better approximate Gaussian distributions, and are unitless. Features found in ESI+ and ESI- modes were combined, and further filtered into two data sets: the primary data set and the sensitivity analysis data set. When considering the percent of samples in which an ion was detected, 40 samples (37 subject, and 3 pooled quality control samples) were included. The primary data set retained only those ions that were found in at least 50% of all samples (n=20) (p=1736 ions), and the sensitivity data set retained those ions that were found in at least 25% of all samples (n=10) (p=3380 ions).After filtering, the primary data and sensitivity analysis data sets were each split into a training and a validation data set as described above.

Statistical Analyses

Discovery of biomarkers of exposure. The primary data set, split into training and validation data sets, was used for the discovery of biomarkers of exposure. Using the training data, relative abundances of all ions were compared between Mn exposed and unexposed workers using a two-sided t-test. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg method to control false discovery rates (FDR). Given the exploratory nature of these analyses and the desire to find identify a larger set of potential ions that could distinguish between groups defined by exposure, an FDR<0.1 was considered to be significant. Ions found to be significantly different between the exposed and unexposed workers in the training group were subsequently tested in the validation set. Since the number of statistical comparisons was more limited in the validation set (n=15), the unadjusted p-values are reported. For those ions in the 50% (primary) data set that were found to be significantly different in the

training group and were also significantly different in the validation group, the exposure-response relationship was explored categorically by comparing relative abundances in the three categories of exposure: No Exposure, Lower Exposure, and Higher Exposure.

Sensitivity analysis. The biomarker discovery process was repeated in the sensitivity data set with a 25% filtering step (i.e., less stringent) to see how changing the filtering criteria affected the number of ions found to be different between Mn exposed and Mn unexposed subjects. For the sensitivity analysis an FDR<0.05 and <0.01 in addition to an FDR<0.1 was considered to be the threshold for significance, to see how changing this criteria affected the number of ions found to be different between Mn exposed and unexposed subjects.

4.3 RESULTS

Subject demographics for the Mn exposed and unexposed workers, stratified by training and validation sets, are presented in Table 9. Unexposed workers were slightly older than the exposed workers. The majority of exposed and unexposed workers self-identified as white, though there were more Hispanic workers in the Mn exposed group than the unexposed group. The unexposed workers were nearly equally divided between first and second shift, whereas the exposed workers were predominantly first shift workers. No Mn unexposed workers wore a respirator, but 12 (60%) of the Mn exposed workers self-reported to have worn an N95 dust mask for part or all of the workday. However, the foundry does not have a formal respiratory protection program, and overall respiratory hygiene at the site was observed to be poor. Thus, their use is unlikely to have a substantial effect on their received Mn dose.

Results from personal inhalable dust monitoring are presented as box plots in Figure 2, with the training and validation sets combined, stratified into the three categories of exposure.

The mean Mn exposure in the Lower Exposure group (n=10) was 192.0 µg/m3 (SD: 98.3, range: 98.5 to 374.3) and the mean Mn exposure in the Higher Exposure group (n=10) was 520.7 µg/m3 (SD: 339.0, range: 180.4 to 1243.3). One worker in the No Exposure group had an inhalable dust measurement (150.8 µg/m3) that was substantially higher than the other workers in the No Exposure group. It is believed this is due to operating a vehicle with windows down near an area where welding was occurring, per worker report. However, this substantially higher exposure could have been due to another exposure source at the scrap yard that was not accounted for. Eighteen of the 34 inhalable Mn samples (53%) exceeded the ACGIH recommended TWA TLV of 100 µg/m3; all of which were collected at the foundry.

Following metabolomics analysis of the urine samples, a total of 1736 ions were found in the primary data set (ions found in at least 50% of the samples). Nineteen ions were found to be significantly different between the Mn exposed and unexposed groups. However in three instances, it was believed multiple isotopologues or fragments of another significant ion were classified as being significantly different between Mn exposed and unexposed groups. For these instances, only the ion with the largest relative abundance was retained and reported herein. Thus, a total of 15 unique ions were found to be significantly different between the Mn exposed and unexposed groups (Table 10). Nine of the 15 ions were replicated in the validation set.

The exposure was stratified into three exposure categories (No Exposure, Lower Exposure and Higher Exposure) to visualize possible exposure-response relationships. The box plots in Figure 3 illustrate the distribution of the abundance of these nine ions in the three exposure groups. Appendix A details the relative abundance of each of the ions stratified by the three exposure groups. Of the nine ions that replicated in the validation set, the abundance of all but one were increased in the exposed group, and were generally comparable in the training and

validation sets. The ion with m/z 415.22 and retention time 8.95 minutes was the only ion of the nine to exhibit a lower abundance in the exposed group than in the unexposed group. Many of the ions exhibited an apparent exposure-response relationship across the three categorical exposure groups.

To determine whether the filtering criteria affected the number of ions found to be different between Mn exposed and Mn unexposed subjects, we compared our original results from the primary data (filtering criteria of ions found in at least 50% of the samples) to the ions identified in the sensitivity data (filtering criteria of ions found in at least 25% of the samples). Table 11 shows the results of the sensitivity analyses when less stringent filtering criteria were used (50% vs. 25% for the primary and sensitivity data sets, respectively) and when considering an FDR threshold of 0.01 and 0.05 as well as 0.1. As 0.1 was chosen a priori for the primary analysis, we wished to explore if more stringent FDR would also produce ions of interest. Using the sensitivity data set, the relative abundances of 22 unique ions were found to differ significantly between Mn exposed and unexposed workers in the training set when using a FDR < 0.1, and 11 of these ions remained significantly different between the exposed and unexposed workers in the validation set. When considering an FDR threshold of 0.05, 12 ions were found to be different between the exposed and unexposed in the 25% data set and eight were found to be different in the 50% data set. In the 25% data set eight ions remained significant at the 0.05 level in the validation set, while seven remained significant in the 50% data set. Considering an even more stringent FDR of 0.01, eight ions were found to be different between the exposed and unexposed in the 25% data set and four were found to be different in the 50% data set. In the 25% data set six ions remained significant at the 0.01 level in the validation set while four remained significant in the 50% data set. One additional ion had a p-value of less than 0.01 in the

validation set, but had an FDR greater than 0.01 (but less than 0.05) in the training set. All ions found to be significant in the primary data set were also found to be significant in the sensitivity data set.

4.4 Discussion

We found nine ions to be significantly different in the training and validation sets between groups defined by Mn exposure status (Table 11). An apparent exposure-response relationship emerged for most of the ions when looking at the relative abundances of these nine ions across three exposure groups, though no formal test of trend was done for the three groups. Despite the relatively modest number of workers participating in this study, these ions may in fact distinguish between groups defined by Mn exposure status in this occupational cohort and also provides evidence that metabolomics can be used as a method of exposure assessment (or in tandem with traditional exposure assessment techniques) in occupational settings. Exposure assessment using metabolomics methods is a novel and burgeoning field, with many as-yet unexplored applications for highly original and multidisciplinary environmental and occupational health research. The findings presented here represent an essential first step in the development and validation of a biomarker of Mn exposure using global metabolomics techniques.

Notably, work presented here does not include identification (by name or empirical formula) of any of the ions which we found to be significantly different between the Mn exposed and unexposed workers. Analytical work to determine the identities of these seven ions through fragmentation, isolation, and additional mass spectrometry and nuclear magnetic resonance methods is ongoing. However, as detailed by others, identification of unknown ions is a major

bottleneck in metabolomics research. Knowing the chemical structure or formula of these promising ions would help to inform the biochemical pathways influenced by Mn exposure and allow us to better elucidate the relationship between exposure and health effect.

A challenge to using metabolomics methods for exposure assessment in occupational cohorts is that any metabolites believed to distinguish between Mn exposure status could be non-specific to Mn exposure. Instead, these metabolites could represent unmeasured confounders that differentiate the two groups, including other workplace exposures. In human cohorts, it is extremely challenging to comprehensively assess all confounding exposures that would be present in an occupational setting. While many of these workplace exposures (e.g. iron, chromium, nickel) would likely be correlated with Mn exposure, others may be related to specific industrial processes or base metals and not correlated with Mn exposure. It is always a limitation with metabolomics studies that ions found to differentiate between groups could actually relate to unmeasured confounders as opposed to the exposure in question. However, including separate training and validation groups minimizes the identification of false positive signals. We found nine ions to be different between groups defined by exposure in the training data set that were replicated in the validation data set. Additional studies in Mn exposed individuals and appropriate controls should be conducted to verify these results.

Additionally, the workplace is only one of many micro-environments in which these workers spend an appreciable amount of time, and while all such micro-environments have historically been thought of separately (including in this manuscript), it is the interaction of work and non-work factors that together contribute to overall human health (Schulte et al. 2012; Schulte and Vainio 2010). Despite classifying persons into groups defined by exposure or disease for metabolomics studies, at its very nature metabolomics is a reflection of exposures

sustained in all micro-environments, and through multiple routes of exposure. While this can make it more challenging to investigate a single exposure, it does make metabolomics a powerful tool for exploring the human exposome, assuming that a life history of exposures can be assessed in a rigorous way.

Despite these inherent limitations and considerations, the novelty of the work presented here should not be minimized. As limits on occupational exposures are becoming increasingly stringent and chronic diseases are being linked to low-level exposures, a new set of exposure assessment tools is necessary to assess how subtle biochemical changes in workers influence their health. Indeed, for an exposure such as Mn, it is likely that the majority of exposure would be sustained in a work environment relative to exposures through dietary or ambient sources, making the workplace a logical setting for investigation of Mn. Research presented here shows that metabolomics is one tool that can be used to find subtle biochemical differences between groups classified by exposure, and would have increased utility in well-characterized prospective longitudinal cohort studies with repeat measures as suggested by Wild, where both work and non-work exposures can be simultaneously assessed, and the contribution of each micro-environment characterized (Wild 2005).

4.5 CONCLUSION

In conclusion, this research presents the first time, to our knowledge, that metabolomics methods have been used in an occupational setting to investigate biochemical differences between Mn exposed and unexposed workers. We hope that, with identification of all or some of these nine promising ions and subsequent interpretation of their biological relevance, a greater understanding of the mechanisms behind manganese's neurotoxic effects will be realized.

Additionally, we hope that work presented here can be used by others to expand the use of metabolomics-based analyses in occupational exposure assessment. Doing so will inform the use of metabolomics in exposome studies looking to integrate work and non-work factors in interpreting human exposure and health.

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CHAPTER 4: FIGURES AND TABLES

Table 9. Subject demographics by Mn exposure status

	Mn Exposed Workers			***Mn Unexposed Workers***		
	All (n=20)	**Training (n=12)**	**Validation (n=8)**	**All (n=17)**	**Training (n=10)**	**Validation (n=7)**
	mean ± SD (range)	**mean ± SD (range)**	**mean ± SD (range)**	**mean ± SD (range)**	**mean ± SD (range)**	**mean ± SD (range)**
Age	41.9 ± 12.8 (21, 66)	41.8 ± 12.6 (21, 62)	42.1 ± 12.6 (29, 66)	48.1 ± 11.3 (27, 60)	48.3 ± 10.8 (31, 60)	47.7 ± 10.8 (27, 60)
Ethnicity	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Hispanic	12 (60.0%)	7 (58.3%)	5 (62.5%)	4 (23.5%)	3 (30.0%)	1 (14.3%)
Not Hispanic	8 (40.0%)	5 (41.7%)	3 (37.5%)	13 (76.5%)	7 (70.0%)	6 (85.7%)
Race	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**
White	15 (75.0%)	9 (75.0%)	6 (75.0%)	15 (88.2%)	9 (90.0%)	6 (85.7%)
Other	5 (25.0%)	3 (25.0%)	2 (25.0%)	2 (11.8%)	1 (10.0%)	1 (14.3%)
Respirator	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Yes	12 (60%)	7 (58.3%)	5 (62.5%)	0 (0%)	0 (0%)	0 (0%)
No	8 (40%)	5 (41.7%)	3 (37.5%)	17 (100%)	10 (100%)	7 (100%)
Shift	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**
First	17 (85.0%)	10 (83.3%)	7 (87.5%)	9 (52.9%)	5 (50.0%)	4 (57.1%)
Second	3 (15.0%)	2 (16.7%)	1 (12.5%)	8 (47.1%)	5 (50.0%)	3 (42.7%)

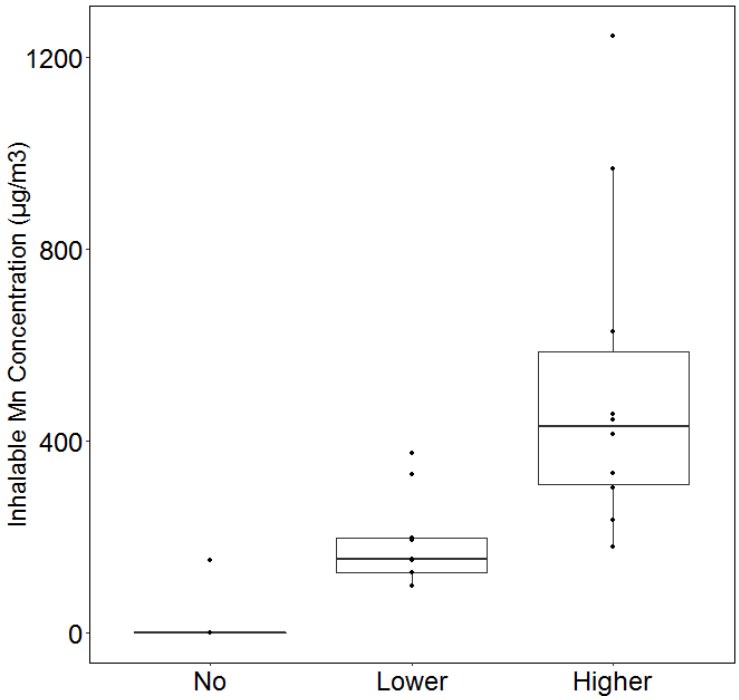

Figure 2. Measured Mn exposure levels by Mn exposure group (in µg/m^3)

For each exposure group, the middle line that divides the box into two parts represents the median value while the top and bottom line of the box represent the 75^{th} and 25^{th} percentiles, respectively. The box represents the interquartile range (IQR) of scores for the group. The whiskers are extended to all values that are no more than 1.5 x IQR from the edge of the box.

