A Gene-environment Study of Metallothionein Single Nucleotide Polymorphisms, Mercury Biomarker Levels and Peripheral Nerve Function

by

Yi Wang

**A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Environmental Health Sciences)
in The University of Michigan
2011**

Doctoral Committee:

**Professor Alfred Franzblau, Chair
Professor Robert A. Werner
Assistant Professor Niladri Basu
Assistant Professor Brenda Wilson Gillespie**

© 2011 Yi Wang
All Rights Reserved

To

My Grandfather

Yongda Chen,

My Father

Yong Wang,

My Mother

Lei Chen,

And

Other Family Members

In Recollection

of My Growth Path

Acknowledgements

I thank everyone that has given me enormous help and encouragement towards the completion of my doctoral dissertation.

Without the help and guidance of my advisor, Dr. Alfred Franzblau, I would not have completed it within the given timeframe.

Dr. Niladri Basu, principle investigator of the project, has provided me with great scholarly guidance and much-needed tips along the way.

Ms. Jaclyn Goodrich, Dr. Basu's doctoral student I closely worked with on the project, has been very helpful and kind with my requests for laboratory-related activities. Without Jaclyn, my path towards completion could have been much more challenging and less fruitful.

I also thank for the statistical guidance provided by Dr. Brenda Gillespie and the clinical guidance offered by Dr. Robert A. Werner.

I appreciate the help I have received from Dr. Howard Hu and Dr. Marie O'Neill when I was in a difficult situation.

I appreciate the efforts the field team has put in during the two Michigan Dental conventions. Without the high quality of the data collected during those conventions, it would have been much more difficult to move forward in the doctoral program.

Table of Contents

Dedication……………………………………………………………………………..…ii
Acknowledgements……………………………………………….…………….iii
List of Figures…………………………………………………………………viii
List of Tables…………………………………………………………….…..….ix
List of Abbreviations……………………………………………………………xi
Abstract……………………………………………………………………………xii
Chapter 1 Introduction……………………………………………………………1
1. Forms and Exposure Routes of Mercury and its Biological Monitoring Indicators……………………………………………………………...…1
2. High-level Exposure to Methylmercury and Elemental Mercury and Peripheral Nerve Function……………………………………………..5
   2.1. Methylmercury………………………………………………….…..5
   2.2. Elemental Mercury………………………………………………………6
3. Low-level Exposure to Methylmercury and Elemental Mercury and Peripheral Nerve Function……………………………………………..8
   3.1. Methylmercury……………………………………………………………8
   3.2. Elemental Mercury…………………………………………………...…..9
4. Influence of Genetic Polymorphisms on Mercury Exposure………………12
   4.1. Metallothionein……………………………………………………………14
5. Nerve Conduction Test and Mercury……………………………………..…16
6. Concordance between Symptoms, Monofilament Testing Results and Sensory Nerve Conduction Function in the Lower Extremities…….……..18
7. Summary……………………………………………………………………..19
Tables…………………………………………………………………………21
Chapter 2 Modifying Effects of Metallothionein Single Nucleotide Polymorphisms on the Association between Elemental Hg Exposure and Urinary Mercury Levels…………………………………………………………………………...33
1. Introduction………………………………………………..………………33
2. Materials and Methods…………………………..…………………………36
   2.1. Collection of Exposure Variables………..……………………………36
      2.1.1. Questionnaires………………………………………………………..36
         2.1.1.1. Elemental Mercury Exposure: Occupational and Personal Exposures from Amalgam………………………….36
         2.1.1.2. Preexisting Kidney Disease…………….…………36
         2.1.1.3. Covariates……………………………………………37
         2.1.1.4. Demographics………………………………………….37
   2.2. Collection and Analysis of Urine Specimen……………………………37
      2.2.1. Urine Specimen Collection……………….……………………………37

2.2.2. Urine Mercury Analysis…………………………………………………..37
2.2.2.1. Total Mercury Measurement Accuracy……………...38
2.2.2.1.1. 2009 Samples……………………………………………38
2.2.2.1.2. 2010 Samples……………………………………………39
2.2.2.2. Total Mercury Measurement Precision…………..…39
2.2.2.2.1. 2009 Samples……………………………………………39
2.2.2.2.2. 2010 Samples……………………………………………40
2.2.2.3. Total Mercury Precision from Samples……………..40
2.2.2.3.1. 2009 Samples……………..……………………………40
2.2.2.3.2. 2010 Samples……………………………………………40
2.2.2.4. Total Mercury Detection Limit………………………..41
2.2.2.4.1. 2009 Samples……………………………………...……41
2.2.2.4.2. 2010 Samples……………………………………………41
2.3. Collection of Analysis of DNA Samples…………………...………41
2.3.1. Selection of Metallothionein SNPs………………………………42
2.3.2. DNA Genotyping……………………..…………………………………42
3. Statistical Methods……………………………………………...…………..44
4. Results……………………………………………………………………………45
4.1. Demographics……………………………………………………………..45
4.2. Level of Urine Biomarker…………………………………………………45
4.3. Level of Exposure to Elemental Hg and Other Covariates………..…45
4.4. Associations between Elemental Hg Exposure and Level of Urinary Mercury……………………………………………………..…………..46
4.5. Associations between SNPs and Levels of Biomarkers (No Adjustment for Exposure to Elemental Hg)………………………….. 47
4.6. Results of Urine Multivariate Linear Regression Models……..………47
5. Discussion. …………………………………………………….……………48
Figures and Tables. ……………………………………………………….....54
Chapter 3 Modifying Effects of Metallothionein Single Nucleotide Polymorphisms on the Association between Methylmercury Exposure and Hair Mercury Levels……………………………………………………………………………..66
1. Introduction……………………………………………………………..…66
2. Materials and Methods……………………………………………..……68
2.1. Collection of Exposure Variables. ………………………………………69
2.1.1. Questionnaires…………………………………………………….69
2.1.1.1. Methylmercury Exposure: Dietary Fish Consumption…………………………………………………69
2.1.1.1.1. Quantification of Daily Methylmercury Intake from Fish Consumption………………………………………70
2.1.1.2. Preexisting Diseases……………………………………70
2.1.1.3. Covariates………………………………………………70
2.1.1.4. Demographics.……………………………………...… 71
2.2. Collection and Analysis of Hair Specimen…………………………..…71
2.2.1. Hair Specimen Collection………………………………………71
2.2.2. Hair Mercury Analysis…………………………………………..72
2.2.2.1. Total Mercury Measurement Accuracy…………..…73

2.2.2.1.1. 2009 Samples……………………………………………73
2.2.2.1.2. 2010 Samples……………………………………………73
2.2.2.2. Total Mercury Measurement Precision……………...74
2.2.2.2.1. 2009 Samples……………………………………………74
2.2.2.2.2. 2010 Samples……………………………………………74
2.2.2.3. Total Mercury Precision from Samples……………..75
2.2.2.3.1. 2009 Samples……………………………………………75
2.2.2.3.2. 2010 Samples……………………………………………75
2.2.2.4. Total Mercury Detection Limit………….……………75
2.2.2.4.1. 2009 Samples……………………………………………75
2.2.2.4.2. 2010 Samples……………………………………………75
2.3. Collection of Analysis of DNA Samples………………………………..76
2.3.1. Selection of Metallothionein SNPs………………………………76
2.3.2. DNA Genotyping…………………………………………………77
3. Statistical Methods……………………………………………………….78
4. Results……………………………………………………………………79
4.1. Demographics…………………………………………………………79
4.2. Level of Hair Biomarker………………………………………………80
4.3. Daily Methylmercury Intake from Fish…………………………………80
4.4. Associations between Methylmercury Exposure and Level of Hair Biomarker………………………………………………………………80
4.5. Associations between SNPs and Levels of Biomarkers (No Adjustment for Methylmercury Exposure)……………………………..81
4.6. Results of Hair Multivariate Linear Regression Models………………81
5. Discussion………………………………………………………………..82
Figures and Tables...………………………………………………………88
Appendix……………………………………………………………………..94
Chapter 4 Nerve Conduction Function, Mercury Levels in Urine and Hair, and Single Nucleotide Polymorphisms in Metabolism-related Genes………………..98
1. Introduction……………………………………………………………..98
2. Materials and Methods…………………………………………………99
2.1. Collection of Exposure Variables……………………………………..100
2.2. Collection and Analysis of Urine and Hair Specimens……………...100
2.3. Measurement of Nerve Conduction Function………………………..100
2.4. Hand Diagram…………………………………………………………101
2.5. Collection and Analysis of DNA Samples……………………………102
2.5.1. Selection of Selenoprotein and GSH-related SNPs…………102
3. Exclusion Criteria………………………………………………………103
4. Statistical Methods………………………………………………………103
5. Results……………………………………………………………………105
5.1. Levels of Urine and Hair Biomarkers…………………………………105
5.2. Associations between Nerve Conduction Measurements and Hg Levels in Urine and Hair (No Adjustment for Covariates)……….105
5.3. Associations between Nerve Conduction Measurements and Covariates. ……………………………………………………..…..105

5.4. Associations between Nerve Conduction Measurements and Hg Levels in Urine and Hair (Adjusted for Covariates)………………106
5.5. Results of Stable Multivariate Regression Models Exploring Effect Modification of Selected SNPs (Adjusted for Hg Levels in Urine and Hair and Covariates)……………………………………...106
5.6. Results of Unstable Multivariate Regression Models Exploring Effect Modification of Selected SNPs (Adjusted for Hg Levels in Urine and Hair and Covariates) ……………..……………………………107
6. Discussion…………………………………………………….………...107
Tables………………………………………………………….…………....114
Appendix………………..……………………………………….…………120
Chapter 5 Agreement between Symptom Survey, Monofilament Testing and Electrodiagnostic Findings Consistent with Neuropathy in the Feet……………125
1. Introduction……………..………………………………………...………125
2. Materials and Methods………………..……………………………...…127
2.1. Electrodiagnostic Testing………………………………………………127
2.2. Self-administrated Symptom Questionnaire and Body Diagram…...128
2.3. Monofilament Testing………………..………………………………..129
3. Statistical Methods……………..…………………………………………..130
4. Results……………..…………………...………….……………..131
5. Discussion……………..…………………………………………………133
Figures and Tables……………………...………….…………..…………138
Appendix……………………………………………………………….……147
Chapter 6 Conclusions…………………………………………………..…..150
1. Objectives……………..………….…..…………………………….…150
2. Methods…………….……………..…………………………………..…152
3. Main Results……………..……………..……………………………..…153
4. Discussion/Conclusions…….…………..………………………………..154
5. Future Research……………..………………………………………….…157

List of Figures

Figure 2.1 Relative Positions of Metallothionein SNPs of Interest on Human Chromosome 16q…………………………………………………………………...59
Figure 2.2: Scatter Plot of Ln-transformed Urine Hg against Number of Personal Amalgams Stratified by MT1M (rs2270836) Genotypes…………………………..60
Figure 2.3: Scatter Plot of Ln-transformed Urine Hg against Number of Amalgams Handled Stratified by MT1M (rs2270836) Genotypes………………..61
Figure 3.1: Scatter Plot of Ln-transformed Hair Hg against Estimated Daily Hg Intake from Fish Stratified by MT1M (rs9936741) Genotypes…………………....91
Figure 3.2: Scatter Plot of Ln-transformed Hair Hg against Estimated Daily Hg Intake from Fish Stratified by MT1E (rs708274) Genotypes……………………...92
Figure 3.3: Scatter Plot of Ln-transformed Hair Hg against Estimated Daily Hg Intake from Fish Stratified by MT1A (rs8052394) Genotypes…………………....93
Figure 5.1a: Peak Latency Stratified by MF………………………………………145
Figure 5.1b: Peak Latency Stratified by BDS or Sx &MF………………………..145
Figure 5.2a: Amplitude Stratified by MF…………………………………………...145
Figure 5.2b: Amplitude Stratified by BDS or Sx &MF……………………………145

List of Tables

Table 1.1 Summary of Previous Literatures on Effects of Low-level Elemental Hg Exposures on CNS and PNS Functions……………………………………..…...21
Table 1.2 Summary of Previous Literatures Associating Hg Biomarkers with SNP Genotypes………………………………………………………………………………23
Table 2.1 Demographics…………………………………………………………..….54
Table 2.2 Numbers and Percentages of Subjects with Pre-existing Diseases…………………………………………………………………………..…54
Table 2.3 List of Selected Metallothionein SNPs Genotyped in 2009 and 2010………………………………………………………………………………..…55
Table 2.4 Urine Mercury (μg/l) Results in the MDA Mercury Study Compared with NHANES (2003-2004)…………………………………………………………..….55
Table 2.5 Descriptive Statistics for Exposure Variables of Elemental Hg……......56
Table 2.6 Arithmetic Mean Urine Hg Levels (μg/l) Stratified by Levels of Elemental Hg Exposures from Occupational Practice and Personal Amalgam...56
Table 2.7 Arithmetic Mean Urine Hg Levels (μg/l) Stratified by Genotypes of Selected Metallothionein SNPs…………………………………………………...57
Table 2.8 Coefficients and p Values from Multivariate Linear Regression Models of Natural Log-transformed Urinary Hg Predicted against Exposure Surrogates of Elemental Hg, SNP Genotypes and Exposure-SNP Interactions………………...58
Table 3.1 Hair Mercury (μg/g) in the MDA Study Compared with NHANES (1999-2000)………………………………………………………………………………….88
Table 3.2 Mean Hair Hg Levels (μg/g) Stratified by Levels of Estimated Methylmercury Intake from Dietary Fish Consumption………………………….....88
Table 3.3 Mean Hair Hg Levels (μg/g) Stratified by Genotypes of Selected Metallothionein SNPs……………………………………………..…………..89
Table 3.4 Coefficients and p Values from Multivariate Linear Regression Models of Natural Log-transformed Hair Hg Predicted by Estimated Methylmercury Exposure, SNP Genotypes and Intake-SNP Interactions……………………..…..89
Table 4.1 Mean Amplitude, Onset and Peak Latencies of Median, Ulnar and Sural Nerves Stratified by Low and High Hg Levels in Urine and Hair………...114
Table 4.2 Coefficients and p Values in Base Models of Multivariate Linear Regression Models of Log-transformed Nerve Conduction Function Independently Predicted by Hg Levels in Urine and Hair and Other Covariates……………………………………………………………………….115

Table 4.3 Coefficients and p Values from Stable Multivariate Linear Regression Models (3 out of 504) of Log-transformed Nerve Conduction Function Predicted by Hg Biomarker Levels in Urine or Hair, SNP Genotypes and SNP-biomarker Interactions……………………………………………………………………………116
Table 4.4 Selected Mean Nerve Conduction Measurements Stratified by Genotypes of Selected SNPs……………………………………………………………117
Table 4.5 Coefficients and p Values from Unstable Multivariate Linear Regression Models (9 out of 504) of Log-transformed Nerve Conduction Function Predicted by Hg Biomarker Levels in Urine or Hair, Selected SNP Genotypes and SNP-biomarker Interactions…………………………………………………………...118
Table 5.1 Demographic Characteristics of Subjects……………………………..138
Table 5.2 Neuropathy Definition from Results of Body Diagram Scores and Symptom Questionnaire…………………………………………………………..138
Table 5.3 Prevalence of Subjects with Findings among All Subjects, Diabetic and Non-diabetic Subjects…………………………………………………………….138
Table 5.4 Agreement of Age- and Temperature-adjusted Neuropathy in Feet (Peak Latency>4.1 or 5ms and amplitude<=6μV or 5μV) with Symptoms and Monofilament Test, and Combinations of Symptoms and Monofilament Test, Respectively for All Subjects, Diabetic and Non-diabetic Subjects…………………………………………………………………………...….139
Table 5.5 Agreement of Age- and Temperature-adjusted Neuropathy in Feet (Peak Latency>4.1 or 5ms or amplitude<=6μV or 5 μV) with Symptoms and Monofilament Test, Respectively for All Subjects, Diabetic and Non-diabetic Subjects……………………………………………………………………………140
Table 5.6 Agreement of Age- and Temperature-adjusted Neuropathy in Feet (Peak Latency>4.1 or 5ms) with Symptoms and Monofilament Test, Respectively for All Subjects, Diabetic and Non-diabetic Subjects…………………………………………………………………..………..141
Table 5.7 Agreement of Age-adjusted Neuropathy in Feet (amplitude<=6μV or 5μV) with Symptoms and Monofilament Test, Respectively for All Subjects, Diabetic and Non-diabetic Subjects………………………………………………..142
Table 5.8 Age- and Temperature- adjusted Neuropathy in Feet (Abnormal Peak Latency>4.1 or 5ms;normal:ude<=4.1 or 5ms) Stratified by Various Combinations of Symptoms and Monofilament Test for All Subjects, Diabetic and Non-diabetic Subjects………………………………………………………………………….....143
Table 5.9 Age-adjusted Neuropathy in Feet (abnormal amplitude<=6μV or 5μV;normal: >6μV or 5μV) Stratified by Various Combinations of Symptoms and Monofilament Test for All Subjects, Diabetic and Non-diabetic Subjects……………………………………………………………………..……...144

List of Abbreviations

ALAD	Aminolevulinate Dehydratase
ARE	Antioxidant Responsive Element
ATSDR	Agency for Toxic Substances and Disease Registry
BBB	Blood-brain Barrier
CDC	Center for Disease Control
CEPH	Centre d'Etude du Polymorphisme Humain
CNS	Central Nervous System
DMA	Direct Mercury Analyzer
DOLT	Dogfish Liver Certified Reference Material for Trace Metals
EPA	Environmental Protection Agency
FFQ	Food Frequency Questionnaire
GCL	Glutamate Cysteine Ligase
GIF	Growth Inhibitory Factor
GRE	Glucocorticoid Responsive Element
GST	Glutathione-S-transferase
HFE	Hemochromatosis
HWE	Hardy-Weinberg Equilibrium
MDA	Michigan Dental Association
MRE	Metal Responsive Element
MT	Metallothionein
MTF-1	Metallothionein Transcription Factor-1
NHANES	National Health and Nutrition Examination Survey
PNS	Peripheral Nervous System
RBC	Red Blood Cell
RFLP	Restriction Fragment Length Polymorphism
SRM	Standard Reference Material
TLV	Threhold Limit Value
TMDL	Theoretical Method Detection Limit
UTR	Untranslated Region

Abstract

Mercury (Hg) is a potent neurotoxicant. Recent studies have suggested that several genes that mediate mercury metabolism are polymorphic in humans. We hypothesized that single nucleotide polymorphisms (SNPs) in metallothionein (MT) genes may underlie inter-individual differences in mercury biomarker levels and peripheral nerve function. We also investigated associations of Hg exposure with nerve function and the agreement between screening procedures for neuropathy in the feet. Dental professionals (n=515) were recruited in 2009 and 2010. Samples and measurements included: hair and urine for biomarker measurements; sensory nerve conduction measurements (onset latency, peak latency and amplitude) of the median, ulnar and sural nerves; monofilament testing of the right great toe; and buccal swabs for DNA. Questionnaires were completed for demographics, exposures to dental amalgam and dietary fish intake, neuropathic symptoms, and confounders. The mean Hg levels in urine (1.06μg/L) and hair (0.51μg/g) were not significantly different from the US population (0.95 μg/L and 0.47μg/g, respectively) (NHANES 1999-2000, 2003-2004). Multivariate regression analysis found subjects with MT1M (rs2270836) AA genotype (n=10) had lower urinary Hg levels than GG after controlling for exposure and potential cofounders. After controlling for methylmercury intake from fish, subjects with MT1A (rs8052394) GA and GG (n=24), MT1E (rs708274)

GT and TT (n=51), MT1M (rs9936741) TT had lower hair Hg levels compared to AA, GG, and TC and CC (n=15), respectively. After accounting for hair Hg levels, increased polyunsaturated fatty acid intake from fish was found to improve nerve function in all nerve measurements except median peak and onset latencies. No consistent relationship was observed for urine Hg levels and nerve function. Only 3 out of a total of 504 multivariate models that investigated effect modification of SNPs on nerve-function-biomarker relationships had stable and statistically significant interaction terms. Overall, the findings suggested that some MT genetic polymorphisms may influence mercury biomarker levels. There might be a beneficial effect of fish consumption on nerve function but little evidence was shown for effect modification of the studied SNPs on nerve-function-biomarker relationships. Poor agreement (Kappa: -0.14 ~ 0.44) between procedures demonstrated the challenges for further development and evaluation of methods in a non-clinical population.

Chapter 1

Introduction

1. Forms and Exposure Routes of Mercury and its Biological Monitoring Indicators

Mercury is a neurotoxicant that is ubiquitous in environmental media and food (especially fish) at concentrations that may adversely affect wildlife and human health (Clarkson, Magos 2006). Anthropogenic mercury emissions, which account for about one- to two-thirds of the total global output, include fossil fuel combustion (e.g. coal-fired power plants), mining, smelting, and solid waste incineration (Swain et al. 2007). Mercury circulates around the globe. The U.S. Environmental Protection Agency (EPA) has estimated that over three-quarters (83 percent) of the mercury deposited in the U.S. originates from international sources, with the remaining 17 percent coming from U.S. and Canadian sources (EPA, 2005a). Several regulatory bodies, including the U.S. EPA (U.S. EPA, 1997) and the U.S. Centers for Disease Control (CDC; ATSDR, 1999), rank this potent neurotoxic agent as a top three priority pollutant of concern. A recent estimate suggests that 7.8 % of mothers and >600,000 newborns in the US have concentrations of mercury in their body that exceed levels deemed safe by regulatory agencies (Trasande, Landrigan & Schechter 2005). By 2020, the world-wide socioeconomic consequences (e.g., loss of intelligence) associated with continuous exposures to methyl mercury through fish consumption have been estimated to be ~$10 billion per year if no remedy is employed (JM Pacyna et al. 2008).

Mercury in the environment exists in a variety of forms such as elemental mercury, inorganic mercury compounds (e.g. mercuric chloride, sulfide, iodide and oxide) and organic mercury (e.g. methylmercury and ethylmercury) (ATSDR, 1999; EPA 1997). Little of the elemental and inorganic mercury released to the air results in direct human exposure (ATSDR, 1999). Most of it is transformed by microorganisms such as bacteria and fungi in marine sediments to methylmercury. Methylmercury in fish is considered to be the dominant source of organic mercury in the general public. The primary sources of elemental mercury include personal dental amalgam and thimerosal-containing vaccines (ATSDR, 1999).

Dental amalgam is comprised of mercury (50% by weight), silver, tin and copper. The safety of amalgam has always been a major controversy, particularly the actual level of mercury released and the effects of such exposure on human health (ADSTR, 1999; US Dept of Health and Human Services, Public Health Service, 1993). Patterson, Weissberg & Dennison 1985 reported a daily dose of mercury vapor of 27 µg in exhaled air before and after teeth brushing for 10% of the most exposed subjects with amalgams. The magnitude of mercury exposure from dental amalgam at this level is considered to be significant (Berglund 1990). Alternatively, an estimated average daily dose of 1.7 µg of mercury vapor inhaled from amalgam has been reported and it only accounts approximately for 1% of the WHO occupational threshold limit value (TLV; 50 $\mu g/m^3$) (Berglund 1990). Such discrepancy from Patterson et al. 1985 may be a result of different methods employed and the different timing of measuring mercury vapor in the mouth. As clinical signs following mercury exposure have generally been associated with exposure to air concentrations greater than 50 $\mu g/m^3$, corresponding to urinary mercury concentrations greater than 100 µg/l (BATES 2006), mercury exposure at this level is presumed safe and would not pose adverse clinical effects (Berglund 1990). Mercury exposure from mercury amalgam has been consistently reported to be at low levels in non-occupationally exposed general

populations (Factor-Litvak et al. 2003, DeRouen et al. 2006, Dye et al. 2005, Kingman, Albertini & Brown 1998, Kingman et al. 2005).

In additional to personal amalgam exposure, dental professionals might have elevated levels of mercury resulting from occupational exposure to mercury when preparing, placing and removing dental amalgams at work (Shapiro et al. 1982). Some studies have investigated the relationships between such exposures to mercury vapor and adverse health effects in dentists and dental hygienists (Langworth et al. 1997, Akesson et al. 1991, Naleway et al. 1985). Compared to chloralkali workers reported by Albers et al, 1982 and Frumkin et al, 2001, dentists have much lower levels of exposure: a median time-weighted average air Hg (TWA) of 1.8 $\mu g/m^3$ for dentists and 2.1 $\mu g/m^3$ for dental hygienists (Langworth et al. 1997). The air Hg concentrations also varied between dental clinics resulting from differences in flooring and ventilation. They reported that there was no association between amalgam work and mercury level in urine. Flooring and ventilation have also been shown to be significant predictors of mercury levels in urine in a study of US dentists (Naleway et al. 1985). (Sällsten et al. 1996) noted that gum-chewing could be a significant factor causing increased release and absorption of inorganic mercury from dental amalgam fillings. Age and sex have also been reported as confounders to the relationship between urine mercury level and exposures to elemental mercury from amalgam (EPA 1997).

The primary rationale behind any choice of biological indicator to accurately reflect mercury exposure is that there are differences in the disposition of different forms of mercury upon entry into the body. The dominant route of exposure to elemental mercury is inhalation (Franzblau and Fromes 2005). Transcutaneous and gastrointestinal absorption of inorganic compounds (e.g. elemental mercury as liquid) are largely negligible (Franzblau and Fromes 2005). Elemental mercury is highly lipophilic. Once absorbed into the body this characteristic enables it to diffuse across cellular membranes of the kidney, liver,

red blood cells and the blood brain barrier (BBB) of the brain where it can be oxidized into divalent mercuric mercury (Clarkson, Vyas & Ballatori 2007). Divalent mercuric mercury is then excreted through bile into feces and through the kidney into urine (Clarkson 2002a). A major fraction of the body burden of inorganic mercury eventually accumulates in the kidney (ATSDR, 1999). A good correlation has been found between mercury level in urine and that in air (Tsuji et al. 2003). Spot urine samples have been found to be a valid surrogate measure of 24-hour urine excretion; creatinine adjustment of mercury concentrations was found to have little effect on variations of mercury levels in urine (Martin et al. 1996). Total mercury in urine serves as an appropriate marker of exposure to inorganic mercury (e.g. dental amalgam) and the overall half-life of mercury is approximately 58 days in the body (Berglund et al. 2005, Clarkson 2002a)). Mercury in hair and blood is mostly in the organic form (mostly methylmercury) and has not been found to be well correlated with exposure to inorganic mercury (George et al. 2010).

Organic mercury exposure enters the human body via dietary fish consumption. Upon forming highly mobile water-soluble methylmercury-L-cysteine complexes in the body, methylmercury is readily distributed across the BBB into the central nervous system and to other tissues such as the kidney and liver (Clarkson 2002a). In the bloodstream, ~90% of methylmercury is bound to hemoglobin in the red blood cells (RBC) while inorganic mercury is more evenly distributed between RBC and plasma (Berglund et al. 2005). The major elimination route of methylmercury is feces via biliary excretion (Clarkson 2002b). The half-life of methylmercury in the human body generally falls in the range of 45-70 days (Clarkson 2002a). Hair accumulates methylmercury via neutral amino-acid carriers in the process of transporting amino acids to support the synthesis of keratins. Approximately 90% of the mercury detected in hair is derived from organic mercury (Berglund et al. 2005, George et al. 2010). Berglund et al. 2005 demonstrated that a small fraction of inorganic mercury in hair is the result of demethylation of methylmercury, rather than a result of incorporation of inorganic

mercury. This finding confirms the results of many studies that total mercury in hair is highly correlated with blood methylmercury level and methylmercury intake from recent fish consumption (Kershaw, Clarkson & Dhahir 1980, Oken, Bellinger 2008, Hightower, Moore 2003). Because hair grows approximately at a rate of 1cm every month, hair mercury could reflect exposure to fish consumption in the last month. Thus, scalp hair mercury is considered an appropriate index of recent exposure (depending on the length of the hair used from the scalp) to organic mercury, mostly methylmercury from dietary fish consumption.

2. High-Level Exposure to Methylmercury and Elemental Mercury and Peripheral Nerve Function

2.1 Methylmercury

The first outbreak of methylmercury poisoning in Minamata, Japan in the 1950s showed high levels of methylmercury exposure lead to clinical signs and symptoms of neurodevelopmental deficits (e.g. disturbance of intelligence and body growth, deformity of limbs, dysarthria and deficits of motor functions) in fetuses (Harada 1978). At the high levels of over 1000 ppb in many cases and up to 293 ppm in cord blood and maternal hair, respectively, the clinical or subclinical manifestation of adverse effects on the central nervous system in fetuses and adults appeared to be dependent on the duration, the intensity and the window of exposure during pregnancy (Harada 1978). Another outbreak of severe methylmercury poisoning in Iraq in the 1970s, as a result of consuming mercury-contaminated bread, also led to similar symptoms and signs (paresthesia, ataxia, and dysarthria, etc) with a latent period of ranging from 16 to 38 days in adults with a wide range of ages (Bakir et al. 1973). At high concentrations up to 500 ppb in blood, the symptoms of severe neurological damages were also manifested in infants (Bakir et al. 1973). In the Iraqi study, researchers were able to establish a dose-response relationship between the onset of mercury poisoning symptoms in persons above 9 years old and the estimated blood mercury burden.

Much of the prior work on the toxicity of high-level methylmercury has focused on the clinical symptoms involving the central nervous system following acute or chronic exposure to methylmercury as seen in Japan and Iraq. No prior study has reported the adverse effects of high-level methylmercury on the peripheral nervous system. Adverse effects involving the peripheral nervous system as a significant target organ of high-level mercury toxicity should have been more widely studied.

It is also worth noting that the prenatal exposure to methylmercury that has occurred to the fetuses in Japan and Iraq was at an extremely high level, which is not relevant to the US general population. However, whether lower levels of exposures might lead to clinical or subclinical changes has remained controversial (Stern et al. 2004). Even at a lower level of exposure to methylmercury, associations of exposure with cognitive function, especially language, memory and attention in children were among the first outcomes to be studied (Grandjean et al. 1997, Davidson et al. 1998). The possible effects of lower levels of exposure to methylmercury have been under extensive investigation ever since (Davidson et al. 1998, Grandjean et al. 1997, Myers et al. 2009, Crump et al. 1998, Myers et al. 2003, Myers et al. 2000).

2.2 Elemental Mercury

Clinically manifested adverse effects in the adult brain following elemental mercury exposure have often occurred among workers in occupational settings such as chloralkali plants (Albers et al. 1988, Bluhm et al. 1992, Hargreaves et al. 1988, Frumkin et al. 2001). Some neurological effects may be persistent and/or may have a late onset up to 30 years after exposure (Albers et al. 1988). Mercury residuals may remain detectable in cerebrum 16 years after the initial exposure (Hargreaves et al. 1988). The level of exposure to elemental mercury in cases ((Bluhm et al. 1992)) was approximately at 50μg/l of blood mercury (assuming

blood and urine Hg in equilibrium), significantly higher than the US general population. It appears that the risk of overt clinical signs of mercury poisoning occurring is low when urine mercury level is below 450μg/l (Albers et al. 1988). Nevertheless, at lower exposures ranging from 30 to 100μg/g creatinine in peak urine Hg elemental Hg may still lead to increased risk of neuropsychomoter effects in adult workers (Langolf et al. 1978, Miller, Chaffin & Smith 1975, Roels et al. 1982). In addition, lower exposures to elemental mercury have also been associated with decreased measures of mood and neurobehavioral test results in dental professionals (Echeverria et al. 2005, Echeverria et al. 2006, Echeverria et al. 2010). It may be important to look into the toxicity of low-level exposures to elemental mercury in dental professionals.

Similar to methylmercury toxicity, much of the prior studies on elemental mercury toxicity have focused on central nervous system. Despite that, some studies have shown that elemental Hg may affect the peripheral nerve system (PNS) at high exposure levels (Kingman et al. 2005, Albers et al. 1988, Albers et al. 1982, Gerr, Letz 2000, Levine et al. 1982, Ellingsen et al. 1993). A good correlation was found between prolonged ulnar sensory distal latency and average urine levels in chloralkali workers at mean urine Hg levels of both 290μg/l and 90μg/l (Kingman et al. 2005, Albers et al. 1988, Albers et al. 1982, Gerr, Letz 2000, Levine et al. 1982, Ellingsen et al. 1993). A recent biopsy and autopsy report of peripheral nerves in a Minamata case found obvious degeneration signs of peripheral nerve including regenerated myelin sheaths and endoneurial fibrosis due to the prior severe exposure (Eto et al. 2002). Despite the fact that such high levels of elemental exposure would not normally be seen in the US general population who is typically exposed to mercury primarily via dental amalgam, more research on the peripheral nervous system effects of elemental Hg exposure are warranted.

3. Low-level Exposure to Methylmercury and Elemental Mercury and Peripheral Nerve Function

3.1 Methylmercury

Studies published from data gathered in the Faroe Islands and the Seychelles Islands have been at the forefront of researching health effects following exposure to methylmercury at levels closer to the US general population than previous outbreak investigations (NRC, 2000). Grandjean et al. 1997 studied neurophysiological and neuropsychological performance of 917 seven-year-old children in the Faroe Islands who were prenatally exposed to methylmercury via dietary consumption of whale meat. The study showed that prenatal exposure to methylmercury, after adjusting for covariates, was associated with decreased cerebral function, especially language, and memory and attention in children (Harada 1978, CHOI 1989, Eto et al. 1992). These findings were evident at an average maternal hair mercury level of 4.27µg/g and cord blood mercury level of 22.9µg/l, which were substantially lower than previous outbreaks in Japan and Iraq but still higher than the US general population (EPA 1997). The New Zealand cohort study, whose exposure level was comparable to those found in the Faroe Islands, also suggested decreased language ability was associated with increased mercury level in maternal hair (Crump et al. 1998). In a report released by the National Research Council in 2000, the committee concluded that low levels of prenatal mercury exposure as seen in the Faroe Islands and other epidemiological studies can cause significant adverse effects on neuropsychological development in children (McKeown-Eyssen, Ruedy & Neims 1983, Steuerwald et al. 2000). However, data published following the Faroe Islands study conducted in Seychelles Islands has led to conflicting results (Davidson et al. 1998). The latter study found no associations in 66-month-old child mother pairs between children’s cognitive and language ability and prenatal exposure to methylmercury via daily consumption of ocean fish at the mean maternal hair Hg level of 6.8 µg/g. The apparent discrepancy of the findings between the Faroe Islands and the Seychelles Islands studies might have been

the result of the difference in sources of methylmercury exposure. Nonetheless, the results described above have clearly showed adverse neurological effects following methylmercury exposure at a low level (Bakir et al. 1973, Grandjean et al. 1997, Davidson et al. 1998). There still needs to have more studies of methylmercury exposure at a level close to the US general population, or lower than what has been seen in the Faroe Islands and the Seychelles Islands. Furthermore, much of the prior work has still focused on central nervous system only. Being one of the susceptible targets of methylmercury toxicity, peripheral nervous system changes needs to be investigated and studies are warranted on peripheral nervous system at a level close to the US general population.

3.2 Elemental Mercury

With elemental mercury exposure from amalgam at levels that are relevant to the US general population, there exists conflicting results regarding the associations of neuropsychological and neurophysiologic test results with amalgam exposure (Factor-Litvak et al. 2003, DeRouen et al. 2006, Dye et al. 2005, Kingman et al. 2005, Bjrkman, Pedersen & Lichtenstein 1996, Saxe et al. 1995). A summary of such studies can be found in Table 1.1. Overall, there was no association between amalgam exposure and neurological signs or clinically evident peripheral neuropathy in four of the seven studies. Kingman et al. 2005 concluded that such association is a sub-clinical finding that is not associated with symptoms, clinically evident signs of neuropathy, or any functional impairment. However, Kingman et al. 2005 also found a significant but weak association between amalgam exposure and the continuous vibrotactile sensation responses of great toes in a sample of 1663 US veterans. At an even lower dose (mean urinary Hg < 5μg/l) closer to the general population than what was reported in the Kingman study, two recent studies have shown significant adverse neuropsychological and neurophysiologic effects (Echeverria et al. 2005, Echeverria et al. 2006, Echeverria et al. 2010). Specifically, a significant positive association between several measurements of cognitive function and mean

urinary Hg was found in male dentists and female dental assistants at a level of 3.32 µg/l and 1.98 µg/l in urinary Hg, respectively. Additionally, the pilot study of dental professionals conducted by our research group has shown a small but statistically significant association between peripheral nerve function and amalgam exposure at a mean urinary Hg 3.7 µg/l that is slightly higher than the US general population but with significant overlap (Ishak et al., in preparation). In light of the conflicting results for the associations of elemental Hg exposure with both CNS- and PNS-based neurological measurements, more studies investigating potential adverse neurological effects of amalgam exposure at low levels relevant to the general population (mean urinary Hg < 5µg/l) are warranted.

