UNIVERSITY OF CALIFORNIA

SANTA CRUZ

HAIR AS A BIOMARKER OF ENVIRONMENTAL MANGANESE EXPOSURE

A thesis submitted in partial satisfaction
of the requirements for the degree of

MASTER OF SCIENCE

in

MICROBIOLOGY & ENVIRONMENTAL TOXICOLOGY

by

Rachel Eastman

September 2012

The Thesis of Rachel Eastman
is approved:

_____
Professor Donald R. Smith, Chair

_____
Professor Roberto G. Lucchini

_____
Professor Karen M. Otteman

_____
Tyrus Miller
Vice Provost and Dean of Graduate Studies

Table of contents

List of Figures

Abstract

The absence of well-validated biomarkers of manganese (Mn) exposure in children remains a major obstacle for studies of Mn toxicity. We developed a hair cleaning methodology to improve the potential utility of hair as an exposure biomarker for Mn and other environmental metals. The cleaned hair was then subjected to analysis by ICP-MS, scanning electron microscopy, and laser ablation ICP-MS. Exogenous, but not endogenous, Mn contamination on hair that was uncontaminated or intentionally contaminated with dust or Mn-contaminated water was effectively removed with a cleaning method using 0.5%Triton X-100 sonication plus 1N nitric acid sonication. This optimal cleaning method was then used on hair samples from children (n=121) in an ongoing study of environmental Mn exposure and related health effects. Mean hair Mn levels were 0.121 μg/g (median = 0.073 μg/g, range = 0.011 – 0.736 μg/g), which are ~4 to 70-fold lower than levels reported in other pediatric Mn studies (*1,2,27,32*). Hair Mn levels were also significantly higher in children living in the vicinity of active, but not historic, ferroalloy plant emissions compared to controls ($P<0.001$). These data show that hair can be effectively cleaned of exogenous contamination, and they provide validation of hair Mn levels as a biomarker of environmental Mn exposure in children.

Dedication

I would like to dedicate this thesis to my parents for their unwavering support of me and my pursuits.

Acknowledgments

This study was supported by the National Institutes of Health (R01ES019222, R01 ES018990, and PO1 ES009605)), and the European Union through its Sixth Framework Programme for RTD (contract no FOOD-CT-2006-016253). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health; the European Commission is not liable for any use that may be made of the information contained therein. We thank Rob Franks, Andrew Dina, and Anastasia Michaels for their analytical expertise.

Introduction

Manganese (Mn) is an essential element required for amino acid synthesis, glycosylation, and the citric acid cycle among other things (*9*). However studies in occupationally exposed adults (*3,5,6,15,17,20,21,25*) and environmentally exposed children (*1,7,12,39,40*) have reported associations between elevated Mn exposure and neurological deficits, though details of the exposure-effect relationship are still being defined (*16,29,35*). In part, this may be due to the challenges in accurately characterizing exposure, and to the fact that there are no well-recognized and validated biological markers of Mn exposure like there are for other trace metals such as lead (Pb) (NRC, 1993). The identification and validation of exposure biomarkers is fundamental to human toxicology and risk assessment, and knowing the dose-response relationship is essential for the demonstration of cause and effect (*14*).

Biological markers of exposure should reflect an integration of the internalized dose over time. Studies in environmentally-exposed children have reported that hair Mn (*1,2,26-28,40*), Mn in the exposure medium (e.g., water) (*1,39*), but not blood Mn were predictors of exposure and/or neurotoxic outcomes. The toxicokinetics of Mn suggest that exposure biomarkers such as blood, saliva and urine may at best reflect recent exposure (i.e., days), while hair may integrate or reflect longer-term exposure (e.g., weeks or months) (22,*33,35,38*). This suggests hair may integrate internal exposure and changes in circulating Mn levels over time better than blood, saliva, or urine (*1,27,40*).

However, analytical challenges associated with the accurate and precise measurement of endogenous hair Mn may confound assessment of internal Mn exposure. The existing literature suggests substantial variability in hair Mn levels in environmentally exposed subjects, though interpreting these differences is difficult since studies have often used different methods for cleaning hair prior to analysis (*1,2,23,24,27,32,40*). For example, Sakai et al. (*32*) reported mean hair Mn levels of 2.3 μg/g (± 0.5 SD) in subjects aged 6 mo – 20 yrs (n=418), where hair was washed with distilled deionized water followed by repeated washing with an ethanol-acetone mixture. Wright et al. (*40)* cleaned hair by sonicating in 1% Triton X-100 for 15 minutes, and

reported mean hair Mn levels of 0.47 μg/g (range 0.089 – 2.15 μg/g, n=31) for children living in the vicinity of the Tar Creek Superfund site. Bouchard et al. (*2*) did not wash hair samples before analyses and reported median hair Mn levels of 5.1 μg/g (range 0.28 - 20.0 μg/g, n=46) for children exposed to well water Mn (160 or 610 μg Mn/L); in a subsequent expanded study they utilized the cleaning method of Wright et al. (*40*) and reported median hair Mn levels of 0.7 μg/g (range 0.1 - 21 μg/g, n=302) from children exposed to Mn in water containing 0.1 – 2,700 μg Mn/L (*1*).

The extent to which hair Mn levels reported in these and other studies reflect the internal systemic dose versus exogenous hair Mn contamination is not clear, in part due to reported differences in cleaning/processing of hair. Unlike the significant efforts devoted to improving blood lead measurements (*8,36*), there has been comparatively little effort to develop a cleaning/processing method to effectively remove exogenous hair Mn without altering endogenous Mn (4*,30,37*). However, hair is highly susceptible to exogenous contamination, since Mn is a relatively common constituent of environmental media, such as soil/dust and can be naturally elevated in well-water (*1,2,37,39*).

Here we developed a hair cleaning methodology that removed exogenous Mn without altering endogenous Mn, so as to improve the potential utility of hair Mn as an exposure biomarker. Cleaning methods using Triton X-100, 1N nitric acid, and sonication in different combinations were explored using hair from human subjects that was uncontaminated or intentionally contaminated with dust or Mn-treated water. Levels of Mn, Pb, chromium (Cr) and copper (Cu) were measured by high resolution ICP-MS. In addition, we used scanning electron microscopy to evaluate hair surface morphology, and laser ablation ICP-MS to specifically evaluate surface versus endogenous hair Mn under different conditions. We then used the developed cleaning method on hair samples from children (n=121) in an ongoing study of environmental Mn exposure and related health effects; results show that hair Mn levels were

significantly associated with levels of Mn exposure, providing validation of hair Mn levels as a biomarker of environmental Mn exposure in children.

Experimental

Study Design, Sample Collection and Treatment. Ten adult volunteers, five male and five female age 22 – 65 yrs, each donated ~2 g of hair to the study. The distal 2 - 3 cm of each subject's hair was clipped with stainless steel scissors and stored in a ziplock bag at room temperature until processing. Hair colors were brown, gray, black and red. Subjects did not use hair products other than shampoo and conditioner. Subsamples of hair from each subject were processed using one of five different hair cleaning/processing methodologies described below, and analyzed for Mn, Pb, Cr, Cu concentrations by ICP-MS.

In addition, hair samples from a subset of five subjects were intentionally contaminated with house dust, or Mn-contaminated water containing 70 or 700 μg Mn/L, and cleaned using the methodologies described below to evaluate the efficacy of each cleaning procedure on highly contaminated hair. For this, hair from each subject was exposed to house dust (sieved < 150 μm) at a ratio of 4:10 (hair:dust, w/w), and thoroughly mixed in a polyethylene bag. Hair was then removed with stainless steel forceps and shaken vigorously to remove excess dust. For Mn water contamination, hair samples were immersed for 2 hours in tap water solutions containing 70 or 700 μg Mn/L, and then rinsed five-times with ultrapure (18 MOhm-cm^2) Milli-Q (MQ) water before cleaning.

Hair Cleaning and Processing. Hair (~50 mg) from each of the 10 subjects was aliquoted in triplicate into 5 mL polypropylene syringe bodies, and cleaned using one of the following five procedures: (1) No cleaning; hair was processed for analyses without cleaning. (2) MQ water rinse; hair was rinsed five-times with ultrapure MQ water. (3) Triton sonication (T); hair was sonicated in 0.5% Triton X-100 for 10 minutes, rinsed five-times with MQ water, rinsed once with

1N trace metal grade (TMG) nitric acid, and rinsed five-times with MQ water. (4) Triton and nitric sonication (TN); hair was sonicated for 20 minutes in 0.5% Triton, rinsed five-times with MQ water, sonicated for 10 minutes in 1N TMG nitric acid, rinsed once with 1N nitric acid, and then rinsed five-times with MQ water. And (5) TN x 3; the Triton and nitric acid sonication (TN) cleaning described above was performed three-times in succession. Hair samples from the subset of five subjects that were intentionally contaminated with dust or Mn-contaminated water were cleaned with the same procedures, except the 'No cleaning' method was omitted. In all cases the final 5x MQ water rinse was performed in a HEPA filtered air cleanroom using trace metal clean procedures (see Appendix Figure 7).

