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The reproducibility of urinary ions in manganese exposed workers

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ABSTRACT

Purpose: Manganese (Mn) is found in environmental and occupational settings, and can cause cognitive and motor impairment. Existing Mn exposure studies have not reached consensus on a valid and reproducible biomarker for Mn exposure.

Methods: Previously, global metabolomics data was generated from urine collected in October 2014 using mass spectrometry (MS). Nine ions were found to be different between persons exposed and unexposed to Mn occupationally, though their identity was not able to be determined. Here, we investigated these nine ions in a follow-up set of urine samples taken from the same cohort in January 2015, and in urine samples from a separate Mn-exposed cohort from Wisconsin. We fit an elastic net model fit using the nine ions found in the October 2014 data.

Results: The elastic net correctly predicted exposure status in 72% of the follow-up samples collected in January 2015, and the area under the curve of the receiver operating characteristic (ROC) curve was 0.8. In the Wisconsin samples, the elastic net performed no better than chance in predicting exposure, possibly due to differences in Mn exposure levels, or unmeasured occupational or environmental co-exposures.

Conclusions: This work underscores the importance of taking repeat samples for replication studies when investigating the human urine metabolome, as both within- and between-person variances were observed. Validating and identifying promising results remains a challenge in harnessing global metabolomics for biomarker discovery in occupational cohorts.

1. Introduction

Manganese (Mn) is an established neurotoxicant that is associated with cognitive [1–4] and motor [5–8] health outcomes. Chronic Mn exposures are most frequently found in metal-working occupational settings (e.g. welders, solderers, brazers, and foundry workers), though workers in other manufacturing environments may also be indirectly or directly exposed to Mn. People living in close proximity to Mn-emitting industries or urban highways with excessive automobile exhaust may also be exposed to elevated airborne Mn [9,10]. Both environmental and occupational Mn exposures have been shown to increase the prevalence of Parkinsonian disorders [11–13]. As yet, scientific consensus has not been reached regarding a reliable, reproducible, and easily accessible biomarker of Mn exposure, though biomarkers have been investigated in matrices such as blood, hair, plasma, and urine, and imaging techniques such as magnetic resonance imaging (MRI) or positron emission tomography (PET) have been explored in the literature to uncover possible biomarkers of Mn exposure [14–21].

Previously, we utilized a global metabolomics approach to explore the relationship between urinary ions and Mn exposure in Puget Sound metalworkers [22]. Mn exposed workers were from a Mn-steel foundry, while Mn unexposed workers were crane or vehicle operators at a scrap metal recycling facility. In this study we divided both the exposed and unexposed samples into a training set and validation set. In machine learning, the training set is the group of samples that is used for setting the parameters of the model and the validation set is the group of samples where the model is tested to determine how well it performs in independent data. In our data, we first made statistical comparisons in the training set, and then tested these findings in the validation set. Utilizing a training and validation set helps increase the validity of findings, and reduces spurious findings, especially when multiple comparisons are being made. In urine samples collected in October 2014, we found nine ions that were significantly different by Mn exposure status in the training set and validation sets of Puget Sound

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Table 1
Subject Demographics by geographic location and Mn exposure status.

	Puget Sound Workers			*Wisconsin Workers*				
		Current Exposure			*Current Exposure*		*Cumulative Exposure*	
	All (n = 37)	Yes (n = 20)	No (n = 17)	All (n = 55)	Yes (n = 14)	No (n = 41)	WWY* > 2.5 (n = 27)	WWY* ≤ 2.5 (n = 28)
Age, mean ± SD	44.0 ± 11.8	41.9 ± 12.8	48.1 ± 11.3	51.0 ± 11.3	51.0 ± 8.8	51.0 ± 12.2	54.0 ± 8.8	48.1 ± 13.0
White **Race,** n (%)	30 (81.0)	15 (75.0)	15 (88.2)	54 (98.2)	14 (100)	40 (97.6)	27 (100)	27 (96.4)
Respirator User, n (%)	12 (32.4)	12 (60.0)	0 (0)	17 (30.9)	10 (71.4)	7 (17.1)	12 (44.4)	5 (17.9)
Male, n (%)	37 (100)	20 (100)	17 (100)	52 (94.5)	14 (100)	38 (92.7)	26 (96.3)	26 (92.9)
Current Smoker, n (%)	7 (18.9)	3 (15.0)	4 (23.5)	14 (25.5)	3 (21.4)	11 (26.8)	7 (25.9)	7 (25.0)