Table 10. Relative abundance of ions found to be significantly different (FDR<0.1) between Mn exposured and unexposed subjects in the training group

			Mn Unexposed (n=10)			Mn Exposed (n=12)			FDR	p-value
m/z	retention time (mins)	mode	mean ± SD	cv	% detected	mean ± SD	cv	% detected	training*	validation**
201.02†	3.56	ESI-	2.05 ± 0.51	24.9%	70%	3.22 ± 0.40	12.4%	100%	<0.01	<0.01
297.10†	3.98	ESI-	1.78 ± 0.29	16.3%	10%	3.23 ± 0.42	13.0%	92%	<0.01	<0.01
553.24	4.03	ESI-	0.76 ± 0.27	35.5%	0%	1.94 ± 0.58	29.9%	75%	<0.01	<0.01
160.08	4.21	ESI-	1.49 ± 0.36	24.2%	40%	2.14 ± 0.34	15.9%	92%	0.04	0.14
205.07	4.21	ESI-	1.78 ± 0.42	23.6%	80%	2.46 ± 0.35	14.2%	92%	0.08	0.13
246.01	4.68	ESI-	0.41 ± 0.76	185.4%	0%	1.99 ± 0.31	15.6%	83%	0.01	0.03
311.12	5.18	ESI-	1.45 ± 0.30	20.7%	10%	2.34 ± 0.34	14.5%	92%	<0.01	<0.01
415.22	8.95	ESI-	1.58 ± 0.16	10.1%	20%	2.04 ± 0.30	14.7%	75%	0.03	0.08
321.10	4.01	ESI+	1.72 ± 0.26	15.1%	0%	2.47 ± 0.36	14.6%	83%	0.03	<0.01
229.07	4.20	ESI+	1.59 ± 0.27	17.0%	10%	2.07 ± 0.26	12.6%	67%	0.08	0.40
177.11†	4.71	ESI+	1.84 ± 0.37	20.1%	30%	2.46 ± 0.25	10.2%	92%	0.08	<0.01
354.23	4.95	ESI+	2.17 ± 0.27	12.4%	50%	2.62 ± 0.21	8.0%	83%	0.08	0.20
311.15	6.83	ESI+	2.42 ± 0.22	9.1%	80%	1.97 ± 0.30	15.2%	17%	0.10	0.16
403.23	7.72	ESI+	2.04 ± 0.18	8.8%	60%	1.69 ± 0.19	11.2%	17%	0.08	0.65
459.22	7.78	ESI+	2.84 ± 0.21	7.4%	100%	2.41 ± 0.24	10.0%	58%	0.08	0.08

m/z: mass to charge ratio

*Benjamini-Hochberg corrected p-value between Mn unexposed (n=10) and Mn exposed (n=12) subjects in training group

**Two-sided t-test between exposed (n=8) and unexposed (n=7) in validation groups

†Had known isotopologues or fragments that were also found in the training group with FDR < 0.1

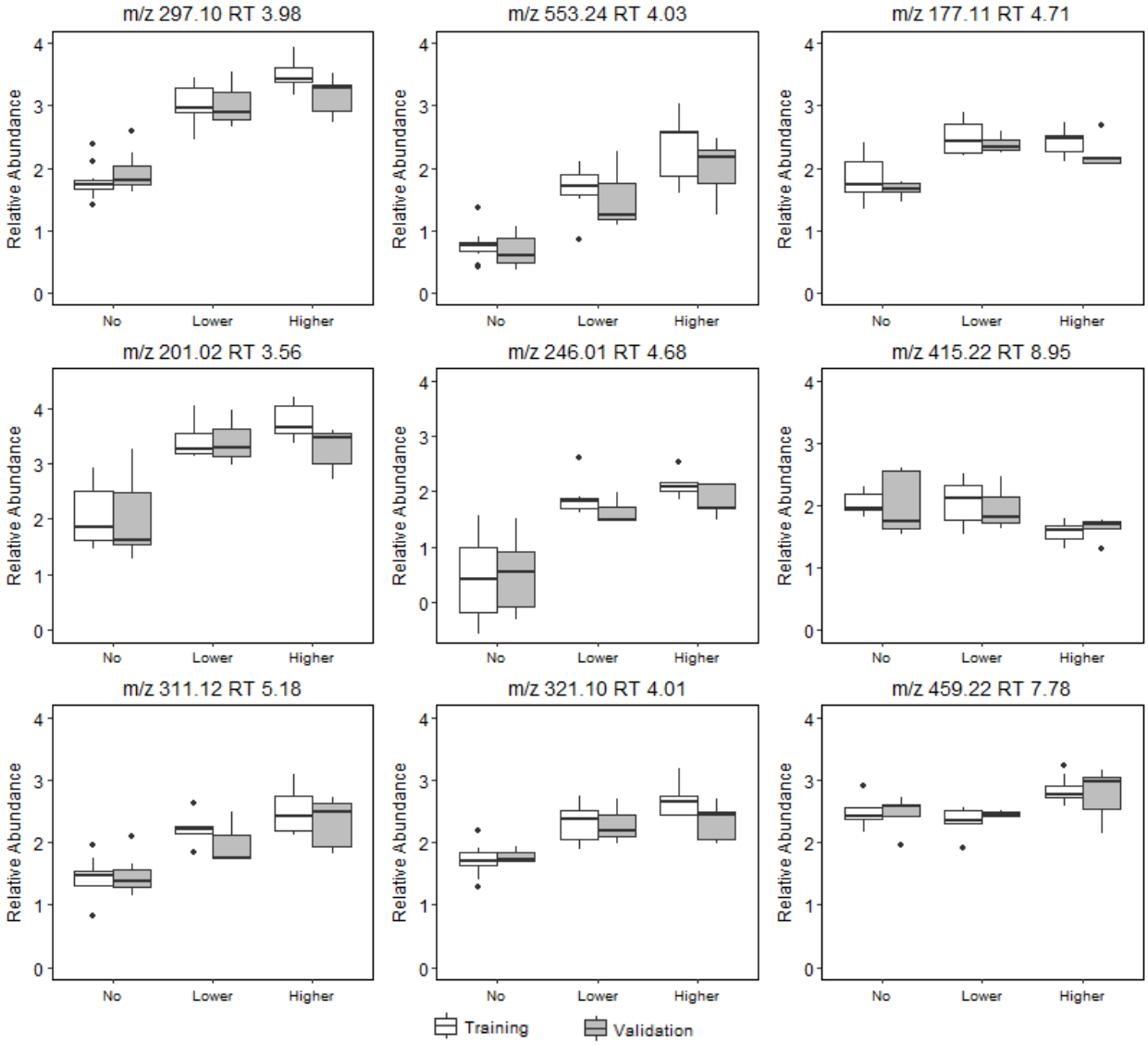

Figure 3. Box plots of exposure-response relationships for ions identified as different bewtween no Mn exposure, lower Mn exposure, and higher Mn exposure in both training and validation sets.

Table 11. Sensitivity Analysis

	Primary data set (50% filtering)	Sensitivity data set (25% filtering)	Overlapping ions
Total ions	1736	3380	--
FDR < 0.1			
n ions found in training set	15	22	15
n ions found in validation set	9	11	9
FDR < 0.05			
n ions found in training set	8	12	8
n ions found in validation set	7*	8	7*
FDR < 0.01			
n ions found in training set	4	8	4
n ions found in validation set	6**	6	6**

*Ion m/z 177.11 RT 4.71 had an FDR<0.05 in the validation set, but had an FDR of 0.08 in the training set

**Ions m/z 177.11 RT 4.71 and m/z 321.10 RT 4.01 had FDR<0.01 in the validation set, but had FDRs of 0.08 and 0.03, respectively, in the training set

Chapter 5. THE REPRODUCIBILITY OF GLOBAL URINARY METABOLOMICS PROFILES IN ASSESSING MN EXPOSURE

5.1 INTRODUCTION

Findings from a global metabolomics analysis, such as presented in Chapter 4, are considered to have greater utility if they can be replicated in independent samples. In Chapter 4, the results from the training group of samples were replicated in a validation group, which consisted of subjects from the same worksites who provided a urine sample on the same day as the training group (October 2014). In this chapter, we look to further replicate the nine ions of interest by investigating if these ions are still associated with Mn exposure in urine samples taken from the same subjects at a different time (January 2015), and if these ions are associated with Mn exposure in samples taken from Mn-exposed workers in a different industry and geographic region (Wisconsin shipyard workers).

In the metabolomics literature, many studies have been conducted on laboratory reproducibility of metabolomics profiles. Generally, the focus has been on different instrument platforms, preparation methods, and storage of biosamples and how these factors can affect the metabolomics profiles when analyzing the same sample (Gika et al. 2007; Gika et al. 2012; Sumner et al. 2007; Dunn et al. 2008). However, few studies have sought to replicate findings from a targeted or global human metabolomics study in other populations. Townsend et al. (2013) investigated the within-person reproducibility of 257 identified metabolites using archived plasma collected 0.8 to 2.3 years apart in two large cohort studies. The authors found that when comparing profiles from the two plasma samples, 90% of the known metabolites had

Spearman correlations ≥0.40, though some classes of metabolites (carbohydrates, amines and purines, pyrimidines) tended to have Spearman correlations <0.4. In a targeted metabolomics study, Chaleckis et al. (2016) collected five serial blood samples over a 24-hour period from four volunteers to assess diel variation of 126 identified blood metabolites. The authors found that in this small number of volunteers, 93% of the identified metabolites (117 of 126) varied less than 2.5-fold on average, over a relatively short time period. The authors also noted that metabolites related to consumption of food, drink, or supplements (e.g., caffeine) varied widely over the day. Both Townsend et al. and Chaleckis et al. used targeted metabolomics methods. In contrast, the replication results presented in this chapter are from global metabolomics studies, where many more (unidentified) ions are being investigated. Townsend et al. and Chaleckis et al. used repeat samples from the same individuals. While this is similar to the urine samples collected from Puget Sound workers in October 2014 and January 2015, the comparisons with urine from the Wisconsin welders represents a different cohort of workers.

It is expected that the metabolome will change over time due to variation in enzymatic activity, subsequent changes of intracellular metabolite concentrations, as well as consumption of exogenous substances (Zhang et al. 2013). To control as much of this innate variability as possible, it has been recommended that repeat samples taken on a different day be taken at the same time of day as the first sample, using the same methods, and stored in the same manner (Zhang tet al. 2013). Additionally, when investigating metabolomics profiles related to short-term environmental or occupational exposure, as we are doing here, variability in short-term exposure levels, and the resultant dose, must also be acknowledged when generalizing or comparing results. Short-term exposures can vary day to day, or even within a work day or work task, due to differences in work tasks, materials being used, environmental conditions (wind,

humidity, temperature), location of work, and exposures generated from other nearby workers. However, all efforts should be taken to ensure comparability between collected replicate measurements, whether they are repeat measurements from the same workers, or samples from a different set of workers. This includes ensuring, as much as possible, that the samples are collected, prepared, and analyzed using the same methods.

In Chapter 4, we found nine ions in urine collected in October 2014 that differentiated between groups defined by Mn exposure (Figure 2 and Table 2 of Chapter 4). In this chapter, we will compare the abundances of these nine ions between groups defined by Mn exposure in a second set of samples collected in January 2015 from the same workers. Additionally, these nine ions and their relation to current Mn exposure will be investigated in urine samples collected from Wisconsin welders in January 2015. A model will be built using the nine ions of interest from the October data, and the model will be subsequently tested in both the January data and Wisconsin welders' data in order to investigate the predictive power of these ions for Mn exposure.

5.2 METHODS

Study population and samples collected: Puget Sound cohort

As previously detailed in Baker et al. (2017), twenty Mn-exposed workers were enrolled from a Mn-steel foundry and 17 crane operators with low exposure to Mn were enrolled from a metal recycling facility, both from workplaces in the Puget Sound region. A single full-shift air sample was taken for each exposed and unexposed subject in October 2014, using IOM Inhalable dust samplers. A spot post-shift urine sample was also collected. After collection of the urine

sample, an exposure questionnaire was administered to assess specific work activities and control measures employed (e.g., ventilation, respiratory protection). Urine samples were transferred to the University of Washington on dry ice. Prior to analysis, both the exposed and unexposed groups were divided into a training set (n=12 and 11 in the exposed and unexposed groups, respectively) and age-, race-, ethnicity-, and work shift-matched validation set (n=8 and 6 in the exposed and unexposed groups, respectively). Results from air monitoring confirmed the exposure difference between the two worksites; the mean 8-hr time weighted average (TWA) Mn exposure at the foundry was 365 $\mu g/m^3$ (SD: 300, range: 98.5, 1243) and the mean Mn exposure for the crane operators was 9.2 $\mu g/m^3$ (SD: 36.5, range: 0.02, 150.8).

A second urine sample was taken from workers at both the foundry and scrap metal recycling center who were still employed in the same role in January 2015. A second personal inhalable air sample was not taken at this time, but a brief questionnaire was used to confirm that job duties (and exposure) remained similar between October 2014 and January 2015. In January 2015, 17 Mn exposed subjects were still employed at the foundry, and 15 Mn unexposed subjects were still employed at the scrap metal recycling yard. The three exposed and two unexposed subjects who were no longer employed in at the foundry and scrap metal recycling yard, respectively, are believe to be missing completely at random though details of their departure from the workplace are not known. Care was taken to collect, transport, store, prepare, and analyze the samples in the same manner as the first set of samples collected in October 2014.

Study population and samples collected: Wisconsin cohort

The source subjects for this aim are from a larger, ongoing welding cohort study conceived of and maintained by Washington University (WU) investigators (Racette et al. 2012).

The larger study includes two shipyards and one fabrication shop located on Lake Michigan in Wisconsin; all subjects are members of the International Brotherhood of Boilermakers (IBB). Both welders and non-welders were recruited from the shipyards and fabrication shop for inclusion in this metabolomics study. In total, 66 shipyard workers were recruited from the existing cohort to provide a urine sample for metabolomics analysis. There was no difference in age, gender, race, or tobacco use between the overall cohort and those recruited to participate for the metabolomics analysis. Participants were re-consented for collection of this study, with IRB approval granted from WU in St. Louis. .

Collaborators from WU and Marissa Baker collected urine samples at the IBB union hall. Many of these samples were collected over a weekend, so workers had not been working on the day that they provided a urine sample. Thirty-four urine samples were collected in the field in January 2015, and 21 urine samples were collected in July 2015. Samples were shipped from Wisconsin to UW on dry ice, and stored at -80°C prior to analysis.

To inform the analyses, and because the shipyards would not allow researchers to conduct personal air sampling, each subject was given an exposure questionnaire in order to refine estimates of cumulative and recent Mn exposure. This questionnaire addressed specific welding activities, environments, and control measures (e.g., ventilation, respiratory protection) for the worker's complete welding exposure history. Special attention was made to recent exposures (within the past week) to obtain the most accurate information possible on the level and timing of Mn exposures that could affect biological responses. Based on this questionnaire results, the metric "weighted welding exposure-years" (WWY) was modeled as a quantitative, continuous measure of cumulative relative exposure to Mn (Racette et al. 2012). This model includes number of years at all previous welding jobs and also measures of duration and intensity

of welding at each job. Based on job title, duties, and types of welding, welders were characterized as full-time welder (40 welding hours/week), part-time welder (20 welding hours/week and 20 around-welding hours/week), or work around welding (0 welding hours/week, 10 around-welding hours/week) for each job they have held. Total welding hours were weighted as 1, while around-welding hours were weighted as 0.25. WWY was calculated as shown in Equation 3 below:

Equation 3:

$$\text{Weighted welding exposure years}_i = \sum_{j=1}^{j} \left[\frac{(\text{welding hours} * 1) + (\text{around-welding hours} * 0.25)}{\text{week}}\right]_{ij} * [\text{weeks in job}]_{ij} * \frac{\text{years}}{2000 \text{ hours}}$$

In addition to WWY as a continuous measure of cumulative exposure to Mn, two binary classifications of exposure status were also considered. For the binary measure "Current Exposure", subjects were coded as exposed if they reported currently being a welder or welders' helper at the time of their urine sample. This measure is the most comparable to the exposure classification used in the Puget Sound cohort. For the binary measure "Cumulative Exposure", subjects were coded as unexposed if they were below the median of the distribution of WWY (i.e., 2.5 years) and exposed if they were above the median of the distribution of WWY.