Hair processing and analysis for metal concentrations. Hair samples were processed for analyses using established trace metal clean techniques in a HEPA filtered-air laboratory following procedures previously described (*36*). Briefly, following the final MQ water rinse, hair samples were dried overnight at 65 ºC, and ~30 mg of dry hair was transferred to a pre-weighed 6 mL polyethylene tube and digested in 0.5 mL 15.7 N double quartz-distilled nitric acid at 80 ºC for 6 h. Following digestion, samples were diluted with 5 mL MQ water, and tubes were capped and vortexed. Rhodium (Rh) and thalium (Tl) were added as internal standards, and samples were analyzed for ^{208}Pb and ^{205}Tl (low resolution), and ^{52}Cr, ^{55}Mn, ^{63}Cu, and ^{103}Rh (medium resolution) by magnetic sector inductively coupled plasma mass spectrometry (Thermo Scientific - Element XR ICP-MS). Metal concentrations were determined by comparison with certified multi-element standards (Spex Industries). The analytical detection limits for Mn, Pb, Cr and Cu were 0.051, 0.003, 0.023, and 0.175 ng/mL, respectively.

Scanning electron microscopy analysis of hair. To evaluate the structural morphology of the hair samples, two representative strands of intentionally contaminated hair (~2 cm each) per treatment/cleaning scheme were selected from three of the subjects. Hair strands were adhered to carbon tape, carbon-coated once inside the SEM instrument, and imaged at 1500X

magnification using an FEI Quanta 200. The working distance was 9.9 mm and accelerating voltage was 8.0 kV. For each hair strand, the entire sample was viewed prior to taking an image to ensure representative pictures were obtained.

Laser ablation ICP-MS. Representative hair strands were analyzed for surface and interior Mn distribution using a Photon Machines Analyte 193 nm ArF excimer laser, interfaced with the XR ICP-MS. Parameters used for analysis were: Laser spot size = 34 μm, laser raster speed = 100 μm/s, and laser power = 20 Hz. Background counts of ^{34}S and ^{55}Mn were collected over a 30 sec interval in the laser off mode, followed by ^{34}S and ^{55}Mn count acquisition with the laser on over a subsequent 170 sec while the laser was rastered back and forth 30-times over a 500 μm section of the hair. Three separate 500 μm sections, separated by ~500 μm were analyzed on each hair strand. Each 500 μm section represented ~2 days of hair growth, based on average adult hair growth rates of ~250 μm/d (*10*).

Hair Mn levels in children exposed to environmental Mn. Hair samples were collected from 121 children age 11 – 13 yrs as part of an on-going epidemiologic study of the health effects of environmental Mn exposure from ferroalloy plant operations in northern Italy (*24*). Briefly, subjects were lifelong residents in one of three study areas: (1) Bagnolo Mella (n= 44), a town with a currently active ferroalloy plant that has been in operation since approximately 1970; (2) Valcamonica (n=41), a region with several historically-active ferroalloy plants that operated for a century, ceasing operation in 2001; and (3) Garda Lake (n=36), a reference region with no history of ferroalloy plant operations. Subjects were recruited through the public school system according to a community-based participatory approach. Full details of subject recruitment, exposure assessments, and health outcome assessments are reported elsewhere (*24*). For each child, ~2 - 3 cm of hair was collected immediately adjacent to the scalp at the nape of the neck, taped together at the distal end, and stored in polyethylene bags at room temperature until analysis.

Hair samples (5 – 30 mg) were processed for analyses using trace metal clean techniques and the Triton + 1N nitric acid sonication cleaning process (TN) detailed above.

Data analyses. Summary data are expressed as mean ± standard error (SE), or mean ± standard deviation (SD), as appropriate. Data were analyzed to test specific hypotheses using one-way analysis of variance (ANOVA) using JMP software (Version 9.0, 2010). Individual cleaning comparisons were done with Tukey's post-hoc test. If necessary, data were square root-transformed or log-transformed to achieve normality and variance equality. A P-value of 0.05 for the various outcomes was considered statistically significant.

Results and Discussion

Cleaning hair with 0.5% Triton + 1N nitric acid sonication optimally cleans exogenous Mn and other metals from hair. In order to determine how cleaning affected hair Mn, Pb, Cr, and Cu concentrations, we cleaned hair from 10 adult subjects with five different cleaning procedures. Results show that there was a significant effect of cleaning procedure on hair Mn levels (ANOVA $F_{(4, 148)} = 38.55$, $p < 0.0001$), which decreased as the apparent strength of cleaning procedure increased in the order: MQ water rinsing (MQ) > Triton sonication + 1N nitric acid rinse (T) > Triton sonication + 1N nitric acid sonication (TN) (Figure 1, Table 1). The latter TN cleaning method reduced hair Mn levels by ~80% compared to uncleaned hair. Similar results were obtained with reductions in Pb, Cu and Cr hair levels (ANOVA $F_{(4, 148)} = 19.77$, $F_{(4, 148)} = 7.87$, and $F_{(4, 148)} = 2.68$, respectively, $P<0.0001$ for Pb and Cu, and $P=0.034$ for Cr), with significant overall reductions of ~70 % compared to uncleaned hair (Table 1). Collectively, these results indicate that the Triton sonication + 1N nitric acid sonication cleaning method reduces significantly more exogenous Mn (and Pb) compared to the less-rigorous cleaning methods evaluated (methods MQ or T). Moreover, without any cleaning or with simple MQ water rinsing, hair Mn levels remained elevated by ~4 – 5-fold over levels in Triton sonication + 1N nitric acid sonication cleaned hair.

TABLE 1. Metal concentrations in uncontaminated and intentionally contaminated hair processed with different cleaning methods (see text).

	Cleaning Method	Mn*	Pb	Cr	Cu
Uncontaminated	No cleaning	0.833 (0.080) [a]	0.796 (0.123)[a]	0.273 (0.064)[a]	73.3 (15.2)[a]
	MQ	0.644 (0.085)[a,b]	0.813 (0.134)[a]	0.241 (0.077)[a,b]	68.2 (18.5)[a]
	Triton	0.419 (0.068) [b]	0.696 (0.137)[a]	0.194 (0.068)[a,b]	45.0 (10.4)[a,b]
	Triton+nitric	0.170 (0.027) [c]	0.250 (0.043)[b]	0.181 (0.070)[a,b]	24.4 (5.20)[b]
	3x Triton+nitric	0.066 (0.012) [c]	0.084 (0.012)[b]	0.134 (0.047)[b]	17.5 (3.45)[b]
Contaminated	*Dust treated*				
	MQ	5.73 (0.632)[a]	1.62 (0.367)[a]	1.07 (0.161)[a]	120.0 (36.4)[a]
	Triton	0.593 (0.108)[b]	0.972 (0.268)[a,b]	0.491 (0.153)[b]	75.7 (20.2)[a,b]
	Triton+nitric	0.238 (0.042)[b]	0.315 (0.068)[b]	0.342 (0.146)[b,c]	36.0 (7.75)[b]
	3x Triton+nitric	0.154 (0.055)[c]	0.0885 (0.013)[c]	0.264 (0.116)[c]	29.7 (7.42)[b]
	70 μg/L water-treated				
	MQ	5.51 (1.55)[a]	0.960 (0.241)[a]	0.312 (0.120)[a]	95.2 (30.4)[a]
	Triton	2.68 (0.667)[a]	0.898 (0.221)[a]	0.237 (0.114)[a]	71.6 (19.6)[a,b]
	Triton+nitric	0.89 (0.258)[a,b]	0.304 (0.056)[b]	0.190 (0.095)[a]	33.0 (7.28)[a,b]
	3x Triton+nitric	0.414 (0.158)[b]	0.098 (0.011)[b]	0.292 (0.112)[a]	29.4 (9.08)[b]
	700 μg/L water-treated				
	MQ	10.5 (3.90)[a]	1.17 (0.315)[a]	0.365 (0.163)[a]	98.6 (25.0)[a]
	Triton	2.31 (0.620)[a,b]	1.09 (0.235)[a]	0.225 (0.112)[a]	66.5 (18.2)[a,b]
	Triton+nitric	0.506 (0.170)[b,c]	0.306 (0.045)[b]	0.246 (0.105)[a]	32.0 (7.14)[b]
	3x Triton+nitric	0.181 (0.066)[c]	0.044 (0.009)[b]	0.230 (0.099)[a]	22.0 (5.48)[b]

* Mn, Pb, Cr, Cu concentrations are μg/g hair; Data are mean (standard error); n = 10 and n = 5 subjects for the 'uncontaminated' and 'contaminated' values, respectively.
[a, b, c] Values with different superscripts are significantly different from one another based on Tukey's posthoc analysis ($P < 0.05$) performed on square root-transformed 'uncontaminated' data and log-transformed 'contaminated' data within metal and contamination category.

