HHS Public Access

Author manuscript

*Muscle Nerve*. Author manuscript; available in PMC 2019 July 04.

# Toenail mercury levels are associated with amyotrophic lateral sclerosis (ALS) risk

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## Abstract

**Introduction—**Mercury is a neurotoxic metal that is potentially a risk factor for amyotrophic lateral sclerosis (ALS). Consumption of methylmercury contaminated fish is the primary source of U.S. population exposure to mercury.

**Methods—**We used inductively coupled plasma mass spectrometry to measure levels of mercury in toenail samples from ALS patients (n=46) and from controls (n=66), as a biomarker of mercury exposure.

**Results—**ALS patients had higher toenail mercury levels (OR 2.49 95%CI 1.18–5.80, *P*=0.024) compared to controls, adjusted for age and gender. We also estimated the amount of mercury consumed from finfish and shellfish and found toenail mercury levels elevated overall among ALS patients and controls in the top quartile for consumption (P=0.018).

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**Competing financial interests:** The authors declare they have no actual or potential competing financial interests.

**Ethical publication statement:** We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

**Author Contributions:**
Dr. Andrew - study concept and design, analysis and interpretation, writing manuscript,
Dr. Chen - critical revision of the manuscript for important intellectual content
Dr. Caller - critical revision of the manuscript for important intellectual content
Dr. Tandan - acquisition of data
Ms. Henegan - acquisition of data
Dr. Jackson - acquisition of data
Dr. Hall - acquisition of data
Dr. Bradley - critical revision of the manuscript for important intellectual content
Dr. Stommel - study concept and design

**Discussion—**Biomarker data show ALS associated with increased with mercury levels, which were related to estimated methylmercury intake via fish. Replication of these associations in additional populations is warranted.

### Keywords

case-control studies; amyotrophic lateral sclerosis; toxicology; neuromuscular disease; mercury; methylmercury; toenail; fish

## Introduction

Causes of amyotrophic lateral sclerosis (ALS) remain largely unknown [1]. Several case-reports of mercury poisoning have demonstrated convincing ALS-like clinical symptoms, leading authors to postulate a causal relationship [2–4]. Methylmercury exposure via fish consumption has also been suggested as an ALS risk factor [5].

Methylmercury is an environmental neurotoxicant associated with a wide range of neurocognitive and behavioral outcomes [6,7]. Methylmercury bioaccumulates through aquatic food chains, binds to proteins and amino acids, and persists in the fish muscle tissue consumed by humans despite cooking [6]. Approximately 95% of the methylmercury in fish fillets is absorbed when eaten [8]. Higher hair mercury level was associated with impaired fine motor speed and dexterity in adults from a Brazilian fishing community [9].

Methylmercury is the main contributor to the mercury levels measured in hair, nails and blood [10], and toenails are considered particularly good biomarkers for mercury exposure from fish consumption [7,11]. Moreover, mercury in toenails is considered to be a stable biomarker of exposure over time [12], and is a biomarker of exposure occurring 6–9 months prior to sampling [13]. Toenail levels of mercury are also highly correlated with chronologically matched hair levels with a slope of 2.79 for hair vs. toenail levels [14]. Levels of total mercury in toenail samples of 28 individuals in an autopsy study were significantly correlated with the level of methylmercury in the brain, as well as with the level of methylmercury in the blood [15].

The objective of this study was to test the *a priori* hypothesis that mercury is associated with ALS risk.

## Methods

Participants were enrolled through the Department of Neurology at Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire and the Department of Neurological Sciences at the University of Vermont Medical Center, Burlington, Vermont. The eligible ALS patients were newly diagnosed cases with either probable or definite ALS according to the Awaji criteria [16]. To decrease the influence of recall bias, we selected a control group consisting of neurology clinic patients with other idiopathic diseases that would prompt them to undertake a similar search for factors in their prior life that might have caused their disease. Diagnoses included multiple sclerosis, brain and spinal cord tumors, adult-onset epilepsy, and non-familial neuromuscular diseases, such as idiopathic peripheral neuropathies. Patients with

neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases were excluded from participation as controls.