Furthermore, in those studies that investigated peripheral nerves, objective measurements of nerve conduction test have never been used; a more sensitive indicator for peripheral nerve impairment, sensory nerve conduction, was not used in any of the studies. More research that employs such sensitive and objective measurements to detect peripheral nerve impairment is also warranted.

There have been four studies exploring the associations of low-level elemental mercury exposures with peripheral nervous system impairment (DeRouen et al. 2006, Kingman et al. 2005, Echeverria et al. 2005, Saxe et al. 1995, Factor-Litvak et al. 1996). A summary of the four studies can be found in Table 1.1. Clinical neurological exams were employed instead of direct measurements of PNS impairments using objective measurements of nerve conduction test. Studies that detect PNS-based impairment using nerve conduction test will be needed as neurological effects of Hg exposure are likely to be sub-clinical (Kingman et al. 2005). The results regarding the associations of elemental Hg exposure with clinically evident neurobehavioral and neuropsychological tests shown in these studies were conflicting. In the study of Air Force veterans by Kingman et al. 2005, no association between amalgam exposure and neurological signs or clinically evident peripheral neuropathy was found in general; neither was such association found in the DeRouen study. However, a

subclinical, statistically significant association was detected between amalgam exposure and the continuous vibrotactile sensation response for non-diabetic participants and controls in Kingman et al. 2005. In the studies conducted by Echeverria et al. 2005 and Echeverria et al. 2006, Hg in urine was associated with impairment of nine clinical exams of attention, memory and coordination in dentists and eight exams of attention, memory, perception and coordination in dental assistants. Given the conflicting results, more studies are warranted even when it comes to assessing clinically evident neurological effects of elemental Hg exposure, and even more so for subclinical effects of such exposure.

In some of these studies, nerve conduction tests were not used as summarized in Table 1.1 (DeRouen et al. 2006, Kingman et al. 2005, Echeverria et al. 2005, Saxe et al. 1995, Factor-Litvak et al. 1996). Nerve conduction tests are considered more sensitive and objective measurements than clinical examinations to detect small impairments of peripheral nerves following acute or chronic exposure to chemicals (Kingman et al. 2005). In particular, studies that involve sensory nerve conduction tests would enhance our ability to detect and quantify impairment.

In the two studies that did incorporate nerve conduction tests, they have only focused on the changes of the motor nerve conduction functions (Factor-Litvak et al. 2003, DeRouen et al. 2006, Kingman et al. 2005, Echeverria et al. 2005). Nerve conduction tests on motor nerve are considered to be less sensitive indicators than that on sensory nerves for detecting impairment of the peripheral nervous system (Albers et al. 1982). These studies were not able to associate amalgam exposure with impairment of motor nerve functions. Specifically, in the randomized clinical trial conducted by DeRouen et al., 2006, no association was found between the z score derived from ulnar motor nerve conduction velocity test and amalgam exposure after comparing children that have amalgams with those that have composites over all 7 years of follow-up. In the study conducted

by Echeverria et al. 2005, no association in urinary Hg was found with the results of ulnar motor nerve conduction tests among dentists and dental assistants.

Importantly, no sensory nerve conduction tests were performed in any of the four studies. Nerve conduction tests on sensory nerves may be more sensitive to the effects of neurotoxins like mercury, and thus better indices of PNS impairment. Although vibration sensitivity of toes and fingers were assessed in three of the studies, vibrotactile measurements are less objective sensory nerve tests compared to sensory nerve conduction tests, particularly in the lower extremities (e.g., the sural nerve). The use of sensory nerves (e.g ulnar and median nerves) in the hand is considered to be a less sensitive index of PNS impairments compared to the lower extremities (e.g sural nerves) as longer axons are typically more susceptible to toxicants, which has been shown in persons with most forms of peripheral neuropathy (Kimura, 1983). A study that assesses the impact of elemental Hg exposure using sural nerve conduction tests is warranted.

In summary, a study that employs objective nerve conduction tests of sensory nerves in the investigation of PNS impairment associated with elemental mercury exposure is warranted. It may be even of greater sensitivity to include sural nerve measurements in addition to ulnar and median sensory measurements to detect small changes in the peripheral nervous system function that would otherwise not be demonstrated.

4. Influence of Genetic Polymorphisms on Mercury Exposure

Individual susceptibility as a result of genetic polymorphism plays an important role in the magnitude and the extent of adverse health effects caused by environmental agents (Olden, Wilson 2000). Lead, a ubiquitous heavy metal in the environment, has been shown to have gene-related differential toxicity in humans (Hu et al. 2001). Polymorphisms in genes such as the vitamin D receptor (VDR), aminolevulinic acid dehydratase (ALAD), and hemochromatosis (HFE)

have been extensively studied and shown to mediate effects of lead in humans (Rajan et al. 2008, Hopkins et al. 2008, Schwartz et al. 2000). Studies of polymorphisms in mercury toxicity have just recently been carried out and more research is warranted considering the ubiquitous existence and potential neurological effects in adults and children.

Studies of effects of single nucleotide polymorphisms (SNPs) in key genes related to mercury metabolism on biomarkers of mercury exposure, or neuropsychological and neurophysiologic function are few (Echeverria et al. 2005, Echeverria et al. 2006, Custodio et al. 2004, Custodio et al. 2005, Schläwicke Engström et al. 2008, Gundacker et al. 2007, Gundacker et al. 2009, Heyer et al. 2008). Table 1.2 summarizes the prior work based on the populations of investigation, the Hg biomarkers employed, the Hg exposure level, SNPs investigated, the results of the studies and the types of statistical analysis conducted. The SNPs that have been previously investigated for modifying the associations between neurological outcomes and mercury exposure are mostly glutathione related genes (Glutathione-S-transferase (GST) and glutamate cysteine ligase (GCL)). In these studies, effect modifications of these SNPs on associations of Hg biomarkers with exposures have been found. For instance, Engström et al, 2008 reported the genotypes of GSTP1-105, GSTP-114 and GCLM-588 modify the effect of methylmercury intake from fish on Hg levels in erythrocytes. Specifically, lower erythrocyte Hg level was seen in subjects, who carry the ValVal allele (v.s. IleIle and IleVal) for GSTP1-105, who carry at least one Val allele (v.s. AlaAla) for GSTP-114, and who carry TT (v.s. CT and CC) for GCLM-588. A separate study conducted by Custodio et al, 2005 found a modified association between elevated urinary Hg level and elemental Hg exposure in gold miners with CT and TT allele (v.s. CC) for GCLM-588.

Even though more than one biomarker were reported to be used in Gundacker et al. 2007, Gundacker et al. 2009, and Custodio et al. 2005, none of these studies has included more than one Hg exposure source (i.e., organic and inorganic) in

one study to assess the effect modifications of the SNPs on the associations of exposures to Hg biomarkers. Due to the dynamic relationship of Hg content in blood, urine and hair, inter-conversion between Hg content of different forms in blood, hair and urine may occur. A single study that includes and adjusts exposure surrogates of both elemental Hg and methylmercury in the attempt to investigate effects of genetic modification of Hg level in hair and urine is warranted. Although Echeverria et al., 2005, 2006 included urinary biomarkers in their investigation of neurogolocial effects associated with elemental mercury exposure and genetic modification of such relationship, objective and sensitive measurements such as nerve conduction tests described above were not used to examine sub-clinical peripheral nerve impairment. In the investigation of the influence of genetic factors on the relationship between methylmercury exposure from dietary fish intake and mercury in erythrocytes by Custodio et al., 2005 and Schläwicke Engström et al. 2008, the level of long-chain n-3 polyunsaturated fatty acids in plasma (P-PUFA), used as a proxy for fish consumption, were accounted. Nonetheless, more research is warranted to account for the actual amount of fish intake (expressed in µg/kg/day) derived from a questionnaire concerning a variety of fish species when we examine the role of genetic polymorphisms in biomarker-exposure relationship.

4.1 Metallothionein

Metallothionein (MT) is a family of thiol-rich, low molecular weight (~7kDa) proteins that are ubiquitously present in humans, microbes and plants, etc. A number of studies have shown that metallothionein can actively bind heavy metals through thiol groups of cysteine residues, thus protecting from heavy metal toxicity and against oxidative stress (Kumari, Hiramatsu & Ebadi 1998, Schurz, Sabater-Vilar & Fink-Gremmels 2000, Aschner et al. 2006).

There are primarily four MT isoforms (MT1, MT2, MT3, and MT4) expressed in the human. MT1 and MT2 are expressed in all tissues, including the brain and

kidney, whereas MT4 is expressed in certain stratified squamous epithelia. MT3, also known as growth inhibitory factor (GIF), is primarily expressed in mammalian brain. The coding sequences of these isoforms are homologously located on human chromosome 16. In all MT sequences, common regulatory elements such as metal responsive elements (MREs), glucocorticoid responsive elements (GREs) and antioxidant responsive elements (AREs) are shared in the upstream promoter region (Karin et al. 1987a). These MREs are the binding locations for heavy metal induction of MT gene expression. Genetic polymorphisms in such locations may significantly affect the MT levels in humans (Karin et al. 1987a). Although glucocorticoids also may induce MT expression, it only acts via binding to GREs in the MT sequences. Oxidative stress induces induction via AREs, independent of the actions of heavy metals.

Besides the upstream regulatory elements, polymorphisms in sequences that code the 3'-untranslated region (3'UTR) of MT mRNA as well as metallothionein transcription factor-1 (MTF-1) may affect the MT levels in humans. The 3'-untranslated region (3'UTR) of MT mRNA has been shown to be associated with signals needed for mRNA localization (Hesketh J. 2004). Cells that do not correctly localize MT mRNA show increased sensitivity to Cd and to oxidative stress (Hesketh J. 2004). Various studies of SNPs within 3'UTR in selenoprotein have highlighted the potential functionality and importance of SNPs within 3'UTR region (Hesketh J. 2004). Thus, SNPs within 3'UTR are worth investigating. The heavy-metal-induced transcription of the MT gene family is regulated by metallothionein transcription factor-1 through the binding of metal at MREs in the promoter regions (Karin et al. 1987b, Palmiter 1994). Thus, genetic polymorphisms located in such regions or nearby may lead to significant changes in transcription level of MT genes, subsequently the binding of heavy metals. In humans, many of the MT genes including MT1A, MT2A, MT1E, and MT1G are functional genes (West et al. 1990).

As summarized in Table 1.2, there were only one study (Gundacker et al. 2009) in which the effects of MT polymorphisms were investigated for the association with level of heavy metals in biological media. In Gundacker et al. 2009, they reported subjects with at least one MT2A (rs10636) homozygote variant (GC and/or CC) have lower blood lead level compared to wildtype GG in a group of Austrian students; they also showed that subjects with MT4 (rs11643815) major homozygote GG have lower hair mercury level compared to GA and AA. The results suggested that metallothionein polymorphisms may play an important role in mediating the effect of heavy metals in various media. More studies are warranted to assess such metallothionein SNPs for their roles. Furthermore, studies on other metallothionein SNPs located in or nearby MREs in the upstream promoter region are also needed in assessing the effect modification of Hg exposures and biomarker levels. In addition, the findings of MT4 and MT2A in the only study that included MT polymorphisms described above were reached through simple bivariate analyses. No exposure variable and covariates were included in the analyses. Thus, studies that adjust for these factors are essential and better in determining the role of MT polymorphisms in modifying relationships between exposure and the level of biomarkers. Also, such gene-exposure interactions would be important in determining the association of Hg level in hair and/or urine with neurological impairment such as altered sensory nerve conduction.

5. Nerve Conduction Test and Mercury

Nerve conduction studies that measure amplitude and latency of peripheral nerves are widely used in clinical practice and epidemiological studies to evaluate peripheral neuropathy and peripheral nerve impairment (Kimura, 1983; 1984). Peak and onset latency (ms) are measured as the time delay between an electrical stimulus of a peripheral nerve and the peak and onset, respectively, of the response signal a recording electrode receives. Amplitude (µV) is measured as the magnitude of the sensory response (Kimura 1984, Salerno D F et al.

1998). (Albers et al. 1982) described sensory nerve conduction test, particularly measurement of distal latencies, as the most discriminative of nerve conduction test for peripheral toxic neuropathy. Reliability of these tests has also been studied among active workers who resemble the general population including dentists (Salerno D F et al. 1999). The results showed that median sensory nerve measures were more reliable than ulnar measures, but both had good to excellent test characteristics; median amplitude and peak latency had higher inter- and intra- examiner reliability.

Several studies has reported prolonged latencies and decreased amplitudes in populations exposed to elemental mercury particularly at higher levels (>50µg/l in urine) (Kingman et al. 2005, Albers et al. 1988, Albers et al. 1982, Levine et al. 1982, Ellingsen et al. 1993, Letz et al. 2000). What was worth noting was the finding of neurological impairment in some of these populations (Kingman et al. 2005, Albers et al. 1988, Albers et al. 1982, Gerr, Letz 2000, Levine et al. 1982, Ellingsen et al. 1993). At high levels (>50µg/l Hg in urine) reported by these studies, abnormalities in both motor and sensory nerves have often been associated with elemental mercury exposure. Albers et al. 1982 found that the number of motor nerve abnormalities was significantly higher in higher- group (defined by the number of mercury peak in urine above 600µg/l) than lower-exposure group in subjects who worked in an industrial plant. Letz 2000 showed a significant difference in ulnar motor nerve conduction velocity between heavy industry workers exposed to elemental mercury 30 years ago and unexposed workers. Albers et al. 1988 reported significantly prolonged ulnar motor and sensory distal latencies and median and sural sensory distal latencies, reduced median sensory conduction velocity in subjects with clinical polyneuropathy compared to normal workers. High correlations of ulnar motor and sensory distal latency with urinary Hg levels were found in 18 mercury cell chlorine plant workers. Nevertheless, in the only study concerning the association of background elemental mercury exposure in the general population (i.e., < 5 µg/L) with nerve conduction test, DeRouen et al, 2006 reported no significant

association between urinary Hg and ulnar motor nerve conduction velocity. The study of Ishak et al (in preparation) shows a small but statistically significant increase in latency with no impact on amplitude in relation to increasing urine mercury concentration. More studies are warranted to use nerve conduction test to investigate the impacts of low-level exposures to mercury, elemental Hg in particular, on peripheral nerve system. When carrying out such investigation, one needs to account for many other factors such as age, sex, anthropometric parameters, and hand temperature as they are important covariates of nerve tests (Kimura 1984, Salerno D F et al. 1998).

6. Concordance between Symptoms, Monofilament Testing Results and Sensory Nerve Conduction Function in the Lower Extremities

Epidemiological studies of Hg on peripheral neuropathy aim to investigate the relations between mercury exposure and potential changes in peripheral nerve function. They also provide a scientific basis for surveillance and monitoring of peripheral neuropathy in the lower extremity. However, the outcomes of the epidemiological research are significantly influenced by the validity, sensitivity, specificity, and positive/negative predictive values of the screening techniques employed. There are different procedures available for screening neuropathy in the lower extremity: physical examination (e.g., monofilament testing of the great toe), symptom surveys and electrodiagnostic testing such as nerve conduction tests. Monofilament testing for loss of sensation in the lower extremity has been shown to be of value in the diagnosis of diabetic neuropathy (Young MJ 1987). Nerve conduction tests have been used to diagnose neuropathy in the lower extremity in diabetic patients (Carrington et al. 2002). Self-reported symptoms, on the other hand, may be more prone to errors of misclassification. One systemic review of the accuracy of monofilament testing against nerve conduction tests concluded that monofilament testing should not be used as the sole diagnostic tool for peripheral neuropathy as the standardization of monofilament testing is lacking (Dros et al. 2009). Nevertheless, monofilament testing may be useful as a screening tool in the setting of epidemiological studies.

Despite the fact that nerve conduction test is an objective measurement for peripheral nerve impairment, there may be evidence suggesting a relatively poor concordance between survey symptoms, physical examinations and nerve conduction function. In the upper extremities concerning carpel tunnel syndrome, a relatively poor overlap was reported between the reported survey symptoms, the physical exam findings, and the electrodiagnostic results consistent with carpel tunnel syndrome (Homan et al. 1999). There has been no study that investigates the concordance between various screening procedures in the lower extremities. Such a study may be warranted for the lower extremities among the general population in relation to mercury exposure.

7. Summary

It has been extensively documented that exposure to mercury (both methylmercury and elemental mercury) at a high level can lead to clinical manifestation of neurological damage in the adult brain and developmental abnormalities in prenatally exposed children. However, the adverse effects of mercury exposure at lower levels, particularly at levels less than 5 µg/l of urinary Hg, remain less clear. Over the past two decades much research has been performed to elucidate the effects of low-level methylmercury exposure but much less is known about low-level elemental mercury exposure, particularly at levels less than 5 µg/l of urinary Hg which reflects background levels in the general population. Further, a majority of mercury studies focus on central nervous system effects and much less is known about changes to the peripheral nervous system. When peripheral nervous system was investigated, only motor nerve conduction function was measured following low-level elemental mercury exposure. Sensory nerve conduction function, a more sensitive measurement for peripheral nerve impairment, was not used. Nerve conduction function in the lower extremities was largely not measured in the investigation of peripheral nerve impairment in relation to mercury. Studies that look at elemental mercury

have never accounted for methylmercury from dietary fish intake in relation to peripheral nerve system effects.

The proposed study is to utilize sensory nerve conduction test to look at effects of both elemental mercury and methylmercury on peripheral sensory nerve function (including the sural nerve) at a level close to the general population. Using Hg levels in urine and hair as biomarkers, the study will assess the two major sources of mercury exposure in one single study: mercury vapor from amalgam and methylmercury from dietary fish consumption. Using sensory nerve conduction tests as an objective measurement for peripheral nerve impairment, the study may be able to detect small but significant adverse changes as a result of mercury exposure. Inclusion of the sural nerve in the lower extremities may enhance detection sensitivity. Single nucleotide polymorphisms (SNPs) of genes related to Hg metabolism may potentially modify the association between Hg biomarkers (Hg in urine and hair) with exposure indices from amalgam and dietary fish consumption. The study will look at SNPs of metallothionine genes, which code proteins that bind to mercury in the body. Such gene-environment interaction in relation to mercury has not been extensively investigated, particularly at levels that are relevant to the general population.

Tables

Table 1.1: Summary of previous literatures on effects of low level elemental Hg exposures on CNS and PNS functions

References	Study populations	Biomarkers	Exposure levels	Clinical exams	Nerve conduction tests(NCV)	Sensory nerves	Sural nerve	Results
Effects of low level exposures to elemental Hg on both PNS and CNS functions								
Kingman et al. 2005	Air Force veterans (n=1663)	None	decayed and filled surfaces ranging from 34.8 to 42.5	clinical signs (peripheral neuropathy);postural tremor (elemental Hg exposure)	None	Not specified (vibration on great toes)	None	Overall, no association of amalgam exposure with clinically evident peripheral neuropathy; yet amalgam exposure associated with continuous vibrotactile sensation response
DeRouen et al. 2006	healthy children of age 8-10 (n=507)	urine	1.8 µg/g creatinine	memory; attention; motor	posterior tibial and ulnar motor nerve velocities	None	None	no association of amalgam exposure with neurobehavioral tests and nerve conduction velocity between amalgam and composite groups
Echeverria et al. 2005	Male dentists (DD; n=194); female assistants (DA; n=233)	Urine	3.32 µg/L (DD); 1.98 µg/L (DA)	memory; attention; perception; coordination	ulnar motor nerve latency and amplitude	Not specified (vibration on fingers)	None	Hg in urine associated with decreased measurements of nine clinical exams of attention, memory and coordination in DDs and eight exams of attention, memory, perception and coordination in DAs; Hg in urine not associated with results of nerve conduction tests and vibration
Echeverria et al. 2006	Male dentists (DD; n=194); female assistants (DA; n=233)	Urine	3.32 µg/L (DD); 1.98 µg/L (DA)	memory; attention; perception; coordination	None	Not specified (vibration on fingers)	None	Hg in urine associated with decreased measurements of nine clinical exams of attention, memory and coordination in DDs and eight exams of attention, memory perception and coordination in DAs; Hg in urine associated with vibration in DAs but only in expected direction in DDs.
Effects of low level exposures to elemental Hg on CNS functions only								
Factor-Litvak et al. 2003	Healthy working adults (n=550)	urine; blood	1.7 µg/g creatinine	memory; attention; fine motor control	None	None	None	no association of amalgam exposure with neuropyschologic tests

Saxes et al.1995	Nuns of age 75-102 (n=129)	None	3 occlusal amalgam fillings	memory; attention; orientation; language	None	None	None	no association of amalgam exposure with cognitive functions
Langworth et al. 1997	Swedish dentists (n=22) and dental nurses (n=22)	whole blood; plasma; urine	18 nmol/L(whole blood);5.1nmol/L(plasma);3 nmol/mmol creatinine (urine)	mood; personality traits	None	None	None	higher number of symptoms seen in personality questionnaire in dental professionals than controls

Table 1.2: Summary of Previous Literatures Associating Hg Biomarkers with SNP Genotypes

References	Study populations	Biomarkers	Exposure levels	Genes/SNPs investigated	Results	Types of Analysis
Gundacker et al. 2007	Austrian students (n=222)	Whole blood; urine; hair	1.73 μg/l (blood);1.94 μg/go (urine); 0.449 μg/g (hair)	GSTT1/GSTM1 deletions; MT	GSTT1 and/or GSTM1 deletions more frequent among those with higher hair Hg; MT1X expression higher in those with wildtypes of GSTT1 and GSTM1	Stratification Analysis
Gundacker et al. 2009	Austrian students (n=324)	Whole blood; urine; hair	median:1.34 μg/l (blood);1.24 μg/gC (urine); 0.202 μg/g(hair)	GSTT1;GSTT1;GSTP1-105;GSTP1-114;GSTA1;GCLC-129;MT1A;MT2A;MT4	GSTP1-114 wildtype, MT4 wildtype, and variants of GSTP1-105/GCLC, GSTP1-105/GSTM1, GSTP1-114/GSTT1 predict Hg level in hair; MT2A GC and/or CC have lower blood lead; MT4 GG have lower hair mercury level	Multivariate linear regression
Engström et al. 2008	Northern Sweden adult fish eaters (n=292)	Blood(erythrocyt e)	5.5 μg/l	GCLC-129; GCLM-588; GSTP1-105;GSTP1-114	GCLM-588 TT had higher Hg compared to CC; GSTP1-105 and -114 Val decrease the slope of Hg regression on P-PUFA	Stratification Analysis; multivariate linear regression
Custodio et al. 2004	Northern Sweden adults (n=365)	Blood(erythrocyt e)	5.0 μg/l	Same as Engström et al. 2008; GSTM1 and GSTT1 deletions; GSTA1	GCLC-129 T and GSTP1-114 Val associated with higher erythrocyte Hg and steeper regression slope on P-PUFA	Stratification Analysis; multivariate linear regression
Custodio et al. 2005	Ecuadorian gold miners and buyers (n=309)	Whole blood; urine; plasma	-	Same as Custodio et al.2004	GCLM-588 T associated with elevated blood plasma, and urine Hg within subgroups based on job	Stratification Analysis
Echeverria et al. 2005	Male dentists (DD; n=194); female assistants (DA; n=233)	Urine	3.32 μg/l (DD);1.98 μg/l (DA)	brain-derived neurotrophic factor (BDNF)-196 G>A	BDNF GA and AA associated with decreased measurements of four clinical exams of memory in DDs and three exams of coordination in DAs	Multivariate linear regression
Echeverria et al. 2006	Male dentists (DD; n=194); female assistants (DA; n=233)	Urine	3.32 μg/l (DD);1.98 μg/l (DA)	Coproporphyrinogen oxidase (CPOX4)-814 A>C	CPOX AC and CC associated with decreased measurements of four clinical exams of memory and attention in DDs and five exams of memory and attention in DAs	Multivariate linear regression
Heyer et al. 2008	Male dentists (DD; n=157); female assistants (DA; n=84)	Urine	2.5 μg/l (DD);1.6 μg/l (DA)	Serotonin Transporter Gene Promoter Polymorphism (5-HTTLPR) insertion/deletion	5-HTTLPR full mutations associated with increased episodes of depression in DDs and DAs	Multivariate linear regression

Bibliography

Akesson, I., Schutz, A., Attewell, R., Skerfving, S., Glantz, P.O., 1991. Status of mercury and selenium in dental personnel: impact of amalgam work and own fillings. Arch. Environ. Health 46, 102-109.

Albers, J.W., Cavender, G.D., Levine, S.P., Langolf, G.D., 1982. Asymptomatic sensorimotor polyneuropathy in workers exposed to elemental mercury. Neurology 32, 1168.

Albers, J.W., Kallenbach, L.R., Fine, L.J., Langolf, G.D., Wolfe, R.A., Donofrio, P.D., Alessi, A.G., Stolp-Smith, K.A., Bromberg, M.B., 1988. Neurological abnormalities associated with remote occupational elemental mercury exposure. Ann. Neurol. 24, 651.

ATSDR. 1999. Toxicological Profile for Mercury. U.S. Department of Health and Human Services, Atlanta, GA.

Aschner, M., Syversen, T., Souza, D., Rocha, J.B.T., 2006. Metallothioneins: mercury species-specific induction and their potential role in attenuating neurotoxicity. Exp. Biol. Med. 231, 1468-1473.

Bakir, F., Damluji, S.F., Amin-Zaki, L., Murtadha, M., Khalidi, A., al-Rawi, N.Y., Tikriti, S., Dahahir, H.I., Clarkson, T.W., Smith, J.C., Doherty, R.A., 1973. Methylmercury poisoning in Iraq. Science 181, 230.

BATES, M., 2006. Mercury amalgam dental fillings: an epidemiologic assessment. Int. J. Hyg. Environ. Health 209, 309-316.

Berglund, A., 1990. Estimation by a 24-hour study of the daily dose of intra-oral mercury vapor inhaled after release from dental amalgam. J. Dent. Res. 69, 1646-1651.

Berglund, M., Lind, B., Björnberg, K.A., Palm, B., Einarsson, O., Vahter, M., 2005a. Inter-individual variations of human mercury exposure biomarkers: a cross-sectional assessment. Environ. Health 4, 20.

Berglund, M., Lind, B., Björnberg, K.A., Palm, B., Einarsson, O., Vahter, M., 2005b. Inter-individual variations of human mercury exposure biomarkers: a cross-sectional assessment. Environ. Health 4, 20.

Bjrkman, L., Pedersen, N.L., Lichtenstein, P., 1996. Physical and mental health related to dental amalgam fillings in Swedish twins. Community Dent. Oral Epidemiol. 24, 260-267.

Bluhm, R.E., Bobbitt, R.G., Welch, L.W., Wood, A.J., Bonfiglio, J.F., Sarzen, C., Heath, A.J., Branch, R.A., 1992. Elemental mercury vapour toxicity, treatment, and prognosis after acute, intensive exposure in chloralkali plant workers. Part I: History,

neuropsychological findings and chelator effects. Human and Experimental Toxicology 11, 201-210.

Carrington, A., Shaw, J., Van Schie, C.H.M., Abbott, C., Vileikyte, L., Boulton, A.J.M., 2002. Can motor nerve conduction velocity predict foot problems in diabetic subjects over a 6-year outcome period? Diabetes Care 25, 2010.

CHOI, B., 1989. The effects of methylmercury on the developing brain. Prog. Neurobiol. 32, 447-470.

Clarkson, T.W., 2002a. The three modern faces of mercury. Environ. Health Perspect. 110 Suppl 1, 11.

Clarkson, T.W., 2002b. The three modern faces of mercury. Environ. Health Perspect. 110 Suppl 1, 11.

Clarkson, T.W., Vyas, J.B., Ballatori, N., 2007. Mechanisms of mercury disposition in the body. Am. J. Ind. Med. 50, 757.

Clarkson, T., Magos, L., 2006. The toxicology of mercury and its chemical compounds. Crit. Rev. Toxicol. 36, 609-662.

Crump, K.S., Kjellstrm, T., Shipp, A.M., Silvers, A., Stewart, A., 1998. Influence of prenatal mercury exposure upon scholastic and psychological test performance: benchmark analysis of a New Zealand cohort. Risk Analysis 18, 701-713.

Custodio, H.M., Broberg, K., Wennberg, M., Jansson, J., Vessby, B., Hallmans, G., Stegmayr, B., Skerfving, S., 2004. Polymorphisms in glutathione-related genes affect methylmercury retention. Arch. Environ. Health 59, 588.

Custodio, H.M., Harari, R., Gerhardsson, L., Skerfving, S., Broberg, K., 2005. Genetic influences on the retention of inorganic mercury. Archives of environmental occupational health 60, 17.

Davidson, P.W., Myers, G.J., Cox, C., Axtell, C., Shamlaye, C., Sloane-Reeves, J., Cernichiari, E., Needham, L., Choi, A., Wang, Y., Berlin, M., Clarkson, T.W., 1998b. Effects of prenatal and postnatal methylmercury exposure from fish consumption on neurodevelopment: outcomes at 66 months of age in the Seychelles Child Development Study. JAMA 280, 701-707.

DeRouen, T., Martin, M., Leroux, B., Townes, B., Woods, J., Leito, J., Castro-Caldas, A., Luis, H., Bernardo, M., Rosenbaum, G., Martins, I., 2006. Neurobehavioral effects of dental amalgam in children: a randomized clinical trial. JAMA 295, 1784-1792.

Dros, J., Wewerinke, A., Bindels, P., van Weert, H., 2009. Accuracy of monofilament testing to diagnose peripheral neuropathy: a systematic review. Annals of family medicine 7, 555-558.

Dye, , Schober, , Dillon, , Jones, , Fryar, , McDowell, , Sinks, , 2005. Urinary mercury concentrations associated with dental restorations in adult women aged 16-49 years: United States, 1999-2000. Occup. Environ. Med. 62, 368.

Echeverria, D., Woods, J.S., Heyer, N.J., Rohlman, D.S., Farin, F.M., Bittner, A.C., Li, T., Garabedian, C., 2005. Chronic low-level mercury exposure, BDNF polymorphism, and associations with cognitive and motor function. Neurotoxicol. Teratol. 27, 781.

Echeverria, D., Woods, J.S., Heyer, N.J., Rohlman, D., Farin, F.M., Li, T., Garabedian, C.E., 2006. The association between a genetic polymorphism of coproporphyrinogen oxidase, dental mercury exposure and neurobehavioral response in humans. Neurotoxicol. Teratol. 28, 39.

Echeverria, D., Woods, J., Heyer, N., Martin, M., Rohlman, D., Farin, F., Li, T., 2010. The association between serotonin transporter gene promotor polymorphism (5-HTTLPR) and elemental mercury exposure on mood and behavior in humans. Journal of toxicology and environmental health.Part A 73, 1003-1020.

Ellingsen, D.G., Mrland, T., Andersen, A., Kjuus, H., 1993. Relation between exposure related indices and neurological and neurophysiological effects in workers previously exposed to mercury vapour. Br. J. Ind. Med. 50, 736-744.

EPA, 1997. Mercury Study Report to Congress. EPA-452/R-97-003, December 1997. Accessible at: www.epa.gov/ttn/oarpg/t3/reports/volume1.pdf (95 pgs., 1.3 MB)

EPA, 2005a. Technical Support Document, Revision of December 2000 Regulatory Finding on the Emissions of Hazardous Air Pollutants From Electric Utility Steam Generating Units and the Removal of Coal- and Oil-Fired Electric Utility Steam Generating Units from the §112(c) List: Reconsideration, Oct. 21, 2005. Accessible at: http://www.epa.gov/ttn/atw/utility/TSD-112-final.pdf

Eto, K., Oyanagi, S., Itai, Y., Tokunaga, H., Takizawa, Y., Suda, I., 1992. A fetal type of Minamata disease. An autopsy case report with special reference to the nervous system. Molecular and chemical neuropathology 16, 171-186.

Eto, K., Tokunaga, H., Nagashima, K., Takeuchi, T., 2002. An autopsy case of minamata disease (methylmercury poisoning)--pathological viewpoints of peripheral nerves. Toxicol. Pathol. 30, 714-722.

Factor-Litvak, P., Kline, J.K., Popovac, D., Hadzialjevic, S., Lekic, V., Preteni-Rexhepi, E., Capuni-Paracka, S., Slavkovich, V., Graziano, J., 1996. Blood lead and blood pressure in young children. Epidemiology 7, 633-637.

Factor-Litvak, P., Hasselgren, G., Jacobs, D., Begg, M., Kline, J., Geier, J., Mervish, N., Schoenholtz, S., Graziano, J., 2003. Mercury derived from dental amalgams and neuropsychologic function. Environ. Health Perspect. 111, 719-723.

Frumkin, H., Letz, R., Williams, P.L., Gerr, F., Pierce, M., Sanders, A., Elon, L., Manning, C.C., Woods, J.S., Hertzberg, V.S., Mueller, P., Taylor, B.B., 2001. Health effects of long-term mercury exposure among chloralkali plant workers. Am. J. Ind. Med. 39, 1.

George, G., Singh, S., Myers, G., Watson, G., Pickering, I., 2010. The chemical forms of mercury in human hair: a study using X-ray absorption spectroscopy. Journal of biological inorganic chemistry.

Gerr, F., Letz, R., 2000. Epidemiological case definitions of peripheral neuropathy: experience from two neurotoxicity studies. Neurotoxicology 21, 761.

Grandjean, P., Weihe, P., White, R.F., Debes, F., Araki, S., Yokoyama, K., Murata, K., Sørensen, N., Dahl, R., Jørgensen, P.J., 1997. Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury. Neurotoxicol. Teratol. 19, 417.

Gundacker, C., Gencik, M., Hikkel, I., Komarnicki, G., Kukuckova, M., Wittmann, K., 2009. Genetic background of lead and mercury metabolism in a group of medical students in Austria. Environ. Res. 109, 786.

Gundacker, C., Komarnicki, G., Jagiello, P., Gencikova, A., Dahmen, N., Wittmann, K.J., Gencik, M., 2007. Glutathione-S-transferase polymorphism, metallothionein expression, and mercury levels among students in Austria. Sci. Total Environ. 385, 37.

Harada, M., 1978. Congenital Minamata disease: intrauterine methylmercury poisoning. Teratology 18, 285.

Hargreaves, R.J., Evans, J.G., Janota, I., Magos, L., Cavanagh, J.B., 1988. Persistent mercury in nerve cells 16 years after metallic mercury poisoning. Neuropathol. Appl. Neurobiol. 14, 443-452.

Heyer, N.J., Echeverria, D., Farin, F.M., Woods, J.S., 2008. The association between serotonin transporter gene promoter polymorphism (5-HTTLPR), self-reported symptoms, and dental mercury exposure. Journal of toxicology and environmental health.Part A 71, 1318.

Hightower, J.M., Moore, D., 2003. Mercury levels in high-end consumers of fish. Environ. Health Perspect. 111, 604.

Homan, M.M., Franzblau, A., Werner, R.A., Albers, J.W., Armstrong, T.J., Bromberg, M.B., 1999. Agreement between symptom surveys, physical examination procedures and

electrodiagnostic findings for the carpal tunnel syndrome. Scandinavian journal of work, environment health 25, 115-124.

Hopkins, M., Ettinger, A., Hernndez-Avila, M., Schwartz, J., Tllez-Rojo, M., Lamadrid-Figueroa, H., Bellinger, D., Hu, H., Wright, R., 2008. Variants in iron metabolism genes predict higher blood lead levels in young children. Environ. Health Perspect. 116, 1261-1266.