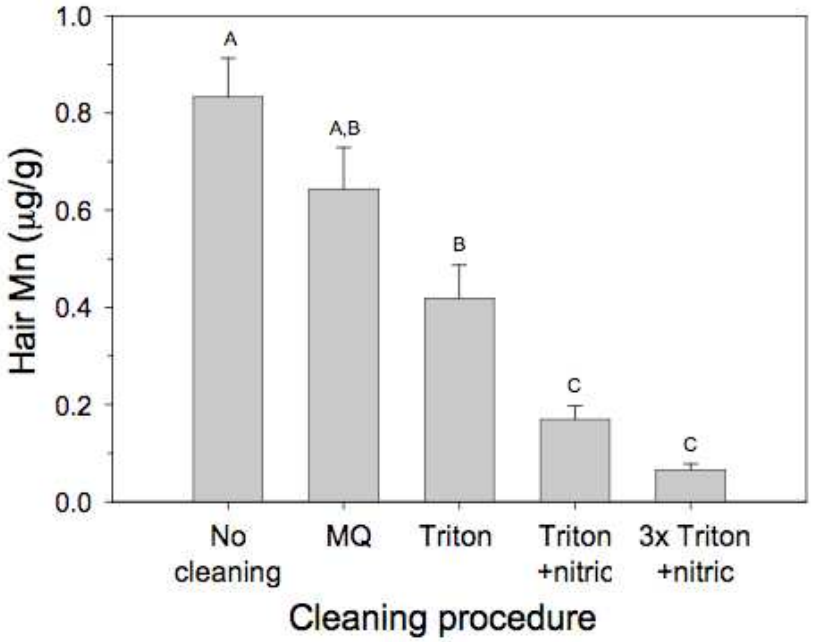

FIGURE 1. Hair Mn levels were reduced with increasing strength of cleaning. Data reflect hair Mn concentrations after cleaning with each procedure. Values are mean (± SE, n=10 subjects). Superscripts denote significant difference between cleaning methods based on Tukey's posthoc analyses performed on square root-transformed data (P < 0.05).

Previous studies that specifically explored cleaning methods to remove exogenous hair Mn were unable to settle on an effective cleaning procedure. Stauber et al. (*37*), using adult hair intentionally contaminated with synthetic sweat and dust, evaluated 20 different cleaning agents, which did not include those evaluated here, and found at best a 15-minute sonication in 0.1 M HCl or 0.1 M cysteine-HCl reduced exogenous Mn 71% and 51%, respectively, in addition to the undesirable effect of removing endogenous hair Mn. Buckley et al. (*4*), using adult hair intentionally contaminated with Mn-contaminated water, tested sonications in Triton X-100, sodium lauryl sulfate or sodium EDTA . Sodium lauryl sulfate removed the most exogenous Mn (~75%), but none of the methods removed all exogenous Mn. Mikasa et al. (*30*), using uncontaminated adult hair, tested procedures using various combinations of acetone, ethanol, polyethylene glycol lauryl ether, HCl, and sonication, and concluded that hair Mn contamination was not effectively removed by any of the procedures tested. These studies underscore the need for an optimized and well-validated hair cleaning methodology that effectively removes

exogenous Mn without altering endogenous Mn so as to improve the potential for hair to serve as a biomarker of internal Mn exposure (*35*).

As with any potential exposure biomarker that is subject to external contamination, it is important to effectively minimize or remove exogenous contamination without 'over-cleaning', i.e., altering endogenous, metabolically incorporated metals that may reflect internal exposure (*35,36*). We evaluated whether the seemingly optimal Triton sonication + 1N nitric acid sonication (TN) cleaning method was possibly over-cleaning hair and removing endogenous hair Mn and other metals. For this, hair samples were cleaned sequentially three-times with the TN cleaning method; if the TN cleaning method was removing endogenous hair Mn, we would expect Mn levels in hair cleaned three-times with the TN method to be significantly lower than in hair cleaned only once. Results show that hair levels of Mn, Pb, Cr, or Cu in the TN versus 3xTN- cleaned were not significantly different from each other, suggesting that the TN cleaning method does not 'over-clean' or remove stably-bound endogenous metals from hair (Figure 1, Table 1).

The TN method cleans highly contaminated hair. Since the potential exists for hair to become highly contaminated from prolonged contact with Mn-contaminated water, dust, and airborne particles (*1,2,24,26,27,35,39*), we investigated whether the seemingly optimal Triton sonication + 1N nitric acid sonication (TN) method would also remove excessive exogenous contamination from hair treated with house dust (hair:dust, 4:10 (w/w)) or Mn-contaminated water at levels of 70 or 700 µg Mn/L. The Mn levels in water were selected to slightly exceed the USEPA secondary standard for Mn in water (50 µg Mn/L) (*34*), or to be comparable to highly elevated Mn levels in well-water reported in several epidemiologic studies of Mn exposure (*1,2,39*). We immersed hair samples in the Mn-contaminated water for 2 hrs, which is significantly longer than a typical shower or bath, but it is slightly less than the ~4 hrs[1] or more of cumulative water contact

[1]The 4 hr estimate of cumulative hair contact time with water assumes a 2 cm segment of hair proximal to the scalp grows in at a rate of 0.75 cm/month (*10*), and that a person's hair contacts water via bathing for ~10 min/day 4 days/wk = 240 average cumulative minutes, or 4 hr for a 2

estimated from routine bathing over the duration of hair growth. These contamination treatments increased hair Mn levels by ~7 to 13-fold (using contaminated MQ-rinsed hair levels) compared to uncontaminated, uncleaned hair (Table 1), and are comparable to hair Mn levels reported for subjects in Mn-contaminated environments (*1,2,13,27,28,31*).

Results show that Mn, Pb, Cr, and Cu levels in hair excessively contaminated with house dust were significantly reduced with increasing rigor of cleaning (ANOVA $F_{(3, 56)}$ = 58.81, $F_{(3, 56)}$ = 19.34, $F_{(3, 56)}$ = 11.89, and $F_{(3, 56)}$ = 5.58, respectively, with P's<0.0001 for Mn, Pb, and Cr, and P=0.002 for Cu) (Table 1). Similarly, Mn levels in hair contaminated with water containing 70 or 700 µg Mn/L were also significantly reduced by cleaning (ANOVA $F_{(3, 68)}$ = 7.298, P=0.0003, and $F_{(3, 67)}$ = 12.29, P<0.0001, respectively), with the TN method again seeming optimal (Table 1).

In order to specifically test whether exogenously contaminated hair could be sufficiently cleaned using the Triton sonication +1N nitric acid sonication (TN) method, we normalized hair Mn levels from each cleaning method to the Mn levels in the uncontaminated, TN-cleaned (UTN) hair sample within subject. We considered the UTN hair sample to best reflect endogenous hair Mn levels in the absence of external contamination. Levels of Mn in dust-contaminated hair cleaned with the TN method were not significantly different than levels in the UTN sample (P= 0.45, based on Tukey posthoc comparison) (Figure 2A); similar results were obtained with Pb, Cr, and Cu (P's ≥ 0.9). However, Mn levels in dust-contaminated hair cleaned with the slightly less-rigorous Triton sonication + nitric acid rinse (T) method remained significantly higher than levels in the UTN sample (P<0.0001 for Mn; P's=0.043, 0.11, and 0.013 for Pb, Cr, and Cu, respectively) (Figure 2A), suggesting this slightly less rigorous 'T' cleaning method may not be adequate for cleaning highly contaminated hair.

Similarly, hair contaminated with water containing 70 or 700 µg Mn/L and cleaned with the TN method were not measurably different from uncontaminated hair cleaned with the TN method (UTN) (P=0.15 and P=0.31 for 70 and 700 µg Mn/L water treatments, respectively, based

---

cm piece of hair; if hair were sampled distal from the scalp, cumulative water contact times could be much higher.

on Tukey's posthoc comparison within subject) (Figure 2B, C). In contrast, however, Mn levels in hair cleaned with the less rigorous Triton sonication + nitric acid rinse (T) method remained significantly elevated compared to Mn levels in the UTN hair sample ($P=0.0005$ and $P=0.0004$ for the 70 and 700 µg Mn/L treatments, respectively). Collectively, these data indicate that the TN cleaning method is able to remove even excessive exogenous contamination with Mn and other metals, and that the TN cleaning method provides significant improvement over the slightly less rigorous Triton sonication + 1N nitric rinse method (T).