* Weighted Welding Years, calculated as described in Racette et al. [8].

workers. When further stratifying exposure into three exposure groups based on job tasks and informed by personal sampling, an exposure-response relationship was apparent for several of these nine ions; however, despite undertaking fragmentation, we were not able to definitively identify any of these nine ions by empirical formula or name. Findings from a global metabolomics analysis have greater utility if they can be replicated in independent samples. In our previous work, the results from the training group of samples were replicated in a validation group, which consisted of workers from the same Puget Sound worksites who provided a urine sample on the same day as the training group in October 2014. In the present work, we sought to further validate the nine ions of interest by investigating if these ions still predict Mn exposure in urine samples taken from the same Puget Sound workers three months later (January 2015), and from Mn-exposed workers in a different Mn industry (Wisconsin shipyard workers). A model was built using the nine ions of interest from the October data that was subsequently tested in both the January data and Wisconsin welders' data in order to investigate the predictive power of these ions for determining Mn exposure.

2. Material and methods

2.1. Study population and samples collected: Puget Sound cohort

All participants in this study gave written, informed consent, and all study protocols were approved by the University of Washington and Washington University IRB, as applicable. We enrolled twenty Mn-exposed workers from a Mn-steel foundry and 17 crane operators with low exposure to Mn from a metal recycling facility, both from workplaces in the Puget Sound region of western Washington State, as previously detailed in Baker et al. [22]. We took a single full-shift air sample for all workers in October 2014, using Institute of Medicine (IOM) Inhalable dust samplers. 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 μg/m^3 (SD: 300, range: 98.5, 1243) and the mean Mn exposure for the crane operators was 9.2 μg/m^3 (SD: 36.5, range: 0.02, 150.8).

A spot urine sample was also collected from each worker on site, post-shift. Subjects were asked to provide a clean catch sample into a 125 mL wide mouth low-density polyethylene bottle (Nalgene), samples were aliquoted on site into 2 mL Safe-Lock Eppendorf tubes. After collection of the urine sample, a brief 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 (UW) in Seattle on dry ice.

Workers at both the foundry and scrap metal recycling center who were still employed in the same role in January 2015 provided a second spot urine sample, post shift, which was collected, transported, and stored the same as the October 2014 urine samples. We did not perform personal inhalable air sampling at this time, but did administer a brief questionnaire to confirm that job duties remained similar between October 2014 and January 2015. In January 2015, 17 (85%) Mn exposed workers were still employed at the foundry, and 15 (88%) Mn unexposed workers were still employed at the scrap metal recycling

2.2. Study population and samples collected: Wisconsin cohort

We also included workers from a larger, ongoing welding cohort study conceived of and maintained by Washington University in St. Louis (WU) investigators [23]. The larger study includes two shipyards and one fabrication shop located in Wisconsin. Both welders and non-welders were recruited from the three worksites for inclusion in this metabolomics study. In total, 55 workers from the existing cohort provided a urine sample for metabolomics analysis. There was no difference in age, sex, race, or tobacco use between the overall cohort and those recruited to participate in the metabolomics analysis. Subject demographics for the Puget Sound and Wisconsin workers, stratified by exposure status, are presented in Table 1.

Urine samples were collected at the IBB union hall. Some samples were collected on a weekend, so workers had not necessarily been working on the day that they provided a urine sample. Like the Puget Sound samples, subjects provided a clean catch sample into a 125 mL wide mouth low-density polyethylene bottle (Nalgene) which were aliquoted into 2 mL Safe-Lock Eppendorf tubes on site. 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.