Hu, H., Wu, M.T., Cheng, Y., Sparrow, D., Weiss, S., Kelsey, K., 2001. The delta-aminolevulinic acid dehydratase (ALAD) polymorphism and bone and blood lead levels in community-exposed men: the Normative Aging Study. Environ. Health Perspect. 109, 827-832.

JM Pacyna, Kyrre Sundseth, Elisabeth G. Pacyna, John Munthe, Mohammed Belhaj, Stefan Åström, Damian Panasiuk, Anna Glodek, 2008. Socio-economic Costs of Continuing the Status-quo of Mercury Pollution. 580.

Ishak MB, Franzblau A, Werner RA, Gillespie BW, Albers JW, Gruninger S, Siew, C, Hamann C.2008. Low-level mercury exposure and peripheral nerve function. [In Preparation]

Karin, M., Haslinger, A., Heguy, A., Dietlin, T., Cooke, T., 1987a. Metal-responsive elements act as positive modulators of human metallothionein-IIA enhancer activity. Mol. Cell. Biol. 7, 606-613.

Karin, M., Haslinger, A., Heguy, A., Dietlin, T., Imbra, R., 1987b. Transcriptional control mechanisms which regulate the expression of human metallothionein genes. Experientia.Supplementum 52, 401-405.

Kershaw, T.G., Clarkson, T.W., Dhahir, P.H., 1980. The relationship between blood levels and dose of methylmercury in man. Arch. Environ. Health 35, 28-36.

Kimura J. 1983 Electrodiagonosis in Diseases of Nerve and Muscle: Principles and Practices. Philadelphia, Davis,

Kimura, J., 1984. Principles and pitfalls of nerve conduction studies. Ann. Neurol. 16, 415.

Kingman, A., Albertini, T., Brown, L.J., 1998. Mercury concentrations in urine and whole blood associated with amalgam exposure in a US military population. J. Dent. Res. 77, 461-471.

Kingman, A., Albers, J.W., Arezzo, J.C., Garabrant, D.H., Michalek, J.E., 2005. Amalgam exposure and neurological function. Neurotoxicology 26, 241.

Kumari J. , Hiramatsu, , Ebadi, , 1998. Free radical scavenging actions of metallothionein isoforms I and II. Free Radic. Res. 29, 93.

Langolf, G.D., Chaffin, D.B., Henderson, R., Whittle, H.P., 1978. Evaluation of workers exposed to elemental mercury using quantitative tests of tremor and neuromuscular functions. AIHA Journal 39, 976-984.

Langworth, S., Sllsten, G., Barregrd, L., Cynkier, I., Lind, M.L., Sderman, E., 1997. Exposure to mercury vapor and impact on health in the dental profession in Sweden. J. Dent. Res. 76, 1397-1404.

Letz, R., Gerr, F., Cragle, D., Green, R.C., Watkins, J., Fidler, A.T., 2000. Residual neurologic deficits 30 years after occupational exposure to elemental mercury. Neurotoxicology 21, 459-474.

Levine, S.P., Cavender, G.D., Langolf, G.D., Albers, J.W., 1982. Elemental mercury exposure: peripheral neurotoxicity. Br. J. Ind. Med. 39, 136.

Martin, M.D., McCann, T., Naleway, C., Woods, J.S., Leroux, B.G., Bollen, A.M., 1996. The validity of spot urine samples for low-level occupational mercury exposure assessment and relationship to porphyrin and creatinine excretion rates. J. Pharmacol. Exp. Ther. 277, 239-244.

McKeown-Eyssen, G.E., Ruedy, J., Neims, A., 1983. Methyl mercury exposure in northern Quebec. II. Neurologic findings in children. Am. J. Epidemiol. 118, 470-479.

Miller, J.M., Chaffin, D.B., Smith, R.G., 1975. Subclinical psychomotor and neuromuscular changes in workers exposed to inorganic mercury. AIHA Journal 36, 725-733.

Myers, G.J., Davidson, P.W., Cox, C., Shamlaye, C., Cernichiari, E., Clarkson, T.W., 2000. Twenty-seven years studying the human neurotoxicity of methylmercury exposure. Environ. Res. 83, 275.

Myers, G., Davidson, P., Cox, C., Shamlaye, C., Palumbo, D., Cernichiari, E., Sloane-Reeves, J., Wilding, G., Kost, J., Huang, L., Clarkson, T., 2003. Prenatal methylmercury exposure from ocean fish consumption in the Seychelles child development study. The Lancet 361, 1686-1692.

Myers, G., Thurston, S., Pearson, A., Davidson, P., Cox, C., Shamlaye, C., Cernichiari, E., Clarkson, T., 2009. Postnatal exposure to methyl mercury from fish consumption: a review and new data from the Seychelles Child Development Study. Neurotoxicology 30, 338-349.

Naleway, C., Sakaguchi, R., Mitchell, E., Muller, T., Ayer, W.A., Hefferren, J.J., 1985. Urinary mercury levels in US dentists, 1975-1983: review of Health Assessment Program. J. Am. Dent. Assoc. 111, 37-42.

NRC, 2000. National Research Council. Toxicological Effects of Methylmercury. Committee on The Toxicological Effects of Methyl-mercury, Board on Environmental Studies and Toxicology. Accessible at: http://books.nap.edu/books/0309071402/html/1.html .

Oken, E., Bellinger, D.C., 2008. Fish consumption, methylmercury and child neurodevelopment. Curr. Opin. Pediatr. 20, 178.

Olden, K., Wilson, S., 2000. Environmental health and genomics: visions and implications. Nature Reviews.Genetics 1, 149-153.

Palmiter, R.D., 1994. Regulation of metallothionein genes by heavy metals appears to be mediated by a zinc-sensitive inhibitor that interacts with a constitutively active transcription factor, MTF-1. Proc. Natl. Acad. Sci. U. S. A. 91, 1219.

Patterson, J.E., Weissberg, B.G., Dennison, P.J., 1985. Mercury in human breath from dental amalgams. Bull. Environ. Contam. Toxicol. 34, 459-468.

Rajan, P., Kelsey, K.T., Schwartz, J.D., Bellinger, D.C., Weuve, J., Spiro, A., Sparrow, D., Smith, T.J., Nie, H., Weisskopf, M.G., Hu, H., Wright, R.O., 2008. Interaction of the delta-aminolevulinic acid dehydratase polymorphism and lead burden on cognitive function: the VA normative aging study. Journal of occupational and environmental medicine 50, 1053.

Roels, H., Lauwerys, R., Buchet, J.P., Bernard, A., Barthels, A., Oversteyns, M., Gaussin, J., 1982. Comparison of renal function and psychomotor performance in workers exposed to elemental mercury. Int. Arch. Occup. Environ. Health 50, 77-93.

Salerno D F, Franzblau A, Werner R A, Bromberg M B, Armstrong T J, Albers J W, 1998. Median and Ulnar Nerve Conduction Studies Among Workers: Normative Values. Muscle & Nerve 21, 1005.

Salerno D F, Werner R A, Albers J W, Becker M P, Armstrong T J, Franzblau A, 1999. Reliability of Nerve Conduction Studies Among Active Workers. Muscle & Nerve 22, 1372.

Sällsten, G., Thorén, J., Barregård, L., Schütz, A., Skarping, G., 1996. Long-term use of nicotine chewing gum and mercury exposure from dental amalgam fillings. J. Dent. Res. 75, 594.

Saxe, S.R., Snowdon, D.A., Wekstein, M.W., Henry, R.G., Grant, F.T., Donegan, S.J., Wekstein, D.R., 1995. Dental amalgam and cognitive function in older women: findings from the Nun Study. J. Am. Dent. Assoc. 126, 1495-1501.

Schläwicke Engström, K., Strömberg, U., Lundh, T., Johansson, I., Vessby, B., Hallmans, G., Skerfving, S., Broberg, K., 2008. Genetic variation in glutathione-related genes and body burden of methylmercury. Environ. Health Perspect. 116, 734.

Schurz, , Sabater-Vilar, , Fink-Gremmels, , 2000. Mutagenicity of mercury chloride and mechanisms of cellular defence: the role of metal-binding proteins. Mutagenesis 15, 525.

Schwartz, B.S., Lee, B.K., Lee, G.S., Stewart, W.F., Simon, D., Kelsey, K., Todd, A.C., 2000. Associations of blood lead, dimercaptosuccinic acid-chelatable lead, and tibia lead with polymorphisms in the vitamin D receptor and [delta]-aminolevulinic acid dehydratase genes. Environ. Health Perspect. 108, 949-954.

Shapiro, I.M., Cornblath, D.R., Sumner, A.J., Uzzell, B., Spitz, L.K., Ship, I.I., Bloch, P., 1982. Neurophysiological and neuropsychological function in mercury-exposed dentists. The Lancet 1, 1147.

Stern, A., Jacobson, J., Ryan, L., Burke, T., 2004. Do recent data from the Seychelles Islands alter the conclusions of the NRC Report on the toxicological effects of methylmercury? Environ. Health 3, 2-2.

Steuerwald, U., Weihe, P., Jrgensen, P.J., Bjerve, K., Brock, J., Heinzow, B., Budtz-Jrgensen, E., Grandjean, P., 2000. Maternal seafood diet, methylmercury exposure, and neonatal neurologic function. J. Pediatr. 136, 599-605.

Swain, E., Jakus, P., Rice, G., Lupi, F., Maxson, P., Pacyna, J., Penn, A., Spiegel, S., Veiga, M., 2007. Socioeconomic consequences of mercury use and pollution. Ambio 36, 45-61.

Trasande, L., Landrigan, P.J., Schechter, C., 2005. Public health and economic consequences of methyl mercury toxicity to the developing brain. Environ. Health Perspect. 113, 590.

Tsuji, J., Williams, P.R.D., Edwards, M., Allamneni, K., Kelsh, M., Paustenbach, D., Sheehan, P., 2003. Evaluation of mercury in urine as an indicator of exposure to low levels of mercury vapor. Environ. Health Perspect. 111, 623-630.

U.S. Department of Health and Human Services. Committee to Coordinate Environmental Health and Related Programs. Subcommittee on Risk Management. Dental amalgam: A scientific review and recommended Public Health Service strategy for research, education and regulation—Final report of the Subcommittee on Risk Management of the Committee to Coordinate Environmental Health and

Related Programs, Public Health Service. Washington: Department of Health and Human Services; 1993:Appendix III:1. DHHS publication 96–0445.

West, A.K., Stallings, R., Hildebrand, C.E., Chiu, R., Karin, M., Richards, R.I., 1990. Human metallothionein genes: structure of the functional locus at 16q13. Genomics 8, 513-518.

Chapter 2

Modifying Effects of Metallothionein Single Nucleotide Polymorphisms on the Association between Elemental Hg Exposure and Urinary Mercury Levels

1. Introduction

Large inter-individual variation has been seen in urinary mercury levels in the general population and workers following exposures to elemental mercury (Tsuji, et al. 2003). At least ten-fold differences in urinary mercury level were reported in past studies which were summarized in Tsuji et al., 2003. It is worth noting that the levels of urinary mercury in those studies were derived from samples obtained in various locations and from measurements of control or unexposed groups in both non-occupational and occupational settings. Considerable inter-individual variation of mercury levels in plasma and blood in human volunteers was seen with time after they were exposed to elemental mercury of the same level for the same duration (Sandborgh-Englund, et al. 1998).

Although variations in sources of exposure (e.g. elemental mercury from dental amalgam) may contribute to the overall inter-individual variations in Hg biomarker levels following exposure to elemental Hg, physiologic differences in mercury disposition may also play an important role in explaining the variations. Such differences in the rate of uptake, absorption, metabolism, distribution and excretion may be attributed to variation in functional efficiency of enzymes and protein products that transport, oxidize and reduce mercury and its metabolites in humans (Gundacker, et al. 2010). Genetic polymorphisms in genes related to the synthesis and transportation of such enzymes and protein products may lead to such variation. In humans, glutathione proteins are crucial for detoxifying a

variety of toxicants including mercury. Five prior studies have shown that single nucleotide polymorphisms (SNPs) in genes that code the enzyme of the rate-limiting step in glutathione synthesis and glutathione conjugation of mercury (glutamate cysteine ligase (GCL); glutathione-S-transferase (GST)) may lead to variations of mercury levels in biomarkers following similar exposures (Custodio, et al. 2004; Custodio, et al. 2005; Gundacker, et al. 2007; Gundacker, et al. 2009; Schläwicke Engström, et al. 2008) For instance, Custodio et al., 2005 reported Ecuadorian gold miners with GCLM-588 CT and TT allele were associated with higher levels of urine Hg compared to those with CC allele. After adjustment for exposure to elemental Hg, the relation of mercury levels in blood and hair with exposure were also seen to be modified among individuals reported in all five studies. Engström et al, 2008 reported that after controlling for methylmercury intake from fish, certain genotypes of GSTP1-105, GSTP-114 and GCLM-588 modify the effects of methylmercury intake from fish on Hg levels in erythrocytes. Specifically, lower erythrocyte Hg levels were seen in subjects who carry the ValVal allele (vs. IleIle and IleVal) for GSTP1-105, those who carry at least one Val allele (vs. AlaAla) for GSTP-114, and those who carry TT (v.s. CT and CC) for GCLM-588. Similarly, GSTT1 and GSTM1 deletions, reported by Gundacker et al., 2007, were more frequently seen among subjects with higher hair Hg levels.

Little is known about the effects of genetic polymorphisms in the family of genes coding metallothionein (MT) proteins that may affect mercury distribution in humans. Metallothioneins are a family of thiol-rich, low molecular weight (~7kDa) proteins that are ubiquitously present in humans, microbes and plants. A number of studies have shown that metallothionein proteins actively bind heavy metals via thiol groups in cysteine residues, which may serve to protect humans from heavy metal toxicity and against oxidative stress (Aschner, et al. 2006; Kumari, et al. 1998; Schurz, et al. 2000). There are four primary MT isoforms (MT1, MT2, MT3, and MT4) expressed in the human. The sequences coding for these isoforms are homologously located on the human chromosome 16. In all of the

MT sequences, there exist common regulatory elements in the upstream promoter region including the metal responsive elements (MREs), glucocorticoid responsive elements (GREs) and antioxidant responsive elements (AREs) (Karin, et al. 1987a). The induction of MT genes occurs via the MREs. Genetic polymorphisms in MREs in humans have been shown to significantly affect MT expression (Karin, et al. 1987a), and this may subsequently influence mercury binding and the amount of mercury available at target organs. In addition to MREs in the upstream promoter sequence, the 3'-untranslated region (3'UTR) of MT mRNA has been shown to be associated with signals needed for mRNA localization (Hesketh 2004). Cells that do not correctly localize MT mRNA show increased sensitivity to Cd and oxidative stress (Hesketh 2004). Furthermore, transcription factors coded by metallothionein transcription factor-1 (MTF-1) regulate the heavy-metal-induced transcription of the MT gene family through the binding of metals at MREs in the promoter region (Karin, et al. 1987b; Palmiter 1994). Genetic polymorphisms located in MTF-1 may also lead to significant changes in transcription level of MT genes (Saydam, et al. 2003), and this may subsequently influence the binding of heavy metals.

To our knowledge no study has ever investigated the potential effect modification of metallothionein SNPs on the relationship of urinary Hg levels with elemental Hg exposure. A prior study investigated MT SNPs in relation to hair mercury levels in subjects exposed to methylmercury and found that subjects with major homozygote GG of MT4 (rs11643815) have lower hair mercury levels compared to GA and AA (Gundacker, et al. 2009). This result suggests that metallothionein SNPs may play an important role in influencing mercury levels in humans. The goal of the present study is to investigate whether SNPs in metallothionein and related genes described above modify the relationships of elemental mercury exposure with urinary Hg level in humans. This study will try to explain the considerable variation seen in subjects exposed to elemental mercury of similar magnitudes from a perspective of genetic polymorphisms in genes potentially important for mercury distribution in humans.

2. Materials and Methods

The subjects in this study were recruited during the Michigan Dental Association (MDA) annual conventions held in 2009 (n=232) and 2010 (n=283). They represent a convenience sample of dental professionals who attended the conventions. Participants were provided written informed consent that had been reviewed and approved by the University of Michigan Institutional Review Board (HUM00027621). Each participate received a confidential letter summarizing and interpreting the results for the samples and measurements they provided.

2.1 Collection of Exposure Variables

Each subject completed a self-administered questionnaire to collect variables that were used as surrogates for recent mercury exposures from different sources. The questionnaire included items for occupational exposure to elemental mercury (how many amalgams placed/removed per week), personal mercury exposure from dental amalgam (how many amalgams in mouth), pre-existing diseases, demographics (race, gender, height weight, age, occupation, etc) and other factors that may influence their exposures to elemental mercury such as gum-chewing, teeth-grinding, and recent chelation activities. Staff members were available to answer any questions subjects had. Questionnaires were reviewed for completeness.

2.1.1 Questionnaires

2.1.1.1 Elemental Mercury Exposure: Occupational and Personal Exposures from Amalgam

The survey included questions related to occupational and personal exposures from amalgam. The variables collected from the questionnaire included average number of amalgams placed/removed per week, and total number of personal amalgams.

2.1.1.2 Preexisting Kidney Diseases

The questionnaire asked about 18 preexisting diseases in all subjects and whether or not the diseases had been diagnosed by a physician (Table 2.2). Eight subjects that reported preexisting kidney diseases (lithiasis, pyelonephritis, orthostatic proteinuria, end stage kidney disease and chronic renal failure) were excluded in the multivariable linear regression analyses.

2.1.1.3 Covariates

Factors that may confound the associations of urine biomarker levels with exposures from occupational practices and personal amalgam were also included in the questionnaire. These potential confounders were surveyed in the following formats: frequency of consuming alcoholic beverages, whether or not subjects reported teeth grinding while asleep, whether or not and how frequently subjects chew gum, and whether or not subjects had recently had chelation therapy.

2.1.1.4 Demographics

Demographic information was also included in the questionnaire. Surveyed variables included age, self-reported height, self-reported weight, BMI, race, gender and occupation (Table 2.1). A dichotomous variable with dentist being the referent as opposed to non-dentist was later used in the multivariate linear regression analyses.

2.2 Collection and Analysis of Urine Specimen

2.2.1 Urine Specimen Collection

Each subject was asked to provide a spot urine sample in a mercury-free container (Becton, Dickinson, and Company; NJ, USA). Urine samples were immediately put on dry ice and then transported back to the lab to be frozen at a temperature below -20°C. Urine samples were not obtained from 3 and 10 subjects in 2009 and 2010, respectively.

2.2.2 Urinary Mercury Analysis

Total mercury content was determined using atomic absorption spectroscopy in the Direct Mercury Analyzer-80 (DMA-80, Milestone Inc., Shelton, CT) based on U.S. EPA Method 7473 as previously described by our laboratory (Paruchuri, et al. 2010). Briefly, 800 µl urine samples were vortexed and placed into quartz boats in the DMA-80. Samples were thermally decomposed into gaseous mercury which was delivered by oxygen to a gold amalgamator that selectively traps mercury. The mercury vapor released by the heated amalgamator is then measured as a function of mercury concentration via absorbance at a wavelength of 253.65nm in the atomic absorption spectrophotometer.

For each measurement, 800 µl of urine was pipetted into a quartz boat. The quartz boat for each measurement was pre-cleaned and periodically sonicated. Empty boats were run as blanks at least every 10 samples, before the first measurement of a day and after the last measurement of a day. As a result, every 11 samples contained at least one blank. To ensure accuracy and precision of measurements, Standard Reference Materials (SRMs) were run every 9 to 18 samples depending on the number of samples in one batch and multiple batches of urine samples were usually run each day. Sample duplicates were also run each day to determine accuracy and precision from samples. The SRMs used in 2009 were ClinCheck urine control (Recipe Chemicals, Germany) and DOLT-3 (National Research Council, Canada). The SRMs used in 2010 were Institut National de Sante Publique Quebec urine standard QMEQAS08U-01 and DOLT-4 (National Research Council, Canada).

2.2.2.1 Total Mercury Measurement Accuracy

2.2.2.1.1 2009 Samples

The SRMs were measured on each day of analysis to determine the validity of the calibration curves. The average recovery of Hg for the ClinCheck SRM for 12 analysis days was 122.89 ± 24.50%. The observed and expected mean Hg concentration was 51.49 ± 10.84µg/l and 42µg/l, respectively. Since new aliquots were made every two weeks, ClinCheck SRM used in the 12 non-consecutive

analysis days was made separately from different aliquots. A large discrepancy was seen between the expected and observed Hg levels as well as a high variation of Hg recovery between aliquots. The manufacturer had reported ClinCheck SRM to be inaccurate after numerous customer complaints. However, ClinCheck SRM still gave precise, albeit inaccurate, results when the aliquots made in the same batch were used. Fortunately, the recovery for DOLT SRM was stable and had much less variability, which thus demonstrated high validity of all urine Hg measurements. The average recovery of Hg for the DOLT SRM for 12 analysis days was 99.49 ± 4.40%. The observed and expected mean Hg concentration was 3342.93 ± 144.56μg/g and 3370μg/g, respectively.

2.2.2.1.2 2010 Samples

The SRMs were measured each day of analysis to determine the validity of the calibration curves. The average recovery of Hg for the INSPQ urine SRMs for 8 analysis days was 71.6 ± 3.9%. The observed and expected mean Hg concentration was 15.7 ± 0.9μg/l and 22.1μg/l, respectively. The average recovery of Hg for the DOLT-4 SRM for 10 analysis days was 90.2 ± 7.2%. The observed and expected mean Hg concentration was 2326.7 ± 186.6μg/kg and 2580μg/kg, respectively. The stable recovery of DOLT-4 SRM gave a good accuracy and provided confidence in the validity of the urine Hg measurements.

2.2.2.2 Total Mercury Measurement Precision

2.2.2.2.1 2009 Samples

Precision (reproducibility) was also measured by within day and between day analysis of SRM and sample replicates. The Relative Standard Deviations (RSDs) (%RSD=SD/ (χ of the replicate values) * 100%) were calculated from replicates of SRMs on the same analysis days to indicate within day precision, and from the SRMs on different analysis days to indicate between day precision. The within day variability of ClinCheck SRM, measured by RSD% of within day analysis, ranged from 2.19 to 4.32%RSD with a mean of 3.29 ± 0.72%RSD for 7 analysis days during which there were replicates of the SRM. The within day

variability of DOLT-3 SRM, on the other hand, was slightly larger, ranging from 0.49 to 9.20%RSD with a mean of 3.97 ± 3.59%RSD for 6 analysis days during which there were replicates of the SRM. The overall data showed good within day precisions. On days when SRMs were analyzed more than once, the within day averages for the SRMs were used to determine the between day average. The between day variability of ClinCheck SRM, measured by %RSD of between day analyses, was 21.05%RSD for 7 analysis days. In contrast, the between day variability of DOLT-3 SRM for 6 analysis days was 4.32 %RSD. DOLT-3 SRM gave good precision measurements for validity of all urine samples measured in 2009.

2.2.2.2.2 2010 Samples

The within day variability of the INSPQ urine SRM, measured by RSD% of within day analysis, ranged from 0.9 to 4.6%RSD for 7 analysis days during which there were replicates of the SRM. The within day variability of DOLT-4 SRM, on the other hand, was slightly larger, ranging from 0.5 to 8.0%RSD for 10 analysis days during which there were replicates of the SRM. The overall data showed good within day precisions. On days when SRMs were analyzed more than once, the within day averages for the SRMs were used to determine the between day average. The between day variability of the INSPQ urine SRM and DOLT-4 SRM, measured by %RSD of between day analysis, were 5.43%RSD and 8.02 %RSD, respectively. Both the INSPQ urine SRM and DOLT-4 SRM gave good precision measurements for validity of all urine samples measured in 2010.

2.2.2.3 Total Mercury Precision from Samples

2.2.2.3.1 2009 Samples

Sample duplicates were run each day for randomly selected samples. The average %RSD for within day sample duplicates were 4.48 ± 3.96 %RSD (n=31) ranging from 0 to 19.32%.

2.2.2.3.2 2010 Samples

Sample duplicates were run each day for randomly selected samples. The average %RSD for within day sample duplicates were 2.98 ± 3.86 %RSD (n=28) ranging from 0 to 16.89%.

2.2.2.4 Total Mercury Detection Limit

2.2.2.4.1 2009 Samples

Samples that had Hg levels below the Theoretical Method Detection Limit, or TMDL were considered below the detection limit. The TMDL is calculated as 3 times the standard deviation of measurement averages of all blanks. In the study of 2009, the TMDL is 0.046 ng. Samples whose Hg levels were below the Practical Method Detection Limit or PMDL (5 x TMDL=0.23 ng) but still above the TMDL were considered to be valid measurements. In the study of 2009, forty-four urine samples had Hg measurements below the PMDL but above the TMDL. These measurements were used without further adjustment in the final analyses. No samples were found below the TMDL.

2.2.2.4.2 2010 Samples

In the study of 2010, the TMDL is 0.011 ng. Samples whose Hg levels were below the PMDL (5 x TMDL=0.055 ng) but still above the TMDL were considered to be valid measurements. Two urine samples had Hg measurements below the PMDL but above the TMDL. These two measurements were used without further adjustment in the final analyses. No samples were found below the TMDL.

2.3 Collection and Analysis of DNA Samples

Buccal swabs were used to collect DNA samples from the subjects based on a published protocol (Min, et al. 2006). A total of four buccal swab samples were collected from each subject. Subjects rubbed the cotton bud of a buccal swab along the inside of their mouth for 30 seconds for each sample and placed the bud in a saline-prefilled tube. The tubes that each contained four buccal swab samples were then stored in lab freezer at –15 to –25 °C. Genomic DNA was isolated within seven days and purified for genotyping using Promega SV

Genomic kit. Briefly, to isolate and purify genomic DNA, buccal swabs were incubated for 1 hr at 55 ºC in storage buffer with 0.032 mg/mL RNase and 0.1mg/mL Proteinase K; then we added 1 mL lysis buffer. We divided each individual sample into two purification columns and eluted the DNA in each column with 150 µL of TE buffer. We then quantified DNA with Picogreen kit and stored at -20ºC until use.

2.3.1 Selection of Metallothionein SNPs

Thirteen metallothionein SNPs were selected for the study based on the following criteria: 1) the significance of a SNP for the functionality and synthesis of metallothionein proteins (e.g. SNPs in metal-responsive-elements (MREs), 3'UTR and coding sequences); 2) SNP prevalence $\geq$ 5% in the Centre d'Etude du Polymorphisme Humain (CEPH) panel as reported in the PubMed SNP database; and 3) previous research results on metallothionein SNPs showing the importance of mRNA localization to the expression of metallothionein proteins (Hesketh 2004). Table 2.3 lists the selected metallothionein SNPs investigated in 2009 and 2010. Among the 13 selected SNPs, 11 MT SNPs are located on Chromosome 16q while two Metallothionein Transcription Factor 1 (MTF1) SNPs are located on Chromosome 1q. The relative positions and lengths of the 11 MT SNPs are shown in Figure 2.1.

2.3.2 DNA Genotyping

Sets of primers and probes for genotyping each SNP were ordered from Applied Biosystems, Inc (Foster City, CA). TaqMan Allelic Discrimination Assay was used for genotyping on ABI 7300 (Carlsbad, CA). Briefly, for every reaction that genotypes one SNP for each subject, we mixed 12.5 µL 2x Taqman Genotyping Master Mix (DNA polymerase, dNTPs, etc) with 1.25 µL 20x SNP assay designated for each SNP (2 primers to amplify gene and 2 probes to detect the 2 alleles of the SNP of interest). We then pipetted 13.75 µL of this mixture into each well of a 96-well semi-skirted plate. All wells contained the same amount of DNA (3 to 20 ng). We then diluted DNA samples in the appropriate amount of

nuclease-free water and pipetted a total of 11.25 µL of water with DNA sample into the plate. For each plate, 4 blank control and 2 sample duplicates were run for quality control. Loaded QPCR seal covered plate into ABI 7300 QPCR machine and performed a pre-read (to measure background fluorescence), an amplification (10 minutes at 95 ºC; 15 sec at 92 ºC and 90 sec at 60 ºC for 40-50 cycles), and a post-read (to measure changes in fluorescence after DNA amplification). We then analyzed the three clusters that represented three genotypes: major homozygotes, heterozygotes, minor homozygotes.

Restriction Fragmentation Length Polymorphism (RFLP) method was used to genotype MT1A (rs8052394). Primers were designed to amplify coding region based on the published gene sequence in Pubmed. The forward and reverse primer sequences were 5`- CAAACTGAGGCCAAGAGTGCACCA-3` and 5`-TGACCTGAGGCAGGTGCCTGATTT-3`, respectively. The primers were designed to introduce a *Pst*I endonuclease restriction site in sequences containing the missense mutation (single nucleotide polymorphism site). PCR was performed using 50 ng genomic DNA template, 1 U DyNAZYME DNA polymerase (FINNZYMES, USA), 5 µl DyNAZYME buffer, 200 µmol/l of deoxynucleotide triphosphate mix (Hamburg, Germany), and 350 nmol/l of each primer in a 50 µl reaction volume. Forty cycles of PCR were performed (2 seconds at 94°C, 60 seconds at 61°C, and 60 seconds at 72°C). The PCR products were then digested with restriction endonucleases *Pst*I. PCR product (25 µl) was added to 20,000 U *Pst*I (New England Biolabs), 1× digest buffer (New England Biolabs), and 100× bovine serum albumin (BSA) (New England Biolabs) to a final volume of 50 µl and incubated at 37°C for two hour. The digestion reactions were heat inactivated by incubation at 80°C for 15 minutes. The products were analyzed by agarose gel electrophoresis using a 1.5% (w/v) Nusieve 3:1 agarose (Eurofins MWG Operon, USA) gel, 1× TAE buffer, and 0.5 µg/ml ethidium bromide. Undigested amplification products were 743 bp in length. After digestion with *Pst*I a product of 405 bp was obtained from mutant sequences. Heterozygote samples produced both products (gel bands) of 743 bp

and 405 bp after digestion while minor homozygote samples produced only products of 405 bp.

Table 2.3 lists allele frequencies of each selected SNP and Hardy-Weinberg Equilibrium (HWE; pass/fail). The allele frequencies of all SNPs are in HWE. Several SNPs were only genotyped in 2009 or 2010. In the multivariable regression analyses, we created two dummy variables for each SNP: heterozygote (major homozygote as referent) and minor heterozygote (major homozygote as referent).

3. Statistical Methods

All statistical analyses were performed in SAS 9.2 (SAS Institute Inc., Cary, NC). Descriptive analyses were performed on BMI, age, occupation, race of the subjects as well as their Hg exposure levels reflected in urinary Hg levels and surrogate variables. Bivariate analyses included age- and occupation- stratified analyses of BMI and age, exposure-stratified urinary Hg levels and SNP genotype-stratified urinary Hg levels. Multivariate regression analyses were conducted in two phases. In the first phase, using multivariate linear regression models, natural log-transformed urinary Hg was regressed against exposure surrogates of elemental Hg (occupational exposure and personal amalgam). In the regression model, covariates that might influence exposure (gum-chewing, teeth-grinding, chelation therapy, occupation, age, race, BMI, gender, etc) were all added to the model. A base model was selected for urine Hg using backward elimination starting from the model including all exposure terms and covariates. The least statistically significant predictor was eliminated in each step and the final base models for urine Hg were derived by retaining all significant predictors ($p<0.05$). In the final model, gender was retained. Because a majority (94.8%) of the non-dentists was female, we fitted the base model with a dentist-only or a nondentist-only sample in order to assess the potential confounding of gender with occupation. Gender was not significant in the dentist-only sample, meaning the gender significance was due to confounding with occupation. Thus, we did

not keep gender in the base models. In the second phase, the base model was combined with main effect and interaction terms between the respective exposure predictors in the base model and dummy variables of each SNP. Such interactions between exposure and genotype were investigated one SNP at a time in separate models.

4. Results

4.1 Demographics

As shown in Table 2.1, the total sample includes 515 subjects. There were 244 dentists (47.4%), 269 non-dentists (52.2%), and 2 unidentified (0.4%). The sample population is predominantly Caucasian (90.5%). There were 198 males (38.5%) and 316 females (61.5%). Thirty-five point nine percent (n=184) of the subjects were male dentists while about 50% (n=255) were female non-dentists. The mean ages for dentists and non-dentists were 56.1 and 48.2 years old, respectively, and this difference was significant. The mean ages for Caucasians and non-Caucasians are 52.5 and 46.8 years old, respectively, and they also differ significantly. The mean BMIs of dentists and non-dentists did not differ significantly and neither did that of Caucasians and non-Caucasians.

4.2 Level of Urine Biomarker

Table 2.4 summarizes the distributions of urine Hg levels for 2009, 2010 and the combined (2009-2010) along with the levels in the US general population reported by NHANES (2003-2004). T-test show no significant difference in both arithmetic and geometric mean urine Hg levels between the two years (p=0.42 and 0.30, respectively). Thus, we combined and used the data obtained from both years for subsequent analyses. There was no significant difference in geometric mean Hg levels in urine in our study population compared to the NHANES reference levels. Overall, the mean level and distribution of urine mercury seen in our study are similar to the US general population.

4.3 Level of Exposure to Elemental Hg and Other Covariates

Table 2.5 summarizes the variables reflecting the level of exposure to elemental Hg from occupational practices and personal amalgams. In the 2009 questionnaire, the variable alcoholic beverage was categorized as follows: didn't drink, drank no more than 1 alcoholic beverage pre week, drank more than 1 but no more than 2 alcoholic beverages per week, and drank no less than 2 alcoholic beverages per week. About 57.74% (n=127) of the subjects did not drink any alcoholic beverages in the last 6 month. About 33.19% (n=77) of the subjects drank no more than 1 alcoholic beverage per week and 9.48% (n=22) of the subjects drank more than 1 but no more than 2 alcoholic beverages per week while the rest 2.59% (n=6) of the subjects drank no less than 2 alcoholic beverages per week. In the 2010 questionnaire, however, we improved the question by switching to the NHANES Food Frequency Questionnaire (FFQ) for alcohol consumption related questions. We instead asked the frequency of consuming alcoholic beverages as defined in number of days per week or per month; we followed up with the question of the number of drinks they usually had on days when they drank. About 26.45% (n=73) of the subjects in 2010 did not drink any alcoholic beverages. The mean consumption was 0.39 alcoholic beverages per day in 2010.

In the two years combined, there were 69 (13.42%) subjects who reported to grind their teeth while asleep when mouthguards were not worn. There were 4 subjects (0.79%) who have had chelation therapy in the past 6 month. The mean numbers of hours of gum-chewing per day were 0.99 ranging from 0 hour (n=255; 50%) to 6 hours (n=1; 0.19%).

4.4 Associations between Elemental Hg Exposure and Level of Urinary Mercury

Table 2.6 shows a clear and significant trend in arithmetic mean urinary Hg levels as elemental Hg exposure increased from both occupational practice and personal amalgam. Cochran-Armitage test of linear trend in mean urinary Hg were significant for from both handling amalgam and personal amalgam.

4.5 Associations between SNPs and Levels of Biomarkers (No Adjustment for Exposure to Elemental Hg)

Table 2.7 summarizes the mean urine Hg levels stratified by three genotypes of the selected matallothionein SNPs after excluding subjects with preexisting kidney diseases. The table also presents the results of ANOVA tests comparing the mean urine Hg levels among SNP genotypes. ANOVA results showed no significant differences in mean urine Hg levels between SNP genotypes for all MT SNPs studied.

4.6 Results of Urine Multivariate Linear Regression Models

Table 2.8 displays multivariate linear models of natural log transformed urinary mercury as predicted by elemental Hg exposure, SNP genotypes and exposure-SNP interactions. In the base model, number of personal amalgams (linear continuous variable) and number of amalgams handled per week (ordinal variable; see categories from Table 2.6) predicted natural log transformed urinary Hg adjusting for occupation (dentist or not, categorical) as shown in the first row of the table. We then added terms of SNP main effects and exposure-SNP interactions to each of the base models as shown in the rest of the table. For all SNPs except for MT1M (rs2270836) and MTF1 (rs473279), no statistically significant interactions were observed. In MT1M 3'UTR (G>A), significant interaction was found between the minor homozygote with both number of personal amalgam and number of amalgam handled. Compared to those with major homozygote (GG) for MT1M 3'UTR (G>A), subjects with minor homozygote (AA) had lower urinary Hg levels (Figure 2.2 and 2.3). In MTF1 3'UTR (G>A), a significant interaction was found between the heterozygote and occupation. Due to small number of subjects with minor homozygotes for MT1A (rs8052394; A>G), MT1G 3'UTR (G>T), MT1E 3'UTR (G>T), MT4 missense (G>A), MT2A 5'UTR (A>G) and MT1A 5'near region (C>G), minor homozygotes for these SNPs were combined with respective heterozygotes in the analyses.