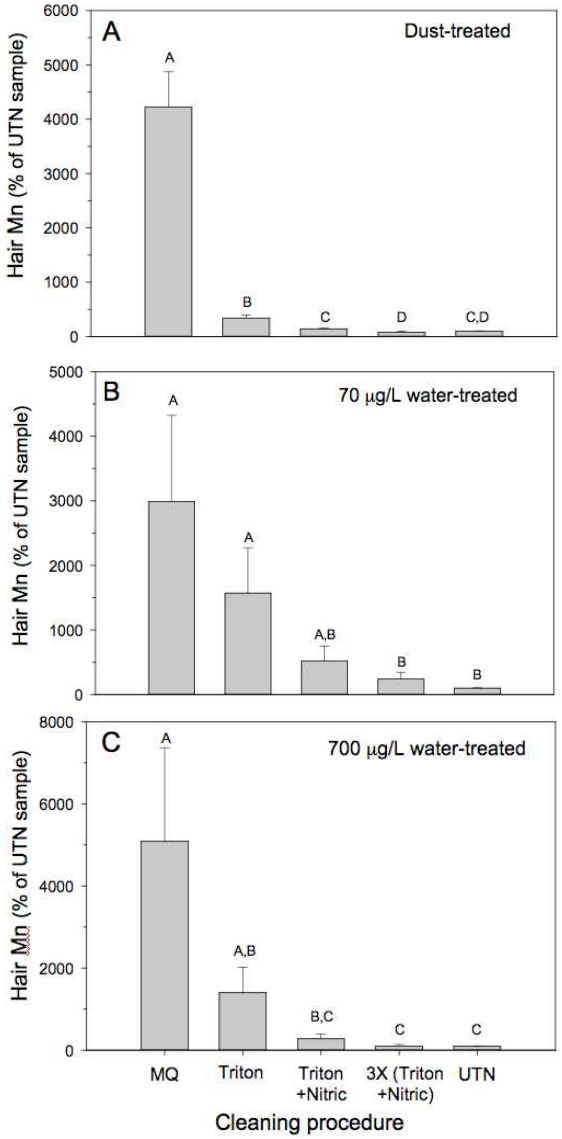

FIGURE 2. Manganese levels in hair that was intentionally contaminated with house dust (A), or water containing either 70 μg Mn/L (B), or 700 μg Mn/L (C), and then cleaned with either MQ water rinsing (MQ), Triton sonication + 1N nitric acid rinsing (Triton), Triton sonication + 1 N nitric acid sonication (Triton + nitric), or the latter TN cleaning performed three times. Manganese levels are expressed as a percent of the uncontaminated hair cleaned with the Triton + nitric method (UTN), calculated within each subject. Values are mean (± SE; n=5 subjects). Superscripts denote significant differences based on Tukey's posthoc analyses performed on log-transformed data ($P < 0.05$).

Individual subjects vary in level of contamination as well as response to cleaning procedures. While only a small number of subjects were utilized in this study, it appeared that hair samples from the different subjects varied in their susceptibility to contamination with Mn-contaminated water, and in their response to cleaning. This is illustrated in hair samples from the five subjects contaminated with 700 μg Mn/L water. Manganese levels in subject 1's hair treated with 700 μg Mn/L water and cleaned with the MQ rinse were actually lower than the levels in his uncontaminated, uncleaned hair. Further cleaning of subject 1's 700 μg Mn/L water-treated hair readily reduced Mn to levels comparable to those in the uncontaminated, Triton + 1N nitric acid sonication-cleaned sample (Figure 3). In contrast the other four subjects were all more susceptible to Mn water contamination when comparing their 700 μg Mn/L water-treated and MQ rinsed sample to their uncontaminated, uncleaned hair sample. Subject 2 shows a ~40 fold increase in hair Mn concentration, while subjects 3, 4, and 5 increase ~5 to 10-fold (Figure 3). The basis for these differences in susceptibility to contamination is not known, but previous studies have reported similar findings. Buckley et al. (*4*) examined metal absorption and removal from hair, specifically zinc (Zn), and found that hair from different individuals absorb Zn to varing degrees when placed in Zn-contaminated water. Metal contamination localizes to specific parts of the hair shaft, and perhaps differences in the composition of high-affinity binding sites for Mn (and other metals) between subjects contributes to the individual susceptibility to contamination that we observed (*18,19*).

Interestingly, subjects also differed in their response to cleaning in a fashion that appeared to be inversely related to individual differences in susceptibility to contamination with the 700 μg Mn/L water. Hair from subject 1 was resistant to contamination and was readily cleaned even with the less rigorous Triton sonication + 1N nitric rinse (T) method used here. In contrast, hair from subjects 2 to 5 was more susceptible to contamination from Mn in water and required more rigorous cleaning to effectively reduce hair Mn to levels comparable with the uncontaminated, Triton sonication + 1N nitric sonication cleaned (UTN) sample within each

subject (Figure 3). These apparent individual differences in susceptibility to contamination and responsiveness to cleaning underscores the need to use a hair cleaning method that is sufficiently rigorous to clean hair that may be both susceptible to contamination and resistant to cleaning.

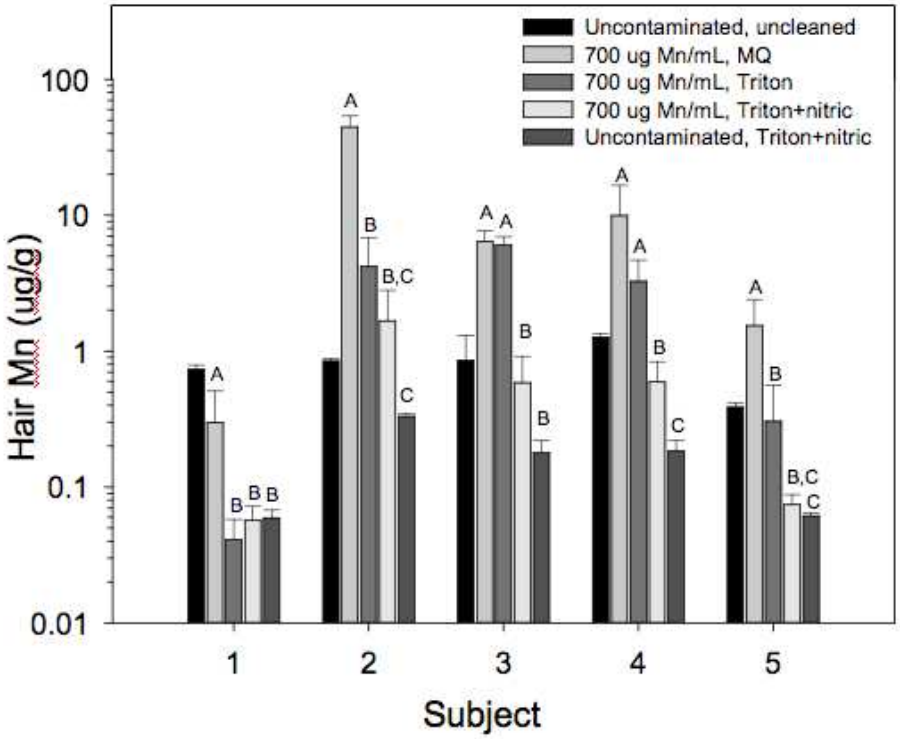

FIGURE 3. Hair from individual subjects varies in susceptibility to contamination and response to cleaning. Hair Mn levels (mean ± SD, n=3 hair samples per bar) in samples from each subject that were uncontaminated and uncleaned, contaminated with 700 μg Mn/L water and rinsed with MQ water, contaminated with 700 μg Mn/L water and cleaned with Triton sonication + 1N nitric acid rinsing (Triton), contaminated with 700 μg Mn/L water and cleaned with Triton sonication + 1N nitric acid sonication (Triton + nitric), or uncontaminated and cleaned with Triton sonication + 1N nitric acid sonication. Superscripts denote significant differences among the latter four bars per subject, based on Tukey's posthoc analysis ($P < 0.05$) (ANOVA $F_{(4,21)} = 15.99$, $F_{(4, 10)} = 35.46$, $F_{(4, 10)} = 33.48$, $F_{(4, 10)} = 34.21$, $F_{(4, 10)} = 29.98$ on log-transformed data for subjects 1 – 5, respectively; $p < 0.0001$ for all). Data from the 'uncontaminated and uncleaned' samples are included for reference only and were not used in the statistical analyses.