As we were unable to conduct air sampling, each worker completed a validated exposure questionnaire at the time of urine sampling in order to estimate cumulative and recent Mn exposure [24]. 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 exposure at the current job to obtain the most accurate information possible on the level and timing of Mn exposures that could affect biological responses. Based on the questionnaire we also estimated the metric "weighted welding exposure-years" (WWY), a quantitative, continuous measure of cumulative relative exposure to Mn previously described by Racette et al. [8]. This estimate includes number of years at all previous jobs with welding fume exposure and measures of intensity of welding fume exposure at each job. Using the questionnaire and calculated WWY, two binary classifications of Mn exposure status were considered. For the binary measure "Current Exposure", workers were coded as exposed if they reported currently being a welder or welders' helper at the time of their urine sample. "Current Exposure" was our primary metric for analysis as it is most comparable to the exposure measurements we made in the Puget Sound cohort. However, as a secondary analysis we also considered the binary measure "Cumulative Exposure". For "Cumulative Exposure", workers were coded as unexposed if they were below the median of the

Table 2
Normalized, log-transformed relative abundances of ions, stratified by Mn exposure status, in samples collected from Puget Sound workers in October 2014 and January 2015, and from Wisconsin workers.

			October 2014 PS workers			January 2015 PS workers			Wisconsin workers		
			Mn exposed (n = 20)	Mn unexposed (n = 19)		Mn exposed (n = 17)	Mn unexposed (n = 15)		Current Mn exposed (n = 15)	Current Mn unexposed (n = 39)	
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	7.80	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; Mn = manganese: RT = retention time (minutes); SD = standard deviation; ESI = electrospray ionization; *m/z* = mass to charge ratio.
* p-value calculated from two-sided *t*-test allowing for unequal variances.

distribution of WWY (i.e., 2.5 years) and exposed if they were above the median of the distribution of WWY.

2.3. Metabolomics data analysis

Global metabolomics profiling was performed on urine samples collected from Puget Sound and Wisconsin workers as detailed in Baker et al. [22] and Tay-Sontheimer et al. [25]. Briefly, 800 µl acetonitrile with deuterated internal standard were combined with 200 µL of urine for protein precipitation. Samples were centrifuged and the supernatants were evaporated under nitrogen gas. Samples were reconstituted in methanol:water containing 0.42% acetic acid, and transferred to glass autosampler vials after vortex and centrifugation. Samples were separated using a 1.8 µm 2.1 x 50 mm Agilent Zorbax SB-Aq analytical column heated to 60 °C on an Agilent (Santa Clara, CA, USA) 1200 HPLC coupled to Agilent 6520 Accurate Mass quadropole time-of-flight (Q-TOF) mass spectrometer. This system was calibrated for accurate masses between *m/z* 118 and 1700. Data were acquired over the 25 min run in electrospray ionization positive (ESI+) and electrospray ionization negative (ESI-) modes to detect both cations and anions, respectively.

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 metabolomics data were combined for data preprocessing, which ensures peak alignment across the sample preparation days. Preprocessing was done using *xcms,* an open-source package developed for R Studio [26,27]. 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, and were $\log_{10}$-transformed to approximate a normal distribution. Normalized, log-transformed relative abundances for the nine ions of interest were compared as described below.

2.4. Statistical analysis

The binary "current exposure" metric for the Wisconsin welders is most comparable to exposure as assessed in the Puget Sound data and is the primary exposure outcome, with the binary "cumulative exposure" metric considered secondarily. Normalized, log-transformed relative abundances of the 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.

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) [28]. 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. 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.

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 packages *ROCR* and *pROC* [29,30].

3. Results

Subject demographics for both the Puget Sound and Wisconsin cohorts are presented in Table 1, stratified by exposure status. Wisconsin workers tend to be slightly older than the Puget Sound workers, and had a higher percentage of workers who are white, and workers that smoke. Table 2 presents normalized, log-transformed relative abundances for the nine ions of interest for both sets of Puget Sound samples and the Wisconsin samples, stratified by Mn exposure status. Four ions were different between exposed and unexposed samples collected from Puget Sound workers in January 2015. None of the nine ions were different between currently exposed and unexposed workers in the Wisconsin dataset. One ion found in the October data set was nearly significantly different between currently exposed and unexposed Wisconsin workers, and was directionally similar to the October Puget Sound samples (*m/z* 459.2202, retention time 7.80, ESI+, $p = 0.08$). However, this ion was not significant in the January Puget Sound data set.