5. Discussion

We investigated the influence of selected MT SNPs on urinary mercury levels in relation to elemental Hg exposure. We tested our hypothesis in a convenience sample of dental professionals whose urinary Hg levels are similar to that found in the US general population. We found significant effect modifications of minor homozygote genotype (AA) of MT1M (rs2270836) on the relationship of urinary Hg level with surrogates of both occupational and personal exposure to elemental Hg after adjusting for covariates. We also detected a significant effect modification of MTF1 (rs473279) on the relationship of urinary Hg level with occupation adjusting for covariates. This latter finding was difficult to interpret in relation to the research question we are addressing in this study and might as well be due to chance as we did not observe effect modification with dental amalgam or occupational amalgam handling. No significant influence from other MT SNPs was found. This study, to our knowledge, is the first to investigate the potential modifying effects of metallothionein SNPs on the relationship of urinary Hg biomarker levels with exposure to elemental mercury. Other findings of the study included that natural log transformed urinary Hg level was predicted by number of personal amalgams, number of amalgams handled per week and occupation.

The urinary Hg levels observed in our study population was consistent with a number of previous reports for both occupationally and non-occupationally exposed populations (DeRouen, et al. 2006; Echeverria, et al. 2005; Echeverria, et al. 2006; Factor-Litvak, et al. 2003; Heyer, et al. 2008). Echeverria et al., (2005, 2006) reported the arithmetic mean urinary Hg levels in male dentists and female assistants were 3.32μg/L and 1.98μg/L, respectively. Heyer et al., 2008 reported the values in male dentists and female assistants were 2.5μg/L and 1.6μg/L, respectively. The overall mean urinary Hg level in this study was 1.01μg/L, which was slightly lower with dentists having a mean of 1.37μg/L and nondentists

(almost all dental hygienists and dental assistants) having a mean of 0.75μg/L. In populations that were not occupationally exposed to mercury, DeRouen et al., 2006 and Factor-Litvak et al., 2003 reported urinary Hg levels in children of age 8 to 10 and healthy working adults were 1.8μg/g and 1.7μg/g creatinine, respectively. As the use of personal protective equipment for dental offices increases and the use of mercury-containing amalgam declines, it may not be surprising to see such a drop in urinary Hg levels.

The associations of urinary Hg level with number of personal dental amalgams have been well established (Dye, et al. 2005; Kingman, et al. 1998; Olstad, et al. 1987; Skare and Engqvist 1994). In a cross-sectional study of 1127 healthy veterans conducted by Kingman et al., 1997, urinary Hg level was predicted by total amalgam surfaces; it was also estimated that, on average, each ten-surface increase in amalgam exposure is associated with an increase of 1μg/L mercury in urine concentration. Similarly, in an assessment of NHANES (1999-2000) data conducted by Dye et al., 2005, total posterior dental surfaces were found to be a significant predictor for log transformed urine Hg level in adult women aged 16-49 years. In our study, we did not take measurements of total amalgam surfaces but rather, we only had subjects' self-reported number of amalgams fillings. We were still able to show that the number of self-reported amalgams among dental professionals was a significant predictor for urinary Hg level. Most importantly, this single predictor explained over 60% of the explained variance in the multivariate linear regression model.

Significant associations of urinary Hg level with occupationally exposure to dental amalgams in dental offices have also been reported (Lehto, et al. 1989; Naleway, et al. 1991). In Naleway et al., 1991, the number of amalgams placed per week by dentists was associated with urinary Hg level. However, the mean urine level was much higher (5.8μg/L in urine) than in the current study. This difference may reflect historical differences in the number of amalgam fillings placed/removed per week and amalgam handling practices. A similar association was also found

by Lehto et al., 1989. In our study, at a mean urine level that was five-fold lower, we were still be able to find significance in the number of amalgams handled per week in predicting urinary Hg level. However, professional handling of amalgam only explained approximately 10% of the total variance of the multivariate regression model. In contrast, personal dental amalgam explained over 60% of the total variance. It is known that the contribution to urinary mercury from professional practice has been significantly reduced in the past decades due to continuous advancement of protective measures and improved workplace regulations (American Dental Association, 2003). Perhaps this could explain the findings in our study that exposure from professional handling of amalgam contributed less to urinary Hg compared to the proportion that was attributed to personal dental amalgam.

Our study is the first to report potential effect modification of any MT SNPs on the relationship of urinary Hg with occupational and non-occupational exposures. Thus, the findings contributed to a small body of previous research on MT SNPs modifying mercury exposure-biomarker relationship in humans. One study reported findings of effect modifications of MT4 (rs11643815), and MT1A (rs11640851) on exposure-biomarker relationship in mercury in hair but not in urine. Gundacker et al., 2009 found subjects with MT4 GG alleles and MT1A AA alleles have lower hair mercury compared to those with GA/AA and AC/CC. Elemental Hg exposure (both personal and occupational) was not examined in their analyses. No prior studies have attempted to assess the interaction of MT SNPs with mercury exposure and the impact on urinary mercury levels. In our study, neither MT4 (rs11643815) nor MT1A (rs11640851) were found to modify elemental mercury exposure-biomarker relationship.

The study found evidence of effect modification of MT1M (rs2270836) SNP on the relationship between urinary Hg and personal and occupational exposures to elemental Hg. Recent studies have showed MT1M expression is associated with cell apoptosis and is significantly upregulated upon *in vitro* treatment of zinc and

copper in various cell lines (prostate cancer cell, bronchial epithelial cell, and Sertoli TM4 cell etc.) (Bigagli, et al. 2010; Kheradmand, et al. 2010; Lin, et al. 2009). Reduced expression of MT1M has also been proposed to be associated with vulnerability to oxidative stress in subjects of major depression (Shelton, et al. 2010). Recent epigenetic research on MT1M also showed a strong correlation between DNA methylation of MT1M genes and duration of smoking history in noncancerous esophageal mucosae of human subjects (Oka, et al. 2009). These results suggested that MT1M may be a functional gene in humans dealing with detoxification of heavy metals and responding to oxidative stress. It is not clear, however, whether this particular SNP (rs2270836) of MT1M gene may be a functional variant. The role of MT1M gene and this particular SNP still warrant further research.

The study suffered from a number of limitations. First, the study had a relatively small sample size of dental professionals available for analysis. For some of the minor homozygote genotypes, for instance, MT1M (rs2270836), there were fewer than 15 subjects with the minor homozygote genotype. That challenged the validity of our findings on MT1M (rs2270836) and also limited the degree of investigation we could perform on potential effect modification of exposure-biomarker relationships by the SNPs of interest for MT1M (rs2270836) as well as some other SNPs. Second, it was difficult to rule out the possibility of false positives due to multiple comparisons in the investigation of interactions. There were 51 comparisons made in the urine models and only 3 of those were statistically significant (Table 2.8). Third, our study group was a convenience sample, not a random sample. Although our study population was a self-selected convenience sample of volunteers, there was no reason to believe that subjects had any prior knowledge of their genotypes or mercury levels. Thus, there was likely to be no bias. Four, instead of using the total amalgam surfaces, a more refined and often more accurate exposure surrogate, the study used the total number of amalgam restorations as the surrogate for personal exposure to elemental Hg. Despite this, total number of personal amalgams was still found to

be a significant predictor for natural log transformed urinary Hg level. Five, self-reported measurements may create recall and misclassification bias. Despite potential bias, our findings on the relationship between urinary Hg levels and exposures from personal amalgams and dental practice were consistent with the results of previous reports. Six, when it comes to the reliability of urinary Hg biomarkers accurately reflecting exposure, day-to-day variability in urinary Hg excretion in addition to inter-individual and intra-individual variability has been reported to be averaged 22% among samples taken on three consecutive days (Ellingsen, et al. 1993). However, this magnitude of variation was modest and the likely impact would be to bias over the results towards the null. Although the study was limited by these factors, it is still the first and largest gene-environment study investigating the potential genetic susceptibility concerning metallothionein genes in humans by associating mercury urinary biomarker level with elemental Hg exposures.

Future work may include *in vitro* exposure studies to confirm the functionality of MT1M genes in a variety of cell lines. The functionality of MT1M (rs2270836) SNP, in particular, requires further research in such studies perhaps by exposing SNP deletion variants to heavy metals (e.g. Hg). The degree of induction of MT genes by mercury depends on the presence and the level of other MT gene inducers as well as the level of enzymes and protein products coded by genes that also involves in disposition of mercury in humans. Because glucocorticoid also induces MT gene expression along with oxidative stress (Karin, et al. 1984), it might help if glucocorticoid levels in blood can be measured in future studies. Genetic polymorphisms in other genes coding glutathione-S-transferase (GST), glutamate cysteine ligase (GCL) and selenoprotein might also be checked simultaneously for possible additive or even synergistic effects on urinary Hg levels. The results of the present study on genetic influence on urinary Hg should not be interpreted that subjects in possession of the MT1M (rs2270836) SNP are more susceptible to elemental mercury exposure. Research on genetic factors that may influence personal susceptibility of an individual to mercury exposure is

still in its early stage and requires much more effort in the future. However, the present study findings on MT1M (rs2270836) SNP modifying the exposure-biomarker relationship certainly requires further confirmatory research. It should also be noted that even though the study has primarily been concerned with dental professionals, the findings may give some insights into factors that may influence mercury metabolism from metallothionein polymorphisms in the US general population as the urinary levels found in the current dental sample overlaps that of the US general population and the SNP frequencies in our subjects were all in HWE. Future work may include examining potential effect modification of MT SNPs together with polymorphisms in other genes that are important for human mercury disposition on exposure-biomarker (e.g. hair and blood) relationships in a larger sample.

Figures and Tables

Table 2.1: Demographics

	N	Age (year;SD)	BMI (kg/m^2;SD)	Female
Occupation				
Dentist	244	56.1 (11.6)*	26.4 (4.04)	60 (24.59%)
Non-dentist	269	48.2 (11.2)*	26.4 (5.28)	255(94.80%)
Subtotal	513			315 (61.40%)
Missing	2			2
Race				
Caucasian	463	52.5 (11.9)ɫ	26.3 (4.57)	
Non-caucasian	49	46.8 (12.50)ɫ	27.1 (6.06)	
Subtotal	512			
Missing	3			

*$p<0.005$;ɫ$p<0.0001$

Table 2.2: Numbers and Percentages of Subjects with Pre-exisiting Diseases

Disease	N (%)
Hypertension	98 (19.03%)
Carpel Tunnel Syndrome	56 (10.89%)
Malignancy	51 (10%)
Hypothyroidism	45 (8.74%)
Head Trauma	32 (6.21%)
Diabetes	23 (4.47%)
Coronary Artery Disease	10 (1.94%)
Tremor	10 (1.94%)
Rheumatoid Arthritis	9 (1.75%)
Lithiasis	6 (1.17%)
Peripheral Vascular Disease	4 (0.78%)
Neurodegenerative Diseases	3 (0.58%)
Stroke	3 (0.58%)
Multiple Sclerosis	3 (0.58%)
Pyelonephritis	1 (0.19%)
Orthostatic Proteinuria	1 (0.19%)
End Stage Kidney Disease	0
Chronic Renal Failure	0

Table 2.3: List of Selected Metallothionein SNPs Genotyped in 2009 and 2010

SNP name	MT2A 3'UTR	MTF1 3'UTR	MTF1 3'UTR	MT1M 3'UTR	MT1M 3'UTR	MT1M missense	MT1G 3'UTR	MT1A missense	MT1E 3'UTR	MT4 missense	MT1A missense	MT2A 5UTR	MT1A 5' near
db SNP	rs10636	rs473279	rs3748682	rs9936741	rs2270836	rs1827210	rs12315	rs11640851	rs708274	rs11643815	rs8052394	rs28366003	rs9922957
Allele wildtype	G	G	T	T	G	A	G	A	G	G	A	A	C
Allele variant	C	A	C	C	A	C	T	C	T	A	G	G	G
Polymorphic site	3'UTR	3'UTR	3'UTR	3'UTR	3'UTR	Lys20Thr	3'UTR	Thr27Asn	3'UTR	Gly48Asp	Lys51Arg	MRE proximity	MRE proximity
Genotyped in 2009			X			X	X		X	X			
Genotyped in 2010											X	X	X
Genotyped in 2009-2010	X	X		X	X			X					

Table 2.4: Urine Mercury (µg/l) Results in the MDA Mercury Study Compared with NHANES (2003-2004)

Sample	Geometric Mean	Arithmetic Mean	50th Percentile	75th Percentile	90th Percentile	95th Percentile
NHANES (n=1,529)	0.50ł	0.95[1]	0.48	1.12	2.20	3.33
2009 Study (n=229)	0.69łł	1.11	0.72	1.37	2.51	3.37
2010 Study (n=273)	0.62łł	1.02	0.62	1.19	2.15	3.74
2009-2010 (n=502)	0.65ł	1.06	0.66	1.29	2.34	3.37

[1] Arithmetic mean calculated using NHANES (2003-2004) data

ł : p=0.19 łł : p=0.77

Table 2.5: Descriptive Statistics for Exposure Variables of Elemental Hg

Variables	N	Maximum	Minimum	Mean	SD	Median
Occupational exposure surrogates:						
Total years in dental practice	514	66.0	0.0	24.0	12.6	25.0
Hours of work per week over career	514	87.0	0.0	32.2	8.4	32.0
Hours of work per week last 6 months	514	76.0	0.0	27.8	11.6	30.0
Number of amalgams handled (placed or removed) per week last 6 months	514	398.0	0.0	25.51	43.93	9.5
Personal exposure surrogates:						
Total number of personal dental amalgams	514	20.0	0.0	4.1	4.1	3.0

Table 2.6 Arithmetic Mean Urine Hg Levels (μg/l) Stratified by Levels of Elemental Hg Exposures from Occupational Practice and Personal Amalgam

Amalgam handled/wk (N)	Urine Hg (SD)(μg/L)*	Personal amalgam (N)	Urine Hg (SD) (μg/L)*
0 (215)	0.70(0.71)	0 (128)	0.61(0.79)
1-20 (102)	1.16(1.48)	1-3 (127)	0.91(0.90)
21-45 (102)	1.36(1.32)	4-6 (132)	1.11(1.32)
>=46 (82)	1.50(1.62)	>=7 (114)	1.67(1.61)

* Cochran-Armitage trend test : $p<0.05$

Table 2.7: Arithmetic Mean Urinary Hg Levels (μg/L) Stratified by Genotypes of Selected Metallothionein SNPs

SNP name	db SNP	Ref MAF (Caucasian)	N	Mean Urinary Hg (μg/L) Major homozygote (N)	Heterozygote (N)	Minor homozygote (N)	ANOVA *p*
SNPs Analyzed in Both 2009-2010							
MT2A 3'UTR(G>C)	rs10636	0.250	456	1.04(250)	1.04(166)	1.21(40)	0.70
MTF1 3'UTR(G>A)	rs473279	0.353	451	1.06(211)	1.07(202)	1.18(38)	0.83
MT1M 3'UTR(T>C)	rs9936741	0.042	457	1.06(441)	1.09(16)	-	0.91
MT1M 3'UTR(G>A)	rs2270836	0.278(JPN)	453	1.04(324)	1.10(118)	0.98(11)	0.88
MT1A missense(A>C)	rs11640851	0.269(AFR+CAU)	434	1.03(187)	1.09(193)	0.97(54)	0.77
SNPs Analyzed in 2009							
MTF1 3'UTR(T>C)	rs3748682	0.246	221	1.17(126)	1.05(81)	1.15(14)	0.78
MT1M missense(A>C)	rs1827210	0.144	223	1.09(162)	1.22(51)	1.14(10)	0.81
MT1G 3'UTR(G>T)	rs12315	0.060	223	1.11(202)	1.18(20)	0.54(1)	0.87
MT1E 3'UTR(G>T)	rs708274	0.100	224	1.12(171)	1.13(49)	0.67(4)	0.77
MT4 missense(G>A)	rs11643815	0.112	222	1.07(168)	1.20(51)	1.96(3)	0.39
SNPs Analyzed in 2010							
MT2A 5'UTR(A>G)	rs28366003	0.045	231	1.04(204)	0.81(25)	0.34(2)	0.47
MT1A missense(A>G)	rs8052394	0.097	132	0.95(109)	0.89(21)	1.19(2)	0.90
MT1A 5'near gene(C>G)	rs9922957	0.170(CHN+JPN)	228	1.03(174)	0.93(51)	0.55(3)	0.69

Note: When number of minor homozygote is smaller than 5, the ANOVA results shown is for comparing major homozygote and the pooled heterozygote and minor homozygote

Table 2.8: Coefficients and p Values from Multivariate Linear Regression Models of Natural Log-transformed Urinary Hg Predicted against Exposure Surrogates of Elemental Hg, SNP Genotypes and Exposure-SNP Interactions

		Base model								SNP main effects				
SNP name	db SNP	R^2	Intercept	Personal Amalgam		Amalgam handled/we ek		Nondentist		Homozygote	Heterozygote		Homozygote variant	
			?	**?**	***p***	**?**	***p***	**?**	***p***	**Ref**	**?**	***p***	**?**	***p***
2009-2010														
Base model	-	0.24	-0.66	0.080	*<0.0001*	0.12	*0.006*	-0.45	*<0.0001*	-	-	-	-	-
MT2A 3'UTR(G>C)	rs10636	0.25	-0.56	0.078	*<0.0001*	0.07	*0.3*	-0.54	*0.0002*	-	-0.21	*0.4*	-0.16	*0.7*
MTF1 3'UTR(G>A)	rs473279	0.24	-0.79	0.085	*<0.0001*	0.18	*0.007*	-0.28	*0.06*	-	0.39	*0.1*	0.29	*0.4*
MT1M 3'UTR(T>C)	rs9936741	0.24	-0.67	0.082	*<0.0001*	0.13	*0.008*	-0.43	*<0.0001*	-	0.51	*0.4*	-	-
MT1M 3'UTR(G>A)	rs2270836	0.25	-0.70	0.084	*<0.0001*	0.13	*0.006*	-0.41	*<0.001*	-	-0.85	0.9	**1.77**	***0.01***
MT1A missense(A>C)	rs11640851	0.25	-0.74	0.085	*<0.0001*	0.15	*0.02*	-0.42	*0.004*	-	0.099	*0.7*	0.37	*0.3*
2009														
MTF1 3'UTR(T>C)	rs3748682	0.24	-0.40	0.070	*<0.0001*	0.043	*0.69*	-0.69	*0.004*	-	-0.63	*0.1*	-0.33	*0.74*
MT1M missense(A>C)	rs1827210	0.24	-0.68	0.070	*<0.0001*	0.14	*0.13*	-0.37	*0.08*	-	-0.33	*0.4*	1.04	*0.2*
MT1G 3'UTR(G>T)	rs12315	0.24	-0.62	0.063	*<0.0001*	0.15	*0.06*	-0.41	*0.02*	-	-0.35	*0.5*	-	-
MT1E 3'UTR(G>T)	rs708274	0.23	-0.74	0.076	*<0.0001*	0.15	*0.09*	-0.32	*0.1*	-	0.22	*0.6*	-	-
MT4 missense(G>A)	rs11643815	0.23	-0.63	0.063	*<0.0001*	0.13	*0.14*	-0.39	*0.04*	-	-0.4	*0.4*	-	-
2010														
MT2A 5'UTR(A>G)	rs28366003	0.26	-0.55	0.090	*<0.0001*	0.07	*0.2*	-0.56	*<0.001*	-	-0.45	*0.3*	-	-
MT1A missense(A>G)	rs8052394	0.24	-0.79	0.127	*<0.0001*	0.06	*0.4*	-0.43	*0.02*		0.46	*0.4*	-	-
MT1A 5'near gene(C>G)	rs9922957	0.26	-0.61	0.100	*<0.0001*	0.052	*0.4*	-0.5	*0.0007*	-	0.016	*0.9*	-	-

Note: When number of homozygote variant is smaller than 5, the ANOVA results shown is for comparing homozygote and the pooled heterozygote and homozygote variant.

(Continued) Table 2.8: Coefficients and p Values from Multivariate Linear Regression Models of Natural Log-transformed Urinary Hg Predicted against Exposure Surrogates of Elemental Hg, SNP Genotypes and Exposure-SNP Interactions

SNP name	db SNP	SNP-exposure interactions											
		Amalgam X heterozygote		Amalgam handled X heterozygote		Nondentist X heterozygote		Amalgam X homozygote variant		Amalgam handled X homozygote variant		Nondentist X homozygote variant	
		?	*p*	?	*p*	?	*p*	?	*p*	?	*p*	?	*p*
2009-2010													
Base model	-	-	-	-	-	-	-	-	-	-	-	-	-
MT2A 3'UTR(G>C)	rs10636	0.0001	*0.99*	0.11	*0.3*	0.24	*0.26*	0.063	*0.1*	0.025	*0.9*	-0.064	*0.9*
MTF1 3'UTR(G>A)	rs473279	-0.011	*0.6*	-0.16	*0.1*	**-0.44**	***0.04***	0.004	*0.9*	-0.15	*0.4*	-0.13	*0.7*
MT1M 3'UTR(T>C)	rs9936741	-0.01	*0.8*	-0.22	0.4	-0.60	0.3	-	-	-	-	-	-
MT1M 3'UTR(G>A)	rs2270836	0.0001	*0.99*	0.09	0.4	-0.03	0.9	**-0.24**	***0.02***	**-0.89**	***0.02***	-0.18	0.8
MT1A missense(A>C)	rs11640851	-0.003	*0.9*	-0.057	*0.6*	-0.04	*0.9*	-0.009	*0.8*	-0.12	*0.4*	-0.17	*0.6*
2009													
MTF1 3'UTR(T>C)	rs3748682	0.011	*0.7*	0.22	*0.2*	0.68	*0.06*	0.02	*0.9*	0.20	*0.6*	0.56	*0.4*
MT1M missense(A>C)	rs1827210	0.009	*0.8*	0.25	*0.2*	0.25	*0.5*	-0.01	*0.9*	-0.55	*0.2*	-0.53	*0.4*
MT1G 3'UTR(G>T)	rs12315	0.06	*0.1*	-0.2	*0.6*	-0.12	*0.8*	-	-	-	-	-	-
MT1E 3'UTR(G>T)	rs708274	-0.009	*0.8*	0.03	*0.9*	-0.24	*0.6*	-	-	-	-	-	-
MT4 missense(G>A)	rs11643815	0.04	*0.2*	0.15	*0.5*	0.16	*0.7*	-	-	-	-	-	-
2010													
MT2A 5'UTR(A>G)	rs28366003	0.03	*0.5*	-0.06	*0.7*	0.47	*0.2*	-	-	-	-	-	-
MT1A missense(A>G)	rs8052394	-0.08	*0.11*	0.03	*0.9*	-0.08	*0.9*	-	-	-	-	-	-
MT1A 5'near gene(C>G)	rs9922957	-0.015	*0.7*	0.024	*0.9*	-0.04	*0.9*	-	-	-	-	-	-

Note: When number of homozygote variant is smaller than 5, the ANOVA results shown is for comparing homozygote and the pooled heterozygote and homozygote variant.

Figure 2.1: Relative Positions of Metallothionein SNPs of Interest on Human Chromosome 16q

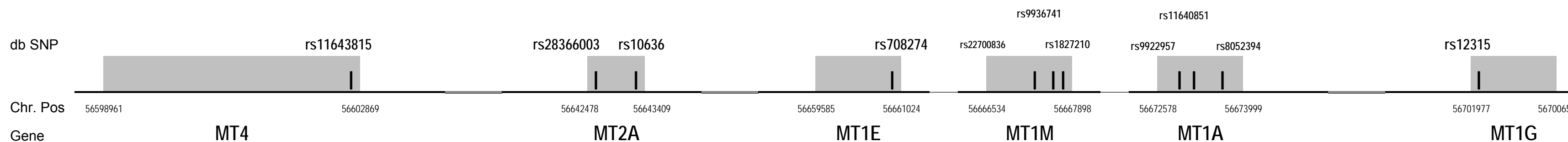


Figure 2.2: Scatter Plot of Ln-transformed Urine Hg against Number of Personal Amalgams Stratified by MT1M (rs2270836) Genotypes

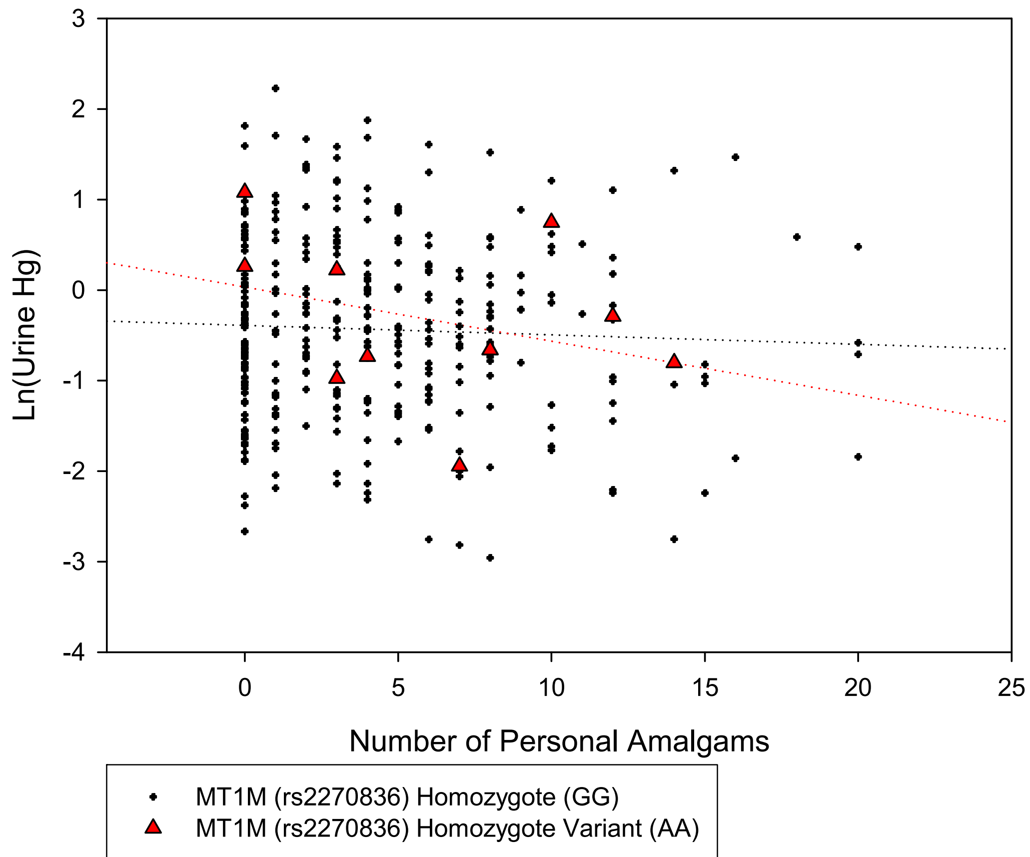
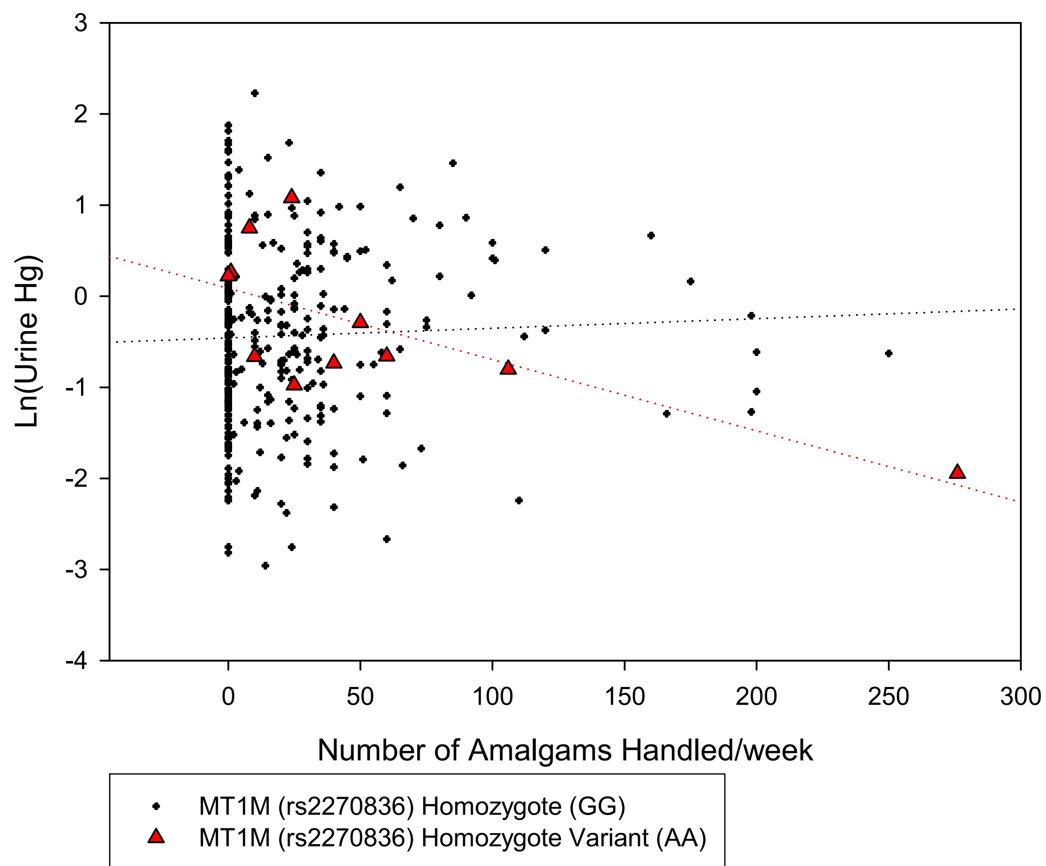


Figure 2.3: Scatter Plot of Ln-transformed Urine Hg against Number of Amalgams Handled Stratified by MT1M (rs2270836) Genotypes



Bibliography

ADA Council on Scientific Affairs, 2003. Dental mercury hygiene recommendations. J. Am. Dent. Assoc. 134, 1498–9.

Aschner, M., Syversen, T., Souza, D., Rocha, J.B.T., 2006. Metallothioneins: mercury species-specific induction and their potential role in attenuating neurotoxicity. Exp. Biol. Med. 231, 1468-1473.

Bigagli, E., Luceri, C., Bernardini, S., Dei, A., Dolara, P., 2010. Extremely low copper concentrations affect gene expression profiles of human prostate epithelial cell lines. Chem. Biol. Interact. 188, 214-219.

Custodio, H.M., Broberg, K., Wennberg, M., Jansson, J., Vessby, B., Hallmans, G., Stegmayr, B., Skerfving, S., 2004. Polymorphisms in glutathione-related genes affect methylmercury retention. Arch. Environ. Health 59, 588.

Custodio, H.M., Harari, R., Gerhardsson, L., Skerfving, S., Broberg, K., 2005. Genetic influences on the retention of inorganic mercury. Archives of environmental occupational health 60, 17.

DeRouen, T., Martin, M., Leroux, B., Townes, B., Woods, J., Leito, J., Castro-Caldas, A., Luis, H., Bernardo, M., Rosenbaum, G., Martins, I., 2006. Neurobehavioral effects of dental amalgam in children: a randomized clinical trial. JAMA 295, 1784-1792.

Dye, , Schober, , Dillon, , Jones, , Fryar, , McDowell, , Sinks, , 2005. Urinary mercury concentrations associated with dental restorations in adult women aged 16-49 years: United States, 1999-2000. Occup. Environ. Med. 62, 368.

Echeverria, D., Woods, J.S., Heyer, N.J., Rohlman, D.S., Farin, F.M., Bittner, A.C., Li, T., Garabedian, C., 2005. Chronic low-level mercury exposure, BDNF polymorphism, and associations with cognitive and motor function. Neurotoxicol. Teratol. 27, 781.

Echeverria, D., Woods, J.S., Heyer, N.J., Rohlman, D., Farin, F.M., Li, T., Garabedian, C.E., 2006. The association between a genetic polymorphism of coproporphyrinogen oxidase, dental mercury exposure and neurobehavioral response in humans. Neurotoxicol. Teratol. 28, 39.

Ellingsen, D.G., Thomassen, Y., Langrd, S., Kjuus, H., 1993. Urinary mercury excretion in chloralkali workers after the cessation of exposure. Scandinavian Journal of Work, Environment Health 19, 334-341.

Factor-Litvak, P., Hasselgren, G., Jacobs, D., Begg, M., Kline, J., Geier, J., Mervish, N., Schoenholtz, S., Graziano, J., 2003. Mercury derived from dental amalgams and neuropsychologic function. Environ. Health Perspect. 111, 719-723.

Gundacker, C., Gencik, M., Hengstschlger, M., 2010. The relevance of the individual genetic background for the toxicokinetics of two significant neurodevelopmental toxicants: Mercury and lead. Mutat. Res. 705, 130-140.

Gundacker, C., Gencik, M., Hikkel, I., Komarnicki, G., Kukuckova, M., Wittmann, K., 2009. Genetic background of lead and mercury metabolism in a group of medical students in Austria. Environ. Res. 109, 786.

Gundacker, C., Komarnicki, G., Jagiello, P., Gencikova, A., Dahmen, N., Wittmann, K.J., Gencik, M., 2007. Glutathione-S-transferase polymorphism, metallothionein expression, and mercury levels among students in Austria. Sci. Total Environ. 385, 37.

Hesketh, , 2004. 3'-Untranslated regions are important in mRNA localization and translation: lessons from selenium and metallothionein. Biochem. Soc. Trans. 32, 990.

Heyer, N.J., Echeverria, D., Farin, F.M., Woods, J.S., 2008. The association between serotonin transporter gene promoter polymorphism (5-HTTLPR), self-reported symptoms, and dental mercury exposure. Journal of toxicology and environmental health.Part A 71, 1318.

Karin, M., Haslinger, A., Heguy, A., Dietlin, T., Cooke, T., 1987a. Metal-responsive elements act as positive modulators of human metallothionein-IIA enhancer activity. Mol. Cell. Biol. 7, 606-613.

Karin, M., Haslinger, A., Heguy, A., Dietlin, T., Imbra, R., 1987b. Transcriptional control mechanisms which regulate the expression of human metallothionein genes. Experientia.Supplementum 52, 401-405.

Karin, M., Haslinger, A., Holtgreve, H., Richards, R.I., Krauter, P., Westphal, H.M., Beato, M., 1984. Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene. Nature 308, 513-519.

Kheradmand, F., Nourmohammadi, I., Modarressi, M., Firoozrai, M., Ahmadi-Faghih, M., 2010. Differential Gene-Expression of Metallothionein 1M and 1G in Response to Zinc in Sertoli TM4 Cells. Iranian biomedical journal 14, 9-15.

Kingman, A., Albertini, T., Brown, L.J., 1998. Mercury concentrations in urine and whole blood associated with amalgam exposure in a US military population. J. Dent. Res. 77, 461-471.

Kumari, , Hiramatsu, , Ebadi, , 1998. Free radical scavenging actions of metallothionein isoforms I and II. Free Radic. Res. 29, 93.

Lehto, T., Alanen, P., Rnnemaa, T., Helenius, H., Kallio, V., 1989. Urinary mercury concentrations in Finnish dentists. Finnish Dental Society Proceedings Supplement 85, 177-185.

Lin, S., Wei, H., Maeder, D., Franklin, R., Feng, P., 2009. Profiling of zinc-altered gene expression in human prostate normal vs. cancer cells: a time course study. J. Nutr. Biochem. 20, 1000-1012.

Min, J.L., Lakenberg, N., Bakker-Verweij, M., Suchiman, E., Boomsma, D.I., Slagboom, P.E., Meulenbelt, I., 2006. High microsatellite and SNP genotyping success rates established in a large number of genomic DNA samples extracted from mouth swabs and genotypes. Twin Research and Human Genetics 9, 501.