Metabolomics data analysis

Global metabolomics profiling was performed on urine samples collected from Puget Sound and Wisconsin participants using the methods detailed in Chapter 4. Urine samples were prepared and analyzed on three occasions: Puget Sound samples collected in October 2014 were prepared and analyzed in November 2014, Puget Sound and Wisconsin samples collected in January 2015 were prepared and analyzed in March 2015, and Wisconsin samples collected in July 2015 were prepared and analyzed in September 2015. Raw data collected during metabolomics analyses across all three time points were combined for data preprocessing, which ensures peak alignment across the sample preparation days, and that the nine peaks of interest would be denoted by the same *m/z* and retention times across data sets. Preprocessing was done using *xcms,* an open-source package developed for R Studio (Smith et al. 2006; Tautenhahn, Böttcher, and Neumann 2008). This package was used for feature detection, retention time alignment, and recursive filling of missing peaks. For each ion detected, relative abundances from the Q-TOF were normalized by dividing by the sum of the abundances of all ions detected, multiplied by 10^6 for convenience, were $\log_{10}$-transformed to approximate a normal distribution, and are unitless. Normalized, log-transformed relative abundances for the nine ions of interest were mined from the ESI negative and ESI positive data sets for further analyses.

Statistical Analysis

The binary "current exposure" metric for the Wisconsin welders is most comparable to exposure as assessed in the Puget Sound data, where exposure levels were assessed over one work day and no work history or historical sampling data were used to develop estimates of

cumulative exposure. Therefore, "current exposure" is considered as the primary exposure outcome in the Wisconsin welder data, with the binary "cumulative exposure" metric considered secondarily. The relative abundance of the nine ions found to be related to Mn exposure in Chapter 4 were categorized by exposure status for urine samples collected from the Puget Sound workers in October 2014 and January 2015, and the urine samples collected from the Wisconsin shipyard workers. Normalized, log-transformed relative abundances of these nine ions were compared between the exposed and unexposed persons using a two-sided t-test. As only nine comparisons were being made, t-tests were not further adjusted for multiple comparisons. A $p<0.05$ was considered to be significant.

Data were visualized using both principal components analysis (PCA) plots, and partial least squares discriminant analysis (PLS-DA) plots. PCA plots were generated using prcomp in base R, and PLS-DA relied on the R package *mixOmics* (Lê Cao, Déjean, and González 2012). While PCA and PLS-DA are commonly used to visualize high dimension data, in this instance they were used for only nine ions. PCA creates new variables (principal components) which are linear combinations of the original variables, with the first principal component explaining the most variation in the data, and the second principal component explaining the most variance in the direction orthogonal to the first principal component (Jolliffe 2002). While PCA is unsupervised (i.e., the algorithm is not informed of group membership), PLS-DA is a supervised method where the algorithm takes into account group membership (Brereton and Lloyd 2014). Both PCA and PLS-DA can be used to see if the data group into patterns based on a binary or categorical characteristic, in this case Mn exposure, and a separation in the plots may be indicative of differences in the metabolomic profiles by group (Want and Masson 2011; Abdi and Williams 2010).

To investigate the predictive power of the nine ions found to be different between Mn exposed and unexposed urine samples collected in October 2014, an elastic net model was fit using the R package *glmnet* with the binomial family option, since exposure was considered as a binomial outcome (1=currently exposed, 0= not currently exposed) (Friedman, Hastie, and Tibshirani 2009). Prior to fitting an elastic net model, the nine ions to be included in the model were checked for collinearity, and those ions with a variance inflation factor (VIF) greater than 10 were excluded from the model. The resulting elastic net model included eight ions, an elastic net mixing parameter α equal to 0.5 and a λ value chosen using leave-one-out cross validation (LOOCV) to minimize mean square error. The elastic net was subsequently tested in the January 2015 Puget Sound samples and in the Wisconsin samples (separately for current and cumulative exposure outcomes) to determine how well exposure status was predicted using these eight ions. A receiver operated characteristic (ROC) curve was produced to visualize the predictive power of these eight ions in the January 2015 Puget Sound samples and in the Wisconsin samples, separately for both current and cumulative exposure, and the area under the ROC curve (AUC) was estimated as a quantitative measure of the predictive ability of these eight ions using the R package *ROCR* (Sing et al. 2005). The AUC can be interpreted as the probability that the model will correctly rank an exposed sample (coded as 1) higher than an unexposed sample (coded as 0) (Fawcett 2006).

5.3 RESULTS

Table 12 presents normalized, log-transformed relative abundances for the nine ions of interest for both sets of Puget Sound samples stratified by Mn exposure status (exposed or unexposed) and the Wisconsin samples stratified by current exposure status. Four ions were

found to be significantly different between exposed and unexposed samples collected from Puget Sound workers in January 2015, and all nine ions seemed to exhibit regression to the mean in the second set of Puget Sound samples, with higher values decreasing and lower values increasing. None of the nine ions were found to be different between currently exposed and unexposed workers in the Wisconsin dataset.For the Puget Sound samples, the nine ions of interest were stratified into three exposure groups (no exposure, lower exposure, higher exposure; see section 4.2 Methods for explanation on how these groupings were created) and visualized with box plots to look for an exposure-response relationship (Figure 4). For most ions, the January 2015 data exhibited no discernable exposure-response relationship.

PCA and PLS-DA plots for the January 2015 Puget Sound samples colored by exposure status are shown in Figure 5A and B, respectively. The principal components represented by these plots were based only on the nine ions found to be most interesting in the October 2014 Puget Sound data. In the Puget Sound January 2015 samples, the first principal component explained 56.3% of the variance, and the second explained 17.3% of the variance; in total, these two principal components explained 73.6% of the variance, though this is 73.6% of the total variance, not variance between the groups. Similarly, PCA and PLS-DA plots for the Wisconsin samples colored by current exposure status are shown in Figures 5C and D, respectively. In the Wisconsin urine data, the first principal component explained 47.3% of the variance, and the second explained 32.6% of the variance, making the total variance explained by these two principal components 79.9%, representing both within- and between-group variance. PCA and PLS-DA plots for the Wisconsin samples colored by cumulative exposure are presented in Appendix B. In the January 2015 data, both the PCA and PLS-DA plots show separation by group, with the PLS-DA plot showing complete separation with no overlapping 95th percent

confidence intervals. However in the Wisconsin data, there is no separation between groups defined by exposure in either the PCA or PLS-DA plot, which is indicative of a different underlying data structure in the Wisconsin samples as compared to the Puget Sound samples in regard to these nine ions.

Using the October 2014 (primary) dataset, the variance inflation factors (VIF) were calculated to assess the nine ions of interest for multicollinearity prior to model building. Of the nine ions of interest, the ion with a *m/z* of 297.10 and a retention time of 4.02 minutes was found to have a VIF=27.8, signaling a high degree of collinearity with other ions. When removing this ion, the eight remaining ions all exhibited a VIF between values of 1 and 8. Subsequent elastic net model building and prediction excluded the ion with a *m/z* 297.10 and a retention time of 4.02 minutes to reduce multicollinearity and variance inflation.

An elastic net model was fit on the October 2014 data setting α to 0.5. Lambda (λ) was chosen through cross validation; λ values were tested over a range of -2 to 10, and λ= 0.00146637 was the value that minimized the mean cross validated error. Classification tables for the January 2015 Puget Sound samples and the Wisconsin samples (using current or cumulative exposure status) are summarized in Table 13. The elastic net model , correctly classified exposure status in 72% (23/32) of January 2015 Puget Sound samples, but only correctly classified current exposure status in 26% (14/54) of the Wisconsin samples, and correctly classified cumulative exposure status in 50% (26/55) of the Wisconsin samples. For the Wisconsin data, while the sensitivity of the predictive model was high (93% when predicting currently exposed and 96% when predicting cumulative exposure), the predictive model classified nearly all Wisconsin individuals as exposed (specificity = 0).

Figure 6 presents the ROC curves for the three datasets. The AUC for the January 2015 samples was 0.80, and the AUCs for the Wisconsin samples were 0.49 for both current and cumulative exposure. An AUC of 0.5, denoted by the solid black line, is what would be expected when classifying exposure status by chance alone. The AUC can be interpreted as the probability that the model will correctly rank an exposed sample (coded as 1) higher than an unexposed sample (coded as 0) (Fawcett 2006). While aggregating the ROC curve into a single summary measure (AUC) does ignore the tradeoffs between sensitivity and specificity plotted along the entirety of the curve (Lobo, Jiménez-Valverde, and Real 2008; Hanczar et al. 2010) the larger AUC for the January 2015 Puget Sound samples relative to the Wisconsin samples can indicate that the elastic net model, and the eight ions included in this model, better classified the January 2015 Puget Sound samples than the Wisconsin samples, whether the Wisconsin samples were classified by current or cumulative exposure status (Hanley and McNeil 1983).

5.4 Discussion

In this chapter, nine ions that differentiated between Mn exposed and unexposed individuals in urine samples collected in October 2014 from Puget Sound workers were further investigated in a second set of samples collected from the same Puget Sound workers three months later (January 2015) and in urine samples collected from Wisconsin shipyard welders in January and July 2015. Four of the nine ions were higher in the Mn exposed individuals compared to the unexposed individuals in the January 2015 Puget Sound cohort (p-value <0.05), while none of the nine ions differed in the Wisconsin shipyard welders, regardless of whether samples were stratified by current exposure or cumulative exposure. The PCA and PLS-DA plots

with the nine ions of interest showed separation by exposure status in the January 2015 Puget Sound samples, while there was no separation in the Wisconsin samples.

It would be expected that the metabolomics profiles of repeat samples on the same individuals would be more similar than when comparing metabolomics profiles of persons from two different cohorts, such as the Puget Sound samples and the Wisconsin samples. However, it is notable that even these nine ions showed differences between exposed and unexposed persons when compared over two time points. In fact, only four of the nine ions were found to be different in the January 2015 Puget Sound samples. Of these four ions, the exposure-response trend that was seen in the October 2014 samples when stratifying exposure into three tiers (no exposure, lower exposure, and higher exposure) was no longer observed in the January 2015 Puget Sound samples. Specifically, the trend in the relative abundance between higher exposure and lower exposure sub-groups was not consistent. This could have been due to changes in work practices or exposures among the exposed persons between October 2014 when the first sample was taken and January 2015 when the follow-up sample as taken. In addition, changes in enzymatic activity or other physiological factors could affect the metabolome between samples. Moreover, while exposure was measured quantitatively for the October 2014 samples using IOM inhalable samplers, exposure was not determined in January 2015 and was assigned based on exposures and job tasks determined at the previous visit. It is possible that exposures or job tasks could have changed during this three month time period leading to exposure misclassification for the January samples. This is one additional challenge that must be noted when investigating ions related to short-term exposure in occupational or environmental settings; day to day variability is expected to be another contributing factor on top of the inherent variability in metabolite levels present due to physiological processes.

Differences in exposure levels and determinants of exposure could also be an explanation for why the nine ions were not related to exposure status in the Wisconsin shipyard workers. As no industrial hygiene measurements for airborne Mn were taken at the Wisconsin shipyards, qualitative measures of relative exposure were developed from questionnaire data. Historical Mn exposure assessments of shipyard welders report Mn exposures ranging from 0.004 to > 2.0 mg/m^3, with the highest levels experienced by welders working in confined or enclosed spaces (Hobson et al. 2011). Of the 14 current Wisconsin shipyard welders included in this analysis, only four individuals(29%) reported welding more than 50% of the workday in an enclosed or confined space, and six individuals (43%) reported doing all welding activities outside. The four welders who spent more than 50% of their workday welding in an enclosed or confined space all reported using either local or general ventilation when welding. Therefore, considering location of the welding (mostly outdoors) and ventilation use of the Wisconsin welders, it might be expected that airborne Mn exposures for these welders would be on the lower end of the range presented for shipyard welders in the literature. Respirator use was also reported to be high in the Wisconsin welders, with all but four welders reporting to wear either a cartridge respirator or powered air purifying respirator (PAPR) at some time (25-50% of the time spent welding). The four welders who did not report wearing a respirator of any type were welders who reported 100% of their welding to be done outdoors. Respirator use would further reduce the dose of Mn received.

In the Puget Sound foundry, measured Mn exposures ranged from 0.10 to 1.2 mg/m^3 (0.37 ± 0.30 mg/m^3). The ventilation in the foundry building where all enrolled subjects worked was limited to general ventilation and natural ventilation via open doors, with no local exhaust ventilation. While the measured exposures in the foundry are comparable to historical shipyard

Mn exposure measurements in the literature, we would expect the foundry Mn exposures to be higher than the Mn exposures experienced by the shipyard welders in Wisconsin due to the reasons discussed previously. Moreover, few foundry workers reported wearing respiratory protection, and the only respiratory protection utilized was either N95 respirators or inadequately protective painter's masks.

Given that the nine ions of interest are unidentified by name or empirical formula, it is impossible to know if the ions investigated in this chapter are related to the Mn exposure or other co-exposures or confounders. Foundry work could have a number of co-exposures that could differ from those encountered by crane operators at a metal recycling center and shipyard workers. These include substantial exposure to silica and carbon monoxide, in addition to polycyclic aromatic hydrocarbons (PAHs), phenol, formaldehyde, isocyanates, and amines (IARC 2012). Both founding and welding workers could also have exposures to other metals such as chromium, nickel, and iron. In these studies, co-exposures were not assessed using either quantitative or qualitative methods. Identification of the ions of interest by name and determination of a mechanism for altered urinary concentrations of the ions following Mn exposure, would increase their credibility as biomarkers of Mn exposure. Identification could also aid in the understanding of why the ions that were predictive of exposure in the Puget Sound workers were not predictive in the Wisconsin shipyard workers.

An ideal biomarker of exposure should be both sensitive and specific, predictive, robust, have good precision (repeatability), and be informative in a variety of populations (Baker 2005; Mayeux 2004). When considered in an elastic net model, eight ions identified in the October 2014 Puget Sound samples exhibited relatively strong sensitivity and specificity in predicting Mn exposure status in a repeat sample collected from the same individuals. However, these ions were

not predictive of Mn exposure status in subjects from a different industry and geographical region, though this could have been due to differences in exposure levels and scenarios. Perhaps these ions found in the Puget Sound cohort only become physiologically perturbed when higher levels of acute Mn exposure are realized, and they would be reproducible in other occupationally-exposed cohorts with higher acute exposure to Mn than the Wisconsin shipyard workers. While an exposure-response relationship for these ions was apparent in the October 2014 Puget Sound samples when stratifying exposure into no exposure, lower exposure, and higher exposure, this relationship was not as apparent in the January 2015 Puget Sound samples. Therefore, without identification of the ions, or deeper understanding of how these ions may be related to Mn or other exposures, their utility as biomarkers of Mn exposure is limited outside of the Puget Sound cohort.

5.5 CONCLUSIONS

This is the first time, to the authors' knowledge, that the reproducibility of the human urine metabolome, and its relation to occupational Mn exposure, has been explored. Of nine ions associated with Mn exposure status in urine samples collected in October 2014, four of the ions continued to differ by exposure status in urine samples collected from the same individuals in January 2015. Moreover, an elastic net model built using the relative abundances of eight of these ions of interest correctly predicted exposure status in 72% of subjects and the AUC of the ROC was 0.8 for the January 2015 samples. As the identity of these ions is not yet known, it is unclear if these ions are related to Mn exposure or other occupational or environmental co-exposures. The results were not replicated in the Wisconsin shipyard workers, possibly due to differences in Mn exposure levels, unmeasured occupational or environmental co-exposures, or

sample collection, storage, and handling procedures. The nine metabolites may be reproducible in other occupationally-exposed cohorts with higher acute Mn exposure than the relatively low exposure levels assumed in the Wisconsin shipyard workers.