The October 2014 Puget Sound samples were stratified into three exposure groups, based on job title and informed by personal air measurements: No exposure (operators at the scrap metal recycling yard, mean measured Mn exposure: 0.01 mg/m^3 ± 0.37), Lower Exposure (forklift operators, foundry helpers, and molders at the foundry, mean measured Mn exposure: 0.19 mg/m^3 ± 0.10), and Higher Exposure (melters or pourers at the foundry, mean measured Mn

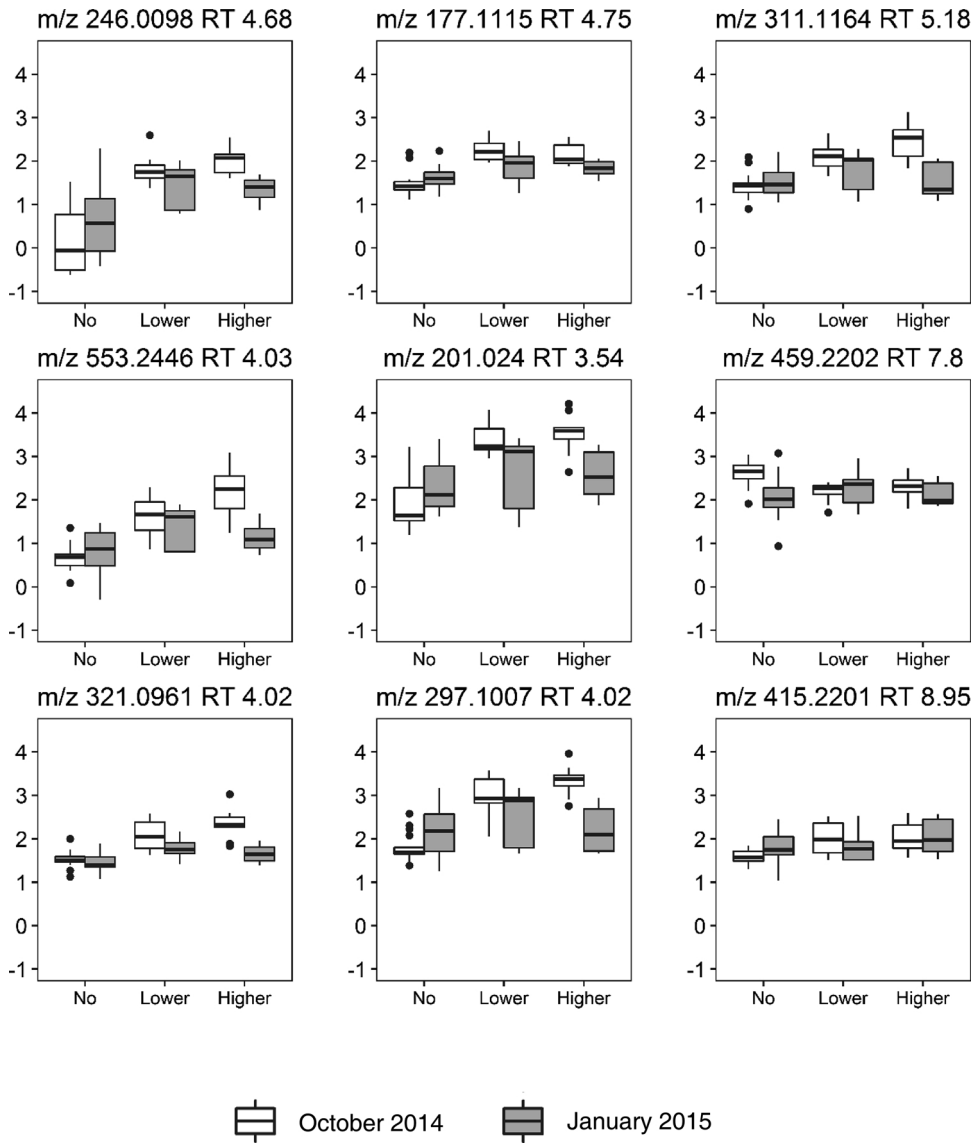

Fig. 1. Box plots of normalized, $\log_{10}$ relative abundances of the nine ions of interest, for both October 2014 Puget Sound and January 2015 Puget Sound samples, stratified by no, lower, and higher manganese exposure.