Naleway, C., Chou, H.N., Muller, T., Dabney, J., Roxe, D., Siddiqui, F., 1991. On-site screening for urinary Hg concentrations and correlation with glomerular and renal tubular function. J. Public Health Dent. 51, 12-17.

Oka, D., Yamashita, S., Tomioka, T., Nakanishi, Y., Kato, H., Kaminishi, M., Ushijima, T., 2009. The presence of aberrant DNA methylation in noncancerous esophageal mucosae in association with smoking history: a target for risk diagnosis and prevention of esophageal cancers. Cancer 115, 3412-3426.

Olstad, M.L., Holland, R.I., Wandel, N., Pettersen, A.H., 1987. Correlation between amalgam restorations and mercury concentrations in urine. J. Dent. Res. 66, 1179-1182.

Palmiter, R.D., 1994. Regulation of metallothionein genes by heavy metals appears to be mediated by a zinc-sensitive inhibitor that interacts with a constitutively active transcription factor, MTF-1. Proc. Natl. Acad. Sci. U. S. A. 91, 1219.

Paruchuri, Y., Siuniak, A., Johnson, N., Levin, E., Mitchell, K., Goodrich, J., Renne, E., Basu, N., 2010. Occupational and environmental mercury exposure among small-scale gold miners in the Talensi-Nabdam District of Ghana's Upper East region. Sci. Total Environ. 408, 6079-6085.

Sandborgh-Englund, G., Elinder, C.G., Johanson, G., Lind, B., Skare, I., Ekstrand, J., 1998. The absorption, blood levels, and excretion of mercury after a single dose of mercury vapor in humans. Toxicol. Appl. Pharmacol. 150, 146-153.

Saydam, N., Steiner, F., Georgiev, O., Schaffner, W., 2003. Heat and heavy metal stress synergize to mediate transcriptional hyperactivation by metal-responsive transcription factor MTF-1. The Journal of biological chemistry 278, 31879-31883.

Schläwicke Engström, K., Strömberg, U., Lundh, T., Johansson, I., Vessby, B., Hallmans, G., Skerfving, S., Broberg, K., 2008. Genetic variation in glutathione-related genes and body burden of methylmercury. Environ. Health Perspect. 116, 734.

Schurz, , Sabater-Vilar, , Fink-Gremmels, , 2000. Mutagenicity of mercury chloride and mechanisms of cellular defence: the role of metal-binding proteins. Mutagenesis 15, 525.

Shelton, R.C., Claiborne, J., Sidoryk-Wegrzynowicz, M., Reddy, R., Aschner, M., Lewis, D.A., Mirnics, K., 2010. Altered expression of genes involved in inflammation and apoptosis in frontal cortex in major depression. Mol. Psychiatry.

Skare, I., Engqvist, A., 1994. Human exposure to mercury and silver released from dental amalgam restorations. Arch. Environ. Health 49, 384-394.

Tsuji, J., Williams, P.R.D., Edwards, M., Allamneni, K., Kelsh, M., Paustenbach, D., Sheehan, P., 2003. Evaluation of mercury in urine as an indicator of exposure to low levels of mercury vapor. Environ. Health Perspect. 111, 623-630.

Chapter 3

Modifying Effects of Metallothionein Single Nucleotide Polymorphisms on the Association between Methylmercury Exposure and Hair Mercury Levels

1. Introduction

Large inter-individual variations have been seen in hair mercury levels in humans following exposures of similar magnitude to methylmercury from dietary fish consumption (Canuel, et al. 2006; Haxton, et al. 1979). Canuel et al., 2006 reported variations up to 14 times between average measured hair Hg level and expected value based on calculated daily oral exposure of methylmercury from fish. Haxton et al., 1979 found hair mercury levels in English fishing villagers who had high consumption of fish to be comparable to those with average or low fish consumption in other populations of Western Europe. Variation was also seen in the elimination half-life of methylmercury in humans ranging from 45 to 70 days (Clarkson 2002).

Although levels of exposures vary, inter-individual physiologic differences in mercury disposition may play an important role in explaining the observed variations. Differences in the rate of uptake, absorption, distribution, and excretion may be attributed to variations in functional efficiency of enzymes and protein products that transport, oxidize and reduce mercury and its metabolites in humans (Gundacker, et al. 2010). Genetic polymorphisms in genes related to the synthesis and transportation of such enzymes and protein products may lead to variations in hair Hg levels following methylmercury exposure. In humans, glutathione proteins are crucial for detoxifying a variety of toxicants including mercury. Five prior studies have shown that single nucleotide polymorphisms (SNPs) in genes that code the enzyme of the rate-limiting step in glutathione

synthesis and glutathione conjugation of mercury (glutamate cysteine ligase (GCL); glutathione-S-transferase (GST)) may lead to variations of mercury levels in biomarkers following similar exposures (Custodio, et al. 2004; Custodio, et al. 2005; Gundacker, et al. 2007; Gundacker, et al. 2009; Schläwicke Engström, et al. 2008). For instance, GSTT1 and GSTM1 deletions or double deletion, reported by Gundacker et al., 2007, were more frequently seen among subjects with higher hair Hg levels. Gundacker et al, 2009 found subjects with GSTP1-114 wildtype CC have higher hair Hg levels compared to those with CT and TT.

Little is known about the effects of genetic polymorphisms in the family of genes coding metallothionein (MT) proteins that may affect mercury biomarker levels in humans. Metallothioneins are a family of thiol-rich, low molecular weight (~7kDa) proteins that are ubiquitously present in humans, microbes and plants. A number of studies have shown that metallothionein proteins actively bind heavy metals via thiol groups in cysteine residues, which may serve to protect humans from heavy metal toxicity and against oxidative stress (Aschner, et al. 2006; Kumari, et al. 1998; Schurz, et al. 2000). There are four primary MT isoforms (MT1, MT2, MT3, and MT4) expressed in humans. The sequences coding for these isoforms are homologously located on human chromosome 16. In all of the MT sequences, there exist common regulatory elements in the upstream promoter region including metal responsive elements (MREs), glucocorticoid responsive elements (GREs) and antioxidant responsive elements (AREs) in the upstream promoter region (Karin, et al. 1987a). The induction of MT genes occurs via the MREs. Genetic polymorphisms in MREs in humans have been shown to significantly affect MT expression (Karin, et al. 1987a), and this may subsequently influence mercury binding and the amount of mercury available at target organs. In addition to MREs in the upstream promoter sequence, the 3'-untranslated region (3'UTR) of MT mRNA has been shown to be associated with signals needed for mRNA localization (Hesketh 2004). Cells that do not correctly localize MT mRNA show increased sensitivity to Cd and oxidative stress (Hesketh 2004). Furthermore, transcription factors coded by metallothionein transcription factor-1

(MTF-1) regulate the heavy-metal-induced transcription of the MT gene family through the binding of metals at MREs in the promoter region (Karin, et al. 1987b; Palmiter 1994). Genetic polymorphisms located in MTF-1 may also lead to significant changes in transcription level of MT genes (Saydam, et al. 2003), and this may subsequently influence the binding of heavy metals.

There is only one study in which the effects of MT SNPs were investigated for the association of methylmercury exposure with hair Hg levels (Gundacker, et al. 2009). The study found subjects with MT4 (rs11643815) wildtype GG were associated with higher hair Hg levels compared to those with GA and AA. This suggests that this particular metallothionein SNP may play an important role in modifying the relationship of hair Hg level with methylmercury exposure. The study, however, did not take into account the large variability of total mercury content of different species of fish when adjusting for Hg intake from fish in their analyses. A study taking account of methylmercury intake from dietary fish consumption with adjustment for differences in mercury content of different species of fish may be warranted to investigate this particular metallothionein SNP as well as many other SNPs in MREs and 3'UTR region or nearby. The goal of the present study is to investigate whether SNPs in metallothionein and related genes described above modify the relationship of methylmercury exposure with hair Hg level in humans. This study will try to explain the considerable variations seen in subjects exposed to methylmercury of similar magnitudes from a perspective of genetic polymorphisms in genes known to be important for mercury distribution in humans.

2. Materials and Methods

The subjects in this study were recruited during the Michigan Dental Association (MDA) annual conventions held in 2009 (n=232) and 2010 (n=283). They represent a convenience sample of dental professionals who attended the conventions. All participants were provided written informed consent that had been reviewed and approved by the University of Michigan Institutional Review

Board (HUM00027621). Each participate received a confidential letter summarizing and interpreting the results for the samples and measurements they provided.

2.1 Collection of Exposure Variables

Each subject completed a self-administered questionnaire to collect variables that were used as surrogates for recent mercury exposures from different sources. Surrogate variables in the questionnaire included variables for methylmercury exposure from dietary fish consumption, pre-existing diseases, demographic information (race, gender, height weight, age, occupation, etc) and other factors that may influence their exposures to methylmercury such as alcoholic consumption. Staff members were available to answer any questions subjects had. Each questionnaire was reviewed for completeness.

2.1.1 Questionnaires

2.1.1.1 Methylmercury Exposure: Dietary Fish Consumption

The questionnaire focused on the 6 month period prior to the date of survey. The variables reflecting methylmercury exposure were the frequency of consumption of twenty eight fish species (Appendix 3.1), the average portion size, and whether overall fish consumption was typical for the past year.

We surveyed the frequency of fish consumption using the scheme adopted from the NHANES Food Frequency Questionnaire (1999-2000). In the 2009 survey, subjects were provided with the following frequency options for each species: less than 1 time per month, 1 time per month, 2-3 times per month, 1 time per week, 2 times per week, 3-4 times per week, 5-6 times per week, 1 time per day, and 2 or more times per day. In the 2010 survey, we added the option “never” to those described above in the 2009 survey. Subjects were given probes for the question of portion size. The portion size options included the following: 1 portion=3 ounces of grilled fish=size of a deck of cards; 2 portions=1 regular 6-ounce can of tuna.

2.1.1.1.1 Quantification of Daily Methylmercury Intake from Fish Consumption

In the subsequent estimation of daily methylmercury intake from fish, a midpoint between the boundaries of a range of fish-eating frequency such as 2-3 times per month and 3-4 times per week as described above was used. For instance, if the subject reported having eaten a certain fish species for 2-3 times per month, the frequency value of 2.5 times per month was used in the estimation of daily methylmercury intake from fish. We combined the "never" and "less than 1 time per month" frequency categories together into one that was later defined as "never" because counting "less than 1 time per month" as "half time per month" may lead to an overestimation of daily methylmercury intake. We also obtained species-specific average mercury concentrations shown in the Appendix. As a result, we were able to estimate daily methylmercury intake (μg/kg-day) from dietary fish consumption based on the formula as follows:

$$\text{Estimated Daily Methylmercury Intake from Fish} = [U^* \sum_{i=1}^{n} P_i * C_i]/ BW/T$$

Estimated Daily Methylmercury Intake from Fish = Estimated Average Daily Methylmercury Intake from Common Fish Species for Each Subject (μg/kg/day)

U = Average Unit Portion Size of Fish Meals (g/portion)

P_i=Frequency of Eating a Particular Fish Species (portions/month) where i = 1, 2, 3…n and n = 28

C_i = Species-specific Average Methylmercury Concentration in Fish Tissues as Listed in the Appendix (μg/g)

BW = Body Weight of the Subject (kg)

T = 30 (day/month)

2.1.1.2 Preexisting Diseases

The questionnaire asked about 18 preexisting diseases in all subjects and whether or not these diseases had been diagnosed by a physician (Table 2.2).

2.1.1.3 Covariates

Frequency of consuming alcoholic beverage was surveyed in both years. In the 2009 questionnaire, the variable alcoholic beverage was categorized as follows: didn't drink, drank no more than 1 alcoholic beverage pre week, drank more than 1 but no more than 2 alcoholic beverages per week, and drank no less than 2 alcoholic beverages per week. About 57.7% (n=127) of the subjects did not drink any alcoholic beverages in the last 6 month. About 33.2% (n=77) of the subjects drank no more than 1 alcoholic beverage per week and 9.5% (n=22) of the subjects drank more than 1 but no more than 2 alcoholic beverages per week while the rest 2.6% (n=6) of the subjects drank no less than 2 alcoholic beverages per week. In the 2010 questionnaire, however, we improved the question by switching to the NHANES Food Frequency Questionnaire (FFQ) for alcohol consumption related questions. We instead asked the frequency of consuming alcoholic beverages as defined by number of days per week or per month; we followed up with the question of the number of drinks they usually had on days when they drank. About 54.7% (n=127) of the subjects in 2010 did not drink any alcoholic beverages. The mean consumption is 0.39 alcoholic beverages per day in 2010.

2.1.1.4 Demographics

Demographic information was also included in the questionnaire. Surveyed variables included age, self-reported height, self-reported weight, Body Mass Index (BMI), race, gender and occupation (Table 2.1). A dichotomous variable with dentist being the referent as opposed to non-dentist was later used in analyses.

2.2 Collection and Analysis of Hair Specimen

2.2.1 Hair Specimen Collection

Each subject was asked to provide a scalp hair sample. A minimum of 10 mg (approximately 10-20 occipital hair strands) of hair was collected by staff holding a hemostat and cutting as close to the scalp as possible from the occipital region of the head. The scalp side of the hair was then placed onto a 1.5 x 2 inch Post-it

(adhesive paper square; 3M, St.Paul, MN), which was subsequently folded in half to tightly wrap up the strands. The strands were then placed in a resealable plastic bag and stored at room temperature. Hair samples could not be obtained from 4 and 6 subjects in 2009 and 2010, respectively.

2.2.2 Hair Mercury Analysis

Total mercury content in hair samples was determined using atomic absorption spectroscopy in the Direct Mercury Analyzer-80 (Milestone Inc., Shelton, CT). This method is endorsed by the US Environmental Protection Agency Method 7473 as previously described by our laboratory (Paruchuri, et al. 2010). Briefly, hair samples were placed in nickel boats and were heated in a controlled manner and subsequently thermally decomposed in a continuous flow of ultra pure oxygen. The remaining decomposition products were delivered to a gold amalgamator that selectively traps mercury. The mercury vapor released by the heated amalgamator was then measured as a function of mercury concentration via absorbance at a wavelength of 253.65nm in the atomic absorption spectrophotometer.

For each measurement, 4-9 mg hair cut from scalp side of the strands were weighed using calibrated analytical balances. All hair samples were handled with clean scissors and tweezers and placed in nickel boats. Nickel boats were pre-cleaned prior to each measurement and periodically sonicated. Empty nickel boats were run as blanks at least every 10 samples, before the first measurement of a day and after the last measurement of a day. As a result, every 11 samples contain at least one blank. To ensure accuracy and precision of measurements, Standard Reference Material (SRM) were run every 4 to 23 samples depending on the number of samples in one batch. Multiple batches of hair samples were usually run each day. Sample duplicates were also run each day to determine accuracy and precision from within samples. The SRM used for hair in 2009 was NIES Japan CRM #13 Human Hair. This SRM was dried about once every two weeks and the total moisture content was taken into account. To

dry the SRM, 50-70 mg were placed in a nickel boat and weighed. The sample was then incubated for 16 hours at 60 °C and reweighed. The average recovery of mercury was 88.9 ± 1.1% for the NIES hair and the within-day and between day variability of the SRM were 0.7% and 1.0%, respectively. We observed good accuracy and precision for the NIES Japan CRM#13 Hair SRM. This gave us confidence in the validity of all the hair Hg measurements. The SRMs used in 2010 were NIES Japan CRM #13 Human Hair and DOLT-4 (National Research Council, Canada).

2.2.2.1 Total Mercury Measurement Accuracy

2.2.2.1.1 2009 Samples

The NIES Japan CRM #13 Hair SRM was measured each day of analysis to determine the validity of the calibration curves. The average recovery of Hg from the SRM for all 12 analysis days was 91.4 ± 6.5%. Expected total Hg in the NIES hair SRM was 4.42 μg/g dry weight and the mean observed Hg was 3.86 ± 0.23 μg/g dry weight. Recovery calculations included adjustments for moisture content of the SRM which ranged from 7 to 9% based on the previously described drying method. However, the SRM was only dried about once every two weeks. Recovery appeared to be lower on days further away from the last drying procedure. Incorporation of moisture over time may have decreased the apparent recovery from this SRM. Accuracy was nonetheless considered to be reasonably good.

2.2.2.1.2 2010 Samples

The average recovery of Hg from the NIES Hair SRM for all analysis days was 88.8 ± 1.1 % (n=12). The expected total Hg in the NIES hair SRM was 4.42 μg/g dry weight and the mean observed Hg was 3.92 ± 0.05 μg/g dry weight. Recovery calculations did not include adjustments for moisture content of the SRM which ranged from 7 to 9% based on drying procedures used in past studies. Recovery would be around 95% if the SRM contained 7% moisture. However, the SRM was not dried prior to these analysis days, and incorporation

of moisture over time may have decreased the apparent recovery. The average recovery from the DOLT-4 SRM was 95.5 ± 1.8% (n=12). The expected total Hg in DOLT4 was 2.58 μg/g dry weight and the observed mean Hg was 2.46 ± 0.05 μg/g.

2.2.2.2 Total Mercury Measurement Precision

2.2.2.2.1 2009 Samples

Precision (reproducibility) was also measured by within day and between day replicate analysis of SRM and samples. The Relative Standard Deviations (RSDs) (%RSD=SD/ (χ of the replicate values)* 100%) were calculated from replicates of SRM on the same analysis days to indicate within day precision, and from the SRM on different analysis days to indicate between day precision. The within day variability of the NIES Japan CRM#13 SRM measured by RSD% of within day analyses ranged from 0.14 to 14.09%RSD with the mean of 5.86 ± 4.6%RSD for 9 analysis days during which there were replicates of the SRM. On days when SRM was analyzed more than once, the within day averages for the NIES Japan CRM#13 SRM was used to determine the between day average. The between day variability of the SRM, measured by %RSD of between day analyses was 5.9%RSD for 11 analysis days.

2.2.2.2.2 2010 Samples

The within day variability of the NIES Hair SRM, measured by RSD% of within day analysis, ranged from 0.19 to 1.78%RSD for 4 analysis days during which there were replicates of the SRM. The within day variability of DOLT-4 SRM was ranging from 1.14 to 2.16%RSD for 4 analysis days during which there were replicates of the SRM. The overall data showed good within day precisions. On days when SRMs were analyzed more than once, the within day averages for the SRMs were used to determine the between day average. The between day variability of the NIES Hair SRM and DOLT-4 SRM, measured by %RSD of between day analysis, were 0.99%RSD and 1.3 %RSD, respectively. Both SRMs

gave good precision measurements for validity of all hair samples measured in 2010.

2.2.2.3 Total Mercury Precision from Samples

2.2.2.3.1 2009 Samples

Sample duplicates were run on a second day only if the measurement of the first day didn’t seem reliable due to the sample being measured right after a SRM or above 2.5 µg/g. The average %RSD for within day sample duplicates were 7.56 ± 7.76 %RSD (n=6).

2.2.2.3.2 2010 Samples

Sample duplicates were run each day for randomly selected samples. The average %RSD for within day sample duplicates were 4.97 ± 4.62 %RSD (n=26) ranging from 0.11 to 20.07%. Eight subjects did not have the minimum sample amount of 4 mg required by the DMA for accurate measurement. 1.1-3.4 mg of hair were measured for these individuals instead.

2.2.2.4 Total Mercury Detection Limit

2.2.2.4.1 2009 Samples

Samples that had Hg levels below the Theoretical Method Detection Limit, or TMDL were considered below the detection limit. The TMDL is calculated as 3 times the standard deviation of measurement averages of all blanks. In the study of 2009, the TMDL is 0.073 ng. Samples whose Hg levels were above TMDL but below the Practical Method Detection Limit or PMDL (5xTMDL=0.265 ng) were considered to have valid measurements. In the study of 2009, twenty-five samples had their hair Hg measurements above TMDL but below PMDL. These measurements were used without further adjustment in the final analyses. No samples were found below TMDL.

2.2.2.4.2 2010 Samples

In the study of 2010, the TMDL is 0.013 ng. Samples whose Hg levels were below the PMDL (5 x TMDL=0.065 ng) but still above the TMDL were considered to be valid measurements. Hg levels in all samples were above the PMDL and TMDL.

2.3 Collection and Analysis of DNA Samples

Buccal swabs were used to collect DNA samples from the subjects based on a published protocol (Min, et al. 2006). A total of four buccal swab samples were collected from each subject. Subjects rubbed the cotton bud of a buccal swab along the inside of their mouth for 30 seconds for each sample and placed the bud in a saline-prefilled tube. The tubes that each contained four buccal swab samples were then stored in lab freezer at –15 to –25 °C. Genomic DNA was isolated within seven days and purified for genotyping using Promega SV Genomic kit. Briefly, to isolate and purify genomic DNA, buccal swabs were incubated for 1 hr at 55 ºC in storage buffer with 0.032 mg/mL RNase and 0.1mg/mL Proteinase K; then we added 1 mL lysis buffer. We divided each individual sample into two purification columns and eluted the DNA in each column with 150 µL of TE buffer. We then quantified DNA with Picogreen kit and stored at -20ºC until use.

2.3.1 Selection of Metallothionein SNPs

Thirteen metallothionein SNPs were selected for the study based on the following criteria: 1) the significance of a SNP for the functionality and synthesis of metallothionein proteins (e.g. SNPs in metal-responsive-elements (MREs), 3'UTR and coding sequences); 2) SNP prevalence ≥ 5% in the Centre d'Etude du Polymorphisme Humain (CEPH) panel as reported in the PubMed SNP database; and 3) previous research results on metallothionein SNPs showing the importance of mRNA localization to the expression of metallothionein proteins (Hesketh 2004). Table 2.3 lists the selected metallothionein SNPs investigated in 2009 and 2010. Among the 13 selected SNPs, 11 MT SNPs are located on Chromosome 16q while two Metallothionein Transcription Factor 1 (MTF1) SNPs

are located on Chromosome 1q. The relative positions and lengths of the 11 MT SNPs are shown in Figure 2.1.

2.3.2 DNA Genotyping

TaqMan Allelic Discrimination Assay was used for genotyping all genes except MT1A (rs8052394) on ABI 7300 (Carlsbad, CA) (Figure 2.1). Sets of primers and probes for genotyping each SNP were ordered from Applied Biosystems, Inc (Foster City, CA). Briefly, for every reaction that genotypes one SNP for each subject, we mixed 12.5 µL 2x Taqman Genotyping Master Mix (DNA polymerase and dNTPs) with 1.25 µL 20x SNP assay designated for each SNP (2 primers to amplify gene and 2 probes to detect the 2 alleles of the SNP of interest). We then pipetted 13.75 µL of this mixture into each well of a 96-well semi-skirted plate. All wells contained the same amount of DNA (3 to 20 ng). DNA samples were then diluted with the appropriate amount of nuclease-free water and were pipetted a total of 11.25 µL of the diluted mixture into the plate. For each plate, four blank controls and two sample duplicates were run for quality control. The QPCR seal covered plate was then loaded into ABI 7300 QPCR machine and a pre-read (to measure background fluorescence), an amplification (10 minutes at 95 ºC; 15 sec at 92 ºC and 90 sec at 60 ºC for 40-50 cycles), and a post-read (to measure changes in fluorescence after DNA amplification) were performed. We then identified each subject sample in the three clusters that represented three genotypes: major homozygotes, heterozygotes, minor homozygotes. I'm

Restriction Fragmentation Length Polymorphism (RFLP) method was used to genotype MT1A (rs8052394). Primers were designed to amplify coding region based on the published gene sequence in Pubmed. The forward and reverse primer sequences were 5'- CAAACTGAGGCCAAGAGTGCACCA-3' and 5'-TGACCTGAGGCAGGTGCCTGATTT-3', respectively. The primers were designed to introduce a *Pst*I endonuclease restriction site in sequences containing the missense mutation (single nucleotide polymorphism site). PCR was performed using 50 ng genomic DNA template, 1 U DyNAZYME DNA

polymerase (FINNZYMES, USA), 5 µl DyNAZYME buffer, 200 µmol/l of deoxynucleotide triphosphate mix (Hamburg, Germany), and 350 nmol/l of each primer in a 50 µl reaction volume. Forty cycles of PCR were performed (2 seconds at 94°C, 60 seconds at 61°C, and 60 seconds at 72°C). The PCR products were then digested with restriction endonucleases *Pst*I. PCR product (25 µl) was added to 20,000 U *Pst*I (New England Biolabs), 1× digest buffer (New England Biolabs), and 100× bovine serum albumin (BSA) (New England Biolabs) to a final volume of 50 µl and incubated at 37°C for two hour. The digestion reactions were heat inactivated by incubation at 80°C for 15 minutes. The products were analyzed by agarose gel electrophoresis using a 1.5% (w/v) Nusieve 3:1 agarose (Eurofins MWG Operon, USA) gel, 1× TAE buffer, and 0.5 µg/ml ethidium bromide. Undigested amplification products were 743 bp in length. After digestion with *Pst*I a product of 405 bp was obtained from mutant sequences. Heterozygote samples produced both products of 743 bp and 405 bp after digestion while minor homozygote samples produced only products of 405 bp.

Table 2.3 display allele frequencies of each selected SNP and Hardy-Weinberg Equilibrium (HWE; pass/fail). The allele frequencies of all SNPs are in HWE. Several SNPs were only genotyped in 2009 or 2010 (Table 2.3). In the multivariable regression analyses, we created two dummy variables for each SNP: heterozygote (major homozygote as referent) and minor heterozygote (major homozygote as referent).

3. Statistical Methods

All statistical analyses were performed in SAS 9.2 (SAS Institute Inc., Cary, NC). Descriptive analyses were performed on BMI, age, occupation, race of the subjects as well as their Hg exposure levels reflected in hair Hg levels and surrogate variables. Bivariate analyses included age- and occupation- stratified analyses of BMI and age, exposure-stratified hair Hg levels and SNP genotype-stratified urinary Hg levels. Multivariate regression analyses were conducted in

two phases. In the first phase, using multivariate linear regression models, natural log-transformed hair Hg was regressed against estimated methylmercury exposure (daily methylmercury intake from fish). In the regression model, covariates that might influence exposure such as gum-chewing, teeth-grinding, chelation therapy, occupation, age, race, BMI, gender, and alcohol consumption were all added to the hair model. A base model was selected for hair Hg biomarker using backward elimination starting from the model including all exposure terms and covariates. The least statistically significant predictor was eliminated in each step and the final base model for hair Hg were derived by retaining all significant predictors ($p<0.05$). In the final model, gender was retained. Because a majority (94.8%) of the non-dentists was female, we fitted each base model with a dentist-only or a nondentist-only sample in order to assess the potential confounding of gender with occupation. Gender was not significant in the dentist-only sample, meaning the gender significance was due to confounding with occupation. Thus, we did not keep gender in the base models.

In the second phase, the base model predicting hair Hg was combined with main effect terms of dummy variables of each SNP and interaction terms between the exposure predictors in the base model and the dummy variables of each SNP. Such interactions between exposure and genotype were investigated one SNP at a time in separate models

4. Results

4.1 Demographics

As shown in Table 2.1, the total sample includes 515 subjects and is predominantly Caucasian (90.45%). Approximately one half of the sample were dentists (47.38%) and 2 subjects (0.39%) did not identify their occupation. There were 198 males (38.5%) and 316 females (61.5%). Thirty six percent (n=184) of the subjects were male dentists while about 50% (n=255) were female non-dentists. The mean ages for dentists and non-dentists were 56.1 and 48.2 years old, respectively, and this difference was significant. The mean ages for

Caucasians and non-Caucasians are 52.5 and 46.8 years old, respectively, and they also differ significantly. The mean BMIs of dentists and non-dentists did not differ significantly and neither did that of Caucasians and non-Caucasians.

4.2 Level of Hair Biomarker

Table 3.1 summarizes the distributions of hair Hg levels for 2009, 2010 and the combined 2009-2010 along with to the US general population reported by McDowell et al. 2004 using the population of NHANES (1999-2000). T-test show no significant difference in arithmetic mean hair Hg levels between the two years (p=0.08). Thus, we combined and used the data obtained from both years for subsequent analyses. There was no significant difference in geometric mean Hg levels in hair in our study population compared to respective NHANES reference levels (Table 3.1). Overall, the mean level and distribution of urine and hair mercury seen in our study are similar to the US general population.

4.3 Daily Methylmercury Intake from Fish

The mean estimated daily methylmercury intake in the study was 0.084 µg/kg/day (SD=0.13 µg/kg/day) ranging from 0 to 0.98 µg/kg/day. The mean reported portion size of fish meals was 4.77 ounces (SD=2.73 ounces) ranging from 0.75 to 12 ounces. About 5.86% (n=30) of the subjects reported they didn't eat fish and their hair Hg levels were significantly different from those fish eaters ($p<0.001$). Among these non-fish eaters, about 90% (n=27) of them reported the diet of the past 6 months was typical. The top five most consumed fish species were salmon (1.62 times per month), canned white/albacore tuna (1.40 times per month), shrimp (1.32 times per month), canned light tuna (0.87 times per month) and cod (0.80 times per month). The three least consumed species were porgy (0.16 times per month), carp (0.16 times per month) and shark (0.17 times per month).

4.4 Associations between Methylmercury Exposure and Level of Hair Biomarker

Table 3.2 showed a clear, significant trend of increase in mean level of hair Hg as methylmercury intake from dietary fish consumption increased. Cochran-Armitage trend test of mean hair Hg was performed on four levels of Hg exposures. In addition, estimated daily methylmercury intakes have a Pearson correlation efficient of 0.52 with the mean hair Hg levels.

4.5 Associations between SNPs and Levels of Biomarkers (No Adjustment for Methylmercury Exposure)

Table 3.3 summarizes the mean hair Hg levels stratified by genotype of the selected matallothionein SNPs. The table also presents the results of ANOVA tests comparing the mean hair Hg levels among each SNP genotype with no adjustment for methylmercury intake. ANOVA results showed no significant difference in mean hair Hg levels between SNP genotypes except MT1M (rs9936471). There was a significant difference in the mean hair Hg level of subjects with TT compared to those with TC.

4.6 Results of Hair Multivariate Linear Regression Models

Table 3.4 displays linear regression models of natural log transformed hair Hg concentration as predicted by estimated daily methylmercury intake from fish, SNP genotypes and intake-SNP interactions. In the base model, estimated daily methylmercury intake from fish (linear continuous) predicted In transformed hair Hg adjusting for occupation (dentist or not, categorical), and age (linear continuous). In the subsequent analyses, we only used estimated daily methylmercury intake as a predictor (as shown in the first row of Table 3.4) as we aimed to investigate the effect modification of SNPs on estimated daily methylmercury intake. We then combined terms of SNP main effects and exposure-SNP interactions with each of the base models. For all SNPs except for MT1A (A>G), MT1M 3'UTR (T>C) and MT1E 3'UTR (G>T), there were no significant interaction terms. In models with MT1A (A>G), MT1M 3'UTR (T>C) and MT1E 3'UTR (G>T), significant interactions were found between heterozygote genotype with estimated daily methylmercury intake compared to

major homozygote genotype. Compared to those with major homozygote (TT) for MT1M 3'UTR (T>C) after controlling for estimated daily methylmercury intake from fish, subjects with heterozygote (TC) had higher hair Hg level (Figure 3.1). Those with at least one copy of homozygote variant (GT or TT) for MT1E 3'UTR (G>T) had lower hair Hg level compared to major homozygote (GG) (Figure 3.2). Those with heterozygote (GA) and minor homozygote (GG) for MT1A (rs8052394) had lower hair Hg level compared to major homozygote (AA) (Figure 3.3).

5. Discussion

We investigated the influence of selected metallothionein SNPs on hair mercury levels in relation to estimated methylmercury exposure from dietary fish consumption. We tested our hypothesis in a convenience sample of dental professionals whose hair Hg levels were similar to that found in the US general population. We found significant effect modifications of the heterozygote genotype (TC) of MT1M (rs9936471), that (GT) of MT1E (rs708274) and the pooled heterozygote (GA) and homozygote variant (GG) of MT1A (rs8052394) on the relationship of hair Hg level with estimated daily methylmercury intake from fish consumption. Overall, the hair Hg levels in our study group were slightly higher than that in the US general population reported by McDowell et al. 2004 based on NHANES (1999-2000). However, they were not statistically different from each other. The estimated daily methylmercury intake in the study was 0.084 μg/kg-day, which was below the current US EPA Reference Dose (RfD) of 0.1 μg/kg-day. It ranged from 0 to 0.98 μg/kg-day with 25% exceeding the RfD. We also found the hair Hg level is predicted by estimated daily methylmercury intake from fish adjusting for occupation and age.

The relatively low hair Hg levels observed in our sample were consistent with what has been reported in North American non-indigenous populations in a number of studies (Canuel, et al. 2006; McDowell, et al. 2004). The estimated daily methylmercury intake from fish in our study was below the US EPA RfD of 0.1 μg/kg/day and was consistent with the levels reported in recent studies where

estimated daily methylmercury intake from fish was 0.068 μg/kg/day with a standard deviation of 0.109 (Canuel, et al. 2006; Mahaffey, et al. 2004). In our study where occupation was a predictor of hair Hg level, it may be that occupation is a surrogate of socioeconomic status (SES) as dentists fall into a higher SES group and are more likely to eat fish than non-dentists. Higher fish consumption leads to higher hair Hg levels as showed by numerous studies. Increasing age has been shown to be associated with increasing methylmercury level in blood (Schläwicke Engström, et al. 2008). Increasing age may also be a reflection of deteriorated functions of Hg metabolism.

Our study extends beyond the work of Gundacket et al. (2009) and is the first to report potential effect modification of any MT SNPs on the relationship of hair Hg with methylmercury exposure after controlling for estimated daily methylmercury intake from fish estimated from fish species-specific Hg levels, portion size and frequency of fish consumption. Thus, our findings contributed to previous research on MT SNPs modifying the exposure-biomarker relationship in humans. Only one study has reported findings of effect modification of MT4 (rs11643815) on methylmercury-hair relationship controlling for Hg intake (Gundacker, et al. 2009). In contrast to our study, Gundacker et al., 2009 found subjects with MT4 GG alleles had lower hair mercury compared to those with GA and AA adjusting for fish consumption (gram per week). However, Gundacker et al., 2009 estimated total fish intake in grams per week without any adjustment for differences in mercury content of different species of fish. The contrasting results of our study with Gundacker et al. 2009 for MT4 may be due to this difference in methodology for estimating methylmercury intake from fish. There was another study that used a relatively reliable and refined exposure surrogate to account for methylmercury exposure from fish, albeit in the investigation of GST related SNPs (Schläwicke Engström, et al. 2008).

Our study is the first to investigate the effect modifications of MT SNPs on mercury exposure-hair relationship adjusting for estimated daily methylmercury

intake from fish consumption calculated based on NHANES Food Frequency Questionnaire (1999-2000) and species-specific Hg levels. Unlike Gundacker et al. 2009, the assumption that all fish has the same level of Hg was not made. We found evidence of effect modifications of MT1A (rs8052394), MT1M (rs9936471) and MT1E (rs708274) SNPs on the relationship of hair Hg level and estimated daily methylmercury intake from fish after adjusting for methylmercury intake. In addition, in our study, ANOVA results on mean Hg level of subjects with major homozygotes compared to the pooled mean Hg level of those with heterozygotes and minor homozygotes did not show any significant difference in most cases (Table 3.3). We saw insignificant changes for MT1A (rs11640851) similar to Gundacker et al. 2009 (Table 3.3). Furthermore, Gundacker et al., 2009 found unadjusted higher levels of hair Hg were associated with MT1A (rs11640851) CC genotype and MT4 (rs11643815) GG and GA genotypes. We then calculated the Mantel-Haenszel statistics for the effects of MT1A (rs11640851) and MT4 (rs11643815) on dichotomized mercury hair levels in our study as described in Gundacker et al. 2009 (data not shown). There was no significant difference in associations of unadjusted hair Hg levels with MT1A (rs11640851) (Chi-square $p=0.73$) and MT4 (Chi-square $p=0.26$). It is not clear why there was such conflicting result when the methylmercury exposure levels in the two study groups are similar as suggested by respective median hair Hg levels (Table 3.1). Last but not the least, we did not find any effect modification of MT4 (rs11643815) on exposure-biomarker relationship after adjusting for estimated daily methylmercury intake in our study, which was inconsistent with the finding of Gundacker et al.2009. This may be the result of using species-specific Hg levels in our calculation of estimated daily methylmercury intake from fish, which was likely a better estimation compared to what was used in their study.