Participants were approached by study staff in the clinic, and were required to be at least 21 years of age and residents of New England or the bordering New York region at the time of enrollment (ALS cases June 2009 – August 2016, controls August 2010 – August 2016). Of the potential participants approached about the study, the questionnaire completion rate was 90% for ALS cases and 52% for controls. Reasons for non-participation in the ALS cohort were effort required (33%), and reluctance to share personal information (67%), while controls cited time involved (33%), reluctance to share information about sickness (32%), reluctance to share personal information (18%), and lack of monetary benefit (17%). Informed consent was obtained from participants and all study procedures were approved by the Committee for Protection of Human Subjects at Dartmouth College and the Committee on Human Research in the Medical Sciences, University of Vermont.

In 2014, research staff began a biorepository, collecting toenail clippings from ALS patients and clinic-based control patients (N=46 ALS cases, N=66 controls). The trace metal analyses were conducted by the Dartmouth Trace Element Analysis Core Facility. For all toenail samples, any visible dirt was removed from the nails, and they were transferred to a 7ml polyethylene vial, 2 ml of acetone was added and the vessel placed in an ultrasonic bath for 20 min, following a wash with 2 ml 1% solution of Triton X-100 in an ultrasonic bath for 20 minutes, after which the toenail sample was washed 5 times with deionized water and dried in a clean dry box. This washing procedure removed all external contamination (nail polish, dirt etc.) without extracting metals from inside the nails.

The washed toenail clippings were then acid-digested with $HNO_3$ using a MARSxpress microwave digestion unit (CEM, Mathews, NC). Trace metals were analyzed by inductively coupled plasma mass spectrometry (ICP-MS, 7700x, Agilent, Santa Clara, CA) following EPA 6020 protocol. Several metals that can be reliably assessed in toenails were measured simultaneously: total mercury, manganese, zinc, arsenic, selenium, copper, cadmium, and lead. The ICP-MS was calibrated using NIST traceable single and multi-element standards containing the analytes of interest. Multi-point calibration curves ($n \geq 5$) were constructed for each analyte with correlation coefficient criteria > 0.995. The calibration was followed by an Initial Calibration Blank (IBC) and an Initial Calibration Verification (ICV). Continuing Calibration Verifications (CCV) were made from a second source of single and multi-element standards and contained all the analytes at concentrations at or below the mid-point calibration range. Acceptance criteria for the ICV and CCV were ± 10%. The CCV was run after every 10 samples. Japanese human hair reference NIES #13 certified at 4.42 μg/g Hg was run as a reference material and average recovery for mercury was 92 ± 9% ($n = 9$).

We sought to identify lifestyle factors and behaviors, including fish consumption, that were associated with high toenail mercury levels using a subset of ALS patients and control subjects who had both toenail analyses and completed life-style questionnaires (26 ALS patients and 28 controls. Fish consumption questions asked about specific dietary patterns in the 10 years prior to diagnosis. Regular consumption of fish and shellfish was also assessed

by asking: “Prior to the Diagnosis Date, did you eat fish or shellfish more than 15 times per year?” to indicate meals consumed more than monthly. Participants were asked to “indicate how often you ate certain types of fish or shellfish” using a chart with finfish groups related to trophic levels (catfish / trout / anchovies / salmon / sardine / grouper), (cod / snapper / perch / halibut / canned tuna / mahi mahi), (tuna / shark / swordfish / mackerel / marlin / tilefish / sea bass). Non-finfish seafood was grouped as: (mussels / clams / oysters), (shrimp / scallops), and (lobsters / crabs). We then used this information to estimate annual methylmercury exposure among fish / seafood consumers by cross-referencing self-reported consumption of fish of each species or trophic category with the corresponding species-specific fish fillet mean methylmercury concentrations based on U.S. market mean mercury levels, multiplied by the frequency of consumption [17].

We evaluated the association with toenail mercury level using ALS case-control status as the outcome in a logistic regression analysis. We applied log transformation of the toenail metal values in order to comply with the normal distribution assumption. We used the questionnaire to assess self-reported job / hobby mercury use, as well as estimated mercury exposure via fish-consumption, in relation to toenail mercury levels (log 10 ug/g) using a generalized linear model. P-values <0.05 were considered statistically significant. These analyses were performed using R: A Language and Environment for Statistical Computing, version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria).