exposure: 0.52 mg/m^3 ± 0.34). These groupings were replicated in the January 2015 data and the nine ions were visualized with box plots to look for an exposure-response relationship (Fig. 1).The January 2015 data exhibited no discernable exposure-response relationships. Linear regression models were fit for each of the nine ions to assess the trend across the three exposure groups (no, lower, higher). Two ions were found to have a statistically significant trend across exposure groups, (*m/z* 246.0098 retention time 4.68, $p = 0.016$ and *m/z* 321.0961 retention time 4.02, $p = 0.041$) and the ion with *m/z* 553.2446, retention time 4.03 was nearing significance with $p = 0.058$. However, for all three of these ions, the significance was driven by a mean difference in relative ion abundance between the no exposure and lower exposure group, with no difference between the lower and higher exposure groups.

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 min 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 min to reduce multicollinearity and variance inflation.

An elastic net model developed using 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 3. 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% (27/54) 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).

Fig. 2 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 = 0.5 can be interpreted to mean the exposure status of a sample

Table 3
Ability of October 2014 Puget Sound worker elastic net model to predict manganese exposure in the January 2015 Puget Sound and Wisconsin workers.

			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					

PPV = positive predictive value.
NPV = negative predictive value.

was predicted no better than would be expected by random chance. The AUC for the January samples was significantly different than an AUC = 0.5 (p = 0.013), whereas the AUCs for the Wisconsin samples were not significantly different than an AUC = 0.5, meaning the elastic net model had limited predictive ability in the Wisconsin samples (Fig. 3).

4. Discussion and conclusion

Four of the nine ions that differentiated between Mn exposed and unexposed Puget Sound workers in October 2014 were higher in the Mn exposed and unexposed workers in the January 2015 Puget Sound cohort ($p < 0.05$), while none of the nine ions differed in the Wisconsin shipyard welders, regardless of whether samples were stratified by current exposure or cumulative exposure. An elastic net model was able to correctly classify exposure status in 72% of the January 2015 Puget