In conclusion, the work presented hear underscores the importance of taking repeat samples when investigating the dynamic human urine metabolome, as both within and between person variances were observed. When possible, when comparing global metabolomics profiles related to exposure both within and between occupational cohorts, a thorough exposure assessment should be undertaken to understand if the occupational cohorts being compared are comparable in terms of received exposure and assumed biological dose. Validating and identifying promising results, both in the same population and other populations, remains a challenge in harnessing global metabolomics methods for biomarker discovery in occupational cohorts.

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CHAPTER 5: FIGURES AND TABLES

Table 12. Normalized, log-transformed relative abundances of ions, stratified by exposure status, in samples collected from Puget Sound workers in October and January, and from Wisconsin shipyard workers

			October PS samples			**January PS samples**			**Wisconsin samples**		
			Mn exposed (n=20)	Mn unexposed (n=19)		Mn exposed (n=17)	Mn unexposed (n=15)		Current Mn exposed (n=14)	Current Mn unexposed (n=41)	
m/z	**RT**	**mode**	**mean ± SD**	**mean ± SD**	***p****	**mean ± SD**	**mean ± SD**	***p****	**mean ± SD**	**mean ± SD**	***p****
201.0240	3.54	ESI-	3.47 ± 0.41	1.91 ± 0.61	**<0.001**	2.60 ± 0.72	2.35 ± 0.58	0.28	2.63 ± 0.48	2.79 ± 0.47	0.18
246.0098	4.68	ESI-	1.91 ± 0.32	-0.10 ± 1.3	**<0.001**	1.42 ± 0.41	0.27 ± 1.47	**0.01**	-0.43 ± 1.62	-0.02 ± 1.32	0.36
297.1007	4.02	ESI-	3.16 ± 0.43	1.80 ± 0.32	**<0.001**	2.39 ± 0.60	2.19 ± 0.56	0.34	2.10 ± 0.35	2.08 ± 0.50	0.82
415.2201	8.95	ESI-	2.02 ± 0.37	1.58 ± 0.15	**<0.001**	1.91 ± 0.38	1.84 ± 0.37	0.59	1.70 ± 0.33	1.86 ± 0.35	0.18
553.2446	4.03	ESI-	1.89 ± 0.57	0.67 ± 0.30	**<0.001**	1.27 ± 0.43	0.79 ± 0.56	**0.01**	0.68 ± 0.92	0.61 ± 1.18	0.94
311.1164	5.18	ESI-	2.29 ± 0.40	1.42 ± 0.29	**<0.001**	1.66 ± 0.45	1.53 ± 0.36	0.35	1.47 ± 0.40	1.61 ± 0.38	0.27
321.0961	4.02	ESI+	2.22 ± 0.36	1.52 ± 0.19	**<0.001**	1.71 ± 0.23	1.48 ± 0.21	**0.005**	1.44 ± 0.22	1.53 ± 0.24	0.23
177.1115	4.75	ESI+	2.20 ± 0.25	1.51 ± 0.33	**<0.001**	1.87 ± 0.29	1.62 ± 0.27	**0.02**	2.12 ± 0.31	2.01 ± 0.44	0.30
459.2202	7.8	ESI+	2.24 ± 0.25	2.61 ± 0.28	**<0.001**	2.18 ± 0.36	2.07 ± 0.53	0.51	2.11 ± 0.28	2.25 ± 0.30	0.08

PS=Puget Sound
m/z: mass to charge ratio
RT: retention time (minutes)
p-value calculated from two-sided t-test allowing for unequal variances

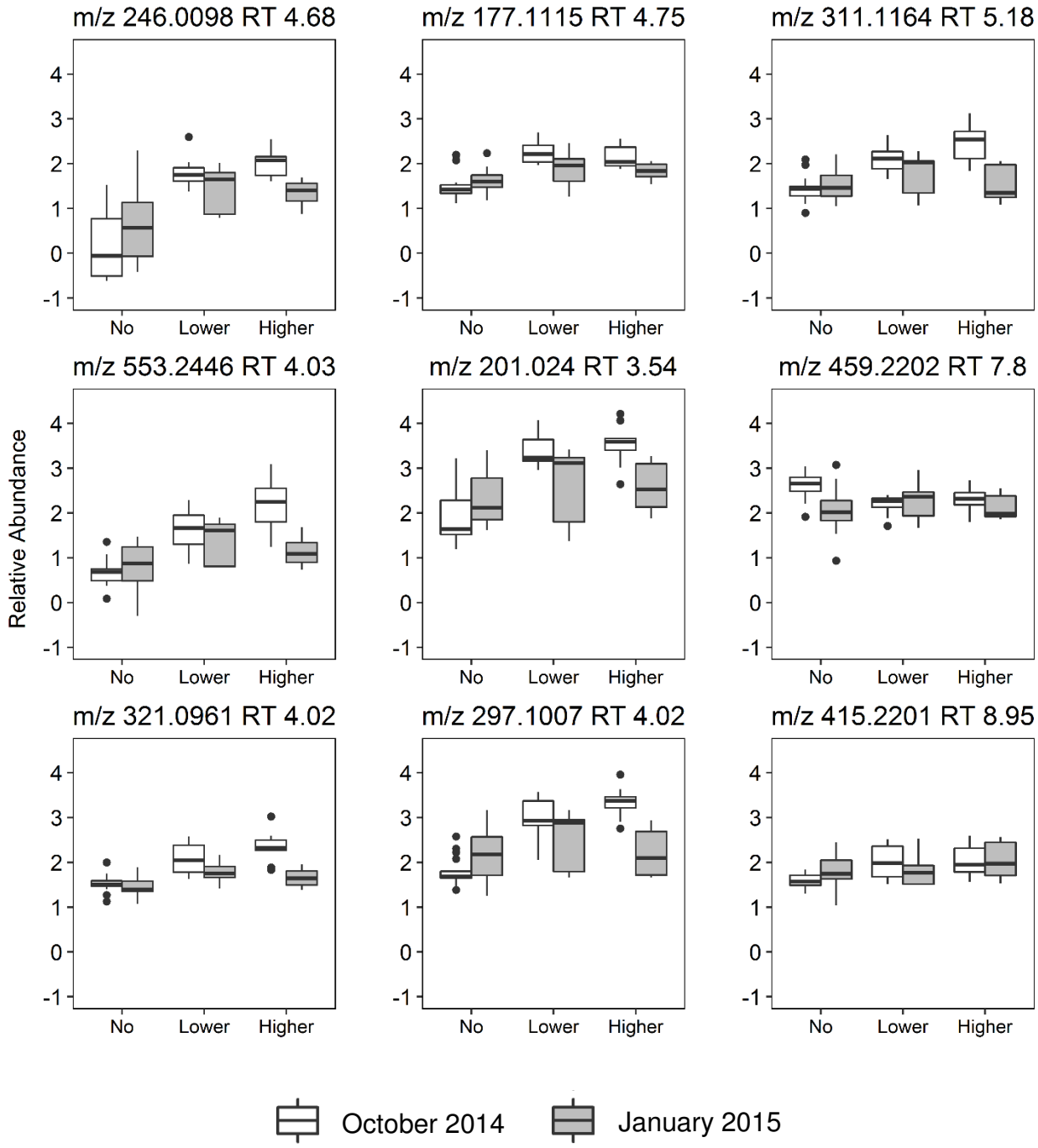

Figure 4. Box plots of normalized, $\log_{10}$ relative abundances of the nine ions of interest, for both October 2014 and January 2015 samples, stratified by no exposure, lower exposure, and higher exposure.

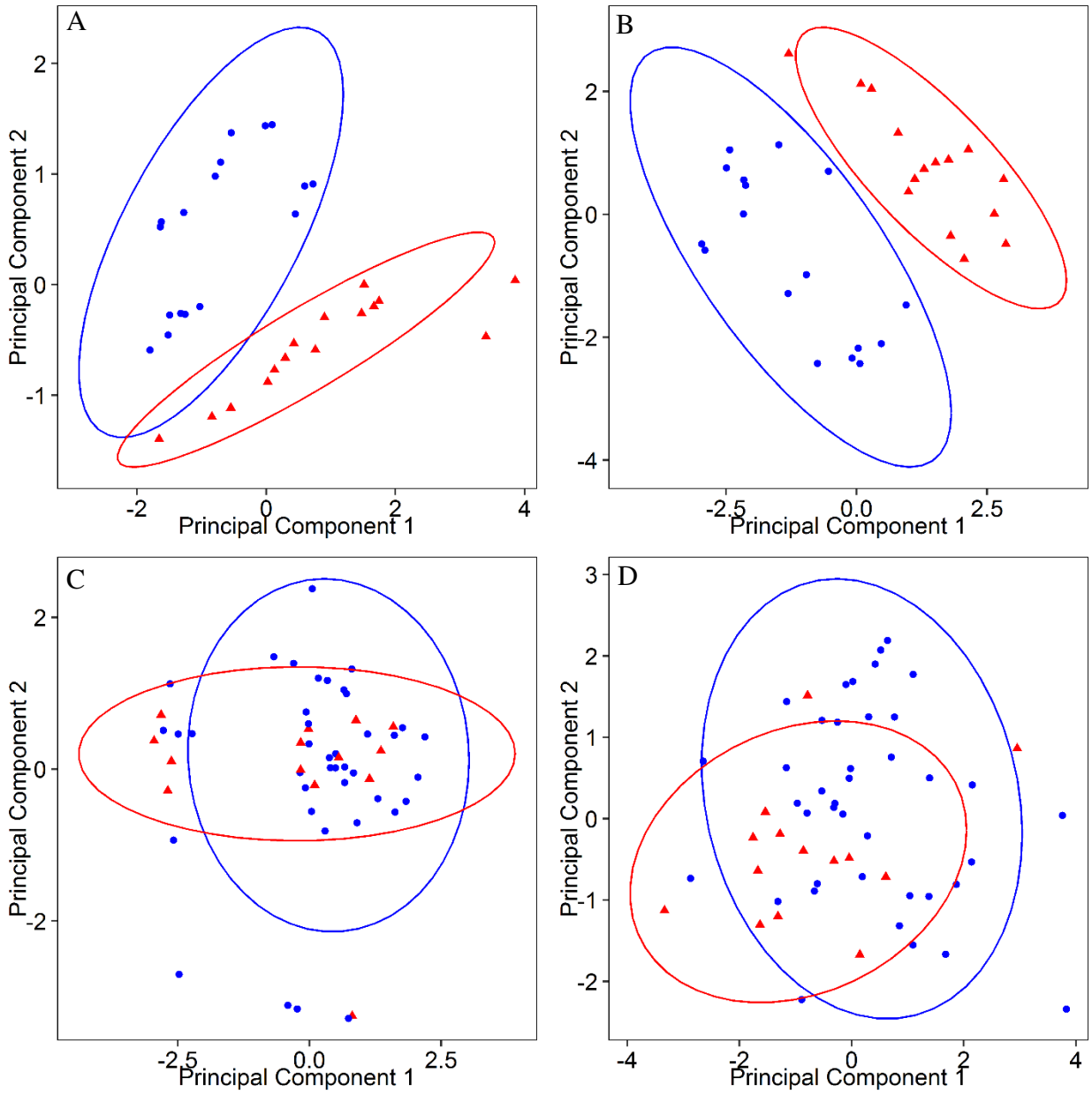

Figure 5 A, B, C, D. PCA and PLS-DA plots for exposed (red) and unexposed (blue) samples from January 2015 in Puget Sound and Wisconsin, considering the 9 ions of interest.

A: PCA for Puget Sound January 2015 Samples
B: PLS-DA for Puget Sound January 2015 Samples
C: PCA for Wisconsin samples (stratified by current exposure)
D: PLS-DA for Wisconsin samples (stratified by current exposure)

Table 13. Diagnostics for elastic net model tested in the January 2015 Puget Sound and Wisconsin samples

			Known						
			Exposed	**Unexposed**	**sensitivity**	**specificity**	**PPV**	**NPV**	**accuracy**
January 2015 Puget Sound	*Predicted*	**Exposed**	13	5	0.76	0.67	0.72	0.71	0.72
		Unexposed	4	10					
Wisconsin Current Exposure	*Predicted*	**Exposed**	14	39	0.93	0.00	0.26	0.00	0.26
		Unexposed	1	0					
Wisconsin Cumulative Exposure	*Predicted*	**Exposed**	27	26	0.96	0.00	0.51	0.00	0.50
		Unexposed	1	0					

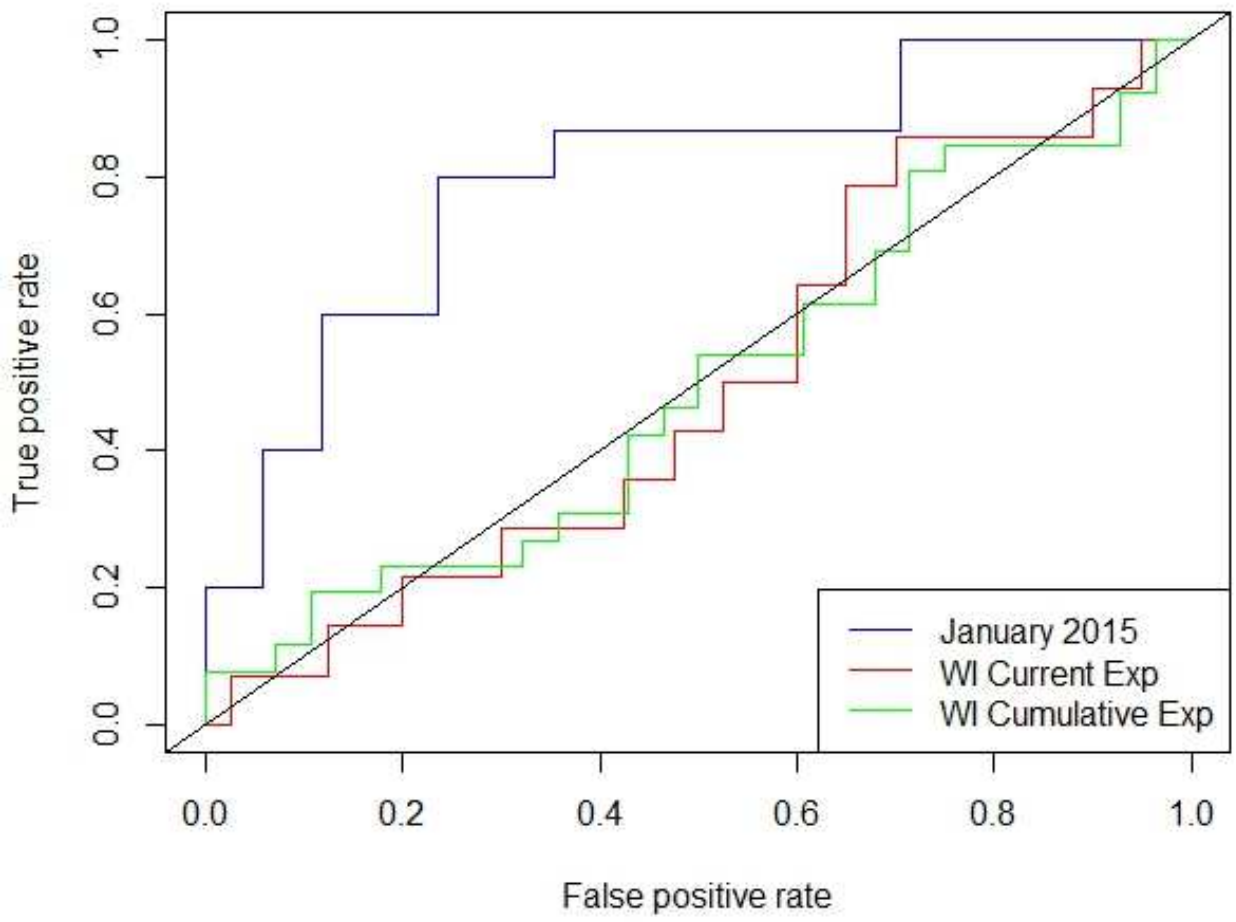

Figure 6. ROC curves for elastic net model as tested in January 2015 Puget Sound samples and Wisconsin samples.