## Results

The characteristics of the 112 patients providing toenail bio-specimens are shown in Table 1. The ALS cases were more likely to be male (65%), and the mean age was 61 years old. Figure 1 and Table 2 show the higher measured levels of total mercury in the toenail samples of the ALS cases, compared to controls. The effect remained significant in a multivariable logistic regression model adjusted for age and gender, with an ALS Odds Ratio ~2.5. We also evaluated the relationship between ALS and toenail mercury level in the context of other metals that we assessed concurrently in the toenail specimens: manganese, zinc, arsenic, selenium, copper, cadmium, and lead (Table 2). The toenail mercury association was not substantially modified by the inclusion of these other metals in the model and remained statistically significant. Zinc levels were also higher among ALS patients in comparison to controls, however this was not our *a priori* hypothesis.

We cross-referenced the questionnaire data for toenail bio-repository participants in order to identify lifestyle factors associated with toenail mercury levels in the subset of subjects in whom we had both toenail and questionnaire data. The demographics of the subset of 54 participants with both questionnaire data and toenail samples are similar to the larger toenail bio-repository (Table 1). Toenail mercury levels (log ug/g) did not differ significantly by gender (P=0.47), age (P=0.41), or smoking (former P=0.48, current P=0.68). Few participants (7%) reported having job or hobby-related mercury exposure, which was not related to higher toenail mercury levels (Table 3). Toenail mercury levels were higher among participants consuming fish or seafood more than once a month (Table 3).

Figure 2 shows that the measured level of toenail mercury was significantly associated with our estimate of the annual mercury consumption from fish. Adjusted for age, gender, and smoking, the participants in the top quartile for estimated annual mercury consumption from fish still had significantly higher measured toenail mercury levels overall (Table 3), and within the ALS case group (adjusted OR 1.72 95%CI 1.09–2.73, P=0.034), but not within the control group (P=0.91).

## Discussion

The relationship between mercury exposure and ALS has been inconsistent among several prior epidemiologic studies, however this past work has focused exclusively on inorganic mercury exposure. There have been many studies showing the neurological and developmental impacts associated with mercury via fish consumption, which results in exposure to the more bioavailable and toxic methylmercury species [18,6,7]. We observed a statistically significant 2.5-fold increase in toenail mercury levels in ALS patients in comparison to controls.

Toenail mercury levels were not elevated among the few reports of job or hobby-related mercury exposure, which typically involves inorganic mercury exposure [10]. Consistent with our prior ALS questionnaire study [19], self-reported mercury exposure was not significantly related to ALS risk in 66 patients vs. 66 controls [20]. No cases of ALS were identified in an occupational cohort of 83 workers with exposure to mercury vapor through mining [21]. In contrast, a case-control study of 77 patients vs. 88 controls in Italy did find increased risk of ALS associated with self-reported mercury exposure [22].

There is evidence of mercury within the brains of patients with ALS and other neurodegenerative diseases. Locus coeruleus and motor neurons had higher levels of silver nitrate autometallography staining (reflecting mercury or bismuth presence) in patients with motor neuron disease, compared to controls [23]. Alzheimer’s disease patients also had a higher level of mercury in the brain microsomes, compared with controls [24]. Residential location analysis identified a significant association between Parkinson’s disease and airborne releases of mercury among never-smokers (Hazard Ratio 1.68 95%CI 1.11–2.25) [25].

Methylmercury is known to bioaccumulate to high levels in the fish fillets of certain species that are consumed regularly [26,27,18]. Overall consumers of fish that are higher on the food chain have higher concentrations of Hg in their blood [28,29]. The fish consuming study participants, particularly those in the top quartile for estimated annual methylmercury intake via fish, had significantly higher measured toenail mercury levels. Fish consumption increased risk of ALS in a multivariate model of dietary factors in Koreans [30]. A prior case-control study in Wisconsin related frequent consumption of fish caught in Lake Michigan with increased risk of ALS [31].

Some species of fish also contain omega-3 polyunsaturated fatty acids (PUFA), which were associated with numerous health benefits including lower risk of ALS in dietary studies of prospective cohorts [32]. For example, fish consumers who eat mostly salmon have higher

blood levels of omega-3 PUFA [28]. Thus, the choice of the type of fish or shellfish consumed appears to be associated with the risk of developing ALS.

In addition to methylmercury, fish may contain other environmental contaminants that could be related to ALS, including polychlorinated biphenyls (PCBs) [33], and cyanobacterial toxins [34]. Mercury is a much more ubiquitous fish contaminant compared to organic contaminants, with 81% of fish consumption advisories nationally established at least in part for mercury [35].