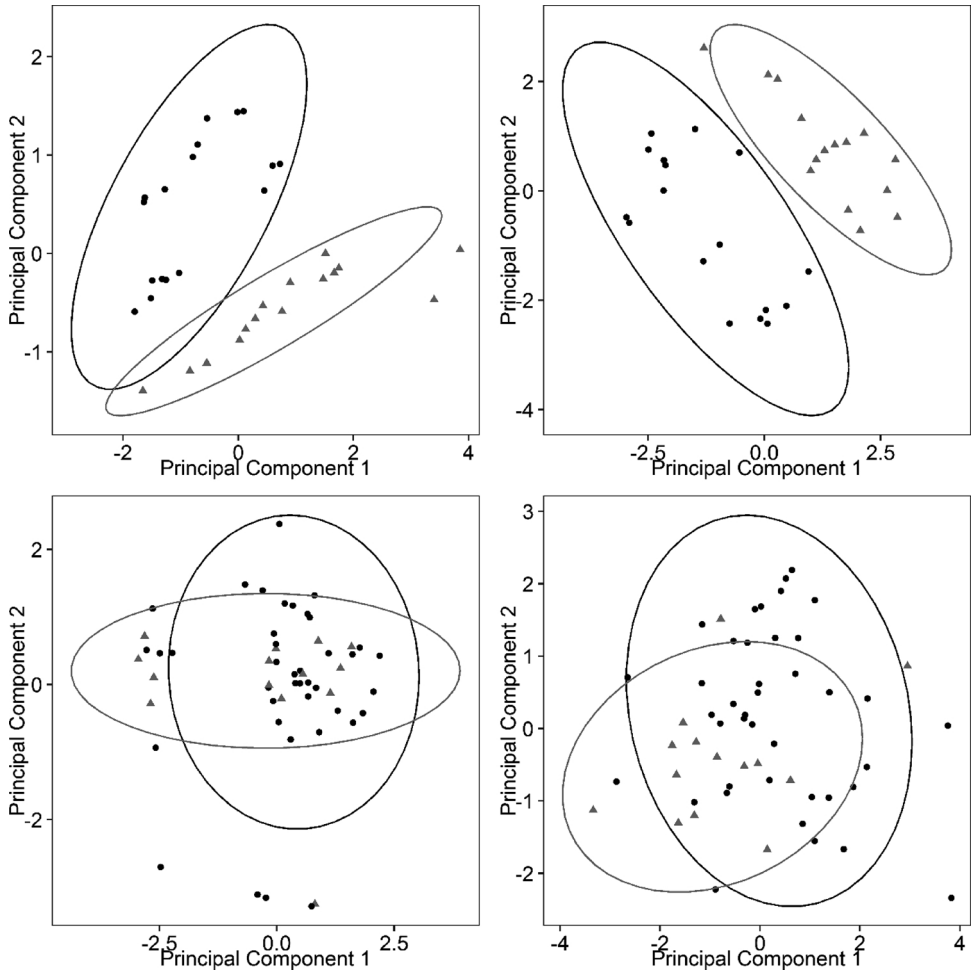

Fig. 2. PCA and PLS-DA plots for manganese exposed (lighter triangles) and unexposed (darker circles) January 2015 Puget Sound and Wisconsin samples, considering the nine ions of interest.
Top left: PCA for January 2015 Puget Sound Samples.
Top right: PLS-DA for January 2015 Puget Sound Samples.
Bottom left: PCA for Wisconsin samples (coded by current exposure status).
Bottom right: PLS-DA for Wisconsin samples (coded by current exposure).

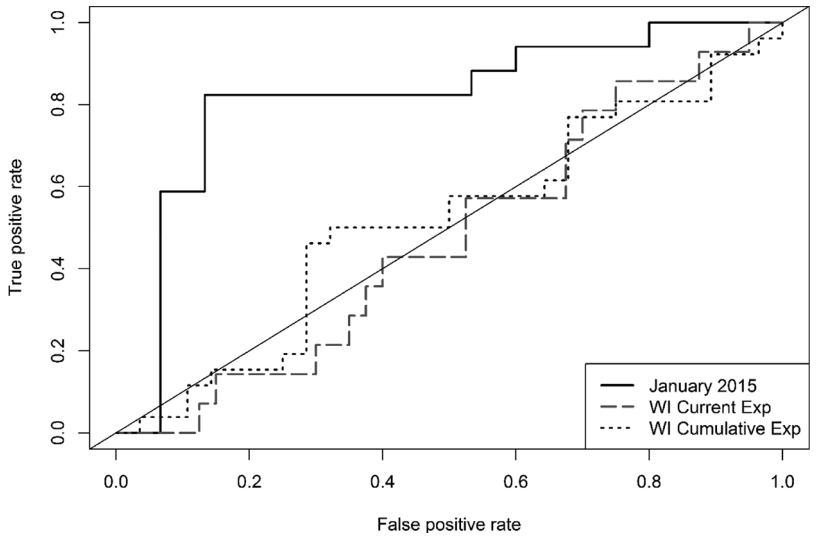

Fig. 3. ROC curves for elastic net model as tested in January 2015 Puget Sound samples and Wisconsin samples.
True positive rate: sensitivity.
False positive rate: 1-specificity.
Area under the curve (AUC) for January 2015 = 0.80.
AUC for Wisconsin current or cumulative exposure = 0.49.

Sound Samples with an AUC of the ROC being 0.80.

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 [31–34]. However, few studies have sought to replicate findings from a targeted or global human metabolomics study in other populations as we have done here.