Cleaning hair with the triton + nitric sonication does not damage the structural integrity of hair. To determine whether the optimal cleaning method or the intentional contamination of hair with Mn-contaminated water visibly altered the morphological appearance, and hence possibly the structural integrity of hair, representative hair samples from three subjects were analyzed by scanning electron microscopy (SEM). Four different treatment/cleaning schemes were selected for analysis: 1) Uncontaminated and uncleaned, 2) uncontaminated and cleaned with Triton sonication + 1N nitric acid sonication, 3) 700 μg Mn/L water-contaminated and rinsed with MQ, and 4) 700 μg Mn/L water-contaminated and cleaned with Triton sonication + 1N nitric acid sonication. Results show that there was no discernible difference in the hair surface morphology across any of the treatment conditions within subject (Figure 4). These images further substantiate that the optimal Triton sonication + 1N nitric acid sonication cleaning method does not visibly damage the structural morphology of hair, consistent with our assessment above that this cleaning method does not 'over-clean' hair and remove endogenous metals - a result that may have been more likely if the structural integrity of hair was damaged by cleaning.

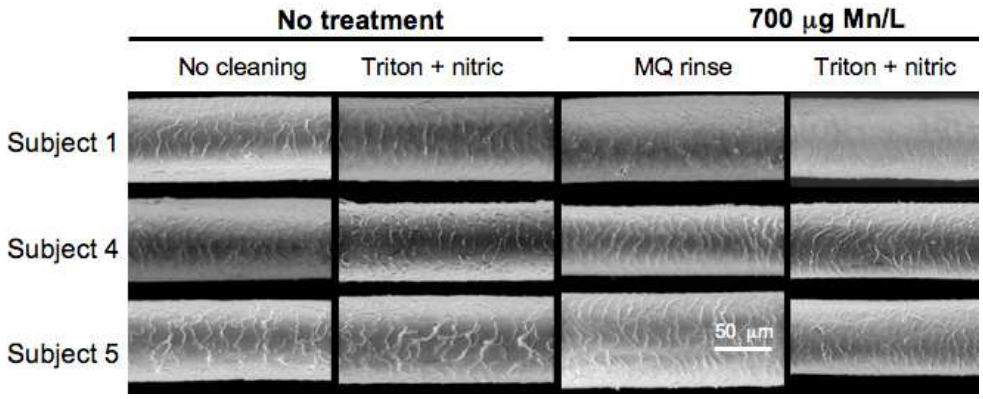

FIGURE 4. Scanning electron microscopy images (1500X magnification) of representative hair strands from three subjects show that hair surface morphology is not visibly altered by optimal cleaning or intentional contamination with 700 μg Mn/L water. Four different treatment/cleaning schemes are shown for each subject: Uncontaminated uncleaned, uncontaminated cleaned with Triton sonication + 1N nitric acid sonication, contaminated with 700 μg Mn/L water and MQ rinsed, and contaminated with 700 μg Mn/L water and cleaned with Triton sonication + 1N nitric acid sonication. Hair diameters vary from ~ 50-80 μm. Scale bar is 50 μm.

Mn contamination from water localizes to both the surface and interior hair shaft, and contamination is removed with the Triton + 1N nitric acid sonication cleaning procedure. We used laser ablation ICP-MS to selectively measure surface versus endogenous hair Mn levels, in order to determine whether the pattern of Mn distribution in hair changes following various contamination and cleaning conditions. Analyses were performed on two representative hair strands from the same three subjects and treatment/cleaning conditions selected for SEM analyses. Representative data from a hair strand that was contaminated with 700 μg Mn/L water and rinsed with MQ before analysis shows ^{34}S counts remained relatively stable over ablation through the hair shaft, while ^{55}Mn counts peaked dramatically coinciding with ablation of the hair's surface, and rapidly declined to asymptotically low counts corresponding to the interior of the hair (Figure 5A).

Subsequently, hair ^{55}Mn counts were normalized to ^{34}S counts (as $^{55}Mn/^{34}S$ ratios), and the relative levels of Mn in surface versus interior hair shaft were evaluated following intentional contamination with 700 μg Mn/L water and cleaning. Notably, contamination of hair with 700 μg Mn/L water led to significant increases in surface Mn, as well as increased Mn throughout the interior of the hair shaft, compared to Mn levels in uncontaminated uncleaned hair (Figure 5B, Table 2). The result showing that the interior of hair was susceptible to Mn contamination from water was somewhat unexpected. Importantly, however, there was no significant difference or increase in surface or interior $^{55}Mn/^{34}S$ ratios in the uncontaminated hair or 700 μg Mn/L water-contaminated hair that was cleaned with the Triton sonication + 1N nitric acid sonication (TN) method (Figure 5B), indicating that the TN cleaning method removed hair contamination from both the surface and interior of the hair shaft.

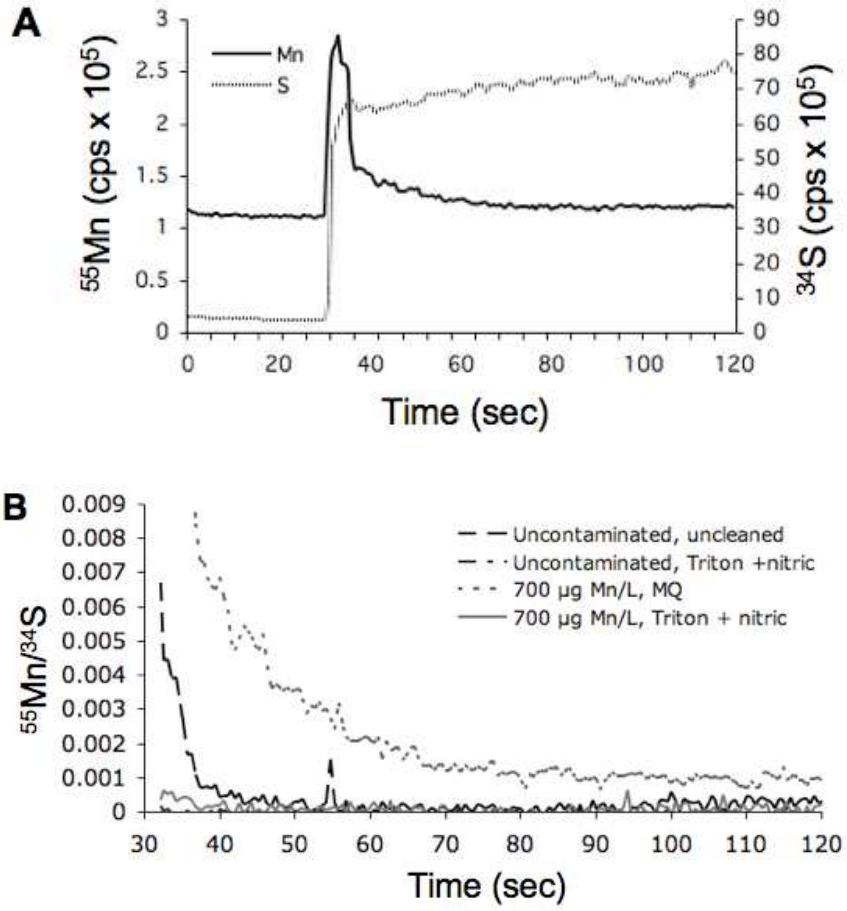

FIGURE 5. A) ^{55}Mn and ^{34}S counts per second (cps) in a representative hair sample from subject 5 contaminated with 700 μg Mn/L and cleaned with MQ water, measured using laser ablation (LA) ICP-MS. B) ^{55}Mn/^{34}S cps ratios measured in hair by LA ICP-MS following four different treatment/cleaning schemes, as follows: Uncontaminated and uncleaned, uncontaminated cleaned with Triton sonication + 1N nitric acid sonication, contaminated with 700 μg Mn/L water and MQ rinsed, and contaminated with 700 μg Mn/L water and cleaned with Triton sonication + 1N nitric acid sonication. The ^{55}Mn/^{34}S cps ratio is plotted versus time starting when the laser was turned on. Data for each hair in 'B' reflect the average ^{55}Mn/^{34}S cps ratio from three 500 μm sections/hair that were each rastered back and forth with the laser at a rate of 100 μm/s (see text for details).

Similar results were obtained in hair from all three subjects (1, 4, and 5) analyzed by LA-ICP-MS. In uncontaminated, uncleaned hair the ^{55}Mn/^{34}S ratios in the surface of the hair shaft were universally higher compared to the interior of the hair. Interestingly, though, this difference in surface versus interior hair Mn levels (as ^{55}Mn/^{34}S ratios) was exacerbated in hair intentionally contaminated with 700 μg Mn/L water and MQ rinsed for subjects 4 and 5, but not subject 1

(Table 2). This between-subject difference in surface versus interior hair shaft Mn levels following water Mn contamination is consistent with the individual subject differences in susceptibility to contamination and response to cleaning reported above, and likely reflects differences in the composition of both the surface and interior of the hair shaft. Importantly, the surface versus interior hair Mn levels of hair contaminated with 700 μg Mn/L water and cleaned with the Triton sonication + 1N nitric acid sonication method were each significantly reduced in all three subjects, and surface versus interior hair Mn levels were not notably different from one another after cleaning (Table 2).