The molecular mechanism for mercury in neurodegenerative disease remains unknown. Mercury uptake at motor nerve terminals in the muscle and retrograde axonal transport to the cell bodies is thought to deposit mercury in the spinal motor neurons, brainstem motor nuclei, and cerebral cortex when mice are dosed with $HgCl_2$ [36]. Methylmercury activates the mitochondrial permeability transition pore and elevates presynaptic Ca2+, leading to enhanced glutamate release in rat neurons [37]. Methylmercury induced expression of the mitochondrial gene *Cox1* and the oxidative stress response gene *SOD1* in the muscle tissue of fish exposed via their diet [38]. Cysteine-s-methylmercury conjugates can also act as molecular mimics of methionine, interfering with amino acid transporter enzymes, such as glutamine transaminase K and cystathione gamma-lyase [39]. A gene-environment interaction mechanism that has been suggested for ALS is supported by the report that SOD1 mutant mice exposed to methylmercury in the drinking water (1–3 ppm/day) showed early onset hind limb weakness and shorter time to rotarod failure, compared to unexposed or wild-type mice [40]. Our results suggest a need for additional work investigating causality and potential mechanisms for ALS induction by methylmercury.

Limitations of our study include relatively small sample sizes that impair statistical power within certain subgroups. A post-hoc power analysis estimated that we could detect a minimum difference in mean toenail mercury level of 0.095 ug/g using our 46 cases and 66 controls (SD=0.18, power 0.8, alpha 0.05). Detailed information on the dates of exposure were not collected, thus we could not calculate latency. Our analyses of risk factors for ALS were based on comparisons of ALS patients with controls recruited from among patients with idiopathic neurological diseases that, as in ALS, often cause patients to search their memories for possible lifetime exposures that might have caused their disease. We believe that the selection of such control subjects and the increased levels of mercury in biosamples make recall bias an unlikely explanation of our findings. Although some of the diagnoses of the clinic-based controls could have risk-factors in common with ALS, such relationships would be expected to bias the results towards the null. Omission of subsets of the clinic-controls e.g. those with epilepsy, or those with neuropathy, did not materially affect the increase in risk of ALS associated with toenail mercury. Toenail levels are representative of metal exposures occurring >6 months prior to sampling, reducing the probability of reverse causation artifacts [13]. The *a priori* hypothesized association between toenail mercury and ALS risk was maintained in a composite model containing other metals assessed in toenails.

Biomarker data and fish consumption frequency data suggest that mercury exposure is associated with ALS. As a cautionary note, prior work on chelation therapy with dimercaptosuccinic acid (DMSA) has not shown clinical benefit for ALS patients, and use of

sodium rather ethylenediamine tetraacetic acid (EDTA) has led to patient mortality [41]. Participants with elevated toenail mercury levels had higher estimated methylmercury intake from fish consumption. Given the neuroprotective chemicals found in certain species [32], the choice of fish and shellfish consumed by individuals may have health-related implications. Our results need confirmation in other large cohorts of ALS patients, including those with various familial mutations, as well as experimental work to demonstrate whether these statistical associations are causal in nature.

## Acknowledgments

We would like to thank the study participants and our sources of funding: ALS Association grants SC5181 and 15-IIP-213; Centers for Disease Control (CDC) / Agency for Toxic Substances and Disease Registry (ATSDR) contract 200-2014-59046; and the Diamond Endowment. The Dartmouth Clinical and Translational Science Institute, under award number UL1TR001086 from the National Center for Advancing Translational Sciences (NCATS) of the National Institutes of Health (NIH). The National Institute of Environmental Health Sciences of the National Institutes of Health under Award Number P42ES007373. We thank Wenyan Zhao, PhD for her statistical review.

## Abbreviations

| | |
|---|---|
| **ALS** | amyotrophic lateral sclerosis |
| **CI** | confidence interval |
| **Hg** | mercury |
| **OR** | odds ratio |
| **SD** | standard deviation |