The study showed evidence of effect modifications of MT1A (rs8052394), MT1M (rs9936471) and MT1E (rs708274) SNPs. One prior study has showed a significant decrease of superoxide dismutase (SOD) activity among diabetic subjects especially those with MT1A (rs8052394) AA genotype presumably

because of increased inflammation as a result of MT under-expression (Yang, et al. 2008). A three-fold increase in risk of oral squamous cell carcinoma was also reported in subjects with MT1A (rs8052394) AA genotype (Zavras, et al. 2010). These results were consistent with our finding. Recent studies have also showed expressions of MT1M and MT1E genes were associated with cell apoptosis and were significantly upregulated upon *in vitro* treatment of zinc and copper in various cell lines (prostate cancer cell, bronchial epithelial cell, HepG2 cell and Sertoli TM4 cell.) (Bigagli, et al. 2010; Chung, et al. 2006; Kheradmand, et al. 2010; Lin, et al. 2009). Recent epigenetic research also showed a strong correlation between DNA methylation of MT1M genes and duration of smoking history in noncancerous esophageal mucosae of human subjects (Oka, et al. 2009). DNA methylation of MT1E gene has also been found in melanoma cell lines and tissues (Faller, et al. 2010). These results suggested that MT1A, MT1M and MT1E may be functional genes in humans responding to oxidative stress as previously reported (Karin, et al. 1984; Karin, et al. 1987a; Karin, et al. 1987b). It is likely that SNPs in MT1A (rs8052394) may be associated with changes in levels of antioxidants based on the consistent results we see in this study and the prior two studies. It is not clear, however, whether the two particular MT1E (rs708274) and MT1M (rs9936471) SNPs are functional variants. The roles of MT1M and MT1E genes and their SNPs warrant further research.

The present study suffers from a number of limitations. First, the study has a relatively small sample size of dental professionals available for analysis. For some of the minor homozygote genotypes, for instance MT1M (rs2270836), there were fewer than 15 subjects with the minor homozygote genotype. The small numbers limit the power of our findings on MT1M (rs2270836) and also limits the degree of investigation we could perform on potential effect modification of exposure-biomarker relationship by the SNPs of interest for MT1M (rs2270836) as well as some other SNPs. Second, it was difficult to rule out the possibility of false positives due to multiple comparisons in the investigation of interactions. There were 19 comparisons made in the hair models and only 3 of those were

statistically significant. Third, our study group was a convenience sample, not a random sample. Although our study population was a self-selected convenience sample of volunteers, there is no reason to believe that subjects had any prior knowledge of their genotypes or hair mercury levels. Thus, there was a low probability of selection bias. Four, the choice of deriving daily methylmercury intake from NHANES Food Frequency Questionnaire (1999-2000) and species-specific Hg levels may create recall and misclassification biases as with any self-reported exposure surrogates. The subjects might be over- or under-reporting the frequency of fish consumption and this will lead to an over- or under-estimation of daily fish methylmercury intake. Five, measurements of 2 cm hair from scalp only reflect the most recent two-month methylmercury exposure from fish or a steady-state body burden of methylmercury from fish. The survey time frame we used for fish frequency was six month before the time of survey and an unbiased reflection of fish methylmercury intake in hair Hg was dependent upon a stead-state body burden of methylmercury. The fish consumption of subjects may individually fluctuate over time two months before the time of survey. However, such fluctuation would bias the study results towards the null as the fish consumption of a subject may increase or decline prior to the two-month-before-survey time point. Despite all the limitations described above, the study is still the first and largest gene-environment study investigating the potential impact of SNPs concerning metallothionein genes in humans on the relationship between mercury hair biomarker levels and methylmercury intake estimated based on fish species-specific Hg levels and NHANES Food Frequency Questionnaire.

Future work may include *in vitro* exposure studies to confirm the functionality of MT1A, MT1M and MT1E genes in a variety of cell lines. The functionality of MT1A (rs8052394), MT1M (rs2270836) and MT1E (rs708274) SNPs, in particular, requires further research in such studies perhaps by exposing SNP deletion variants to heavy metals (e.g. Hg). The degree of induction of MT genes by mercury depends on the presence and the level of other MT gene inducers as well as the level of enzymes and protein products coded by genes that are also

involved in disposition of mercury in humans. Because glucocorticoid also induces MT gene expression along with oxidative stress (Karin, et al. 1984), it might improve study results by measuring glucocorticoid levels in blood in future studies. Genetic polymorphisms in other genes coding glutathione-S-transferase (GST), glutamate cysteine ligase (GCL) and selenoprotein might also be checked simultaneously for possible additive or even synergistic effects on hair Hg level. The results of the present study on genetic influences on hair Hg should not be interpreted that subjects in possession of MT1A (rs8052394), MT1M (rs2270836) or MT1E (rs708274) SNPs are more susceptible to methylmercury exposure. Research on genetic factors that may influence personal susceptibility to mercury exposure is still in an early stage and requires much more effort in the future. The present study findings on MT1A (rs8052394), MT1M (rs2270836) and MT1E (rs708274) SNPs modifying exposure-biomarker relationship certainly requires further confirmatory research. It should also be noted that even though the study has primarily been concerned with dental professionals, the findings may give some insights into the factors that may influence methymercury metabolism in relation to metallothionein polymorphisms in the US general population as the hair Hg levels found in the current dental sample overlaps that in the US general population. Future work may include examining potential effect modification of MT SNPs together with polymorphisms in other genes that are important for human mercury disposition on exposure-biomarker (e.g. blood) relationships in a larger sample.

Figures and Tables

Table 3.1: Hair Mercury (µg/g) in the MDA Study Compared with NHANES (1999-2000)

Sample	Arithmetic Mean	Geometric Mean	50th Percentile	75th Percentile	90th Percentile	95th Percentile
NHANES (n=1,726)	0.12*	0.47	0.19	0.42	1.11	1.73
2009 Study (n=226)	0.30‡	0.55	0.29	0.66	1.36	1.92
2010 Study (n=279)	0.27‡	0.45	0.28	0.54	1.07	1.33
2009-2010 (n=505)	0.28*	0.51	0.29	0.58	1.17	1.49

* : p=0.29 ‡:p=0.90

Table 3.2 Mean Hair Hg Levels (µg/g) Stratified by Levels of Estimated Methylmercury Intake from Dietary Fish Consumption

	Daily MeHg intake I (µg/kg-day) (N)	Mean (SD)*
Hair Hg (µg/g)	0<=I<0.01 (145)	0.22 (0.28)
	0.01<=I<0.05 (137)	0.39 (0.39)
	0.05<=I<0.12 (112)	0.56 (0.48)
	I>=0.12 (109)	0.91 (1.02)

* Cochran-Armitage linear trend test: p<0.05

Table 3.3: Mean Hair Hg Levels (μg/g) Stratified by Genotypes of Selected Metallothionein SNPs

SNP name	db SNP	Ref MAF (Caucasian)	N	Mean Hair Hg (μg/g)			
				Major homozygote (N)	Heterozygote (N)	Minor homozygote (N)	ANOVA *p*
SNPs Analyzed in both 2009 and 2010							
MT2A 3'UTR(G>C)	rs10636	0.250	465	0.54(253)	0.45(169)	0.57(43)	0.41
MTF1 3'UTR(G>A)	rs473279	0.353	460	0.50(213)	0.55(208)	0.44(39)	0.59
MT1M 3'UTR(T>C)	rs9936741	0.042	466	0.50(451)	1.03(15)	-	**0.005**
MT1M 3'UTR(G>A)	rs2270836	0.278(JPN)	462	0.48(332)	0.60(120)	0.53(10)	0.30
MT1A missense(A>C)	rs11640851	0.269(AFR+CAU)	441	0.45(191)	0.57(196)	0.56(54)	0.26
SNPs Analyzed in 2009 only							
MTF1 3'UTR(T>C)	rs3748682	0.246	222	0.50(128)	0.63(81)	0.44(13)	0.39
MT1M missense(A>C)	rs1827210	0.144	224	0.50(165)	0.67(50)	0.61(9)	0.37
MT1G 3'UTR(G>T)	rs12315	0.060	224	0.54(203)	0.56(20)	1.71(1)	0.28
MT1E 3'UTR(G>T)	rs708274	0.100	225	0.55(174)	0.50(47)	0.98(4)	0.45
MT4 missense(G>A)	rs11643815	0.112	223	0.54(167)	0.55(52)	0.33(4)	0.84
SNPs Analyzed in 2010 only							
MT2A 5'UTR(A>G)	rs28366003	0.045	239	0.48(211)	0.51(26)	0.43(2)	0.97
MT1A missense(A>G)	rs8052394	0.097	137	0.47(113)	0.44(22)	1.93 (2)	0.53
MT1A 5'near gene(C>G)	rs9922957	0.170(CHN+JPN)	236	0.50(178)	0.43(55)	0.27(3)	0.71

Note: When number of minor homozygote is smaller than 9, the ANOVA results shown is for comparing major homozygote and the pooled heterozygote and minor homozygote

Table 3.4: Coefficients and p Values from Multivariate Linear Regression Models of Natural Log-transformed Hair Hg Predicted by Estimated Methylmercury Exposure, SNP Genotypes and Intake-SNP Interactions

		Base model				SNP main effects					SNP-intake interactions			
SNP Name	db SNP	R^2	Intercept	Estimated Daily MeHg Intake		Homozygote	Heterozygote		Homozygote variant		Intake X Heterozygote		Intake X homozygote variant	
			6	6	p	Ref	6	p	6	p	6	p	6	p
2009-2010														
Base model	-	0.19	-1.6	3.62	<0.0001	-	-	-	-	-	-	-	-	-
MT2A 3'UTR(G>C)	rs10636	0.20	-1.5	3.37	<0.0001	-	-0.15	0.2	-0.12	0.5	0.69	0.39	0.54	0.6
MTF1 3'UTR(G>A)	rs473279	0.20	-1.6	4.09	<0.0001	-	0.09	0.4	-0.24	0.3	-0.94	0.2	1.13	0.5
MT1M 3'UTR(T>C)	rs9936741	0.21	-1.57	3.63	<0.0001	-	-0.04	0.9	-	-	**19.3**	**0.02**	-	-
MT1M 3'UTR(G>A)	rs2270836	0.20	-1.58	4.01	<0.0001	-	-0.02	0.88	-0.14	0.7	-0.7	0.3	-1.17	0.3
MT1A missense(A>C)	rs11640851	0.19	-1.55	3.84	<0.0001	-	-0.005	0.9	-0.09	0.6	-0.36	0.7	-0.63	0.5
2009														
MTF1 3'UTR(T>C)	rs3748682	0.25	-1.65	4.05	<0.0001	-	0.26	0.1	0.15	0.7	-0.71	0.5	2.15	0.5
MT1M missense(A>C)	rs1827210	0.25	-1.59	4.4	<0.0001	-	0.05	0.8	-0.02	0.9	-0.61	0.6	-1.67	0.2
MT1G 3'UTR(G>T)	rs12315	0.24	-1.58	3.88	<0.0001	-	0.28	0.4	-	-	-1.12	0.6	-	-
MT1E 3'UTR(G>T)	rs708274	0.26	-1.59	4.79	<0.0001	-	-0.04	0.8	-	-	**-1.76**	**0.05**	-	-
MT4 missense(G>A)	rs11643815	0.25	-1.51	3.62	<0.0001	-	-0.25	0.2	-	-	1.08	0.3	-	-
2010														
MT2A 5'UTR(A>G)	rs28366003	0.16	-1.52	3.58	<0.0001	-	-0.25	0.1	-	-	-0.7	0.5	-	-
MT1A missense(A>G)	rs8052394	0.20	-1.63	4.04	<0.0001	-	0.29	0.2	-	-	**-2.76**	**0.03**	-	-
MT1A 5'near gene(C>G)	rs9922957	0.15	-1.54	3.14	<0.0001	-	-0.13	0.6	-	-	1.71	0.4	-	-

Note: When number of homozygote variant is smaller than 9, the ANOVA results shown is for comparing homozygote and the pooled heterozygote and homozygote variant.

Figure 3.1: Scatter Plot of Ln-transformed Hair Hg against Estimated Daily Hg Intake from Fish Stratified by MT1M (rs9936741) Genotypes

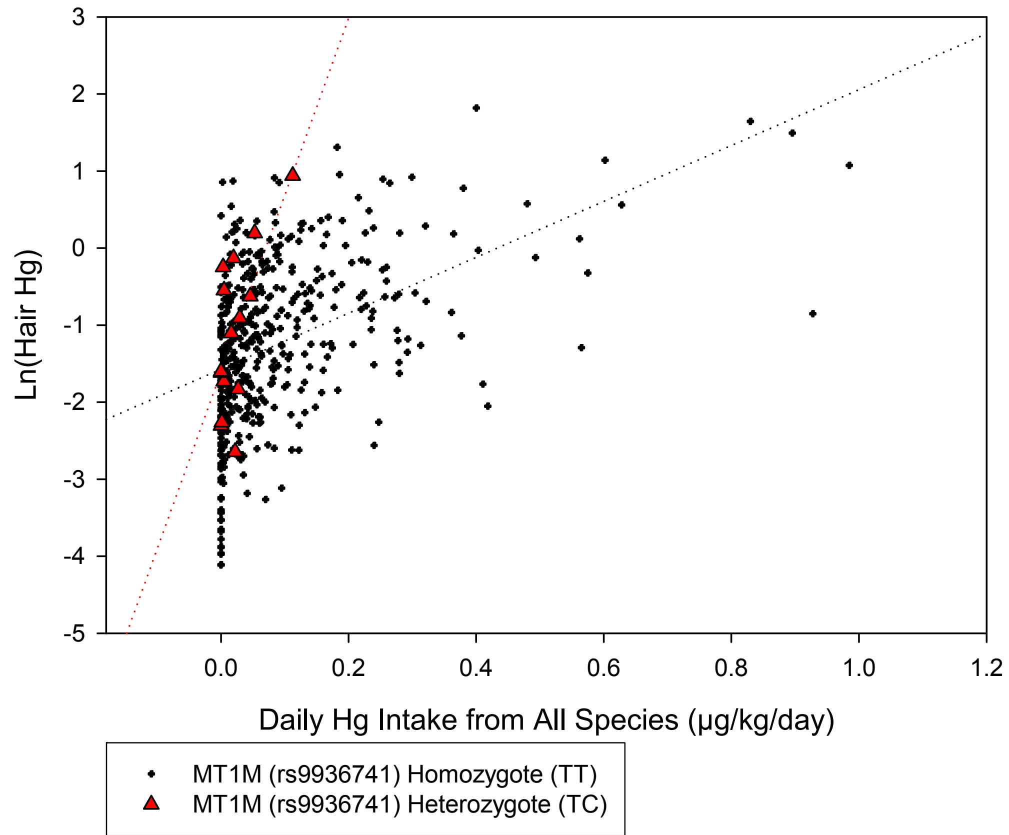


Figure 3.2: Scatter Plot of Ln-transformed Hair Hg against Estimated Daily Hg Intake from Fish Stratified by MT1E (rs708274) Genotypes

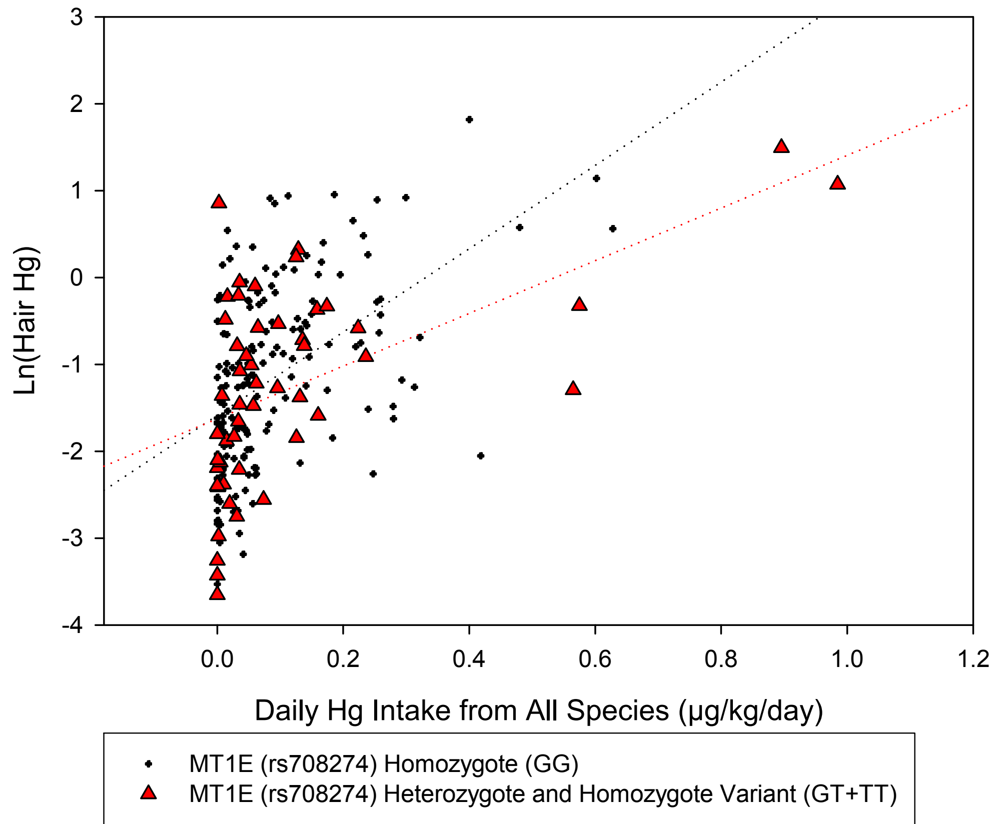
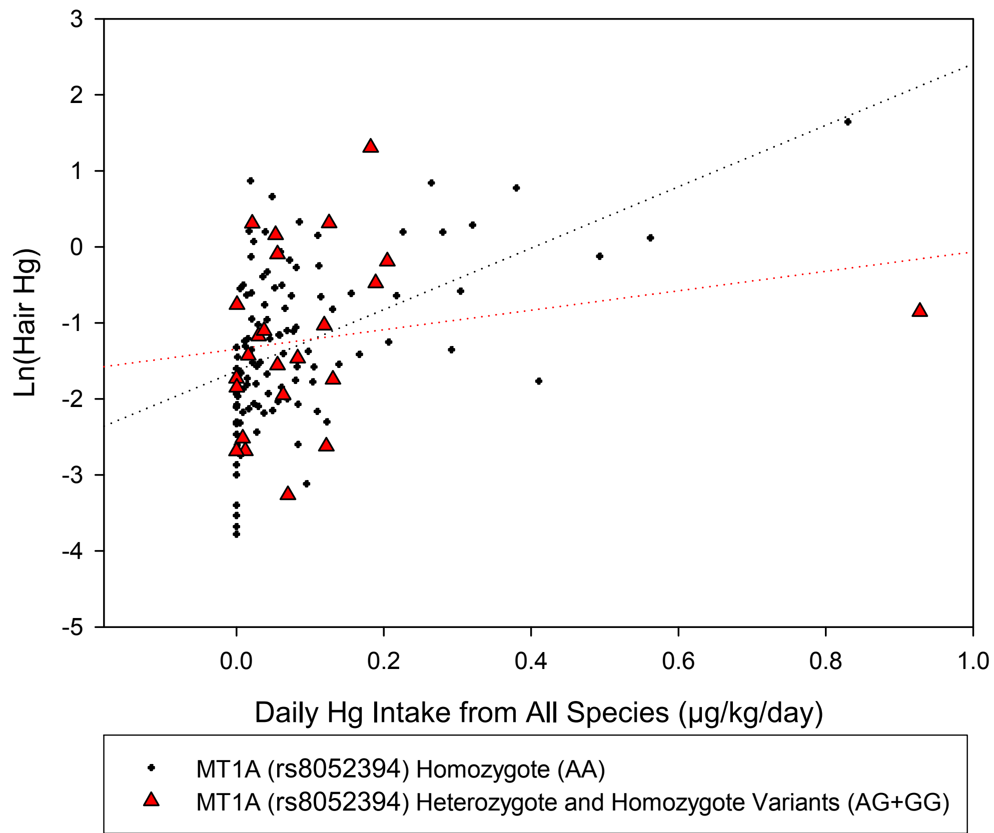


Figure 3.3: Scatter Plot of Ln-transformed Hair Hg against Estimated Daily Hg Intake from Fish Stratified by MT1A (rs8052394) Genotypes



Appendix

Appendix 3.1: Summary of Average Hg Concentrations in Fish Species

Fish Species	Average Concentration (μg Hg/g)	Source	Year of Data	Number of Samples Averaged
Tuna White(canned)	0.356	FDA Monitoring Program	2003	27
Tuna Light(canned)	0.258	FDA monitoring program	2003	12
Tuna Fresh	0.647	FDA monitoring program	2001-2004	9
Salmon	0.015	FDA monitoring program	2003	1
Shrimp	0.028	FDA monitoring program	1993-1995	2
Cod	0.084	FDA monitoring program	2004	19
Crab	0.049	FDA monitoring program	2004-2005	4
Scallop	0	FDA monitoring program	1991	1
Mussel	0.091	Mierzykowski et al. 2001		
Halibut	0.232	FDA monitoring program	2004	14
Lobster	0.224	FDA monitoring program	2004	10
Clam	0	FDA monitoring program	1991-1993	6
Oyster	0.014	FDA monitoring program	2004	4
Perch	0.005	FDA monitoring program	1991, 1994	6
Perch Freshwater	0.141	FDA monitoring program	1991, 1995	5
Trout	0.111	FDA monitoring program	2002-2004	17
Carp	0.271	FDA monitoring program	1993	1
Walleye	0.065	FDA monitoring program	1991-1994	4
Seabass	0.679	FDA monitoring program	1996-1997	3
Fresh Seabass	0.38	Bahnick et al. 1994		
Pike	0.31	Bahnick et al. 1994		
Swordfish	1.312	FDA monitoring program	2002-2004	13
Red Snapper	0.193	FDA monitoring program	2002-2004	36
Shark	1.086	FDA monitoring program	1992-2000	29
King Mackerel	0.73	AHA*		
Porgy	0.522	NMFS Report 1978**		
Tilapia	0.01	FDA monitoring program	1991-1992	9
Whitefish	0.074	FDA monitoring program	2002-2004	19

Note: *

American Heart Association weblink: http://www.peripheralarterydisease.com/presenter.jhtml?identifier=3013797 (accessed 11/04/2010)

**NMFS National Marine Fisheries Service Report 1978

*** FDA monitoring program: http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/FoodbornePathogensContaminants/Methylmercury/ucm191007.htm (accessed 11/04/2010)

Bibliography

Aschner, M., Syversen, T., Souza, D., Rocha, J.B.T., 2006. Metallothioneins: mercury species-specific induction and their potential role in attenuating neurotoxicity. Exp. Biol. Med. 231, 1468-1473.

Bigagli, E., Luceri, C., Bernardini, S., Dei, A., Dolara, P., 2010. Extremely low copper concentrations affect gene expression profiles of human prostate epithelial cell lines. Chem. Biol. Interact. 188, 214-219.

Canuel, R., de Grosbois, S., Atikess, L., Lucotte, M., Arp, P., Ritchie, C., Mergler, D., Chan, H., Amyot, M., Anderson, R., 2006. New evidence on variations of human body burden of methylmercury from fish consumption. Environ. Health Perspect. 114, 302-306.

Chung, M., Hogstrand, C., Lee, S., 2006. Cytotoxicity of nitric oxide is alleviated by zinc-mediated expression of antioxidant genes. Exp. Biol. Med. 231, 1555-1563.

Clarkson, T.W., 2002. The three modern faces of mercury. Environ. Health Perspect. 110 Suppl 1, 11.

Custodio, H.M., Broberg, K., Wennberg, M., Jansson, J., Vessby, B., Hallmans, G., Stegmayr, B., Skerfving, S., 2004. Polymorphisms in glutathione-related genes affect methylmercury retention. Arch. Environ. Health 59, 588.

Custodio, H.M., Harari, R., Gerhardsson, L., Skerfving, S., Broberg, K., 2005. Genetic influences on the retention of inorganic mercury. Archives of environmental occupational health 60, 17.

Faller, W., Rafferty, M., Hegarty, S., Gremel, G., Ryan, D., Fraga, M., Esteller, M., Dervan, P., Gallagher, W., 2010. Metallothionein 1E is methylated in malignant melanoma and increases sensitivity to cisplatin-induced apoptosis. Melanoma Res. 20, 392-400.

Gundacker, C., Gencik, M., Hengstschlger, M., 2010. The relevance of the individual genetic background for the toxicokinetics of two significant neurodevelopmental toxicants: Mercury and lead. Mutat. Res. 705, 130-140.

Gundacker, C., Gencik, M., Hikkel, I., Komarnicki, G., Kukuckova, M., Wittmann, K., 2009. Genetic background of lead and mercury metabolism in a group of medical students in Austria. Environ. Res. 109, 786.

Gundacker, C., Komarnicki, G., Jagiello, P., Gencikova, A., Dahmen, N., Wittmann, K.J., Gencik, M., 2007. Glutathione-S-transferase polymorphism, metallothionein expression, and mercury levels among students in Austria. Sci. Total Environ. 385, 37.

Haxton, J., Lindsay, D.G., Hislop, J.S., Salmon, L., Dixon, E.J., Evans, W.H., Reid, J.R., Hewitt, C.J., Jeffries, D.F., 1979. Duplicate diet study on fishing communities in the United Kingdom: mercury exposure in a "critical group". Environ. Res. 18, 351-368.

Hesketh, , 2004. 3'-Untranslated regions are important in mRNA localization and translation: lessons from selenium and metallothionein. Biochem. Soc. Trans. 32, 990.

Karin, M., Haslinger, A., Heguy, A., Dietlin, T., Cooke, T., 1987a. Metal-responsive elements act as positive modulators of human metallothionein-IIA enhancer activity. Mol. Cell. Biol. 7, 606-613.

Karin, M., Haslinger, A., Heguy, A., Dietlin, T., Imbra, R., 1987b. Transcriptional control mechanisms which regulate the expression of human metallothionein genes. Experientia.Supplementum 52, 401-405.

Karin, M., Haslinger, A., Holtgreve, H., Richards, R.I., Krauter, P., Westphal, H.M., Beato, M., 1984. Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene. Nature 308, 513-519.

Kheradmand, F., Nourmohammadi, I., Modarressi, M., Firoozrai, M., Ahmadi-Faghih, M., 2010. Differential Gene-Expression of Metallothionein 1M and 1G in Response to Zinc in Sertoli TM4 Cells. Iranian biomedical journal 14, 9-15.

Kumari, , Hiramatsu, , Ebadi, , 1998. Free radical scavenging actions of metallothionein isoforms I and II. Free Radic. Res. 29, 93.

Lin, S., Wei, H., Maeder, D., Franklin, R., Feng, P., 2009. Profiling of zinc-altered gene expression in human prostate normal vs. cancer cells: a time course study. J. Nutr. Biochem. 20, 1000-1012.

Mahaffey, K.R., Clickner, R.P., Bodurow, C.C., 2004. Blood organic mercury and dietary mercury intake: National Health and Nutrition Examination Survey, 1999 and 2000. Environ. Health Perspect. 112, 562.

McDowell, M.A., Dillon, C.F., Osterloh, J., Bolger, P.M., Pellizzari, E., Fernando, R., Montes de Oca, R., Schober, S.E., Sinks, T., Jones, R.L., Mahaffey, K.R., 2004. Hair mercury levels in U.S. children and women of childbearing age: reference range data from NHANES 1999-2000. Environ. Health Perspect. 112, 1165.

Min, J.L., Lakenberg, N., Bakker-Verweij, M., Suchiman, E., Boomsma, D.I., Slagboom, P.E., Meulenbelt, I., 2006. High microsatellite and SNP genotyping success rates established in a large number of genomic DNA samples extracted from mouth swabs and genotypes. Twin Research and Human Genetics 9, 501.

Oka, D., Yamashita, S., Tomioka, T., Nakanishi, Y., Kato, H., Kaminishi, M., Ushijima, T., 2009. The presence of aberrant DNA methylation in noncancerous esophageal

mucosae in association with smoking history: a target for risk diagnosis and prevention of esophageal cancers. Cancer 115, 3412-3426.

Palmiter, R.D., 1994. Regulation of metallothionein genes by heavy metals appears to be mediated by a zinc-sensitive inhibitor that interacts with a constitutively active transcription factor, MTF-1. Proc. Natl. Acad. Sci. U. S. A. 91, 1219.

Saydam, N., Steiner, F., Georgiev, O., Schaffner, W., 2003. Heat and heavy metal stress synergize to mediate transcriptional hyperactivation by metal-responsive transcription factor MTF-1. The Journal of biological chemistry 278, 31879-31883.

Schläwicke Engström, K., Strömberg, U., Lundh, T., Johansson, I., Vessby, B., Hallmans, G., Skerfving, S., Broberg, K., 2008. Genetic variation in glutathione-related genes and body burden of methylmercury. Environ. Health Perspect. 116, 734.

Schurz, , Sabater-Vilar, , Fink-Gremmels, , 2000. Mutagenicity of mercury chloride and mechanisms of cellular defence: the role of metal-binding proteins. Mutagenesis 15, 525.

Yang, L., Li, H., Yu, T., Zhao, H., Cherian, M.G., Cai, L., Liu, Y., 2008. Polymorphisms in metallothionein-1 and -2 genes associated with the risk of type 2 diabetes mellitus and its complications. American journal of physiology: endocrinology and metabolism 294, E987-E992.

Zavras, A.I., Yoon, A.J., Chen, M.K., Lin, C.W., Yang, S.F., 2010. Metallothionein-1 Genotypes in the Risk of Oral Squamous Cell Carcinoma. Annals of Surgical Oncology.

Chapter 4

Nerve Conduction Function, Mercury Levels in Urine and Hair, and Single Nucleotide Polymorphisms in Metabolism-related Genes

1. Introduction

Much of the work on the peripheral neurological impact of mercury has been conducted using clinical neurological examinations of the peripheral nervous system at high levels of exposure (Albers, et al. 1982; Albers, et al. 1988; Ellingsen, et al. 1993; Letz, et al. 2000; Levine, et al. 1982). However, clinical neurological examinations may not be adequate to detect subclinical effects of low-level exposure to methylmercury and elemental mercury. Nerve conduction tests, widely used in clinical practice and research to evaluate peripheral neuropathy, can serve as an objective measure of peripheral nerve impairment in relation to mercury at low levels.

There are four prior studies of low-level elemental Hg exposure in which clinical neurological examinations were used to associate signs and symptoms of peripheral neuropathy with elemental Hg exposures (DeRouen, et al. 2006; Echeverria, et al. 2005; Echeverria, et al. 2006; Kingman, et al. 2005). None of these studies assessed polymorphisms in genes important for Hg metabolism in relation to said associations. Two of these studies that employed nerve conduction tests focused solely on motor nerve conduction. Motor nerve conduction is generally considered a less sensitive indicator of peripheral nervous impairment than sensory nerve conduction (DeRouen, et al. 2006; Echeverria, et al. 2005; Echeverria, et al. 2005; Kingman, et al. 2005). Furthermore, ulnar nerve conduction in the hand was the only measurement

used in those two studies. However, the use of the sural nerve in the lower extremities is likely to be more sensitive to the effects of peripheral neurotoxicants than the ulnar or median nerves in the hand as nerves with longer axons are typically more susceptible to neurotoxicants, which has been shown in patients with most forms of neurotoxin- or neurotoxicant-induced peripheral neuropathy (Kimura, 1983). Although three of the four studies reported vibration sensitivity of toes and fingers in relation to elemental Hg exposure (Echeverria, et al. 2005; Echeverria, et al. 2006; Kingman, et al. 2005), sensory nerve conduction is considered a more objective index of peripheral nervous impairment compared to vibrotactile measurement in the lower extremities. A study that employs nerve conduction of sensory nerves (including the sural nerve) in the investigation of the association of peripheral nerve function with elemental mercury exposure is warranted. There has been no prior study of low-level methylmercury Hg exposure in relation to the peripheral nervous system.

The goal of the present study is two-folded: 1) to measure sensory nerve conduction function in the median and ulnar nerves in the hand and the sural nerve in the foot of subjects exposed to methyl- and elemental mercury at low levels similar to the U.S general population; 2) to assess the association of the measurements of onset and peak latencies and amplitude with urinary Hg (a marker of elemental Hg exposure) and hair Hg (a marker of methylmercury Hg exposure). The study will also explore the potential effect modification of single nucleotide polymorphisms in genes related to mercury metabolism on the associations between nerve conduction and Hg levels in urine and hair, respectively.

2. Materials and Methods

The subjects in this study were recruited during the Michigan Dental Association (MDA) annual conventions held in 2009 (n=232) and 2010 (n=283). They represent a convenience sample of dental professionals who attended the

conventions. Each participant signed a written informed consent approved by the University of Michigan Institutional Review Board (HUM00027621).

2.1 Collection of Exposure Variables

Each subject completed a self-administered questionnaire to collect variables that were used as surrogates for recent exposures to elemental mercury and methylmercury. Using the same methods of estimation for daily methylmercury intake, we also estimated daily intake of polyunsaturated fatty acids (PUFA) from fish (mg/kg/day) with specie-specific PUFA content obtained from the US Department of Agriculture National Nutrient Database for Standard Reference (http://www.nal.usda.gov/fnic/foodcomp/search/). The PUFA is a widely used measure of omego-3 fatty acid intake. The questionnaire also included items for pre-existing diseases, pregnancy, and demographics (race, gender, height, weight, age, occupation, hand dominance etc). The details of these items were reported in Chapter Two and Three. Staff members were available to answer any questions subjects had. Questionnaires were reviewed for completeness before subjects left.

2.2 Collection and Analysis of Urine and Hair Specimens

Each subject was asked to provide a spot urine sample in a mercury-free container (Becton, Dickinson, and Company; NJ, USA) and a scalp hair sample that was a minimum of 10 mg (approximately 10-20 occipital hair strands). The method details and results of urine and hair specimen collection are reported in Chapters Two and Three, respectively. Urine samples were not obtained from 3 and 10 subjects in 2009 and 2010, respectively. Hair samples were not obtained from 4 and 6 subjects in 2009 and 2010, respectively.

2.3 Measurement of Nerve Conduction Function

Sensory nerve conduction was performed on each subject and included measurements of amplitude, onset latency and peak latency of the median and ulnar sensory nerves in the right wrist and the sural nerve in the right ankle,

respectively. The temperature of the right midpalm and midfoot at the time of measurements were also recorded. Hands and/or feet were warmed with electric heating pads if the limbs were initially below 32 °C.

Antidromic stimulation was applied 14cm proximal to standard ring-shaped recording electrodes, separated by a distance of 3cm and placed on digits II and V of the right upper extremity for median and ulnar sensory nerves, respectively. A standard device, TECA Synergy (Oxford Instrument, Hawthorne, NY), was used to record the amplitude, onset latency and peak latency upon the stimulation over the median and ulnar sensory nerves. For measuring nerve conduction of the sural sensory nerve, antidromic stimulation was applied on the posterior aspect of the right calf, 14cm proximal to the recording electrode placed behind the lateral malleolus in the lower extremity. The peak and onset latencies (milliseconds-ms) were defined as the time required for an electrical stimulus to initiate the first peak of an action potential waveform and the time to deflect from baseline of waveform, respectively. The amplitude (microvolts-μV) was defined as the baseline-to-peak voltage difference on a waveform. All parameters were recorded in accordance with the guidelines outlined by the American Association of Electrodiagnostic Medicine (American Association of Electrodiagnostic Medicine 2002).