It would be expected that the metabolomics profiles of repeat samples on the same workers would be more similar than the metabolomics profile of different workers. However, it is notable that even these nine ions showed differences between exposed and unexposed workers 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. For most biological compartments, Mn exhibits a long half-life [16]. Thus, it is reasonable to assume that the body burden of Mn in the Puget Sound workers wouldn't change appreciably between the two time points we studied, even if acute exposures did change. Therefore, the ions we investigate here are more likely related to acute Mn exposure, though we did not consider any metrics related to chronic exposure (e.g. job history) in the Puget Sound cohort.

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 subgroups 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 was taken, or simply batch effects due to the samples being analyzed at different times

While exposure was measured quantitatively for the October 2014 samples using IOM inhalable samplers, exposure was not measured again in January 2015 and instead 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 exposed workers' 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. That is, changes in enzymatic activity or other physiological factors could affect the metabolome between samples; if these are changes due to circadian variability in the metabolite levels, taking samples at the same time of day can reduce some of this variability.

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 workers. As no industrial hygiene measurements for airborne Mn were taken at the Wisconsin worksites, estimates of relative exposure were developed from questionnaire data. Racette et al. (2017) estimated that in the larger Wisconsin welder cohort, the mean time-weighted average Mn exposure for a welder was 0.14 mg/m^3, based on models developed and validated previously, and informed by specific information on welding type and location of welding [24,35]. Higher levels could be experienced by welders working in confined or enclosed spaces, and doses could be reduced with the use of respiratory protection. Respirator use was commonly reported in the currently exposed Wisconsin welders (n = 14), with all but four (n = 10) 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 internal dose of Mn received, though this would vary based on adherence to proper respirator hygiene.

In the Puget Sound foundry, measured Mn exposures ranged from 0.10 to 1.2 mg/m^3 (mean: 0.37 ± 0.30 mg/m^3), making exposures generally higher than the estimated exposures in the larger Wisconsin cohort. The ventilation in the foundry building where all enrolled individuals worked was limited to general ventilation and natural ventilation via open doors, with no local exhaust ventilation. The estimated exposure (mean: 0.14 mg/m^3) for the larger cohort of Wisconsin welders is on the low end of the measured Mn concentrations in the foundry. Moreover, few foundry workers reported wearing respiratory protection, and the only respiratory protection utilized was either N95 respirators or inadequately protective painter's masks, in contrast to the more effective protective equipment sometimes used in the Wisconsin worksites. Thus, it is likely that Mn exposures were lower in the Wisconsin welders relative to the Puget Sound foundry workers.

Given that the nine ions of interest are unidentified by name or empirical formula, it is impossible to know if the ions investigated here are related to the Mn exposure or other co-exposures or confounders, including those that might not have occurred at the Wisconsin worksites. 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 phenol, formaldehyde, isocyanates, and amines at levels higher than would be encountered with typical welding operations [36]. In these studies, co-exposures were not assessed using either quantitative or qualitative methods. Identification of each of the nine ions by name, and determination of a mechanism for altered urinary concentrations of the ions following Mn exposure, would be important steps necessary to further assess each ion's credibility as a biomarker 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 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 [37,38]. 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 workers 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 at higher Mn exposures and these results would be reproducible in other higher acute Mn occupationally-exposed cohorts than the Wisconsin shipyard workers. While we validated that four of the nine ions were different in January 2015 Puget Sound samples, further work will need to be done to identify the ions, to provide a deeper understanding of how these ions may be related to Mn or other exposures as their utility as biomarkers of Mn exposure is limited outside of the Puget Sound cohort.

This study provided a unique opportunity to explore the reproducibility of the human urine metabolome, and its relation to occupational Mn exposure. The work presented underscores the importance of taking repeat samples as replication studies when investigating the 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. Additionally, confounding sources of exposure should be assessed, and other external factors known to be sources of metabolome variability such as diet, and timing of sample should be controlled to the extent possible. While these particular confounders were not assessed in our study, controlling for known and assumed sources of metabolome variability increases the ability to find true differences related to exposure. Validating and replicating promising results, both in the same population and other populations, remains a challenge in harnessing global metabolomics methods for biomarker discovery in occupational cohorts.

Conflict of interest

The authors have no conflicts of interest to report.

Acknowledgments

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