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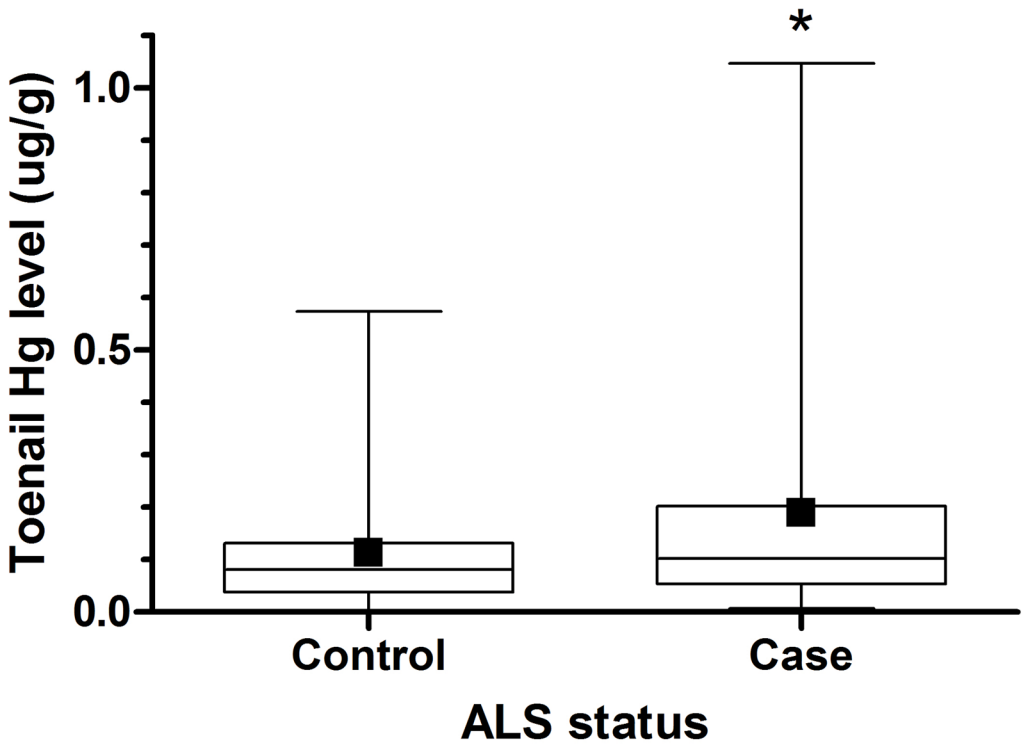


**Figure 1. Higher mercury levels in toenails of ALS cases vs. controls**

The graph shows the measured toenail mercury level on the y-axis according to case-control status (■ = mean ug/g, box represents the 95%CI of the mean, whiskers show the min. to max. range). The ALS cases had significantly higher toenail mercury levels (mean 0.19 95%CI 0.12–0.26 ug/g; maximum 1.05 ug/g), compared to the controls (mean 0.11 95%CI 0.084–0.14 ug/g; maximum 0.57 ug/g) (* t-test P-value=0.022 on log10 ug/g). The effect remained significant in a multivariable logistic regression model adjusted for age and gender, with an ALS Odds Ratio (OR) of 2.49 95%CI 1.18–5.80 for a ten-fold elevation in toenail mercury level (log10 ug/g) ($P$=0.024).

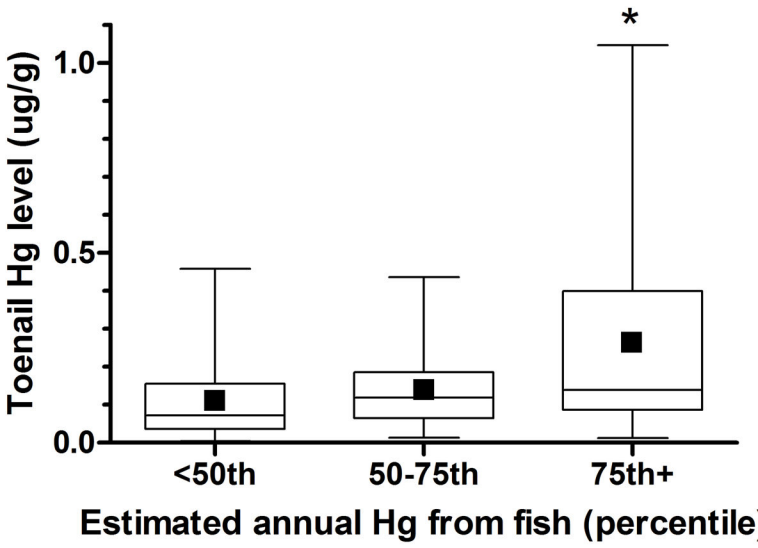


**Figure 2. Higher toenail mercury levels associated with estimated annual mercury consumption from fish**

The graph shows the measured toenail mercury level on the y-axis according to the percentile of estimated annual mercury consumed from fish ((■ = mean ug/g, box represents the 95%CI of the mean, whiskers show the min. to max. range). The toenail mercury levels for annual consumption in the 50–75$^{th}$ percentile (mean 0.14 95%CI 0.074–0.21 ug/g) did not differ significantly from those below the median (mean 0.11 95%CI 0.064–0.15 ug/g) (univariate P=0.25). Participants in the top quartile (upper 75$^{th}$ percentile) for estimated annual mercury consumed from fish had significantly higher measured toenail mercury levels (mean 0.27 95%CI 0.087–0.44 ug/g) compared to participants below the median (univariate P=0.035).