2.4 Hand Diagrams

A standard self-administrated hand diagram was given to each participate (Katz and Stirrat 1990; Katz, et al. 1990). Subjects were asked to shade the areas where numbness, tingling, burning or pain had occurred more than three times, or lasting more than one week in the six months prior to the measurement. The diagrams were then reviewed and scored for symptoms consistent with Carpel Tunnel Syndrome (CTS) independently by two experienced physician-raters and the results were compared for discrepancies. Discrepancies were reconciled between the two raters through consensus.

2.5 Collection and Analysis of DNA Samples

Buccal swabs were used to collect DNA samples from the subjects based on a published protocol (Min, et al. 2006). A total of twenty-eight SNPs in genes important for mercury metabolism such as metallothionein (MT), selenoprotein and glutathioine (GSH) -related proteins were genotyped using TaqMan Allelic Discrimination Assay (Applied Biosystems, Foster City, CA), Restriction Fragmentation Length Polymorphism (RFLP) method and a PCR-based deletion-detecting method (Appendix 4.1). The rationale of selecting MT SNPs and the methods of genotyping those SNPs was reported in Chapter Two and Three.

2.5.1 Selection of Selenoprotein and GSH-related SNPs

Mercury is eliminated following conjugation with glutathione (ROONEY 2007), a process that may involve glutathione s-transferases (GSTs; GSTT1, GSTM1, GSTP1, etc.) and indirectly depends on enzymes of the glutathione synthesis pathway (GSR, GCLC, GCLM, GSS). Selenoproteins (e.g. SEPP1, glutathione peroxidases) combat the oxidative stress created by mercury and bind the toxicant directly via selenocysteine residues (Chen, et al. 2006). Genes coding for proteins above are highly polymorphic and may modify mercury metabolism and elimination in the human body. A total of fifteen SNPs were selected for this study (Appendix 4.1). Six of those SNPs (GSTP1-105, GSTP1-114, GSTT1 deletion, GSTM1 deletion, GCLM -588, and GCLC -129) have been previously reported to significantly modify the mercury biomarker-exposure relationship in humans (Custodio, et al. 2004; Custodio, et al. 2005; Gundacker, et al. 2007; Gundacker, et al. 2009; Schläwicke Engström, et al. 2008). The nine remaining SNPs were chosen based on: 1) the importance of the gene category to which they belong in mercury metabolism (glutathione s-transferases, enzymes involved in glutathione synthesis, or selenoproteins); 2) SNP minor allele frequency $\geq$ 5% in the Centre d'Etude du Polymorphisme Humain (CEPH) panel as reported in the PubMed SNP database; 3) a known or hypothesized effect of SNP on enzyme function or gene expression (e.g., the SNP encodes an amino

acid change or a change in an important regulatory region such as the 5'UTR or 3'UTR).

3. Exclusion Criteria

Different exclusion criteria were applied for models according to the specific analyses involved. In all analyses, one subject with Charcot-Marie-Tooth (CMT) and three pregnant women were excluded. When urinary Hg was included in the analyses, we excluded 8 subjects with preexisting kidney diseases (lithiasis, pyelonephritis, orthostatic proteinuria, end stage kidney disease or chronic renal failure). We excluded an additional 24 subjects with self-reported history of stroke or diabetes in addition to the above exclusion, resulting in a sample size of 479 for modeling nerve conduction of ulnar and sural sensory nerves in relation to urinary Hg. For analyses where nerve conduction of the median sensory nerve was modeled in relation to urinary Hg, we excluded 48 additional subjects with a self-reported history of CTS or rheumatiod arthritis along with 11 additional subjects diagnosed with CTS from the hand diagram, resulting in a sample size of 420. For analyses of ulnar or median sensory nerves in relation to hair Hg, only subjects with stroke or diabetes were excluded in addition to CMT and pregnant subjects. When the median nerve was modeled, the 48 additional subjects with a history of CTS or rheumatoid arthritis along with the 11 additional subjects diagnosed with CTS from the hand diagram were also excluded.

4. Statistical Methods

All statistical analyses were performed in SAS 9.2 (SAS Institute Inc., Cary, NC). Descriptive analyses of the sample population were performed and the demographic results are reported in Chapters Two and Three. Bivariate analyses included stratification analyses of nerve conduction measurements of all three nerves using the cutoff of median Hg levels in urine and hair, respectively. Statistical comparisons were made for the mean nerve conduction measurements between low- and high- Hg strata (Table 4.1).

The multivariate linear regression analyses were conducted in three phases. In the first phase, using multivariate linear regression models, each log-transformed nerve conduction measurement was regressed against covariates including race, gender, height, weight, age, occupation, dominant hand, hand temperature and foot temperature. A base model predicting each nerve measurement was then formed using the statistically significant predictors derived from the first phase of analyses. A total of nine base models were developed for amplitude, onset latency and peak latency of the median, ulnar and sural nerves (Table 4.2). In the second phase, urine and hair Hg biomarker levels were independently added to each of the nine base models resulting in eighteen models (Table 4.2). We also assessed the net effects of PUFA intake from fish on the nerve function after accounting for hair Hg levels in the models (Table 4.2). In the third phase of the analyses, for each SNP, the main effects and interaction terms with the Hg biomarker using dummy variables for the heterozygote and minor homozygote with the major homozygote as referent were added to the eighteen models (Table 4.3 and 4.5). Such interactions were investigated one SNP at a time in separate models, resulting in a total of 504 different models. For each of the models (12 out of 504) that achieved statistical significance in SNP-biomarker interaction terms, a sensitivity analysis was performed. In each of the 12 models, a subject was considered a potentially influential observation if the absolute value of the DFBETA statistic of a biomarker-dependent variable (either urine or hair Hg biomarker) in relation to the respective modeled nerve conduction measurement was equal to/greater than the conventional cutoff value (2/sqrt(regression model sample size)). These influential observations were ranked in descending order according to the DFBETA statistics and were excluded if the Cook's D statistic of the observations exceeded the conventional cutoff value (4/ (regression model sample size)) in the sensitivity analysis. Models were labeled as "stable" if exclusion of any or all influential observations did not affect the significance or parameter estimate direction of interaction terms (Table 4.3). Models were labeled "unstable" if such exclusion resulted in changes in significance or parameter estimate direction of interaction terms (Table 4.5).

Sensitivity analyses were performed in the same fashion with the models that assessed the net effects of PUFA intake after accounting for hair Hg levels.

5. Results

5.1 Levels of Urine and Hair Biomarkers

Table 2.4 and Table 3.1 summarize the distributions of urine and hair Hg levels for 2009, 2010 and the combined (2009-2010) along with the corresponding levels in the US general population. Both the geometric mean levels of urinary and hair mercury in our study are higher than that in the US general population but there are considerable overlaps of the distributions for both urine and hair Hg. Overall, the urine and hair Hg levels among subjects in our study are similar to what has been reported in the US general population.

5.2 Associations between Nerve Conduction Measurements and Hg Levels in Urine and Hair (No Adjustment for Covariates)

Table 4.1 shows the overall mean results of nerve conduction measurements (amplitude, onset latency and peak latency) for all three nerves tested (median, ulnar and sural). Stratification analyses revealed a consistent relationship of worse nerve conduction measurements with higher Hg levels in urine and hair, respectively across all nerves, some of which were significant.

5.3 Associations between Nerve Conduction Measurements and Covariates

The log-transformed nerve conduction measurements of all three nerves were predicted by different sets of covariates in multivariate linear regression models (Table 4.2). The respective parameter estimates of the covariates for all three nerve measurements were similar in magnitude across all nerve models.

Age was shown to be inversely associated with measured nerve function across all nerve conduction measurements. “Better” ulnar nerve function was observed in females compared to males for all nerve measurement models in which gender was a significant covariate. BMI was inversely associated with median

nerve function in all models. Height was inversely associated with sural nerve function in all models. Limb temperature was inversely associated with latencies in all three nerves and ulnar nerve amplitude but not significantly associated with amplitude in the median or sural nerves. Left-handed subjects were shown to have higher median and ulnar amplitudes in the right hand compared to right-handed subjects. However, the same relationship was not seen in models of sural amplitude in the left-handed subjects.

5.4 Associations between Nerve Conduction Measurements and Hg Levels in Urine and Hair (Adjusted for Covariates)

Table 4.2 also shows the associations of Hg levels in urine and hair, respectively with nine log-transformed nerve conduction measurements in multivariate analyses accounting for covariates described above. Statistical significance was only achieved in urine Hg models for sural onset latency and ulnar peak latency. Models with hair Hg were significant for sural onset and peak latencies and median amplitude. Although only 3 out of 9 hair Hg models were significant, there was consistency in the relationship between increased hair Hg levels and improved nerve function across all nerve measurements. No consistent relationship was observed in urine Hg models.

In assessing the net effects of potentially beneficial PUFA intake after accounting for hair Hg levels(Table 4.2), we modeled PUFA intake and hair Hg levels in one model because the correlation between the two was not high (r=0.41). We observed the same relationship between increased PUFA intake and improved nerve function for all nerve measurements except median peak and onset latencies.

5.5 Results of Stable Multivariate Regression Models Exploring Effect Modification of Selected SNPs (Adjusted for Hg levels in Urine and Hair and Covariates)

Only 3 out of 504 multivariate linear regression models of log-transformed nerve conduction measurements achieved stable, statistical significance for the SNP-biomarker interaction terms (Table 4.3). Compared to those with CC alleles, subjects with CT alleles of the GPX1 (rs1050450) SNP had lower ulnar peak latency for the same increment of urine Hg levels. Subjects with GA alleles of the GCLC 5'UTR (rs17883901) SNP had higher sural amplitude compared to those with GG alleles for the same increment of hair Hg levels. Subjects with GT or TT alleles of the MT1E 3'UTR (rs708274) had higher ulnar onset latency compared to those with GG alleles for the same increment of hair Hg levels. A stratification analysis of respective nerve measurements by genotypes showed no statistical significance between genotypes (Table 4.4).

5.6 Results of Unstable Multivariate Regression Models Exploring Effect Modification of Selected SNPs (Adjusted for Hg levels in Urine and Hair and Covariates)

Nine other multivariate linear regression models (out of 504 models) of log-transformed nerve conduction measurements also achieved statistical significance for the SNP-biomarker interaction terms (Table 4.5). However, these results were unstable and did not hold after excluding potentially influential observations.

6. Discussion

We measured sensory nerve conduction function including amplitude, onset latency and peak latency in median, ulnar and sural nerves, respectively in a dental population exposed to elemental and methyl- mercury at levels similar to the US general population. We also associated Hg levels in urine and hair with the nine nerve conduction parameters in multivariate linear regression models that accounted for covariates such as age, gender, BMI, height, weight, dominant hand, and hand and/or foot temperature. Furthermore, we explored whether SNPs in genes related to Hg metabolism may modify the association of Hg levels in urine and hair, respectively with nerve conduction measurements in

multivariate linear regression models. We found higher hair Hg levels were associated with better nerve function (shorter onset and peak latencies and larger amplitudes of all nerves) after taking covariates into account (Table 4.2). Similar consistent relationships were not observed in models of urine Hg levels with nerve function. Higher urine Hg levels were associated with worse ulnar nerve function (amplitude, onset and peak latencies). For median and sural nerves, higher urine Hg levels were associated with improved function in measurements of onset and peak latencies but worse amplitudes. Nerve function deteriorated with increasing age. Only 3 out of 504 multivariate models that investigated effect modification of SNPs achieved stable significance in those interaction terms (Table 4.3). In the other 9 out of 504 models, unstable significant interaction terms were found and such effect modification did not hold after sensitivity analyses were performed on those models (Table 4.5).

The nerve conduction values of median and ulnar nerves in our study were within the range of the normative values previously reported in workers but with a mean age 16 years younger than the present study (Salerno D F, et al. 1998). Because of the large age difference, all of our mean values were expectedly worse. The sural nerve values were also within the normal range (Schuchmann 1977). Coefficient of covariate factors in models predicting nerve parameters, such as age, gender, height, weight, and temperature, were similar in magnitude and direction to those reported in prior studies (Fujimaki, et al. 2009; Greathouse, et al. 1989; Kimura 1984; Letz and Gerr 1994; Rivner, et al. 2001; Robinson, et al. 1993; Salerno D F, et al. 1998; Tong, et al. 2004; Trojaborg, et al. 1992; Werner and Franzblau 1996; Werner 2006).

High-level exposures to elemental Hg (≥ 50 μg/L in urine) have been previously shown to alter median and sural sensory nerve conduction in occupationally exposed populations (Shapiro, et al. 1982; Zampollo, et al. 1987). Thermometer factory workers with chronic high-level elemental mercury exposures had electrophysiological signs of peripheral neuropathy in most cases. A good

correlation between changes in sural nerve conduction velocity and H index demonstrated the sensory nerve involvement of the damages due to mercury (Zampollo, et al. 1987).

Four prior studies have explored the association between low-level mercury exposure and signs and symptoms of peripheral neuropathy using clinical neurological examinations (DeRouen, et al. 2006; Echeverria, et al. 2005; Echeverria, et al. 2006; Kingman, et al. 2005). In these studies, only elemental Hg exposure was studied and none of them looked at methylmercury exposure or dietary fish intake. None of them included sensory nerve conduction studies, which was also identified as a limitation by Kingman et al 2005. Although DeRouen et al. 2006 looked at motor nerve function in relation to urinary Hg, none of them investigated the role of Hg biomarkers using Hg levels in urine and hair simultaneously as used in this study to predict adverse peripheral nerve outcomes; nor did any of them take into account covariates important for peripheral nerve impairment in multivariate regression analyses. Unlike these previous studies, we used sensory nerve conduction studies, a more objective measure of peripheral nerve function compared to clinical exams, to look at how Hg biomarkers reflecting both elemental Hg and methylmercury exposures predict changes in sensory nerve conduction, adjusting for covariates. In an unpublished work by our group examining the association of latencies and amplitudes of median and ulnar sensory nerves with urine Hg concentration similar to the level in this study in a convenience sample of 2,767 dentists and hygienists, median and ulnar amplitude were not found to be significantly associated with urine Hg level. This was consistent with our results. However, they found median peak latency but not ulnar peak latency was stably associated with urine Hg level. In the present study, we found borderline significance between urine Hg level and ulnar peak latency rather than median peak latency. The conflicting result of median peak latency might be due to the fact that in the previous study gender was found to be a significant covariate for predicting median peak latency, but our study did not. The previous study found an

unstable relationship for the ulnar nerve that was driven by a single observation. The relationship for the ulnar nerve in the present study was borderline significant despite not being driven by one observation.

Even though motor nerve conduction was measured in DeRouen et al 2006, no sensory nerves were involved and only ulnar motor nerve was measured compared to a more comprehensive approach in our study where sural and median sensory nerves in addition to ulnar sensory nerve were measured. The results of the DeRouen et al 2006 showed there was no significant difference in ulnar motor nerve conduction velocity between children with dental amalgams and those with composite from baseline year through the follow-up at year seven. It is unclear whether the nerve conduction measurements taken were on motor or sensory nerves in Echeverria et al 2005.

We observed a counterintuitive and consistent relationship between increased hair Hg levels and improved sensory nerve conduction function across all nerves tested after controlling for covariates. Relationships between maternal fish consumption and in utero neurodevelopment have long been under intense debate, and beneficial effects of fish consumption on central nervous system have been previously reported (Mozaffarian and Rimm 2006; Oken and Bellinger 2008). Hair Hg serves as a surrogate of fish consumption and possibly consumption of omega-3 fatty acids. Omega-3 fatty acids are widely known to decrease the risk of cardiovascular diseases and may favorably affect other clinical outcomes (Oken, et al. 2005). However, a potential, though indirect beneficial effect of fish consumption on peripheral nerve function has not been reported previously. A recent clinical case study of peripheral nerve conduction in a CTS subject given a high dose of omega-3 oil suggested that omega-3 fatty acids may improve median sensory nerve conduction even nine months after the treatment (Ko, et al. 2010). We speculate that the present finding may be attributed to the concurrent intake of beneficial omega-3 fatty acids with methylmercury via dietary fish consumption. Thus, we modeled the effects of

PUFA intake from fish on nerve function after accounting for hair Hg level for each of the nine nerve measurements (Table 4.2). We observed a similar relationship between improved nerve function and increased PUFA intake after accounting for hair Hg levels for most nerve measurements except for median onset latency and peak latency. This suggested a possible, albeit not strong, beneficial effect of omega-3 intake on nerve function. Further research on the relationship is warranted.

Unlike the consistent relationship seen for hair Hg levels, a mixed result of both detrimental and beneficial relationship of urine Hg levels with sensory nerve function was observed. Such mixed results seem to be consistent with previous findings at both high- and low- levels of exposure to elemental Hg (DeRouen, et al. 2006; Echeverria, et al. 2005; Shapiro, et al. 1982; Zampollo, et al. 1987). At low levels, DeRouen et al. 2006 reported no association between elemental mercury exposure from dental amalgam and motor ulnar nerve conduction, while Echeverria et al. 2005 reported ulnar motor nerve latency was associated with urine Hg levels in both directions. Even at high levels, the conflicting results existed. Zampollo et al. 1987 found no correlation between urine Hg levels and any of the nerve conduction measurements including median sensory amplitude and sural sensory velocity whereas Shapiro et al. 1982 suggested a positive relationship between slower sural sensory and median motor conduction velocity and higher mercury levels. Our mixed findings are observed across all sensory nerve conduction parameters but it may not be possible to rule out that these mixed results might be attributed to random noise in measurements.

No prior study has looked at the effect of genetic polymorphisms on the associations between nerve conduction studies and Hg levels in urine or hair. Given the small number of stable and significant interaction terms (n=3) and the large number of models tested (n=504), it is not possible to rule out that the effect modification of GPX1 (rs1050450), GCLC 5'UTR (rs17883901) and MT1E 3'UTR (rs708274) SNPs may be false positives as a result of multiple comparisons.

Therefore, caution should be exercised in interpreting these results. Despite that, the protein products of these genes have all been found to serve as anti-oxidants in humans (Aschner, et al. 2006; Custodio, et al. 2004; Custodio, et al. 2005; Gundacker, et al. 2009; Kumari, et al. 1998; Ravn-Haren, et al. 2006; Schläwicke Engström, et al. 2008; Schurz, et al. 2000). The results seen here may be explained by anti-oxidant mechanism in which these proteins reduce oxidative stress and subsequently contribute to improve nerve conduction function.

The study suffers from a number of limitations in addition to the ones described in Chapters Two and Three (small sample size and self-selected convenience sampling design, etc). The selection of SNPs was based on their relationships with Hg exposures rather than adverse neurological outcomes as described in Chapters Two and Three. It may not be surprising to have found so few stable and significant results out of all 504 models tested given that the SNPs were not oriented to neurological outcomes. Despite the fact that nerve conduction is a sensitive and objective technique for assessing peripheral nerve impairment, variability and errors in those measurements may still contribute to the inconsistent results for sural nerve measurements (Kimura 1984). In our study, the sural nerve did not appear to be particularly more susceptible than ulnar or median nerve according to the respective number of significant Hg biomarker predictors for all nerve measurements of ulnar (n=1), median (n=1) and sural (n=2) (Table 4.2). Greater variability may exist in the sural measurements compared to those for median and ulnar nerves.

We did not correct for potential multiple comparison errors due to potential high correlations between nerve measurements ($r>0.85$ within the same nerve; $r<0.5$ within the same type of measure) in different nerves when analyzing modifying effects of MT SNPs on the relationship of nerve function with Hg levels in hair and urine. Since there were so few significant interaction terms showing modifying effects, we would not have drawn a conclusion that was any different from the current one had we made such correction. Any correction for multiple

comparisons would only have decreased the default cutoff p value (p=0.05) and thus resulted in even fewer significant results.

Regardless, the study is still the first to examine low-level Hg in both urine and hair, respectively with objective sensory nerve conduction measurements in median, ulnar and sural nerves. We performed the study in a population with Hg exposure levels close to the US general population. We may not be able to conclude positive findings of significant effect modification of selected exposure-driven SNPs on biomarker-outcome relationships. Future research is needed to fully investigate the role of genetic polymorphisms in the development of adverse neurological outcomes related to Hg exposures. Future research is also needed to explore the relationship between improved nerve conduction and hair Hg levels. Using blood PUFA levels, we may further explore the potentially beneficial effects of fish consumption on peripheral nerve function after accounting for hair Hg levels.

Tables

Table 4.1: Mean Amplitude, Onset and Peak Latencies of Median, Ulnar and Sural Nerves Stratified by Low and High Hg Levels in Urine and Hair

	Median-cutoff	Onset Latency (msec)			Peak Latency (msec)			Amplitude (μV)		
	Quantiles	Median (N)	Ulnar (N)	Sural (N)	Median (N)	Ulnar (N)	Sural (N)	Median (N)	Ulnar (N)	Sural (N)
Overall Mean		2.65(405)	2.51(474)	3.02(465)	3.41(405)	3.24(474)	3.81(465)	29.35(405)	27.85(474)	13.12(465)
Urine Hg	Low	2.63(197)	2.45(233)*	2.98(226)	3.37(197)	3.16(233)*	3.75(226)†	31.63(197)*	30.41(233)*	13.71(226)
	High	2.68(196)	2.57(229)*	3.05(227)	3.46(196)	3.31(229)*	3.87(227)†	26.91(196)*	25.29(229)*	12.52(227)
Hair Hg	Low	2.64(197)	2.48(235)	3.00(230)	3.40(197)	3.20(235)†	3.78(230)	31.78(197)*	30.42(235)*	13.61(230)
	High	2.66(202)	2.53(232)	3.01(228)	3.42(202)	3.28(207)†	3.83(228)	27.18(202)*	25.39(207)*	12.67(228)

*p<0.0001; †p<0.01

Table 4.2: Coefficients and p Values in Base Models of Multivariate Linear Regression Models of Log-transformed Nerve Conduction Function Independently Predicted by Hg Levels in Urine and Hair and Other Covariates

	Adj-R^2	Intercept	Age		Female		BMI		Hand Temperature		Left-handed		Weight		Height		Foot Temperature		Urine		Hair		PUFA	
		6	**6**	***p***	**6**	***p***	**6**	***p***	**6**	***p***	**6**	***p***	**6**	***p***	**6**	***p***	**6**	***p***	**6**	***p***	**6**	***p***	**6**	***p***
Log(Median Onset Latency)	0.21	1.25	0.005	*<0.0001*			0.006	*<0.0001*	-0.021	*0.003*									-0.006	*0.26*				
	0.21	1.26	0.004	*<0.0001*			0.007	*<0.0001*	-0.021	*0.004*											-0.01	*0.27*		
	0.23	1.33	0.004	*<0.0001*			0.006	*<0.0001*	-0.023	*0.004*											-0.01	*0.27*	0.0004	*0.66*
Log(Ulnar Onset Latency)	0.30	1.89	0.002	*<0.0001*	-0.06	*<0.0001*			-0.03	*<0.0001*									0.004	*0.25*				
	0.29	1.92	0.002	*0.0011*	-0.07	*<0.0001*			-0.03	*<0.0001*											-0.01	*0.08*		
	0.30	1.9	0.002	*<0.0001*	-0.07	*<0.0001*			-0.03	*<0.0001*											-0.01	*0.13*	-0.0003	*0.64*
Log(Sural Onset Latency)	0.25	1.72	0.001	*0.008*											0.004	*<0.0001*	-0.04	*<0.0001*	**-0.009**	***0.04***				
	0.22	1.69	0.001	*0.007*											0.004	*<0.0001*	-0.04	*<0.0001*			**-0.02**	***0.02***		
	0.22	1.69	0.001	*0.007*											0.004	*<0.0001*	-0.04	*<0.0001*			**-0.02**	***0.05***	-0.0002	*0.74*
Log(Median Peak Latency)	0.21	1.72	0.004	*<0.0001*			0.006	*<0.0001*	-0.03	*<0.0001*									-0.002	*0.71*				
	0.22	1.73	0.004	*<0.0001*			0.006	*<0.0001*	-0.03	*<0.0001*											-0.01	*0.25*		
	0.25	1.81	0.004	*<0.0001*			0.005	*<0.0001*	-0.03	*<0.0001*											-0.01	*0.17*	0.0002	*0.77*
Log(Ulnar Peak Latency)	0.36	2.12	0.002	*<0.0001*	-0.049	*0.0001*	-0.001	*0.09*	-0.031	*<0.0001*									**0.006**	***0.05***				
	0.34	2.12	0.002	*<0.0001*	-0.057	*0.0001*	-0.002	*0.04*	-0.03	*<0.0001*											-0.008	*0.16*		
	0.37	2.17	0.002	*<0.0001*	-0.057	*0.0001*	-0.002	*0.005*	-0.03	*<0.0001*											-0.008	*0.21*	-0.0005	*0.31*
Log(Sural Peak Latency)	0.26	1.85	0.002	*<0.0001*											0.004	*<0.0001*	-0.04	*<0.0001*	-0.003	*0.43*				
	0.26	1.83	0.002	*<0.0001*											0.004	*<0.0001*	-0.04	*<0.0001*			**-0.022**	***0.003***		
	0.26	1.83	0.002	*<0.0001*											0.004	*<0.0001*	-0.04	*<0.0001*			**-0.022**	***0.008***	-0.0001	*0.85*
Log(Median Amplitude)	0.46	4.4	-0.02	*<0.0001*	0.31	*<0.0001*	-0.02	*<0.0001*			0.2	*0.01*							-0.03	*0.10*				
	0.47	4.31	-0.02	*<0.0001*	0.35	*<0.0001*	-0.02	*<0.0001*			0.2	*0.005*									**0.06**	***0.05***		
	0.47	4.31	-0.02	*<0.0001*	0.35	*<0.0001*	-0.02	*<0.0001*			0.2	*0.005*									0.06	*0.09*	0.0007	*0.77*
Log(Ulnar Amplitude)	0.53	5.4	-0.02	*<0.0001*	0.51	*<0.0001*	-0.01	*0.0003*	-0.045	*0.02*	0.16	*0.006*							-0.007	*0.65*				
	0.53	5.25	-0.02	*<0.0001*	0.53	*<0.0001*	-0.01	*0.0005*	-0.042	*0.002*	0.17	*0.003*									0.04	*0.13*		
	0.53	5.25	-0.02	*<0.0001*	0.53	*<0.0001*	-0.01	*0.0005*	-0.042	*0.002*	0.17	*0.003*									0.04	*0.21*	0.0008	*0.72*
Log(Sural Amplitude)	0.25	4.83	-0.02	*<0.0001*									-0.007	*<0.0001*	-0.005	*0.05*			-0.0003	*0.99*				
	0.25	4.92	-0.02	*<0.0001*									-0.007	*<0.0001*	-0.006	*0.03*					0.07	*0.06*		
	0.25	4.91	-0.02	*<0.0001*									-0.007	*0.0002*	-0.006	*0.02*					0.04	*0.34*	0.006	*0.06*

Table 4.3: Coefficients and p Values from Stable Multivariate Linear Regression Models (3 out of 504) of Log-transformed Nerve Conduction Function Predicted by Hg Biomarker Levels in Urine or Hair, SNP Genotypes and SNP-biomarker Interactions

				Base model																	
	SNP Name	db SNP	Year of Data	R^2	Intercept	Age		Female		BMI		Hand Temperature		Weight		Height		Urine		Hair	
					β	**β**	***p***	**β**	***p***	**β**	***p***	**β**	***p***	**β**	***p***	**β**	***p***	**β**	***p***	**β**	***p***
Log(Ulnar Peak	Base model	-	2009-2010	0.36	2.12	0.002	*<0.0001*	-0.049	*0.0001*	-0.001	*0.09*	-0.031	*<0.0001*					0.006	*0.05*		
Latency)	GPX1(C>T)	rs1050450	2009	0.31	2.16	0.002	*<0.0001*	-0.046	*<0.0001*	-0.003	*0.02*	-0.031	*0.0002*					0.009	*0.07*		
Log(Ulnar	Base model	-	2009-2010	0.29	1.92	0.002	*0.0011*	-0.07	*<0.0001*			-0.03	*<0.0001*							-0.01	*0.08*
Onset Latency)	MT1E 3'UTR(G>T)	rs708274	2009	0.31	2.13	0.002	*0.0006*	-0.07	*<0.0001*			-0.04	*<0.0001*							-0.019	*0.04*
Log(Sural	Base model	-	2009-2010	0.25	4.92	-0.02	*<0.0001*							-0.007	*<0.0001*	-0.006	*0.03*			0.07	*0.06*
Amplitude)	GCLC 5' UTR(G>A)	rs17883901	2009	0.34	5.26	-0.01	*<0.0001*							-0.008	*0.0003*	-0.008	*0.01*			0.03	*0.54*

Note:For MT1E, pooled heterozygotes and minor homozygotes were compared to major homozygotes; Genotyping of the three SNPs were only done in 2009

(Continued) Table 4.3: Coefficients and p Values from Stable Multivariate Linear Regression Models (3 out of 504) of Log-transformed Nerve Conduction Function Predicted by Hg Biomarker Levels in Urine or Hair, SNP Genotypes and SNP-biomarker Interactions

				SNP main effects					SNP-Urine interactions				SNP-Hair interactions			
	SNP Name	db SNP	Year of Data	Major Homozygote	Heterozygote		Minor Homozygote		Urine X Heterozygote		Urine X Minor homozygote		Hair X Heterozygote		Hair X Minor homozygote	
				Ref	**β**	***p***	**β**	***p***	**β**	***p***	**β**	***p***	**β**	***p***	**β**	***p***
Log(Ulnar	Base model	-	2009-2010	-	-	-	-	-	-	-	-	-				
Peak	GPX1(C>T)	rs1050450	2009	-	0.017	*0.23*	-	-	**-0.016**	***0.05***	-	-				
Log(Ulnar	Base model	-	2009-2010	-	-	-	-	-					-	-	-	-
Onset	MT1E 3'UTR(G>T)	rs708274	2009	-	-0.048	*0.01*	-	-					**0.033**	***0.05***	-	-
Log(Sural	Base model	-	2009-2010	-	-	-	-	-					-	-	-	-
Amplitude)	GCLC 5' UTR(G>A)	rs17883901	2009	-	-0.19	*0.09*	-	-					**0.32**	***0.01***	-	-

Note:For MT1E, pooled heterozygotes and minor homozygotes were compared to major homozygotes; Genotyping of the three SNPs were only done in 2009

Table 4.4: Selected Mean Nerve Conduction Measurements Stratified by Genotypes of Selected SNPs

SNP name	db SNP	Ref MAF (Caucasian)	N	Mean Nerve Conduction Functions			
				Major homozygote (N)	Heterozygote(N)	Minor homozygote(N)	ANOVA *p*
2009							
				Ulnar Peak Latency (msec)			
GPX1(C>T)	rs1050450	0.120	207	3.26(107)	3.23(79)	3.18(21)	0.54
				Ulnar Onset Latency (msec)[1]			
MT1E 3'UTR(G>T)	rs708274	0.100	216	2.51(168)	2.46(48)	-	0.22
				Sural Amplitude (μV)[1]			
GCLC 5' UTR(G>A)	rs17883901	0.060	210	13.23(183)	12.78(27)	-	0.74

Note: [1] Due to small size in minor homozygote, the results shown are pooled results of minor homozygote with heterozygote.