True positive rate: sensitivity,

False positive rate: 1-specificity

Area under the curve (AUC) for January 2015 = 0.8

AUC for Wisconsin current or cumulative exposure = 0.49

Chapter 6. THE USE OF NMR FOR GLOBAL AND TARGETED METABOLOMICS FOR MN EXPOSURE STUDIES

6.1 INTRODUCTION

In the preceding chapters, mass spectrometry (MS) was used to generate global metabolomics data. Inn this chapter, NMR data was used to explore both targeted and global metabolomics profiles of the Puget Sound and Wisconsin urine samples. When the field of metabolomics was becoming established, NMR was the predominant analytical approach (German, Hammock, and Watkins 2005). However, NMR methods are generally less sensitive with higher limits of detection than MS methods, and for targeted analyses, fewer metabolites have been identified for NMR than for MS (Emwas 2015). Thus, MS has surpassed NMR as the predominant analysis approach in the recent metabolomics literature (Gowda and Raftery 2015). Despite the growing popularity of MS for metabolomics profiling, metabolomics data generated from NMR are more reproducible and reliable, are more easily quantitated, and identification of unknowns from NMR peaks is often more straightforward than identification of unknowns from MS data (Markley et al. 2017; Emwas 2015; Alonso, Marsal, and Julià 2015). Additionally, for NMR, sample preparation is simpler, the specimens remain intact during analysis, and all metabolites can be detected during a single run, without having to change chromatographic conditions, as is the case for MS metabolomics (Emwas 2015). This makes NMR an attractive analytical approach to use either instead of or in tandem with MS for metabolomics profiling.

We choose to pursue global NMR as a complementary analysis to the global MS analysis already discussed. Due to differences in the platforms, and differences in how the data are analyzed, we thought that NMR may provide additional compounds of interest that were not found with the MS analysis. While the same compounds may be found between the two

platforms, this would not be known unless the compounds were identified by name or empirical formula. Additionally, we choose to pursue targeted NMR as a means to investigate perturbations in pathways and common metabolites between the groups defined by exposure. With targeted metabolomics, we would be able to better interpret the physiological reasons for the differences and have an idea as to whether or not the metabolite could be related to Mn exposure, or if levels differed between the groups due to other reasons. NMR was chosen over MS for targeted metabolomics due to its stability and reproducibility, especially with urine samples collected and analyzed over multiple time points.

Some differences between analysis of MS chromatograms and NMR data must be noted. In MS chromatograms, each unique ion is indicated by a single peak (though naturally occurring isotopologues of an ion or fragmentation of an ion could cause its peak to show up multiple times), which is denoted by *m/z* ratio and retention time; the x-axis of the chromatogram uses retention time as the unit of measure. The relative abundance of each ion is related to the peak height or area under the peak. In NMR spectra, each chemical is indicated by multiple peaks across the full NMR spectrum. With NMR, the peak heights in the spectra are proportional to the concentration of the metabolite in the biofluid. The x-axis of the NMR spectra is in units of ppm. When referring to NMR, it is important to note that ppm is not a concentration, but a measure of the chemical shift. That is, the resonant frequency of a nucleus relative to a standard in a magnetic field. Because a single chemical has multiple signals across the full spectrum (0-10 ppm), regions of the spectrum are binned (typically into 0.005 to 0.1 ppm sections) for global analyses. This means that each bin may include signals from multiple unique chemicals, and signals from the same chemical typically appear in multiple bins. Moreover, signals can overlap, and strong signals can distort nearby, weaker signals. While analytical tools exist to attempt to

account for these considerations, these tools were not used with the NMR data presented here (Dieterle et al. 2006; Savorani, Tomasi, and Engelsen 2010). As the distribution of peaks is characteristic for each metabolite, compound libraries can be used to identify and quantify particular metabolites, thereby allowing targeted metabolomics to be performed using NMR data.

As with MS metabolomics, NMR metabolomics have largely been utilized to explore differences between persons with a disease and healthy controls, and for disease biomarker discovery. Urinary metabolites have long been recognized as clinically relevant for diagnosing or monitoring diseases, such as urinary glucose tests for monitoring diabetes (Marshall et al. 2015), or urinary creatinine to measure kidney function (Tynkevich et al. 2014). It has been NMR, and the quantitation it affords, that has been used to successfully discover and validate the above mentioned biomarkers, as well as to discover other putative urinary biomarkers for diseases such as pancreatitis (Lusczek et al. 2013), a variety of cancers (Srivastava et al. 2010; Ladep et al. 2014; Davis et al. 2012), gestational diabetes (Diaz et al. 2011), and measures of neonatal health (Dessì et al. 2011; Moltu et al. 2014) .

While the use of NMR for comparing metabolomics profiles between persons defined by disease status is the most common in the literature, NMR methods have also been used to investigate metabolomics profiles related to exposure, which is most of interest to this dissertation. The majority of NMR metabolomics studies investigating differences due to exposure have been done in plant or animal models, such as cadmium exposure in silene flowers (Bailey et al. 2003) or environmental estrogen exposures in fish (Samuelsson et al. 2006). Other studies have looked at a dietary exposure, such as gluten (Sellitto et al. 2012). Regarding human exposure studies, Kuo et al. (2012) used global NMR methods to compare urine metabolomics

profiles of workers exposed to welding fumes (n=35) and those employed as office workers (n=16) in a Taiwanese shipyard, one of the few human exposure studies utilizing NMR metabolomics. In the shipyard personal exposure to respirable Mn ranged from 35 $\mu g/m^3$ to over 2000 $\mu g/m^3$ (mean: 70 $\mu g/m^3$ in the manufacture area, and 1230 $\mu g/m^3$ in the assembly area). Mn levels for the office workers were not reported. After analyzing and identifying promising peaks from binned global NMR data, the authors concluded that welders exhibited significantly higher levels of glycine, taurine, betaine/TMAO, serine, S-sulfocysteine, hippurate, gluconate, creatinine and acetone, and significantly lower levels of creatine relative to the office workers. The authors hypothesized that increased levels of glycine, taurine, and betaine were related to modulating inflammatory and oxidative tissue injury processes, while a decrease in creatine was due to reactive oxygen species and free radicals decreasing creatine kinase activity in the welders, though further biochemical studies were needed to verify these hypotheses. While Wang et al. investigated a similar occupational exposure to that presented here, they only collected one urine sample from each subject, and thus could not assess the reproducibility of their findings as is done in this chapter.

In this chapter, global urine NMR metabolomics profiles are generated for the Puget Sound and Wisconsin cohorts as a complement to the MS analyses undertaken in Chapters 4 and 5, an as an attempt to find other ions that may be related to Mn exposure in these cohorts. As in Chapter 5, ions related to exposure are investigated in the primary data set (Puget Sound data collected in October 2014) and subsequently tested in the second set of Puget Sound samples (collected January 2015) and in the samples collected from the Wisconsin shipyard. This chapter also investigates a set of 59 metabolites identified by NMR and their biological pathways to determine if any metabolites or pathways may differ between workers defined by exposure

status. Knowing the name of the metabolites and pathways will allow us to further investigate if there is a biological reason for them to be related to Mn exposure.

6.2 METHODS

Occupational settings and urine collection

The samples analyzed using NMR were previously described in Chapter 5 (see "Study population and samples collected: Puget Sound cohort" and "Study population and samples collected: Wisconsin cohort" in Chapter 5 methods section, 5.2). In brief, we analyzed samples from two occupational cohorts: (1) urine samples collected from Puget Sound foundry workers and scrap metal recyclers in October 2014 (n=37, the primary dataset); (2) a second set of urine samples collected from the same Puget Sound foundry workers and scrap metal recyclers in January 2015 (n=32); (3) urine samples collected from Wisconsin shipyard workers on two occasions (n=61).

Puget Sound workers were classified as "exposed" if they worked at the foundry and "unexposed" if they were employed as a crane operator at the scrap metal recycling yard. Wisconsin shipyard workers were classified as "currently exposed" if they reported being a welder or welders' helper at the time of their urine sample, a classification most similar to the exposure classification used in the Puget Sound cohort. Additionally, Wisconsin shipyard workers were coded as "cumulatively exposed" if they were above the median weighted welding years for the cohort (WWY > 2.5, see Equation 3, Chapter 5, for additional information on the calculation of WWY).

NMR sample analysis

Urine samples were aliquoted into in 1 mL Eppendorf tubes, and stored at -80 °C until analyzed at the Northwest Metabolomics Research Center using urine NMR protocols established by Beckonert et al. (2007) and analyzed using a Bruker Avance III 800 MHz spectrometer (Massachusetts, USA) featuring a cryogenically cooled probe and Z-gradients suitable for inverse detection. Briefly, 300 µL urine was mixed with 300 µL of 0.1 M phosphate buffer. The samples were run as ^{1}H NMR using the one-pulse or NOESY pulse sequence, along with the CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence, with water suppression using presaturation. Bruker AMIX software (Massachusetts, USA) was used for correcting phase and baseline distortion, quantitation, and binning the peaks. The 0.0 to 10 ppm spectral region was binned, creating 2000 bins of equal width (0.005 ppm). The bins were further collapsed prior to statistical analysis to yield 250 bins of 0.04 ppm (Brindle et al. 2002; Odunsi et al. 2005). While an increased bin size reduces the resolution of each bin, previous studies have shown that bins of 0.04 ppm can accommodate small pH-related shifts better than smaller bins, and fewer bins reduces the number of multiple comparisons for biomarker discovery (Holmes et al. 1998; Holmes et al. 2000).

During data preprocessing NMR metabolomics data is commonly normalized and scaled. Normalization applies a mathematical adjustment to each sample in order to make the samples more comparable to each other and better align the underlying probability distributions of each sample. In the case of urine samples, normalization can account for differences in hydration between the subjects, in addition to other parameters. Scaling is performed on each bin or metabolite that was identified and is done to normalize the ranges of the data (Craig et al. 2006). For these analyses, NMR data were normalized by sum; (i.e. the total sum of each observation

(total spectrum) was calculated, and the intensity in each of the 250 bins was divided by this sum to represent a fraction of the total sum). The data were also Pareto scaled (i.e. for each bin, data were mean centered and divided by the square root of the standard deviation for that bin). Pareto scaling is done to better preserve the data structure, and reduce the relative influence of the larger values, as larger fold changes are decreased more than smaller fold changes (Worley and Powers 2013; Eriksson 1999; van den Berg et al. 2006). Data from October 2014 Puget Sound samples, the January 2015 Puget Sound samples, and the Wisconsin shipyard samples were normalized and Pareto scaled together. Ample sample was volume was used to ensure that if the metabolite was present, it would be able to be detected. Ions that were not detected were labeled as 0 intensity prior to binning. After binning the data, no bins had 0 intensity.

To generate the targeted metabolomics data, the library of 67 peaks previously validated in Nagana et al. (2014) were identified from the generated spectra, and each identified compound was quantified using Bruker AMIX software. In the Puget Sound and Wisconsin metalworkers data, 59 identified peaks were quantified. These identified peaks were normalized and Pareto scaled as discussed above. Metabolites that were not detected were labeled as 0 intensity. In the targeted data set, N-methylnicotinamide was not detected in one Wisconsin sample and Trans-aconitic acid were not detected in one sample from the Puget Sound collected in January.

Statistical analysis

Global NMR Analysis. The primary data set (Puget Sound samples collected in October 2014) was split into the same training and validation data sets as was done with the MS data in Chapter 4. The training binned data were visualized using PCA and PLS-DA, to look for separation between groups defined by exposure. Using the training data, integral intensities of all n=250

spectral bins was compared between Mn exposed and unexposed workers using a Wilcoxon rank-sum test, which does not assume normality of the underlying distributions. The p-values calculated from the Wilcoxon rank-sum were adjusted for multiple comparisons using the Benjamini-Hochberg method to control false discovery rates (FDR). As the lowest FDR that was found in the training data set was 0.33, we investigated the five most different training set bins (as determined by FDR) in the October 2014 validation set, in the full set of Puget Sound data collected in January 2015, and in the full set of Wisconsin data, stratified both by current exposure and cumulative exposure, using an unadjusted Wilcoxon rank-sum test.

Targeted NMR analysis. For targeted NMR analyses, the primary data set was not split into a training and validation set, given that fewer comparisons were being made. As was done for the global NMR data, the October 2014 and January 2015 Puget Sound data and the Wisconsin data were visualized using PCA and PLS-DA, prior to hypothesis testing (see Section 5.2 "Statistical Analyses" for more discussion on PCA and PLS-DA). For all 59 identified metabolites, the October 2014 Puget Sound data and the Wisconsin data were subject to Benjamini-Hochberg adjusted Wilcoxon rank-sum tests between groups defined by current exposure status. In the October 2014 Puget Sound data, five metabolites were tied for the lowest FDR of 0.28; these metabolites were tabulated and further visualized with box plots. The top five metabolites were also tabulated and visualized with box plots for the Wisconsin data set to compare current exposure status groups.

Using the October 2014 Puget Sound data, an elastic net model was fit on all 59 metabolites using the R package *glmnet* with the binomial family option (Friedman, Hastie, and Tibshirani 2009). The resulting elastic net model included an elastic net mixing parameter α

equal to 0.5, and a λ value chosen using leave one out cross validation (LOOCV) to minimize mean square error. The elastic net model was subsequently tested in the January 2015 Puget Sound samples and in the Wisconsin samples (separately for current and cumulative exposure outcomes) to determine how well exposure status was predicted. The area under the ROC curve (AUC) was estimated as a quantitative measure of the predictive ability of the elastic net model for exposures in the January 2015 Puget Sound samples and current and cumulative exposures in the Wisconsin samples using the R package *ROCR* (Sing et al. 2005).

Pathways enrichment analysis. To identify pathways that may have been perturbed between individuals exposed and unexposed to Mn, pathways enrichment analysis using 59 metabolites was carried out using MetaboAnalyst 3.0 (Xia et al. 2015; Xia and Wishart 2016) for the October Puget Sound data, and the Wisconsin data, separately. This MetaboAnalyst module combines an enrichment analysis which calculates whether pathways differ significantly between groups defined by exposure, and a pathways topology analysis which assigns an impact score to each pathway, with a higher score indicative of not only significant perturbations in the pathway, but also biologically meaningful changes in the measured metabolites (Draghici et al. 2007).

The identified metabolites were matched to their Human Metabolome Database identifier for upload to MetaboAnalyst 3.0, and were compared to the KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolites pathway library for *homo sapiens*. The Global Test (Xia and Wishart 2010) option in MetaboAnalyst 3.0 was used to evaluate concentration changes among groups of metabolites to calculate adjusted statistical significance, and the betweeness centrality option (shortest path between nodes) was used to calculate metabolite importance (Aittokallio and Schwikowski 2006).