**Table 1**

Population characteristics comparing controls and ALS cases by gender and age.

| | | Toenail biorepository | | | Toenail subset with questionnaires | | |
|---|---|---|---|---|---|---|---|
| | | Clinic controls | ALS Cases | P-value* | Clinic controls | ALS Cases | P-value* |
| **Characteristic** | | **N=66 (%)** | **N=46 (%)** | | **N=28 (%)** | **N=26 (%)** | |
| Gender | Female | 28(42) | 16(35) | | 14(50) | 9(35) | |
| | Male | 38(58) | 30(65) | 0.42 | 14(50) | 17(65) | 0.25 |
| Age | mean±SD | 61.74±11.75 | 61.31±10.86 | 0.72 | 61.39±9.23 | 63.50±9.96 | 0.42 |

*Chi-square or t-test.

Abbreviation: Standard deviation (SD)

**Table 2**

Multivariable model of toenail mercury and ALS status, adjusted for other metals.

| | Clinic controls | | ALS cases | | |
|---|---|---|---|---|---|
| | **N=66 mean** | **95%CI of mean** | **N=46 mean** | **95%CI of mean** | **Multivariable P-value*** |
| Mercury level (ug/g) | 0.11 | 0.12–0.26 | 0.19 | 0.084–0.14 | 0.044 |
| Age | 61.74 | 58.18–64.00 | 61.31 | 58.71–64.99 | 0.89 |
| Male gender | n=38 (58%) | | n=30 (65%) | | 0.078 |
| Zinc level (ug/g) | 99.07 | 93.23–104.9 | 112.74 | 93.08–132.4 | 0.039 |
| Manganese level (ug/g) | 0.57 | 0.29–0.85 | 0.49 | 0.056–0.84 | 0.1 |
| Arsenic level (ug/g) | 0.064 | 0.043–0.085 | 0.089 | 0.034–0.14 | 0.26 |
| Copper level (ug/g) | 4.38 | 3.35–5.41 | 4.04 | 3.20–4.87 | 0.36 |
| Cadmium level (ug/g) | 0.009 | 0.0051–0.013 | 0.0061 | 0.0039–0.0082 | 0.36 |
| Lead level (ug/g) | 0.33 | 0.061–0.60 | 0.2 | 0.070–0.33 | 0.87 |
| Selenium level (ug/g) | 0.88 | 0.84–0.91 | 0.96 | 0.78–1.13 | 0.93 |

*Adjusted model includes: age, gender, log10 ug/g mercury, zinc, manganese, arsenic, copper, cadmium, lead, selenium.

Abbreviation: 95% confidence interval (95%CI)

**Table 3**

Assessment of toenail mercury level by exposure source.

| | | | | Toenail Hg (ug/g) | | Multivariable analysis | |
|---|---|---|---|---|---|---|---|
| | | N=54 | % | mean | 95%CI of mean | P-value* | P-value** |
| Self-reported job/hobby mercury exposure | No | 40 | 93% | 0.2 | 0.14–0.27 | | |
| | Yes | 3 | 7% | 0.059 | −0.069–0.19 | 0.095 | 0.12 |
| Eat fish at least monthly | No | 23 | 43% | 0.11 | 0.057–0.16 | | |
| | Yes | 31 | 57% | 0.21 | 0.12–0.29 | 0.067 | 0.021 |
| | percentile: | | | | | | |
| Estimated annual mercury from fish consumption | <50th | 26 | 50% | 0.11 | 0.064–0.15 | | |
| | 50–75th | 13 | 25% | 0.14 | 0.074–0.21 | 0.42 | 0.33 |
| | 75th + | 13 | 25% | 0.27 | 0.087–0.44 | 0.052 | 0.018 |

Log10 ug/g mercury model adjusted for *age, *gender, and **smoking.

Abbreviations: 95% confidence interval (95%CI)