TABLE 2. Average hair Mn concentrations (μg/g), hair surface and interior $^{55}Mn/^{34}S$ ratios, and the surface minus interior $^{55}Mn/^{34}S$ ratios, for three subjects. #

	Uncleaned	TN-cleaned	Mn-contaminated, MQ-cleaned	Mn-contaminated, TN-cleaned
Subject 1				
Hair [Mn]	0.734	0.059	0.300	0.057
Surface Mn/S	0.00166	<DL*	0.00053	<DL
Interior Mn/S	0.00017		0.00014	
Surf. – Int. Mn/S	0.00149		0.00040	
Subject 4				
Hair [Mn]	1.26	0.184	9.93	0.591
Surface Mn/S	0.00792	<DL	0.06873	0.00162
Interior Mn/S	0.00550		0.02169	0.00087
Surf. – Int. Mn/S	0.00243		0.04704	0.00075
Subject 5				
Hair [Mn]	0.386	0.061	1.54	0.074
Surface Mn/S	0.00311	<DL	0.01316	<DL
Interior Mn/S	0.00043		0.00124	
Surf. – Int. Mn/S	0.00284		0.01192	

Total hair Mn concentrations by hair digestion and ICP-MS analyses. $^{55}Mn/^{34}S$ ratios measured by laser ablation ICP-MS. For the latter, two hairs for each treatment with three 500 μm sections laser-rastered on each hair were assessed per subject (see text).
* <DL. Detection limits for $^{55}Mn/^{34}S$ ratios were estimated as 0.00017, 0.00053 and 0.00089 for subjects 1, 4 and 5, respectively.

Hair morphology and composition differ somewhat in surface (cuticle) versus interior regions, with hair surface being rich in cystienes as well as amino and carboxyl functional groups, while the interior is largely cysteine-rich (*18,19*). How these differences affect Mn binding and susceptibility to contamination and/or cleaning is not known. Studies on hair calcium (Ca) and magnesium (Mg), which as Mn homologues may provide some clues on Mn binding in hair, have shown that Ca and Mg bind to amino and carboxyl functional groups in cuticle scales on the surface of hair (*18,19*), and co-localize with melanin molecules in the interior of the hair shaft (*18*).

Children's hair Mn levels reflect environmental exposures. In order to better validate the use of hair Mn as a biomarker of environmental Mn exposure, we applied the Triton sonication + 1N nitric acid sonication cleaning method to hair samples collected from children age 11- 14 yrs living in the vicinity of active ferroalloy plant emissions (Bagnolo Mella, n=44), a historic but currently inactive ferroalloy plant (Valcamonica, n=41), and a reference region with no history of industrial activity (Garda Lake, n=36) in northern Italy. Importantly, recent reports on children living in the Valcamonica versus Garda Lake region have shown significant impairment of motor coordination, hand dexterity, and odor identification associated with environmental Mn levels, while motor tremor intensity was positively associated with hair Mn levels (*24*).

Here, there was a significant effect of exposure region on hair Mn levels (ANOVA $F_{(2,117)}$ = 8.28, P<0.001), with levels in children from the active ferroalloy emission site (Bagnolo Mella) having significantly higher hair Mn levels (median = 0.134 µg/g, range 0.011 – 0.736 µg/g) compared to children from the reference Garda Lake region (median = 0.060 µg/g, range 0.023 – 0.344 µg/g) (P<0.001) or historically active Valcamonica region (median = 0.070 µg/g, range 0.025 – 0.642 µg/g (P=0.019) (Figure 6). Hair Mn levels were no different between children living in the Garda Lake versus Valcamonica regions (P=0.43, all based on Tukey's posthoc on log-transformed data). These data substantiate that endogenous hair Mn levels are significantly associated with environmental Mn exposure, and they support the validity of hair Mn levels as a biomarker of environmental Mn exposure in children.

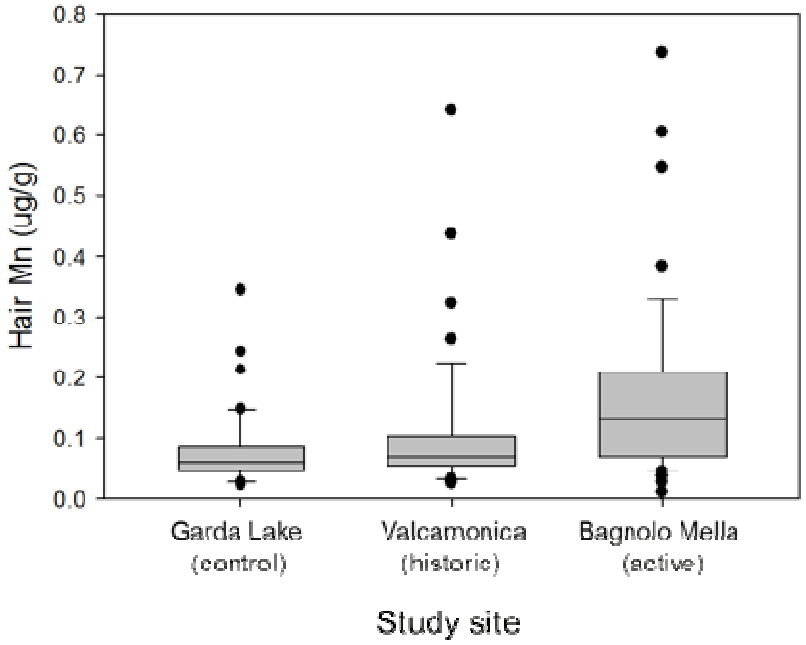

FIGURE 6. Hair Mn concentrations in children living in the vicinity of active ferroalloy plant emissions (Bagnolo Mella, n=44), a historic but currently inactive ferroalloy plant (Valcamonica, n=41), and a reference region with no history of industrial activity (Garda Lake, n=36). There is a significant effect of exposure region on hair Mn levels ($F_{(2,117)}$ = 8.28, P<0.001), with levels in children from the active ferroalloy emission site (Bagnolo Mella) having significantly higher hair Mn levels compared to children from the reference Garda Lake region (P<0.001) and historically active Valcamonica region (P=0.019). Hair Mn levels are not different between Garda Lake and Valcamonica (P=0.43, all based on Tukey's posthoc on log-transformed data).

Hair Mn levels across the cohort of 121 children averaged 0.121 µg/g (median=0.073 µg/g, range 0.011 – 0.736), which is ~4 – >6-fold lower than levels in referent and environmentally exposed children reported in other recent studies (*1,2,11,13,26,27,40*). For example, Haynes et al. (*11*) investigated manganese exposure in children living near a ferromanganese plant in the USA and reported mean hair Mn levels of 0.47 µg/g (0.3 SD, range 0.085 – 1.25 µg/g, n=38). Similarly, Menezes-Filho et al. (*26*) investigated hair Mn levels in children living in the vicinity of a ferromanganese plant in Brazil, and reported geometric mean hair Mn levels of 1.37 µg/g in reference (unexposed) children (median 1.19, range 0.39 – 8.58 µg/g, n=43), and mean levels of 9.61 µg/g in children living 1-2 km downwind of the

ferromanganese plant (median = 9.68, range 1.1 – 46.2 µg/g, n=75). Wright et al. (*40*) reported mean hair Mn levels of 0.47 μg/g (range 0.089 – 2.15 μg/g, n=31) in children living in the vicinity of the Tar Creek Superfund site, while Bouchard et al. (*1*) reported mean hair Mn levels of 0.7 µg/g (range 0.1 – 21 µg/g, n=302) in children exposed to Mn-contaminated well water (range 1 – 2700 µg Mn/L). All of these studies cleaned hair via a 15 minute sonication in 1% Triton X-100 and rinse with MQ or distilled deionized water – a method that is likely less aggressive than the 0.5% Triton sonication + 1N nitric acid sonication (TN) method used to clean the children's hair in the present study, or even less optimal and less aggressive than the 0.5% Triton sonication + 1N nitric acid rinse (T) method that our data indicated did not reliably clean Mn-contaminated hair (Table 1, Figure 2). Thus, the extent that the differences in hair Mn reported in these studies versus the present study reflects differences in hair cleaning and processing versus differences in internalized Mn exposure is not clear.