6.3 RESULTS

Global NMR Results

Binned data for the October 2014 training group samples, colored by exposure status are shown in the PCA and PLS-DA plots presented in Figure 7A and B, respectively. In these data, the first principal component explained 21.8% of the variance, the second explained 17.9% of the variance, and these two principal components explained 39.7% of the total variance. The majority of variance (representing both within-group and between-group sources of variation) is not captured in these two principal components. It can be seen that the PCA plot (7A) showed no separation by exposure group, while the PLS-DA plot showed nearly complete separation. For both plots, the distribution of unexposed samples tended to be more dispersed, suggesting more variability in levels of ions measured by NMR relative to the levels measured in the exposed workers, and suggesting differences in binned metabolomic profiles of Mn exposed and unexposed workers.

Table 14 lists the top five spectral bins that differentiated exposed and unexposed groups in the October training set of data, and their p-value in the October validation set when using a Wilcoxon rank-sum test. None of the five bins had an FDR < 0.3 in the training set, and none were significant (defined as $p < 0.1$) when tested in the validation set of October data. Similarly none of these top five bins were significant in either the January Puget Sound data or the Wisconsin data set (data not presented). Given the lack of significantly different bins in the training set, and that none of the top five bins in the training set were significant in the validation set or the other data sets, no further global analyses were explored for this chapter. It is possible

that the bin size employed for these analyses (0.04 ppm) may have reduced the resolution such that global differences between the exposed and unexposed were obscured.

Targeted NMR Results

Using the 59 metabolites, PCA and PLS-DA analyses were conducted for the October 2014 Puget Sound and Wisconsin data (colored by current exposure) (Figure 8). For the October 2014 Puget Sound data (Figure 8 A, B), the first principal component explained 43.2% of the variance, the second principal component explained an additional 12.4%, and these two components together explained more than half the total variance (55.6%). In PCA analysis, there was no separation between subjects defined by exposure. However, when exposure group was considered as with PLS-DA, the groups were incompletely separated. For the Wisconsin data (Figure 8 C, D), the first principal component explained 32.2% of the variance, the second principal component explained 14.2%, and these two components explained a total of 46.4% of the. Like with the October 2014 Puget Sound data, no separation was apparent in the PCA, however some separation was apparent in the PLS-DA, though it appeared to be less separation than was seen in the Puget Sound data (Figure 8B). Both PLS-DA graphs (8 B, D) suggest that metabolomic differences exist between Mn exposed and unexposed individuals.

Table 15 summarizes the five metabolites that differed between groups defined by current exposure in each of the Puget Sound October 2014 data and the Wisconsin data. None of the top five metabolites overlapped between the Puget Sound samples and the Wisconsin samples. There was only one metabolite with a FDR < 0.1 based on the Wilcoxon rank-sum test in either the Puget Sound or Wisconsin data. In the Wisconsin data, isobutyrate was lower in Mn exposed subjects (-1.36 ± 0.98) compared to unexposed subjects (1.20 ± 5.48, FDR = 0.06). Isobutyrate is

a metabolite of isobutyric acid, found in alcoholic beverages and other dietary sources. The relative intensities of these ten metabolites (identified in the Puget Sound and Wisconsin data) are shown stratified by exposure status (Figures 9 and 10). The abundances of these metabolites in the January 2015 Puget Sound samples are plotted with the October 2014 Puget Sound samples in Figure 9 for comparison. The abundances of these metabolites differed between the October 2014 and January 2015 collection timepoints, though it is unknown if these changes were due to differences in the physiology of the individuals, changes to environmental or dietary exposures, or differences in sample collection and handling.

As a final analysis for the targeted NMR data, an elastic net model was fit to 59 identified metabolites in the October 2014 Puget Sound data. The elastic net model was fit using an $\alpha = 0.5$. Lambda (λ) was set to 0.2108499 which minimized the mean cross validated error after testing λ over a range from -2 to 10. The resultant model fit in the October 2014 dataset included n=10 metabolites (model presented in Appendix C). This model was tested for its ability to classify exposure status in the January 2015 Puget Sound samples, the Wisconsin samples stratified by current exposure status, and in the Wisconsin samples stratified by cumulative exposure status (Table 16). In the January 2015 Puget Sound sample, 78% (25/32) of the samples were correctly classified using the October 2014 model with a model sensitivity and specificity of 76% and 80%, respectively. In the Wisconsin data, the accuracy in classifying current exposure status was 52% (28/54) and in classifying cumulative exposure status was 48% (26/54).

The AUC for ROC curves was 0.79 for the January 2015 data and 0.56 for both current and cumulative exposure in the Wisconsin data (Figure 11). The model developed using October 2014 data was able to classify exposure in the January 2015 Puget Sound samples, but was no better than chance in the Wisconsin samples, regardless of how exposure was considered.

Pathways Enrichment Analysis Results

Pathways enrichment analysis was undertaken in the October 2014 Puget Sound data and the Wisconsin data (stratified by current exposure status and cumulative exposure status). Forty-five pathways were identified that included at least one of the 59 metabolites. In the Puget Sound samples, the top four pathways that were most different between groups had a high FDR (between 0.38 and 0.44; Table 17). However, all four of these pathways contained at least 4 metabolites, which is considered the minimum number for meaningful pathway analysis (Navarro et al. 2016; Maudsley et al. 2011). The top four pathways identified were: tyrosine metabolism; valine, leucine, and isoleucine degradation; glyoxylate and dicarboxylate metabolism; and phenylalanine metabolism, though their impact scores suggest that they are not particularly meaningful (impact scores <0.16, when values closer to 1 are considered to be more meaningful). The pathway with the highest impact score in the Puget Sound data was synthesis and degradation of ketone bodies (impact score of 0.7; FDR = 0.51). Ketone bodies are produced during times of fasting, or physical activity. Given the differences in work demands between the exposed and unexposed persons (exposed persons perform much more physically intense work), it is likely that differences in this pathway between work sites is not due to Mn exposure. As a lack of compelling differences between exposed and unexposed persons and overall low impact scores for pathways were observed, no further pathway analysis was conducted for the Puget Sound samples.

In the Wisconsin data for current or cumulative exposure, no pathways containing at least 4 metabolites had an FDR < 0.5. As in the Puget Sound data, the only pathway with an impact

score >0.5 was the synthesis and degradation of ketone bodies. Therefore, no further pathway enrichment analysis was conducted on the Wisconsin data.

6.4 DISCUSSION

In this chapter, a variety of analyses for global and targeted NMR data are presented for three sets of data. Comparing the findings from global NMR to global MS, no NMR spectral bins differed between exposed and unexposed cohort in the October 2014 training or validation set, while nine ions met these criteria when using global MS methods. However, without knowing the identities of the ions it is unknown if any of the peaks found by MS were present in the NMR data as global MS and NMR data are not directly comparable. In the MS data, many more comparisons were being made; 1736 and 3380 ions were compared when applying 50% and 75% filtering, respectively (allowing for up to 50% or 75% of the samples to have missing values for each ion), whereas 250 bins of 0.04 ppm were used for the NMR data. The selected number of bins may have reduced the resolution in the data such that any true differences could not be detected with the small sample size.

NMR data for each sample received from the Northwest Metabolomics Research Center were normalized by sum. While typically this is performed to account for urinary concentration differences due to hydration or other situations (e.g., drugs, treatments, food depravation) that can cause increases or decreases in urinary volume or osmolality, normalization by total sum can also introduce bias into the underlying data (Craig et al. 2006; Dieterle et al. 2006). As Dieterle et al. (2006) explains, dilution is assumed to change the concentration of all metabolites found in the urine by the same factor, whereas enzymatic activity and fluxes (or perhaps, exposure) would only change some metabolites, and change them by different factors. Therefore, normalizing all

signals by a single value (the total sum) is not universally valid, as differences in metabolite levels is not only due to dilution. Moreover, a high concentration of a particular metabolite, such as glucose, would influence the total sum. This limitation affects the accuracy and robustness of the resultant data, and can influence multivariate and correlation analyses (Dieterle et al. 2006). Dieterle et al. found that probabilistic quotient normalization, which uses a reference spectrum to scale samples based on the most probable dilutions, perform the best to account for different dilutions of urine. As no reference samples were run with these NMR samples (interestingly, this is typical of NMR data analysis) these data were not able to be normalized using probabilistic quotient normalization. For future studies, it is recommended that NMR data, particularly for urine, be normalized using probabilistic quotient normalization with reference spectra run with the samples to inform the normalization process.

The metabolomics portion of this dissertation (Chapters 4 through 6) analyzed samples from the Puget Sound workers and the Wisconsin shipyard workers. The sample size of the original Puget Sound cohort (which was conceived as a pilot study) likely was a major contributor to the lack of power, and this limitation must be acknowledged. MetaboAnalyst 3.0 includes a Power Analysis module which calculates the number of samples that would be needed in each group (exposed, unexposed) to detect differences at a user-specified FDR and power using techniques originally developed for power calculations of gene expression data (Van Iterson et al. 2009). Based on the binned NMR data from exposed and unexposed individuals, over 1000 samples in each group would be needed to detect differences at a FDR < 0.25 and power of 0.8 when using univariate analysis techniques (such as a t-test or Wilconxon rank sum test). Based on the targeted NMR data, approximately 400 samples would be needed in each group to detect differences at a FDR <0.25 and power of 0.8 using univariate analysis

techniques. In the primary data set (October 2014 Puget Sound samples), the training group consisted of 12 exposed and 10 unexposed individuals, whereas the entire October 2014 data set, which was used for the targeted analysis, consisted of 20 exposed and 17 unexposed individuals. It must be noted, these power calculations were based on the global NMR binned data, where no convincing differences were found between groups defined by exposure. Thus, while ideally a larger cohort study is necessary to detect univariate differences in the urine metabolome related to Mn exposure with and FDR<0.25 and power of 0.8, large differences could be detected with smaller sample sizes, as was the case with the MS data presented in Chapter 4. Given the challenges of enrolling worksites and workers to participate in occupational health research, these numbers may not be realistic unless data can be combined or compared from multiple ongoing studies. However, even with smaller sample sizes than may be ideal based on the power calculations presented, metabolomics could still be used a means of hypothesis generation and for biomarker discovery though as yet its use will be limited to occupational health researchers, and not practitioners.

Even when the number of samples is much lower than the number of observations (e.g. bins or peaks), an elastic net model can still be informative (Zou and Hastie 2005). The elastic net model using the targeted NMR data was built in the October 2014 Puget Sound data, and tested in the January 2015 Puget Sound and Wisconsin data to assess how well Mn exposure was classified in the other data sets. Like with the MS data, the elastic net model performed fairly well, classifying persons as exposed or unexposed with 78% accuracy in the January 2015 data. Sensitivity and specificity were also moderately high, (76% and 80%, respectively). However, the elastic net model was unable to predict exposure in the Wisconsin data by current or cumulative exposure status. Similar to conclusions with the MS data, samples taken from the

same individuals (in October 2014 and January 2015) were more similar than samples taken from a different cohort. NMR as an analysis platform is more reproducible than MS, as MS instrument or environmental conditions can affect retention times, the precision of the *m/z* ratio measurement, and measured abundances while NMR is less sensitive to these changes (Gowda and Raftery 2015). Therefore, the differences observed between the cohorts when using NMR data are likely due to differences in environmental and dietary exposures, other unmeasured confounders, or underlying physiological changes or differences over time.

As previously described, Wang et al. (2012) investigated metabolomics profiles of workers exposed to welding fumes using global and targeted NMR methods. Wang et al. measured Mn in welding fume, with measured levels being comparable to those measured in the foundry, but much likely higher than Mn exposures in the Wisconsin shipyard (see 5.4 Discussion). Wang et al. found several spectral bins that differed significantly ($p<0.05$ and FDR<0.2) between non-smoking and non-alcohol drinking welders (n=10) and controls (n=6) at a Taiwanese shipyard, using an adjusted two-sided t-test. Identifying specific peaks from the bins, Wang et al. report acetone, creatinine, TMAO/betaine, taurine, glycine, gluconate, serine, hippurate, and s-sulfocysteine to all be significantly higher in welders than in controls, and creatine to be significantly lower in welders In the October Puget Sound samples, like in the Taiwanese shipyard, hippurate and acetone were higher in Mn exposed subjects than unexposed subjects. In the January 2015 samples, hippurate, but not acetone was also higher in exposed versus unexposed individuals. As yet, there does not seem to be an obvious biological reason for increased urine hippurate in relation to Mn or welding fumes exposure.

Notably, Wang et al. only included subjects who did not smoke cigarettes or drink alcohol to control for potential co-exposures, whereas this was not a consideration with the Puget

Sound and Wisconsin studies. Given the small sample size of the Wang study (10 welders and 6 controls), it is somewhat surprising that differences were identified based on exposure. Thus, it is possible that larger effect sizes were present in the Taiwan cohorts, compared to relatively minor differences in our data. Differences in normalization and scaling methods between the Wang et al. study and ours make direct comparisons challenging.

Examining the workflow of occupational metabolomics studies, many decisions are made that could affect findings from metabolomics studies (e.g., sample collection, data preprocessing and analysis). Therefore, conclusions drawn from studies with adequate sample sizes that can be reproduced both in the same subjects and in independent cohorts are ultimately most compelling. While global metabolomics allows many more potential metabolites to be found that differentiate between exposed and unexposed persons, identification of these metabolites and interpretation of their biological plausibility, remains a challenge in global metabolomics. With targeted NMR, absolute quantitation of the identified metabolite is possible, but fewer metabolites can be identified compared with MS. Our NMR study was able to identify 59 metabolites, whereas many MS libraries can identify 200-300 metabolites. Therefore, it is a combination of MS and NMR techniques that may be the most valuable for metablomics studies, using the strengths of each approach to discover potential biomarkers of Mn exposure.

6.5 CONCLUSIONS

This chapter used global and targeted NMR metabolomics to investigate urinary biomarkers of Mn exposures. For binned global NMR data, no bins were found to be differentiate between exposed and unexposed individuals. This could be due to small sample sizes (in the training and validation sets) and limited power, lack of resolution in NMR data due

to binning, or a true lack of differences between the exposed and unexposed persons in the Puget Sound cohort.

For the 59 metabolites identified by NMR, several metabolites were weakly suggestive of a difference between exposed and unexposed persons. Following correction for multiple comparisons, glycolic acid and hippurate differed by exposure in the October 2014 (FDR = 0.28) and January 2015 ($p < 0.05$) Puget Sound data.

An elastic net model was fit to the 59 identified metabolites in the October 2014 Puget Sound data, and tested on the other samples. The elastic net model had fairly good predictive power in the repeat (January) samples, but no real utility in the Wisconsin samples. A convincing biomarker of Mn exposure would be sensitive to varying levels of Mn exposure, over different exposed cohorts, which was not found to be the case for the targeted NMR metabolites explored in this chapter. In conclusion, to continue to investigate metabolites related to Mn exposure, a larger cohort study would be warranted, with care given to sample collection and preprocessing, and where MS and NMR are used in tandem to investigate and quantitate metabolites that may distinguish between groups defined by exposure.

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CHAPTER 6: FIGURES AND TABLES

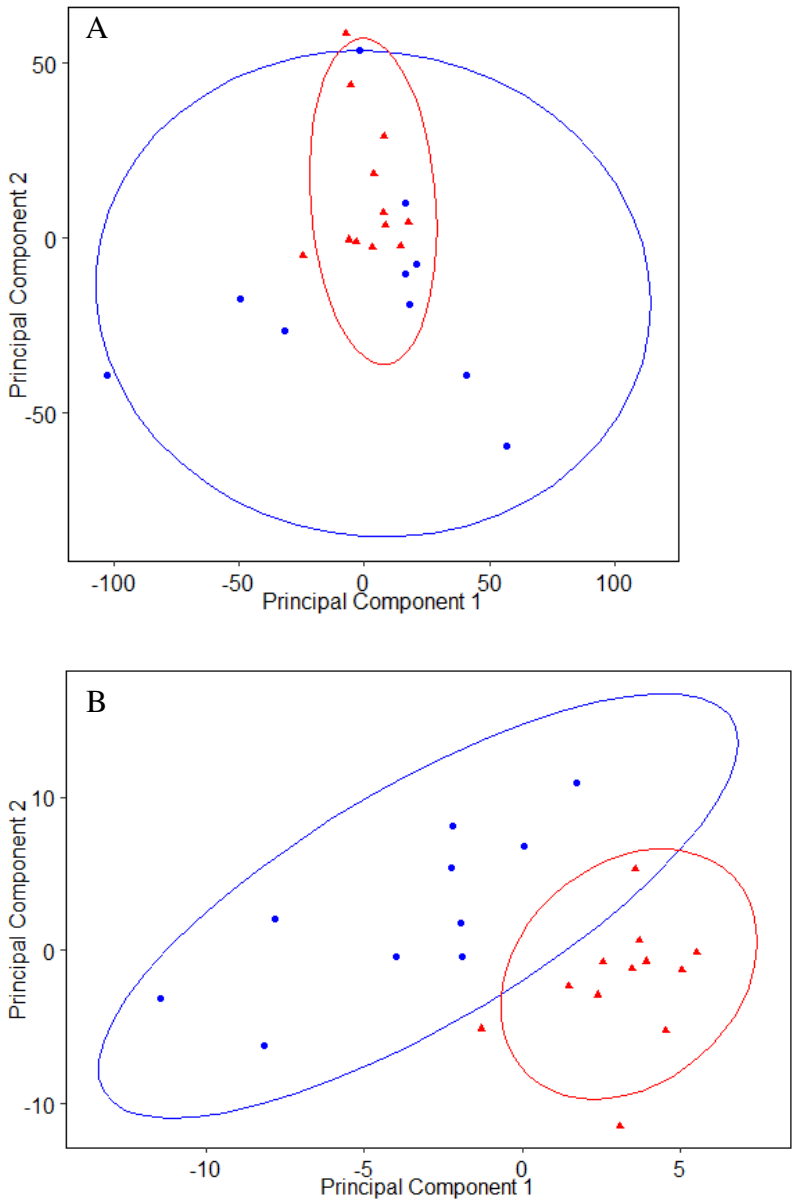


Figure 7 A&B. PCA and PLS-DA plots for exposed (red) and unexposed (blue) samples from the October 2014 Puget Sound training set samples, considering 250 global NMR bins.

A: PCA
B: PLS-DA

Table 14. Relative bin intensities for the top five bins differentiating between Mn exposed and unexposed subjects in the October 2014 Puget Sound training and validation set

	October PS training samples				
	Mn exposed (n=12)	Mn unexposed (n=10)			
Bin (ppm)	**mean ± SD†**	**mean ± SD†**	***p training***	***FDR training****	***p validation*****
2.2665006	0.47 ± 1.64	-2.83 ± 2.23	0.002	0.33	0.54
7.4515005	1.35 ± 2.16	-2.43 ± 2.58	0.003	0.33	0.40
2.3065006	4.59 ± 6.22	-2.26 ± 2.84	0.005	0.45	0.78
7.3715005	2.84 ± 5.99	-2.28 ± 2.97	0.007	0.45	0.96
2.9865006	2.37 ± 3.02	-0.97 ± 1.91	0.009	0.45	0.34

*****Benjamini-Hochberg corrected p-value between Mn unexposed (n=10) and Mn exposed (n=12) subjects in training group, from Wilcoxon rank-sum test
**Wilcoxon rank-sum test between exposed (n=8) and unexposed (n=7) in validation groups
†Total relative intensities for the 0.04 ppm bin, which were normalized by sum and Pareto scaled prior to analyses

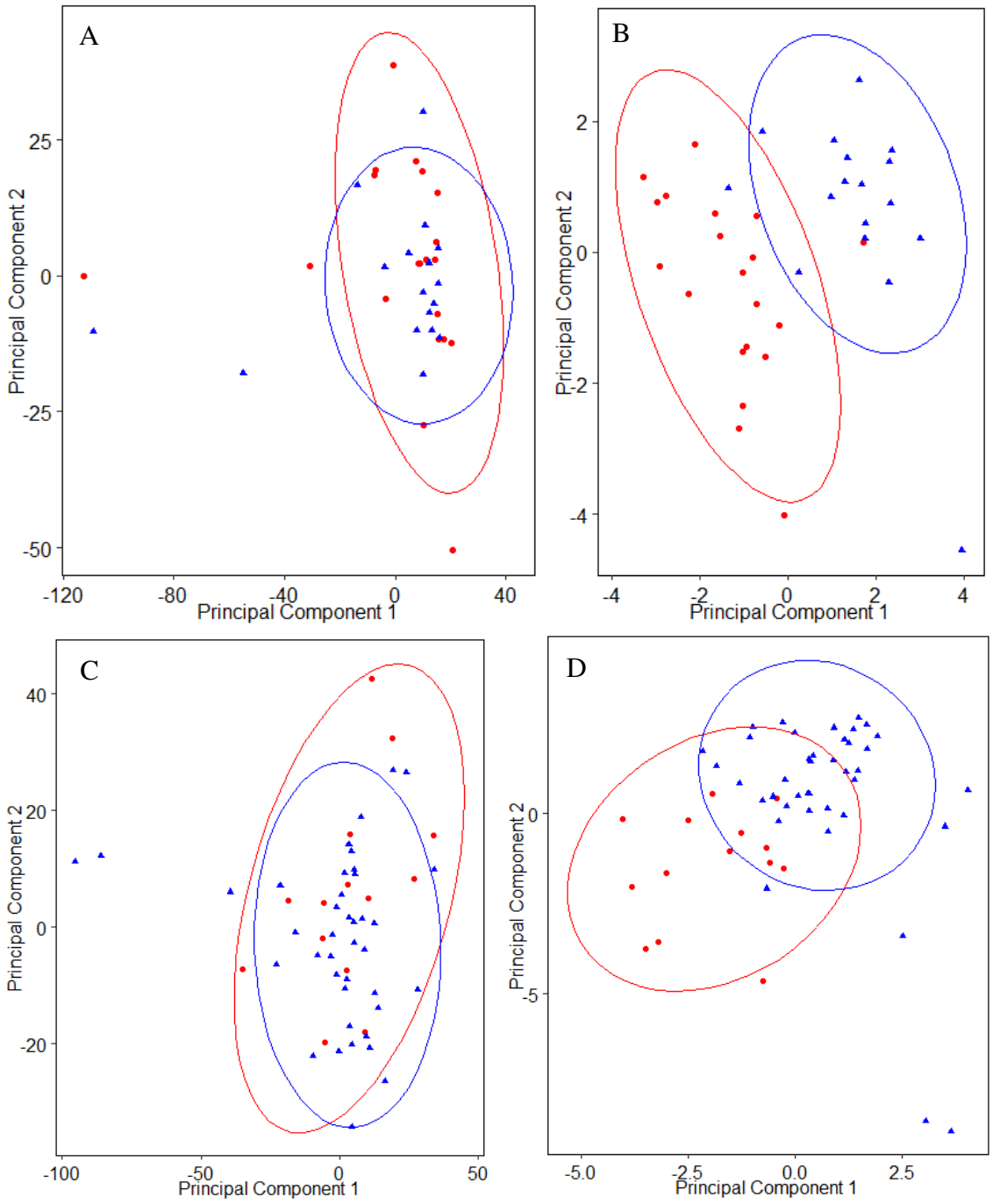


Figure 8 A, B, C, D. PCA and PLS-DA plots for exposed (red) and unexposed (blue) samples from October 2014 Puget Sound and Wisconsin data sets, considering the 59 metabolites identified from NMR.

A: PCA for Puget Sound 2014 Samples
B: PLS-DA for Puget Sound 2014 Samples
C: PCA for Wisconsin samples (stratified by current exposure)
D: PLS-DA for Wisconsin samples (stratified by current exposure)

Table 15. Relative metabolite intensities for the top five metabolites differentiating between currently Mn exposed and unexposed subjects in the Puget Sound samples and the Wisconsin samples.

	October PS samples				
	Mn exposed (n=20)	Mn unexposed (n=17)			
Metabolite	**mean ± SD**	**mean ± SD**	***p***	***FDR****	***p* January****
Glycolic acid	2.04 ± 5.51	-1.60 ± 5.63	0.02	0.28	0.02
Hippurate	4.68 ± 11.3	-2.65 ± 9.58	0.02	0.28	0.04
Prolinebetaine	5.63 ± 12.9	-1.55 ± 1.90	0.02	0.28	0.63
3-hydroxybutyrate	1.46 ± 4.84	-0.09 ± 3.68	0.02	0.28	0.37
Acetone	-0.98 ± 0.94	-1.30 ± 1.25	0.02	0.28	0.38

	Wisconsin samples			
	Mn exposed (n=14)	Mn unexposed (n=40)		
Metabolite	**mean ± SD**	**mean ± SD**	**p**	**FDR***
Isobutyrate	-1.36 ± 0.98	1.20 ± 5.48	<0.0001	0.06
N-methylnicotinamine	0.67 ± 1.12	-0.05 ± 0.86	0.02	0.49
Betaine	3.39 ± 11.2	-1.15 ± 4.39	0.03	0.49
Histidine	0.63 ± 2.09	-0.50 ± 1.86	0.05	0.69
Isoleucine	-0.90 ± 0.26	-0.71 ± 0.42	0.06	0.69

*****Benjamini-Hochberg corrected p-value between Mn unexposed and Mn exposed subjects, from Wilcoxon rank-sum test

**Unadjusted p-value between Mn unexposed and Mn exposed subjects from Wilcoxon rank-sum test, for the January 2015 Puget Sound samples

Units are relative intensities for the identified metabolite; values have been normalized by sum and Pareto scaled prior to analyses

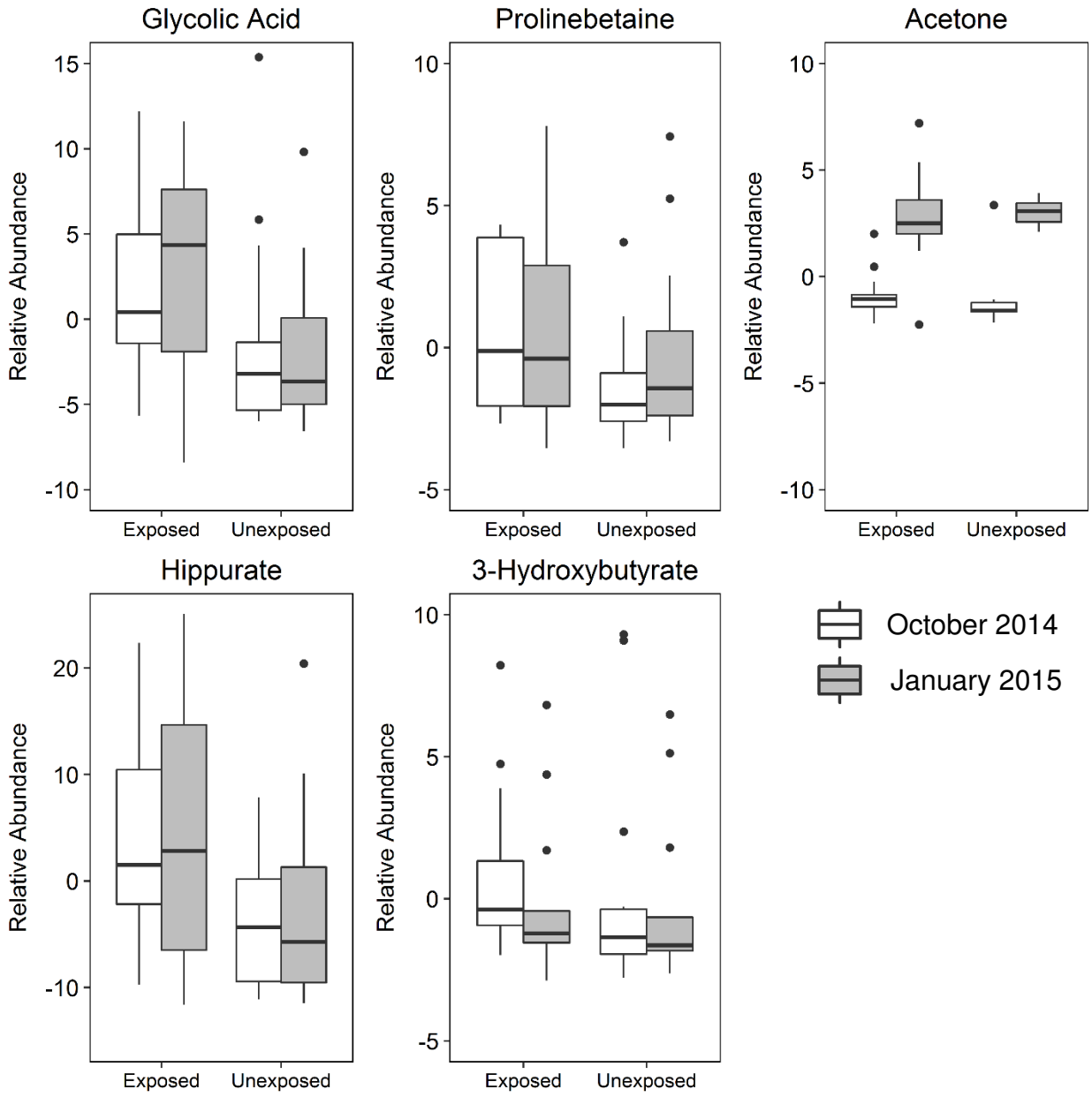


Figure 9. Box plots of the relative abundances of the top five metabolites differentiating between exposed and unexposed samples collected from Puget Sound workers in October 2014 (white) based on Wilcoxon rank-sum test. January 2015 samples (grey) are included to visualize the distribution of these five metabolites over time.