Several of these previous studies that used a 1% Triton X-100 sonication and water rinse cleaning method (*1,27,40*) did report associations between hair Mn levels and health effects in children as a result of increased environmental exposure to Mn. Again, however, the extent that reported hair Mn levels reflect the internalized Mn dose versus external environmental Mn contamination of hair is not clear; certainly both endpoints are of interest in the environmental epidemiology of Mn toxicity, but a biomarker of the internalized Mn dose, integrated over the duration of hair growth may be expected to better predict health risk than measures of exogenous environmental Mn. Notable, our recent study reported an association between hair Mn levels and tremor intensity in children age 11 – 14 yrs exposed to environmental Mn from historic ferroalloy plant emissions (*24*). This latter study also used the 0.5% Trition sonication + 1N nitric acid sonication (TN) method in a similar but distinct pediatric group from what we report here, and reported median hair Mn levels of 0.11 µg/g (mean 0.16, range 0.02 – 3.45 µg/g, n=258 children); levels that were quite similar to those we report in the present study.

Appendix

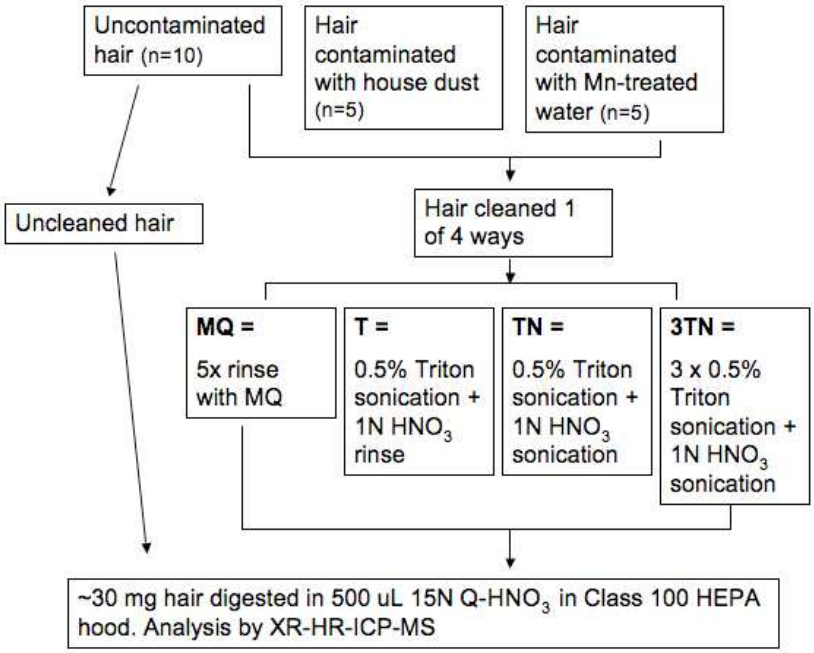

Figure 7. Hair processing schematic. Contaminated or uncontaminated hair was cleaned or uncleaned then analyzed on the ICP-MS.

Initial Studies that Informed Development of The Optimal Hair Cleaning Method Reported Above. Initial experiments that I performed suggested that hair samples contaminated with 700 μg Mn/L water could not be adequately cleaned, even with the TN cleaning method described above (Figure 8) (ANOVA $F_{(4,40)} = 44.82$, $p < 0.0001$, on log-transformed data). All samples contaminated with 700 μg Mn/L water and cleaned with one of the procedures above had Mn levels above the UTN sample (uncontaminated and cleaned with Triton + 1N nitric acid sonication). As a result, I explored my method of hair processing to see if changes could be made that would ensure removal of high levels of Mn contamination to the UTN level. In the study from Figure 8, triplicate hair samples were contaminated and cleaned together, i.e., in the same syringe body placed in the cleaning solution. To test how this type of processing affected hair Mn, a set of samples was processed in triplicate while another set was processed individually (each sample in its own syringe body) and all were analyzed for Mn concentrations. Figure 9 shows samples processed separately have more consistent hair Mn levels, while samples processed in triplicate within the same syringe body have a larger variation in hair Mn, suggesting that bulk-cleaned samples are not cleaned as consistently and reliably as samples cleaned individually. As a result all future experiments processed triplicate samples individually to ensure equal contamination and cleaning.

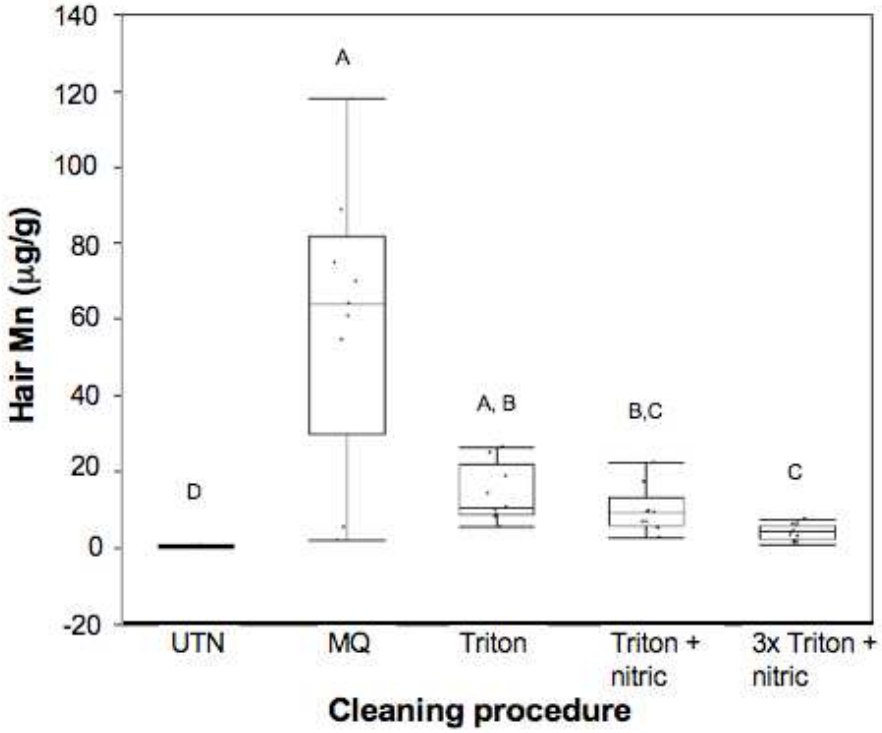

Figure 8. Triton + 1N nitric acid sonication does not remove exogenous hair Mn. Box plot of hair Mn of three subjects after contamination with 700 μg Mn/L water and cleaning with the above procedures (n=9 samples per bar, 3 per subject). The UTN sample (uncontaminated sample cleaned with Triton + 1N nitric acid sonication) was added for comparison and is part of the statistics. Superscripts denote significant differences based on Tukey's posthoc analysis (P < 0.05) on log-transformed data.

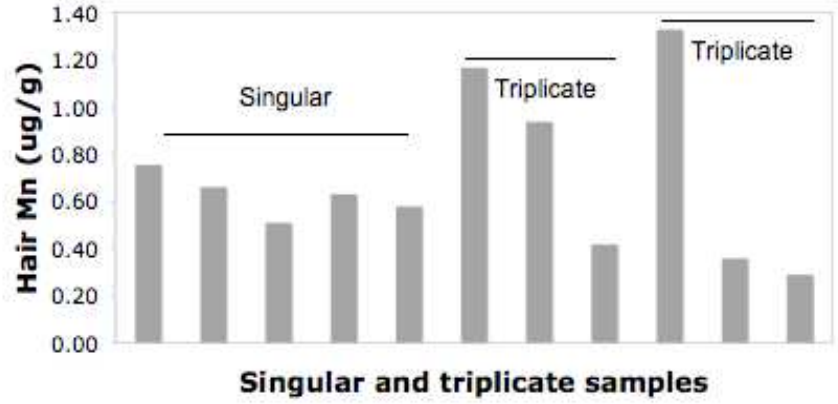

Figure 9. Larger variation in hair Mn is seen when triplicate hair samples are contaminated and cleaned together. Hair samples from one subject were contaminated and cleaned in triplicate (in the same syringe body) or individually (in separate syringe bodies). Two sets of triplicate samples and five individual samples were processed. Each bar represents one hair replicate sample.

References

1. Bouchard, M. F., Sauve, S., Barbeau, B., Legrand, M., Brodeur, M.E., Bouffard, T., Limoges, E., Bellinger, D. C., and Mergler, D. (2011) Intellectual impairment in school-age children exposed to manganese from drinking water., *Environmental health perspectives 119,* 138-43.

2. Bouchard, M., Laforest, F., Vandelac, L., Bellinger, D., and Mergler, D. (2006) Hair Manganese and Hyperactive Behaviors: Pilot Study of School-Age Children Exposed through Tap Water, *Environmental Health Perspectives 115*, 122-127.

3. Bowler, R. M., Roels, H. a, Nakagawa, S., Drezgic, M., Diamond, E., Park, R., Koller, W., Bowler, R. P., Mergler, D., Bouchard, M., Smith, D., Gwiazda, R., and Doty, R. L. (2007) Dose-effect relationships between manganese exposure and neurological, neuropsychological and pulmonary function in confined space bridge welders., *Occupational and environmental medicine 64*, 167-77.

4. Buckley, R. A, and Dreosti, I. E. (1984) Radioisotopic studies concerning the efficacy of standard washing procedures for the cleansing of hair before zinc analysis., *The American journal of clinical nutrition 40*, 840-6.

5. Cook, D.G., Fahn, S., Brait, K. A. (1974) Chronic manganese intoxication. *Arch Neurol 30,* 59–64.

6. Dietz, M. C., Ihrig, a, Wrazidlo, W., Bader, M., Jansen, O., and Triebig, G. (2001) Results of magnetic resonance imaging in long-term manganese dioxide-exposed workers., *Environmental research 85*, 37-40.

7. Ericson, J. E., Crinella, F. M., Clarke-Stewart, K. A., Allhusen, V. D., Chan, T., and Robertson, R. T. (2007) Prenatal manganese levels linked to childhood behavioral disinhibition., *Neurotoxicology and teratology 29*, 181-7.

8. Flegal, A. R., and Smith, D. R. (1992) Current needs for increased accuracy and precision in measurements of low levels of lead in blood., *Environmental research 58*, 125-33.

9. Fraústo da Silva, J. J. R. and Williams, R. J. P. *The Biological Chemistry of the Elements.* Oxford: Clarendon Press, 1993. Print.

10. Harkey, M. R. (1993) Anatomy and physiology of hair., *Forensic Science International 63*, 9-18.

11. Haynes, E. N., Heckel, P., Ryan, P., Roda, S., Leung, K., Sebastian, K., and Succop, P. (2011) OHIO : A PILOT STUDY, *Neurotoxicology 31*, 468-474.

12. Henn, B. C., Ettinger, A. S., Schwartz, J., Tellez-Rojo, M. M., Lamadrid-Figueroa, H., Hernandez-Avila, M., Schnaas, L., Amarasiriwardena, C., Bellinger, D. C., Hu, H., and Wright, R. O. (2010) Early Postnatal Blood Manganese Levels and Children's Neurodevelopment, *Epidemiology 21*, 433-439.

13. Hernandez-Bonilla, D., Schilmann, a, Montes, S., Rodriguez-Agudelo, Y., Rodriguez-Dozal, S., Solis-Vivanco, R., Rios, C., and Riojas-Rodriguez, H. (2011) Environmental exposure to manganese and motor function of children in Mexico., *Neurotoxicology*.

14. Hill, A. B. (1965) The Environment and Disease: Association or Causation?, *Proceedings of the Royal Society of Medicine 58,* 295-300.

15. Huang, C.C. (2007) Parkinsonism induced by chronic manganese intoxication—an experience in Taiwan. *Chang Gung Med J 30,* 385–395.

16. Hudnell, H. K. (1999) Effects from environmental Mn exposures: A review of the evidence from non-occupational exposure studies., *Neurotoxicology 20,* 379-398.

17. Josephs, K. A, Ahlskog, J. E., Klos, K.J., Kumar, N., Fealey, R.D., Trenerry, M. R. (2005) Neurologic manifestations in welders with pallidal MRI T1 hyperintensity. *Neurology 64*, 2033–2039.

18. Kempson, I. M., Skinner, W. M., and Kirkbride, K. P. (2006) Advanced analysis of metal distributions in human hair., *Environmental science & technology 40*, 3423-8.

19. Kempson, I. M. and Skinner, W. M. (2005) ToF-SIMS analysis of elemental distributions in human hair., *Science of the Total Environment 338*, 213-227.

20. Kenangil, G., Ertan, S., Sayilir, I., Ozekmekçi, S. (2006) Progressive motor syndrome in a welder with pallidal T1 hyperintensity on MRI: a two year follow-up. *Mov Disord 21,* 2197–2200.

21. Lee, J.W. (2000) Manganese intoxication*. Arch Neurol 57*, 597–599.

22. Li, G. J., Zhang, L.-L., Lu, L., Wu, P., and Zheng, W. (2004) Occupational Exposure to Welding Fume among Welders: Alterations of Manganese, Iron, Zinc, Copper, and Lead in Body Fluids and the Oxidative Stress Status, Journal of *Occupational and Environmental Medicine 46*, 241-248.

23. Loranger, S., and Zayed, J. (1995) Environmental and occupational exposure to manganese: a multimedia assessment., *International archives of occupational and environmental health 67*, 101-10.

24. Lucchini, R. G., Guazzetti, S., Zoni, S., Donna, F., Peter, S., Zacco, A., Salmistraro, M., Bontempi, E., Zimmerman, N. J., and Smith, D. R. (2012) NeuroToxicology Tremor , olfactory and motor changes in Italian adolescents exposed to historical ferro-manganese emission, *Neurotoxicology*, 1-10.

25. Mena, I., Marin, O., Fuenzalida, S., Cotzias, G.C. (1967) Chronic manganese poisoning. Clinical picture and manganese turn-over. *Neurology 17*, 128–136.

26. Menezes-filho, J. A., Paes, C. R., Pontes, Â. M. D. C., Moreira, J. C., Sarcinelli, N., and Mergler, D. (2010) High levels of hair manganese in children living in the vicinity of a ferro-manganese alloy production, *Neurotoxicology 30*, 1207-1213.

27. Menezes-Filho, J. A., Novaes, C. D. O., Moreira, J. C., Sarcinelli, P. N., and Mergler, D. (2010) Elevated manganese and cognitive performance in school-aged children and their mothers., *Environmental research.*

28. Menezes-Filho, J. a, Bouchard, M., Sarcinelli, P. D. N., and Moreira, J. C. (2009) Manganese exposure and the neuropsychological effect on children and adolescents: a review., *Revista panamericana de salud publica 26*, 541-8.

29. Mergler, D. and Baldwin, M. (1997) Early manifestation of manganese neurotoxicity in humans: an update, *Environmental Research 73*, 92-100.

30. Mikasa, H., Suzuki, Y., and Fujii, N. (1988) Adsorption and Eiution of Metals on Hair, *Biological Trace Element Research 16*, 59-66.

31. Riojas-Rodríguez, H., Solís-Vivanco, R., Schilmann, A., Montes, S., Rodríguez, S., Ríos, C., and Rodríguez-Agudelo, Y. (2010) Intellectual Function in Mexican Children Living in a Mining Area and Environmentally Exposed to Manganese, *Environmental Health Perspectives 118*, 1465-1470.

32. Sakai, T., Wariishi, M., and Nishiyama, K. (2000) Changes in trace element concentrations in hair of growing children., *Biological trace element research 77*, 43-51.

33. Schroeter, J. D., Nong, A., Yoon, M., Taylor, M. D., Dorman, D. C., Andersen, M. E., and Clewell, H. J. (2011) Analysis of manganese tracer kinetics and target tissue dosimetry in monkeys and humans with multi-route physiologically based pharmacokinetic models., *Toxicological sciences 120*, 481-98.

34. "Secondary Drinking Water Regulations: Guidance for Nuisance Chemicals." *United States Environmental Protection Agency*. Web. 30 May 2012. <http://water.epa.gov/drink/contaminants/secondarystandards.cfm>.

35. Smith, D., Gwiazda, R., Bowler, R., Roels, H., Park, R., Taicher, C., and Lucchini, R. (2007) Biomarkers of Mn Exposure in Humans, *American Journal of Industrial Medicine* 1-11.

36. Smith, D. R., Osterloh, J. D., Niemeyer, S., and Flegal, a R. (1992) Stable isotope labeling of lead compartments in rats with ultralow lead concentrations., *Environmental research 57*, 190-207.

37. Stauber, J. L., Florence, T. M., and Mail, P. (1989) Manganese in Scalp Hair: Problems of Exogenous Manganese and Implications for Manganese Monitoring in Groote Eylandt Aboringines, *Science 83,* 85-98.

38. Wang, D., Du, X., and Zheng, W. (2008) Alteration of saliva and serum concentrations of manganese, copper, zinc, cadmium and lead among career welders, *Toxicology Letters 176*, 40-47.

39. Wasserman, G. a., Liu, X., Parvez, F., Ahsan, H., Levy, D., Factor-Litvak, P., Kline, J., Geen, A. V., Slavkovich, V., LoIacono, N. J., Cheng, Z., Zheng, Y., and Graziano, J. H. (2005) Water Manganese Exposure and Children's Intellectual Function in Araihazar, Bangladesh, *Environmental Health Perspectives* 124-129.

40. Wright, R. O., Amarasiriwardena, C., Woolf, A. D., Jim, R., and Bellinger, D. C. (2006) Neuropsychological correlates of hair arsenic, manganese, and cadmium levels in school-age children residing near a hazardous waste site., *Neurotoxicology 27*, 210-6.