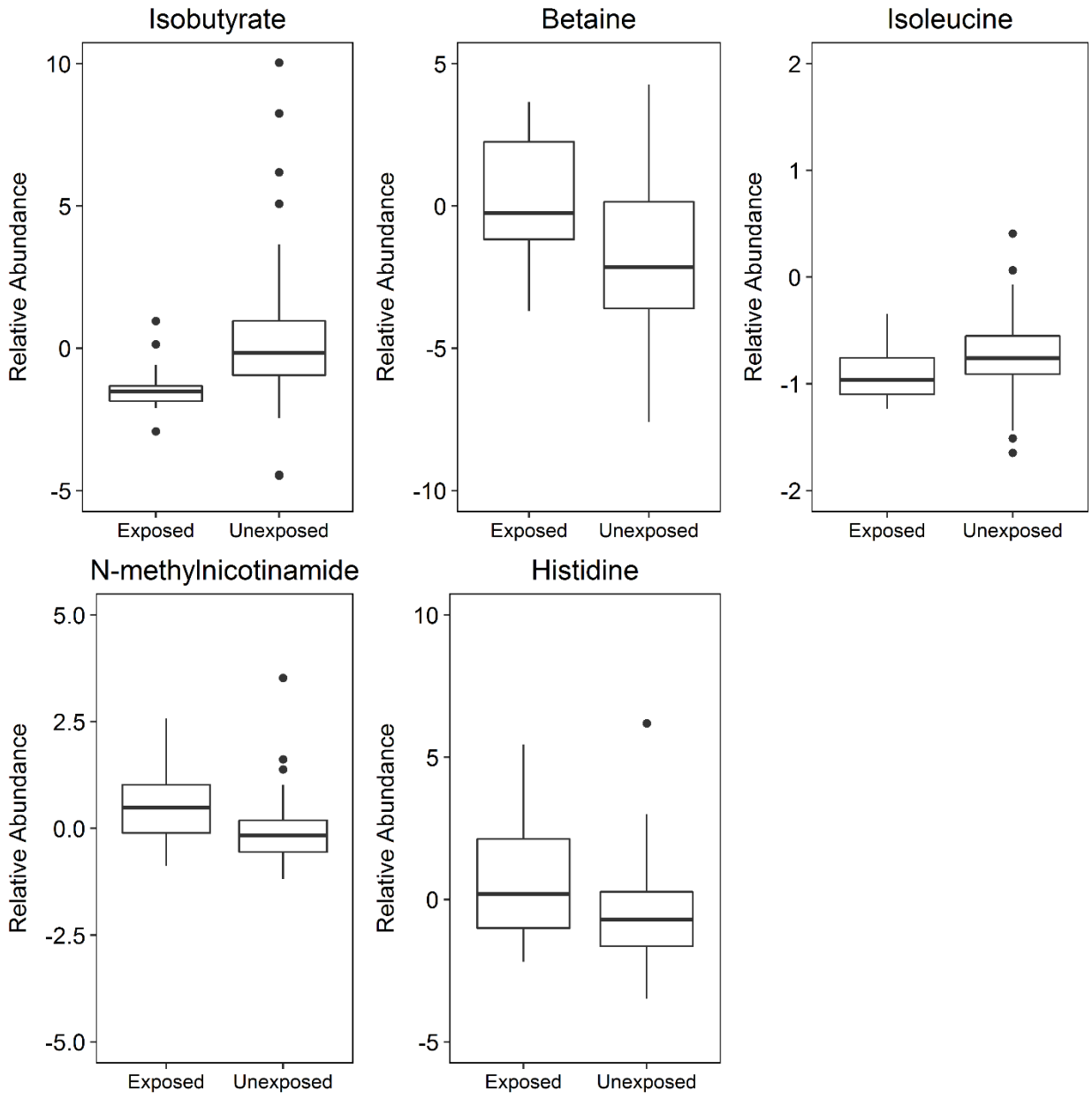


Figure 10. Box plots of the relative abundances of the top five metabolites differentiating between currently exposed and unexposed samples collected from Wisconsin shipyard workers based on Wilcoxon rank-sum test.

Table 16. Diagnostics for elastic net model tested in the January 2015 Puget Sound and Wisconsin samples

			Known						
			Exposed	**Unexposed**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**accuracy**
January 2015 Puget Sound	*Predicted*	**Exposed**	13	3	0.76	0.80	0.81	0.75	0.78
		Unexposed	4	12					
Wisconsin Current Exposure	*Predicted*	**Exposed**	4	16	0.29	0.60	0.20	0.71	0.52
		Unexposed	10	24					
Wisconsin Cumulative Exposure	*Predicted*	**Exposed**	9	11	0.35	0.61	0.45	0.50	0.48
		Unexposed	17	17					

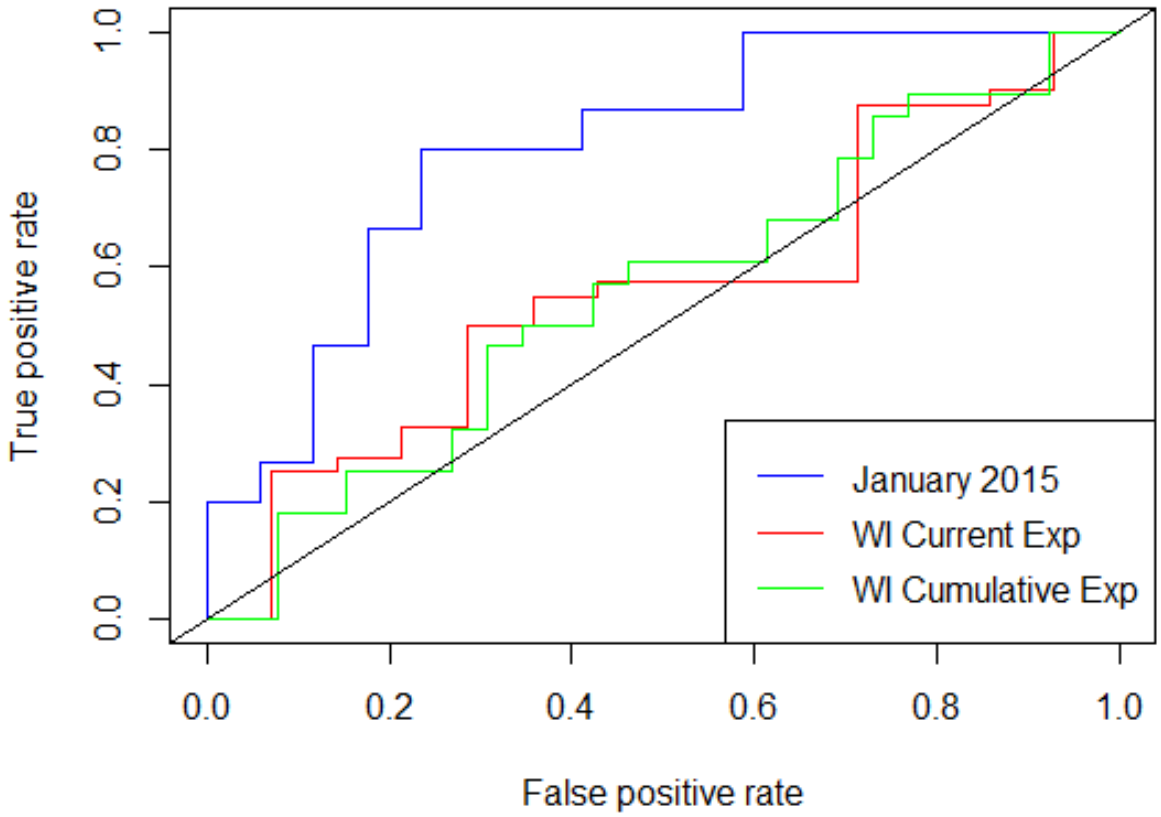


Figure 11. ROC curves for elastic net model as tested in January 2015 Puget Sound samples and Wisconsin samples.

True positive rate: sensitivity

False positive rate: 1-specificity.

Area under the curve (AUC): 0.79 for the January 2015 samples and 0.56 for current and cumulative exposure for the Wisconsin samples.

Table 17. Top pathways, their significance, and impact from pathway enrichment analyses performed in the October 2014 Puget Sound samples

Pathway Name	**Total Metabolites**	**Matched Metabolites**	***p-value***	**FDR**	**Impact†**
Tyrosine metabolism	76	5	0.02	0.38	0.07
Valine, leucine and isoleucine degradation	40	4	0.02	0.38	0.02
Glyoxylate and dicarboxylate metabolism	50	6	0.03	0.38	0.16
Phenylalanine metabolism	45	5	0.04	0.44	0.03
Synthesis and degradation of ketone bodies	6	3	0.10	0.51	0.70

*Total number of metabolites in the pathway
**Number of metabolites in the pathway which were found in the October 2014 Puget Sound samples
†Pathway impact value on Mn exposure calculated from pathway topology analysis

APPENDIX A

Relative abundance of ions found to be significantly different (FDR<0.1) between Mn exposed and unexposed subjects in the training group, stratified by three Mn exposure levels

			No Exposure (n=10)			Lower Exposure (n=7)			Higher Exposure (n=5)		
m/z	retention time (mins)	mode	mean ± SD	cv	% detected	mean ± SD	cv	% detected	mean ± SD	cv	% detected
201.02†	3.56	ESI-	2.05 ± 0.51	24.9%	70%	3.42 ± 0.35	10.2%	100%	3.76 ± 0.35	9.3%	100%
297.10†	3.98	ESI-	1.78 ± 0.29	16.3%	10%	3.04 ± 0.35	11.5%	86%	3.51 ± 0.29	8.3%	100%
553.24	4.03	ESI-	0.76 ± 0.27	35.5%	0%	1.66 ± 0.41	24.7%	71%	2.34 ± 0.59	25.2%	80%
160.08	4.21	ESI-	1.49 ± 0.36	24.2%	40%	2.03 ± 0.40	19.7%	86%	2.30 ± 0.18	7.8%	100%
205.07	4.21	ESI-	1.78 ± 0.42	23.6%	80%	2.34 ± 0.41	17.5%	86%	2.63 ± 0.19	7.2%	100%
246.01	4.68	ESI-	0.41 ± 0.76	185.4%	0%	1.89 ± 0.33	17.5%	71%	2.13 ± 0.25	11.7%	100%
311.12	5.18	ESI-	1.45 ± 0.30	20.7%	10%	2.21 ± 0.23	10.4%	86%	2.52 ± 0.41	16.3%	100%
415.22	8.95	ESI-	1.58 ± 0.16	10.1%	20%	2.04 ± 0.37	18.1%	71%	2.03 ± 0.20	9.9%	80%
321.10	4.01	ESI+	1.72 ± 0.26	15.1%	0%	2.31 ± 0.33	14.3%	71%	2.70 ± 0.30	11.1%	100%
229.07	4.20	ESI+	1.59 ± 0.27	17.0%	10%	1.99 ± 0.31	15.6%	57%	2.19 ± 0.14	6.4%	80%
177.11†	4.71	ESI+	1.84 ± 0.37	20.1%	30%	2.49 ± 0.28	11.2%	100%	2.42 ± 0.24	9.9%	80%
354.23	4.95	ESI+	2.17 ± 0.27	12.4%	50%	2.65 ± 0.16	6.0%	86%	2.58 ± 0.28	10.9%	80%
311.15	6.83	ESI+	2.42 ± 0.22	9.1%	80%	1.85 ± 0.28	15.1%	14%	2.12 ± 0.27	12.7%	20%
403.23	7.72	ESI+	2.04 ± 0.18	8.8%	60%	1.64 ± 0.20	12.2%	0%	1.76 ± 0.16	9.1%	40%
459.22	7.78	ESI+	2.84 ± 0.21	7.4%	100%	2.35 ± 0.22	9.4%	43%	2.49 ± 0.28	11.2%	80%

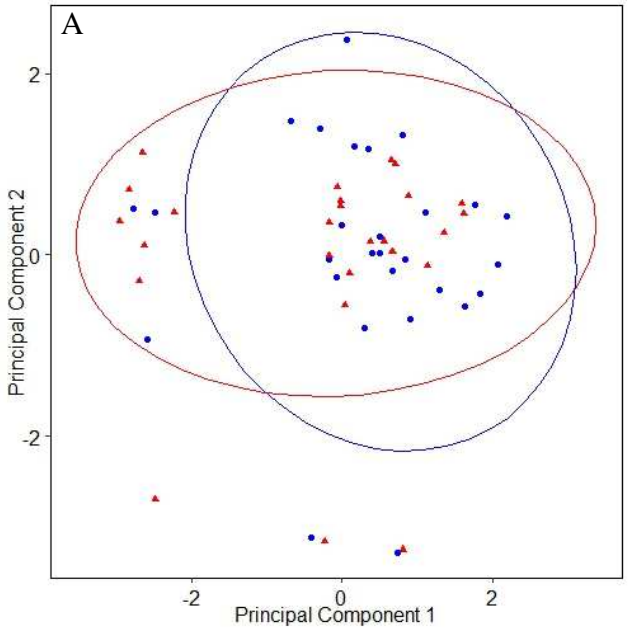
m/z: Mass to charge ratio

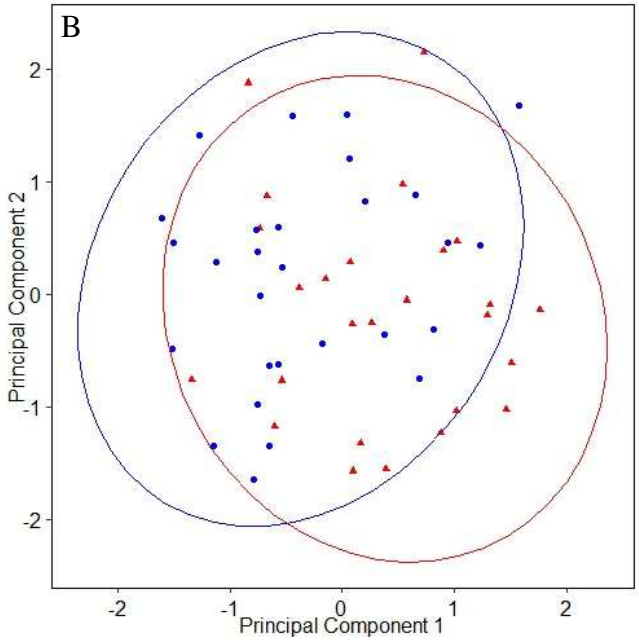
†Had known isotopologues or fragments that were also found in the training group with FDR < 0.1

APPENDIX B

PCA and PLS-DA plots for cumulatively exposed (blue) and unexposed (red) samples from the Wisconsin shipyard workers, considering the 9 ions of interest.

A: PCA for Wisconsin samples (stratified by cumulative exposure)
B: PLS-DA for Wisconsin samples (stratified by cumulative exposure)





APPENDIX C

Elastic net model fit in the October 2014 Puget Sound samples, using the n=59 identified metabolites from NMR.

Metabolite	coefficient
(Intercept)	-0.024
Hippurate	-0.014
Citrate	0.013
Glycolic Acid	-0.008
Prolinebetaine	-0.009
Acetoacetate	-0.084
Tyrosine	0.050
Choline	-0.0007
Fumarate	0.124
Isobutyrate	0.007
Creatine	-0.013

Note: Standard errors and confidence intervals are not considered to be meaningful for strongly biased estimates such as those arising from an elastic net or other penalized regression, and are therefore not reported (See: Goeman, Jelle J. "L1 penalized estimation in the Cox proportional hazards model." Biometrical journal 52.1 (2010): 70-84)

VITA

Marissa Baker is a Washington state native. She obtained her BA in Biological Sciences from Northwestern University in Evanston, IL in 2007, and her MS in Environmental and Occupational Exposure Sciences from University of Washington in 2011. Prior to starting her PhD, Marissa worked as both a Research Coordinator and Research Scientist in the Department of Environmental and Occupational Health Sciences at University of Washington. In 2017, Marissa served on the International Agency for Research on Cancer (IARC) monograph assessing the carcinogenicity of welding fumes and other related chemicals. She was proud to be named the 2017 DEOHS Outstanding PhD Student, and be awarded the 2016 IPA/DGUV Award for Young Exposure Scientists from the International Society of Exposure Science. Marissa lives in West Seattle with her husband, Elliot, and her son, Stellan.