Table 4.5: Coefficients and p Values from Unstable Multivariate Linear Regression Models (9 out of 504) of Log-transformed Nerve Conduction Function Predicted by Hg Biomarker Levels in Urine or Hair, Selected SNP Genotypes and SNP-biomarker Interactions

	SNP Name	db SNP	# of Potentially Influential Outliers exluded	Year of Data	Base model R^2	Intercept	Age		Female		BMI		Hand Temperature		Left-handed		Weight		Height		Foot Temperature		Urine		Hair	
						6	6	*p*	6	*p*	6	*p*	6	*p*	6	*p*	6	*p*	6	*p*	6	*p*	6	*p*	6	*p*
	Base model	-		2009-2010	0.47	4.31	-0.02	*<0.0001*	0.35	*<0.0001*	-0.02	*<0.0001*			0.2	*0.005*									0.06	*0.05*
Log(Median	GSTM3(G>A)	rs7483		2010	0.44	4.37	-0.02	*<0.0001*	0.25	*0.0007*	-0.01	*0.03*			0.2	*0.12*									0.03	*0.55*
Amplitude)			1		0.50	4.4	-0.02	*<0.0001*	0.26	*0.0002*	-0.01	*0.03*			0.2	*0.1*									0.04	*0.42*
	Base model	-		2009-2010	0.53	5.25	-0.02	*<0.0001*	0.53	*<0.0001*	-0.01	*0.0005*	-0.042	*0.002*	0.17	*0.003*									0.04	*0.13*
Log(Ulnar	SEPP1 3'UTR	rs7579		2009-2010	0.54	5.67	-0.02	*<0.0001*	0.52	*<0.0001*	-0.01	*0.0004*	-0.054	*0.007*	0.18	*0.003*									0.05	*0.07*
Amplitude)			7	2009-2010	0.55	5.6	-0.02	*<0.0001*	0.53	*<0.0001*	-0.01	*0.0004*	-0.052	*0.008*	0.18	*0.003*									0.05	*0.06*
	Base model	-		2009-2010	0.26	5.44	-0.02	*<0.0001*									-0.01	*<0.0001*	-0.008	*0.015*					0.06	*0.11*
	GSR(G>T)	rs1002149		2010	0.27	4.94	-0.02	*<0.0001*									-0.006	*0.02*	-0.006	*0.24*					-0.005	*0.94*
			6	2010	0.30	4.63	-0.02	*<0.0001*									-0.008	*0.002*	-0.003	*0.55*					-0.006	*0.99*
Log(Sural Amplitude)	GSTT1(deletion)	-		2009	0.33	5.12	-0.01	*<0.0001*									-0.008	*0.0008*	-0.008	*0.02*					0.08	*0.53*
			7	2009	0.33	4.99	-0.01	*<0.0001*									-0.008	*0.0003*	-0.008	*0.03*					0.07	*0.06*
	MT4 missense(G>A)	rs11643815		2009	0.33	5.24	-0.01	*<0.0001*									-0.009	*0.0002*	-0.008	*0.02*					-0.0005	*0.99*
			3	2009	0.33	5.34	-0.01	*<0.0001*									-0.008	*0.0003*	-0.008	*0.01*					0.03	*0.57*
	Base model	-		2009-2010	0.22	1.73	0.004	*<0.0001*			0.006	*<0.0001*	-0.03	*<0.0001*											-0.01	*0.25*
Log(Median	MT1E 3'UTR(G>T)	rs708274		2009	0.31	2.55	0.004	*<0.0001*			0.006	*0.0003*	-0.05	*<0.0001*											-0.02	*0.15*
Peak			3	2009	0.31	2.51	0.004	*<0.0001*			0.006	*0.0003*	-0.05	*<0.0001*											-0.02	*0.08*
Latency)	GPX1(C>T)	rs1050450		2009	0.29	2.66	0.004	*<0.0001*			0.005	*0.0018*	-0.05	*<0.0001*											0.02	*0.41*
			2	2009	0.30	2.48	0.004	*<0.0001*			0.004	*0.015*	-0.05	*<0.0001*											0.02	*0.18*
Log(Median	Base model	-		2009-2010	0.21	1.26	0.004	*<0.0001*			0.007	*<0.0001*	-0.021	*0.004*											-0.006	*0.26*
Onset	GSTM3(G>A)	rs7483		2010	0.2	0.93	0.005	*<0.0001*			0.006	*0.02*	-0.01	*0.22*											-0.009	*0.62*
Latency)			2	2010	0.24	1.46	0.005	*<0.0001*			0.005	*0.03*	-0.02	*0.03*											-0.02	*0.29*
Log(Sural	Base model	-		2009-2010	0.25	1.72	0.001	*0.008*											0.004	*<0.0001*	-0.04	*<0.0001*	-0.009	*0.04*		
Onset	GSTT1(deletion)	-		2009	0.25	2.7	0.001	*0.11*											0.004	*<0.0001*	-0.07	*<0.0001*	-0.009	*0.21*		
Latency)			1	2009	0.25	2.7	0.001	*0.12*											0.004	*<0.0001*	-0.07	*<0.0001*	-0.009	*0.19*		

(Continuted)Table 4.5: Coefficients and p Values from Unstable Multivariate Linear Regression Models (9 out of 504) of Log-transformed Nerve Conduction Function Predicted by Hg Biomarker Levels in Urine or Hair, Selected SNP Genotypes and SNP-biomarker Interactions

					SNP main effects					SNP-Hair interactions							
	SNP Name	db SNP	# of Potentially Influential Outliers exluded	Year of Data	Major Homozygote	Heterozygote		Minor Homozygote		Urine X Heterozygote		Urine X Minor homozygote		Hair X Heterozygote		Hair X Minor homozygote	
					Ref	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
	Base model	-		2009-2010	-	-	-	-	-					-	-	-	-
Log(Median Amplitude)	GSTM3(G>A)	rs7483		2010	-	-	-	-0.65	*0.0004*					-	-	**1.18**	***0.01***
			1		-	-	-	-0.25	*0.16*					-	-	0.39	*0.38*
	Base model	-		2009-2010	-	-	-	-	-					-	-	-	-
Log(Ulnar Amplitude)	SEPP1 3'UTR	rs7579		2009-2010	-	-	-	0.12	0.21					-	-	**-0.42**	**0.05**
			7	2009-2010	-	-	-	0.12	*0.35*					-	-	-0.07	*0.85*
	Base model	-		2009-2010	-	-	-	-	-					-	-	-	-
	GSR(G>T)	rs1002149		2010	-	-0.54	*<0.0001*	-	-					**0.53**	***0.02***	-	-
Log(Sural Amplitude)			6	2010	-	-0.43	*0.001*	-	-					0.41	*0.17*		
	GSTT1(deletion)	-		2009	-	0.08	*0.53*	-	-					**-0.34**	***0.06***	-	-
			7	2009	-	0.08	*0.58*	-	-					-0.4	*0.12*		
	MT4 missense(G>A)	rs11643815		2009	-	-0.09	0.27	-	-					**0.15**	***0.06***	-	-
			3	2009	-	-0.09	*0.3*	-	-					0.14	*0.34*	-	-
	Base model	-		2009-2010	-	-	-	-	-					-	-	-	-
Log(Median Peak Latency)	MT1E 3'UTR(G>T)	rs708274		2009	-	-0.05	*0.01*	-	-					**0.04**	***0.07***	-	-
			3	2009	-	-0.058	*0.02*	-	-					0.062	*0.25*		
	GPX1(C>T)	rs1050450		2009	-	0.032	0.09	-	-					-0.03	*0.18*	-	-
			2	2009	-	0.029	*0.1*	-	-					**-0.04**	***0.06***	-	-
Log(Median Onset Latency)	Base model	-		2009-2010	-	-	-	-	-					-	-	-	-
	GSTM3(G>A)	rs7483		2010	-	-	-	0.14	*0.03*					-	-	**-0.33**	**0.05**
			2	2010	-	-	-	0.05	*0.49*					-	-	-0.23	0.32
Log(Sural Onset Latency)	Base model	-		2009-2010	-	-	-	-	-	-	-	-	-				
	GSTT1(deletion)	-		2009	-	-0.03	*0.27*	-	-	**0.045**	***0.06***	-	-				
			1	2009	-	-0.02	*0.54*	-	-	0.02	*0.58*	-	-				

Appendix

Appendix 4.1: List of Selected Selenoprotein and Glutathione-related Protein SNPs, their Year Genotyped and the Methods Used

SNP name (Major>minor)	dbSNP ID	Full gene name	Category	Year genotyped	Method
GSTP1 105 (a>g)	Rs1695	Glutathione s-transferase pi 1	glutathione	2009-2010	Taqman
GSTP1 114 (c>t)	Rs1138272	Glutathione s-transferase pi 1	glutathione	2009-2010	Taqman
GCLM 588 (c>t)	Rs41303970	Glutamate cysteine ligase, modifier subunit	glutathione	2009-2010	Taqman
SEPP1-234 (c>t)	Rs3877899	Selenoprotein p1	selenoprotein	2009-2010	Taqman
SEPP1 3'UTR (c>t)	Rs7579	Selenoproteins p1	selenoprotein	2009-2010	Taqman
GPX4 (c>t)	Rs713041	Glutathione peroxidase 4	selenoprotein	2009	Taqman
GPX1-198 (c>t)	Rs1050450	Glutathione peroxidase 1	selenoprotein	2009	Taqman
GCLC 129 (g>a)	Rs17883901	Glutamate cysteine ligase, catalytic subunit	glutathione	2009	Taqman
GSTT1 del (+>-)	N/A	Glutathione s-transferase theta 1	glutathione	2009	Taqman *
GSTM1 del (->+)	N/A	Glutathione s-transferase mu 1	glutathione	2009	Gel-based†
GGT1 intron (t>c)	Rs5751901	Gamma-glutamyltransferase 1	glutathione	2010	Taqman
GSTM3-224 (g>a)	Rs7483	Glutathione s-transferase mu 3	glutathione	2010	Taqman
GSS 5'UTR (g>c)	Rs3761144	Glutathione synthetase	glutathione	2010	Taqman
GSR 5'UTR (g>t)	Rs1002149	Glutathione reductase	glutathione	2010	Taqman
GSR intron (t>a)	Rs2911678	Glutathione reductase	glutathione	2010	Taqman

*Probes/primers from Taqman were used for genotyping of GSTT1. However, since this is not a SNP, the probe was there to look for the presence of a region of the gene (either the gene was there, or it was not. I could not distinguish heterozygotes for this gene. Only +/? vs. -/-). The primers/probes were taken from this reference:
Mordukhovich I., Wilker E., Suh H., Wright R., Sparrow D., Vokonas P.S., and Schwartz J. 2009. Black carbon exposure, oxidative stress genes, and blood pressure in a repeated-measures study. *Environmental Health Perspectives*. 117: 1767-1772.

†This is a deletion polymorphism (like GSTT1). I cannot distinguish heterozygotes with this method. This is a gel based method where I am looking for the presence or absence of the band. It is not an RFLP, though, because I do not cut it first with a restriction enzyme. The method is based off of the method from this paper:
Lee B., Hong Y., Park H., Ha M., Koo B.S., Chang N., Roh Y., Kim B., Kim Y., Kim B., Jo S., and Ha E. 2009. Interaction between GSTM1/GSTT1 polymorphism and blood mercury on birth weight. *Environmental Health Perspectives*. doi: 10.1289/ehp.0900731.

Bibliography

Albers, J.W., Cavender, G.D., Levine, S.P., Langolf, G.D., 1982. Asymptomatic sensorimotor polyneuropathy in workers exposed to elemental mercury. Neurology 32, 1168.

Albers, J.W., Kallenbach, L.R., Fine, L.J., Langolf, G.D., Wolfe, R.A., Donofrio, P.D., Alessi, A.G., Stolp-Smith, K.A., Bromberg, M.B., 1988. Neurological abnormalities associated with remote occupational elemental mercury exposure. Ann. Neurol. 24, 651.

American Association of Electrodiagnostic Medicine, 2002. Practice parameter for electrodiagnostic studies in carpal tunnel syndrome: summary statement. Muscle Nerve 25, 918.

Aschner, M., Syversen, T., Souza, D., Rocha, J.B.T., 2006. Metallothioneins: mercury species-specific induction and their potential role in attenuating neurotoxicity. Exp. Biol. Med. 231, 1468-1473.

Chen, C., Yu, H., Zhao, J., Li, B., Qu, L., Liu, S., Zhang, P., Chai, Z., 2006. The roles of serum selenium and selenoproteins on mercury toxicity in environmental and occupational exposure. Environ. Health Perspective. 114, 297-301.

Custodio, H.M., Broberg, K., Wennberg, M., Jansson, J., Vessby, B., Hallmans, G., Stegmayr, B., Skerfving, S., 2004. Polymorphisms in glutathione-related genes affect methylmercury retention. Arch. Environ. Health 59, 588.

Custodio, H.M., Harari, R., Gerhardsson, L., Skerfving, S., Broberg, K., 2005. Genetic influences on the retention of inorganic mercury. Archives of environmental occupational health 60, 17.

DeRouen, T., Martin, M., Leroux, B., Townes, B., Woods, J., Leito, J., Castro-Caldas, A., Luis, H., Bernardo, M., Rosenbaum, G., Martins, I., 2006. Neurobehavioral effects of dental amalgam in children: a randomized clinical trial. JAMA 295, 1784-1792.

Echeverria, D., Woods, J.S., Heyer, N.J., Rohlman, D.S., Farin, F.M., Bittner, A.C., Li, T., Garabedian, C., 2005. Chronic low-level mercury exposure, BDNF polymorphism, and associations with cognitive and motor function. Neurotoxicol. Teratol. 27, 781.

Echeverria, D., Woods, J.S., Heyer, N.J., Rohlman, D., Farin, F.M., Li, T., Garabedian, C.E., 2006. The association between a genetic polymorphism of coproporphyrinogen oxidase, dental mercury exposure and neurobehavioral response in humans. Neurotoxicol. Teratol. 28, 39.

Ellingsen, D.G., Mrland, T., Andersen, A., Kjuus, H., 1993. Relation between exposure related indices and neurological and neurophysiological effects in workers previously exposed to mercury vapour. Br. J. Ind. Med. 50, 736-744.

Fujimaki, Y., Kuwabara, S., Sato, Y., Isose, S., Shibuya, K., Sekiguchi, Y., Nasu, S., Noto, Y., Taniguchi, J., Misawa, S., 2009. The effects of age, gender, and body mass index on amplitude of sensory nerve action potentials: multivariate analyses. Clinical neurophysiology 120, 1683-1686.

Greathouse, D.G., Currier, D.P., Joseph, B.S., Shippee, R.L., Matulionis, D.H., 1989. Electrophysiologic responses of human sural nerve to temperature. Phys. Ther. 69, 914-922.

Gundacker, C., Gencik, M., Hikkel, I., Komarnicki, G., Kukuckova, M., Wittmann, K., 2009. Genetic background of lead and mercury metabolism in a group of medical students in Austria. Environ. Res. 109, 786.

Gundacker, C., Komarnicki, G., Jagiello, P., Gencikova, A., Dahmen, N., Wittmann, K.J., Gencik, M., 2007. Glutathione-S-transferase polymorphism, metallothionein expression, and mercury levels among students in Austria. Sci. Total Environ. 385, 37.

Katz, J.N., Stirrat, C.R., 1990. A self-administered hand diagram for the diagnosis of carpal tunnel syndrome. J. Hand Surg. 15, 360-363.

Katz, J.N., Stirrat, C.R., Larson, M.G., Fossel, A.H., Eaton, H.M., Liang, M.H., 1990. A self-administered hand symptom diagram for the diagnosis and epidemiologic study of carpal tunnel syndrome. J. Rheumatol. 17, 1495-1498.

Kimura, J., 1984. Principles and pitfalls of nerve conduction studies. Ann. Neurol. 16, 415.

Kingman, A., Albers, J.W., Arezzo, J.C., Garabrant, D.H., Michalek, J.E., 2005. Amalgam exposure and neurological function. Neurotoxicology 26, 241.

Ko, G., Nowacki, N., Arseneau, L., Eitel, M., Hum, A., 2010. Omega-3 fatty acids for neuropathic pain: case series. Clin. J. Pain 26, 168-172.

Kumari, , Hiramatsu, , Ebadi, , 1998. Free radical scavenging actions of metallothionein isoforms I and II. Free Radic. Res. 29, 93.

Letz, R., Gerr, F., 1994. Covariates of human peripheral nerve function: I. Nerve conduction velocity and amplitude. Neurotoxicol. Teratol. 16, 95-104.

Letz, R., Gerr, F., Cragle, D., Green, R.C., Watkins, J., Fidler, A.T., 2000. Residual neurologic deficits 30 years after occupational exposure to elemental mercury. Neurotoxicology 21, 459-474.

Levine, S.P., Cavender, G.D., Langolf, G.D., Albers, J.W., 1982. Elemental mercury exposure: peripheral neurotoxicity. Br. J. Ind. Med. 39, 136.

Min, J.L., Lakenberg, N., Bakker-Verweij, M., Suchiman, E., Boomsma, D.I., Slagboom, P.E., Meulenbelt, I., 2006. High microsatellite and SNP genotyping success rates established in a large number of genomic DNA samples extracted from mouth swabs and genotypes. Twin Research and Human Genetics 9, 501.

Mozaffarian, D., Rimm, E., 2006. Fish intake, contaminants, and human health: evaluating the risks and the benefits. JAMA (Chicago, Ill.) 296, 1885-1899.

Oken, E., Bellinger, D., 2008. Fish consumption, methylmercury and child neurodevelopment. Curr. Opin. Pediatr. 20, 178-183.

Oken, E., Wright, R., Kleinman, K., Bellinger, D., Amarasiriwardena, C., Hu, H., Rich-Edwards, J., Gillman, M., 2005. Maternal fish consumption, hair mercury, and infant cognition in a U.S. Cohort. Environ. Health Perspect. 113, 1376-1380.

Ravn-Haren, G., Olsen, A., Tjnneland, A., Dragsted, L., Nex, B., Wallin, H., Overvad, K., Raaschou-Nielsen, O., Vogel, U., 2006. Associations between GPX1 Pro198Leu polymorphism, erythrocyte GPX activity, alcohol consumption and breast cancer risk in a prospective cohort study. Carcinogenesis 27, 820-825.

Rivner, M.H., Swift, T.R., Malik, K., 2001. Influence of age and height on nerve conduction. Muscle Nerve 24, 1134-1141.

Robinson, L.R., Rubner, D.E., Wahl, P.W., Fujimoto, W.Y., Stolov, W.C., 1993. Influences of height and gender on normal nerve conduction studies. Arch. Phys. Med. Rehabil. 74, 1134-1138.

ROONEY, J., 2007. The role of thiols, dithiols, nutritional factors and interacting ligands in the toxicology of mercury. Toxicology 234, 145-156.

Salerno D F, Franzblau A, Werner R A, Bromberg M B, Armstrong T J, Albers J W, 1998. Median and Ulnar Nerve Conduction Studies Among Workers: Normative Values. Muscle & Nerve 21, 1005.

Schläwicke Engström, K., Strömberg, U., Lundh, T., Johansson, I., Vessby, B., Hallmans, G., Skerfving, S., Broberg, K., 2008. Genetic variation in glutathione-related genes and body burden of methylmercury. Environ. Health Perspect. 116, 734.

Schuchmann, J.A., 1977. Sural nerve conduction: a standardized technique. Arch. Phys. Med. Rehabil. 58, 166-168.

Schurz, , Sabater-Vilar, , Fink-Gremmels, , 2000. Mutagenicity of mercury chloride and mechanisms of cellular defence: the role of metal-binding proteins. Mutagenesis 15, 525.

Shapiro, I.M., Cornblath, D.R., Sumner, A.J., Uzzell, B., Spitz, L.K., Ship, I.I., Bloch, P., 1982. Neurophysiological and neuropsychological function in mercury-exposed dentists. The Lancet 1, 1147.

Tong, H., Werner, R., Franzblau, A., 2004. Effect of aging on sensory nerve conduction study parameters. Muscle Nerve 29, 716-720.

Trojaborg, W.T., Moon, A., Andersen, B.B., Trojaborg, N.S., 1992. Sural nerve conduction parameters in normal subjects related to age, gender, temperature, and height: a reappraisal. Muscle Nerve 15, 666-671.

Werner, R.A., Franzblau, A., 1996. Hand dominance effect on median and ulnar sensory evoked amplitude and latency in asymptomatic workers. Arch. Phys. Med. Rehabil. 77, 473-476.

Werner, R.A., 2006. Evaluation of work-related carpal tunnel syndrome. J. Occup. Rehabil. 16, 207-222.

Zampollo, A., Baruffini, A., Cirla, A.M., Pisati, G., Zedda, S., 1987. Subclinical inorganic mercury neuropathy: neurophysiological investigations in 17 occupationally exposed subjects. The Italian journal of neurological sciences 8, 249-254.

Chapter 5

Agreement between Symptom Survey, Monofilament Testing and Electrodiagnostic Findings Consistent with Neuropathy in the Feet

1. Introduction

Peripheral neuropathy in the lower extremity is often characterized by alteration or loss of sensation in the feet, e.g. tingling and numbness. The detection of mild peripheral neuropathy may require careful clinical examinations and/or the use of electrodiagnostic testing. Peripheral neuropathy in the lower extremity is clinically important in the general population, especially in subjects with diabetes (Thoma et al). Peripheral neuropathy often results from nerve damage caused possibly by exposure to toxicants, metabolic disorders and infection. Quick and accurate screening and clinical diagnosis of peripheral neuropathy in the lower extremity relies on high sensitivity and specificity of the methods employed. The sensitivity and specificity of the techniques employed significantly affects the outcomes of clinical screening and are also important in epidemiological research of peripheral neuropathy in the lower extremity.

Several tools and procedures have been employed in the screening and epidemiological research of peripheral neuropathy. They include electrodiagnostic testing (Schuchmann 1977; Buschbacher 2003), physical examination procedures (Feng, et al. 2009; Herman and Kennedy 2005; Jirkovsk, et al. 2001; Rahman, et al. 2003), and body diagram and symptom questionnaire (Leonard, et al. 2005; Margolis, et al. 1988). Each of these tools and procedures has strengths and weaknesses. There are other quantitative sensory testing methods of assessing nerve function (e.g. examination of pinprick and vibration

perception, etc) (Boulton et al., 2005). The advantage of electrodiagnostic testing is that it provides an objective measure of peripheral nerve function, which clinical psychophysical methods do not offer. Among lower extremity nerves, the sural nerve is one of the most commonly studied nerves because it is distal sensory nerve. Sural nerve conduction testing is considered to be an objective and sensitive tool to detect peripheral neuropathy in the lower extremities (Kimura 1984). However, it requires specific equipment and training for testers.

There is no universally agreed set of criteria for defining peripheral neuropathy (Buschbacher 2003; Schuchmann 1977; Truong, et al. 1979). One criterion that is typically used to define peripheral neuropathy is an abnormality of a distal sensory nerve. An abnormal sural sensory nerve peak latency is typically set at two standard deviations above the population mean. Cutoffs have been defined by Schuchmann 1977 as >4.1msec and by Wainapel, et al. 1978 as >4.3msec. The amplitude of the sural sensory response can also be used to define a peripheral neuropathy but the amplitudes are relatively small compared to sensory responses in the upper extremity and may be affected by 'noise' more than sensory nerve conduction measurements in the upper extremity (Blando 1998). Monofilament testing (one type of quantitative sensory testing that uses psychophysical techniques for detecting peripheral neuropathy in the feet) has been considered a useful noninvasive tool for detecting peripheral neuropathy especially in diabetic subjects (Jirkovsk, et al. 2001; Rahman, et al. 2003). It is very inexpensive and simple to perform. The Semmes-Wienstein monofilaments employ a large number of filaments to test the sensory threshold to light touch but many clinicians use a single filament to determine if there is a clinically relevant loss of light touch that places a patient at risk for developing a foot ulcer. However, various definitions of abnormal cases exist as a result of a variety of test methodologies employed by medical professionals and researchers (Feng, et al. 2009). Furthermore, poor agreement has been previously reported between physician diagnoses of neuropathy in the lower extremity and monofilament test results (Herman and Kennedy 2005). The under- and over- diagnosis of

peripheral neuropathy in the lower extremity may be significant given the measurement errors of these tools and procedures.

The objective of this study was to assess the agreement between electrodiagnostic testing, monofilament testing and a lower extremity symptom survey in identification of possible peripheral neuropathy in the feet. The analysis for the assessment will be carried out in a population of dental professionals.

2. Materials and Methods

The subjects in this study were recruited during the Michigan Dental Association (MDA) annual conventions held in 2009 (n=232) and 2010 (n=283). They represented a convenience sample of dental professionals who attended the conventions and were recruited for a gene-environment study that investigated the relationship of mercury exposure and nerve conduction testing with urinary and hair biomarkers in dental professionals. Each participant signed a written informed consent approved by the University of Michigan Institutional Review Board (HUM00027621). Each participant received a confidential letter summarizing and interpreting the results for their samples and measurements.

2.1 Eletrodiagnostic Testing

Nerve conduction tests were performed on each subject and included measurements of amplitude and peak latency of the sural nerve in the right ankle. Feet were warmed with electric heating pads if the limb temperature was initially below 32 °C. The temperature of the right midfoot was then recorded at the time of measurement. Stimulation was applied on the posterior aspect of the right calf, 14cm proximal to the standard recording electrode placed behind the lateral malleolus in the lower extremity (Schuchmann 1977). TECA Synergy (Oxford Instrument, Hawthorne, NY) was used to record the amplitude and peak latency using antidromic stimulation of the sural nerve. The peak latency (milliseconds-ms) was defined as the time required for an electrical stimulus to reach peak deflection from baseline of an action potential waveform. The amplitude

(microvolts-μV) was defined as the baseline-to-peak voltage difference of the waveform. All parameters were recorded in accordance with the guidelines outlined by the American Association of Electrodiagnostic Medicine (American Association of Electrodiagnostic Medicine 2002).

Abnormal sural nerve function was defined using two separate criteria: 1) peak latency >4.1ms (20-60 years old) or >5ms (>60 years old); 2) amplitude <=6μV (20-60 years old) or <=5μV (>60 years old). These age-adjusted cutoffs are used by the University of Michigan Electroneuromyography Laboratory. The sural nerve peak latency was also adjusted to a standard temperature of 32 °C based on the following formula: $latency_{corrected}$=$latency_{initial}$-0.3msec*(32°C-temperature in °C) (Tong et al. 2004). Due to missing values (n=56) for foot temperature, we have a sample size of peak latency smaller than amplitude.

2.2 Self-administrated Symptom Questionnaire and Body Diagram

We asked each subject to complete a self-administered questionnaire to collect information on demographics and current symptoms of the lower extremities along with pre-existing diseases, pregnancy and other factors potentially related to mercury exposure. Data on pregnancy and other mercury-related factors were not included in the subsequent analyses. If subjects reported any symptoms in their feet in the week prior to the survey, subjects were asked to report the duration of time they felt numbness and/or tingling in their feet in the following format: less than 6 weeks, 6-12 weeks, or more than 12 weeks (Sx). In the subsequent analyses, results from this question were dichotomized into duration <= 12 weeks and >12 weeks. Staff members were available to answer any questions subjects had. Questionnaires were reviewed for completeness before subjects left.

A self-administrated body symptom diagram displaying the outlines of full front and back views of the human body was given to each participant. They were asked to shade the areas where numbness, tingling, burning or pain had

occurred more than three times, or lasting more than one week in the previous six months. This instrument collected information on the area of body surface with symptoms and the location of the symptoms. It has been shown to be a reliable diagnostic tool among chronic pain patients (Margolis, et al. 1988). For the present analyses, only areas at or below the ankle were reviewed and scored for symptoms consistent with neuropathy in the feet independently by two raters. The results were compared for discrepancies and any discrepancies were reconciled between the two raters through consensus. The results of body diagrams (BDS) were classified into three categories with respect to symptom distribution consistent with neuropathy in the feet, including probable, possible, and unlikely (See Appendix 5.1 for specific definitions). In this analysis, symptoms consistent with neuropathy in the feet were defined in five different ways using different combinations of the body diagram and symptom questionnaire (Table 5.2).

2.3 Monofilament Testing

There are many quantitative sensory testing protocols that incorporate psychophysical testing (e.g. monofilament testing, vibratory sensory testing) to detect peripheral nerve impairment in the lower extremity. In this study, we chose monofilament testing recommended by the American Diabetes Association standard of care guidelines (American Diabetes Association, 2004). Semmes-Weinstein nylon monofilament testing is comprised of a total of 20 monofilaments of equal length but not of equal diameter. They can be used to measure loss of sensation in the lower extremity. In this study, the plantar surface of the great toe on the right foot was tested using a 5.07-gauge Semmes-Weinstein nylon monofilament (Wound Central, Aurora, IL) (Herman and Kennedy 2005). We chose one monofilament instead of all 20 monofilament in order to simplify the data collection by minimizing the time spent on one subject and maximizing the sample size of the study. The use of a single monofilament resulted in a single outcome ("positive" or "negative"), not identification of a specific threshold that the full set of Semmes-Weinstein would allow. Before the test, patients were

asked to feel the monofilament on their finger tip. The monofilament was then applied up to three times to the right great toe with sufficient force to bend the filament. Patients were asked to indicate when a touch occurred. The test result was recorded as abnormal if a subject did not indicate a monofilament touch on two consecutive tries.

3. Statistical Methods

Since nerve conduction and monofilament tests were conducted only on the right foot, all analyses describe results in only the right foot. All analyses were performed using SAS 9.2 (SAS Institute, Inc. Cary, NC). Agreement of electrodiagnostic findings with symptoms consistent with neuropathy in the feet and monofilament results, respectively was assessed by kappa coefficient. Separate kappa coefficients were calculated for all subjects, the diabetic subpopulation, and the non-diabetic subpopulation. Kappa results were interpreted as excellent (>0.75), fair to good (0.40~0.75) and poor (<0.40) (Streiner and Norman 1994). Pearson chi-square tests or Fisher's exact tests were also performed to assess the association of electrodiagnostic findings with symptoms consistent with neuropathy in the feet and monofilament results. Using sural nerve function (peak latency and/or amplitude) as the gold standard, sensitivity and specificity of various combinations of the other tests (body diagram, symptom questionnaire, and monofilament) were calculated. Although many clinicians would define peripheral neuropathy as an abnormality in 2 distal lower limb nerves, we chose to use only one sensory nerve for the gold standard because we only measured the sural sensory nerve to minimize the time spent on each subject and therefore maximize the study sample size. Alternatively we could use the median or ulnar nerve as a second nerve in defining the gold standard for peripheral neuropathy but we would lose sensitivity since this is a more proximal nerve. To reflect the clinical relevance of the nerve function as continuous measurements, we also calculated the mean nerve function stratified by various combinations of tests discussed above. The mean nerve function was compared between test combination strata using t-tests.

4. Results

In our dental cohort, the prevalence of self-reported diabetes was approximately 4% in the entire population and was similar across different occupations (Table 5.1). Diabetic subjects were significantly older and had higher BMI than non-diabetic subjects (Table 5.1). Females constituted the majority of non-dentists (dental hygienists, dental assistant and others) in our population (Table 5.1).

Table 5.2 summarizes the various definitions of symptoms consistent with neuropathy based on the body diagram and the symptom questionnaire. Table 5.3 summarizes the prevalence of cases in all subjects, diabetic subjects, and non-diabetic subjects using the various criteria. The proportions of cases for different criteria seen in all subjects followed a similar pattern to those in the diabetes-stratified subjects. Except for peak latency, the proportions of cases in the diabetic subjects were consistently higher than those in the non-diabetic subjects regardless of the criteria used. One of the biggest difference (three-fold) between the proportions for diabetic and non-diabetic subjects was found in cases that had both abnormal peak latency and amplitude. Except for peak latency, the proportions of cases with abnormal peak latency were higher than abnormal amplitude among all subjects, diabetic subjects and non-diabetic subjects. Table 5.3 also summarizes the prevalence of abnormal monofilament cases. The proportion of cases with abnormal monofilament results was much lower than those of abnormal peak latency and amplitude in all subjects. For symptoms consistent with neuropathy, the proportions of cases varied according to the criteria employed in comparison with abnormal nerve function (peak latency and/or amplitude). The proportions of cases in criteria involving BDS or Sx were also approximately three-fold higher in diabetic subjects than non-diabetic subjects. Not surprisingly, the combination of cases from BDS and Sx led to a lower prevalence (5.24%) while a much higher proportion was seen in the cases of either BDS or Sx (14.56%). A higher prevalence was seen for Sx (>12) (3.69%) than Sx (<=12) (1.55%) in combination with BDS.

Table 5.4 summarizes the kappa coefficients and confidence intervals separately for the entire study population, diabetic subjects and non-diabetic subjects for each symptom consistent with neuropathy in the feet alone and also in combination with monofilament test results in comparison with abnormal peak latency and amplitude. The kappa coefficients in all subjects and non-diabetic subjects were similar, but varied in comparison to kappa values for diabetic subjects. All the kappa values showed poor agreement ranging from -0.12 to 0.44. The highest kappa values were observed in diabetic subjects between the body diagram results alone or combined with the symptom questionnaire (any Sx or Sx>12) in comparison with age- and temperature-adjusted neuropathy defined by the combination of both abnormal amplitude and abnormal peak latency (kappa=0.44, 95% CI:-0.21-1). The lowest kappa value was observed in diabetic subjects between monofilament findings alone in comparison with the combination of abnormal amplitude and abnormal peak latency (kappa=-0.12, 95% CI:-0.23- -0.007). In general, a higher degree of agreement was observed in body diagram results alone, body diagram results in combination with the symptom questionnaire (any Sx or Sx>12), monofilament results alone regardless of stratification. Among all subjects, lower kappa values (-0.004-0.09) were observed in body diagram findings and symptom questionnaire results in combination with monofilament results. Using sural nerve test results as the gold standard, the specificity of various combinations of tests was mostly high (>90%) but the sensitivity was mostly low (<30%). The relative relationships of sensitivity and specificity among diabetic, non-diabetic and all subjects followed the same patterns as seen for kappa values. Using the results of body diagram and the symptom questionnaire against nerve function yielded the highest sensitivity (50.00%) and the lowest specificity (64.71%) in Table 5.4.

Three other tables presented results for the combinations of monofilament findings and symptom results in comparison with the other three criteria of abnormal nerve function listed in Table 5.3 (Table 5.5-5.7). We observed similar

patterns in the kappa coefficients, sensitivity and specificity as described above for Table 5.4. Most of the kappa values fell under 0.40, reflecting poor agreement and ranged from -0.14 to 0.35 with the highest obtained in diabetic subjects for BDS or Sx (>12) with monofilament in comparison with age-adjusted neuropathy defined by abnormal amplitude (Table 5.7). The highest sensitivity (50%) and the lowest specificity (37.50%) appeared when positive results of body diagram or the symptom questionnaire were measured against abnormal amplitude (Table 5.7).

In addition, Table 5.4 included Pearson Chi-square statistics and p-values for testing the degree of association between various symptom definitions, monofilament test results and various combinations of both. Chi-square test statistics and p-values showed that significant associations in all subjects occurred only in those combinations whose kappa values were among the highest as described above. Similar patterns were observed in all three other tables (Table 5.5-5.7). No significant association was observed in diabetic subjects for all four tables.

We also calculated the average peak latency and amplitude in each cell of the 2 x 2 tables constructed with stratification of the various combinations of tests (Table 5.8-5.9). There was no significant between-strata difference in mean peak latency for most combinations of tests. Significant differences in both peak latency and amplitude were, however, seen in stratification of monofilament test alone and in combination with BDS or Sx for total and non-diabetic subjects but not diabetic subjects. For mean amplitude, we observed significant differences in all stratification of symptoms except for BDS & Sx (<=12).

5. Discussion

We assessed the agreement between electrodiagnostic testing results, monofilament findings and symptoms consistent with neuropathy in the feet in a nonclinical convenience sample of dental professionals. The electrodiagnostic

testing included sural sensory nerve peak latency and amplitude. We asked subjects to shade the areas below the ankle where numbness, tingling, burning or pain had occurred more than three times, or lasting more than one week in the six months prior to the measurement. We also asked the subjects to report the duration of time they felt numbness and/or tingling in their feet (less than 6 weeks, 6-12 weeks, more than 12 weeks) if they had felt symptoms during the week prior to the survey. We found the kappa coefficients in diabetic subjects were usually higher than those in non-diabetic subjects, who had similar kappa coefficients to those observed in all subjects combined. In general, a higher degree of agreement was observed with body diagrams alone and body diagrams in combination with symptom questionnaires (any duration or symptom lasting longer than 12 weeks) regardless of population stratification. Kappa values in all subjects and respective subpopulations (diabetic and non-diabetic) in Table 5.4 were among the highest when various combinations of body diagram findings, symptoms and monofilament testing results were compared with covariate-adjusted electrodiagnostic testing results defined by both abnormal sural peak latency and amplitude. When compared with abnormal electrodiagnostic testing results defined by such criteria, body diagram findings and symptoms in combination with monofilament test results in all subjects were among the lowest degree of agreement (kappa: -0.004-0.09). Chi-square test statistics and p values showed that significant associations occurred only in those combinations where kappa values were among the highest.

Not surprisingly, the lower degree of agreement was found when all three tools and procedures (body diagram, symptom survey questionnaire, monofilament testing and electrodiagnostic testing) overlapped. The intersection of body diagram and survey symptom (Sx<=12) for all subjects yielded one of the lowest kappa values (kappa=-0.02, 95% CI: -0.03-0.01) when compared to abnormal peak latency and amplitude (Table 5.4). After the addition of abnormal monofilament testing results, the kappa values did not improve. Similar low kappa values were also observed for the intersection of body diagram and survey

symptom (Sx<=12) elsewhere. This suggested that short-term symptoms that last for less than 12 weeks might not be a good tool for screening neuropathy in the feet. Overall, the kappa values may be unstable due to the small number of cases involved, and therefore should be interpreted with caution.

Using nerve conduction as the gold standard, the sensitivity and specificity of various combinations of tests were mostly low and high, respectively. The overall high specificity indicated that the ability of these tests to detect true negatives were high and could therefore be used in great confidence to detect subjects with true normal nerve function in feet. The tests with low sensitivity were not good for detecting subjects with underlying abnormal nerve function because a subject could potentially have negative results for body diagram, symptom questionnaire and/or monofilament testing but still has abnormal electrodiagnostic results. The results of mean differences in nerve function (Table 5.8-5.9) did not follow the pattern of kappa values discussed above. The discrepancies highlighted the influence of nerve function cutoff values and the potential importance of using nerve function as a continuous measurement in comparing test procedures. It appeared that compared with other combinations, monofilament test alone or in combination with BDS or Sx along with the sural peak latency or amplitude may serve as effective tools for screening for neuropathy in the lower extremity. Figure 5.1a-5.2b gave the graphical representation of the significant differences in the means of peak latency and amplitude in all subjects. On the other hand, as seen in overall low sensitivity and kappa values, there were significant overlaps in both peak latency and amplitude between the strata in the those figures. This demonstrated a low discriminatory power between the screening procedures for identifying cases in a non-clinical population.

In addition, poor agreement between the procedures might be explained by the differences in what each procedure measures. Abnormal sural nerve conduction results in the right foot are indicative of mononeuropathy but not necessarily polyneuropathy in the feet. In contrast, monofilament testing on great toe, body

diagram and symptom survey do not discriminate symptoms of mononeuropathic origin with those of polyneuropahtic origin or of trauma or other etiology. As a result, subjects who reported symptoms might not have abnormal sural nerve conduction. Furthermore, body diagrams and symptom questionnaires surveyed the time duration of up to six months prior to the electrodiagnostic testing. Subjects who were asymptomatic at the time of survey may possibly have abnormal nerve impairment reflected by abnormal nerve conduction.

It is not surprising that a somewhat higher degree of agreement was achieved in diabetic subjects than non-diabetic subjects or all subjects. Diabetes is one of the most common risk factors for neuropathy in the feet. In our study, a higher degree of agreement was usually observed in body diagram alone and body diagram in combination with the symptom questionnaire (any duration or symptom lasting longer than 12 weeks) regardless of population stratification. However, given the small numbers of cases related to most case definitions, most kappa values may be unstable and should be interpreted with caution as discussed before. The low prevalence of cases also highlighted the challenge of developing a screening tool for peripheral neuropathy for use in non-clinical populations because positive predictive value and negative predictive value are a function of prevalence, and not just sensitivity and specificity. Unlike other clinical diagnosis where a result is often characterized as either positive or negative, nerve conduction function is a continuum. Peripheral neuropathy, defined by continuous nerve function, is therefore a continuum. With the same nerve function data, different criteria for dichotomizing may result in different conclusions because a universally accepted cutoff for defining peripheral neuropathy has yet been established. This might help explain the poor agreement between the screening procedures in our study.

Although there is no prior study on agreement between different screening tools and procedures in the lower extremity in a non-clinical population, one similar study that investigated such agreement in the upper extremity also reported poor

degree of agreement between physical examinations, electrodiagnostic findings and symptom consistent with the carpal tunnel syndrome (Homan, et al. 1999). It is worth noting that we conducted this study using a convenience sample of dental professionals who were exposed to low-level mercury. Mercury exposure at high levels may lead to symptomatic peripheral nerve impairment (Albers, et al. 1982; Albers, et al. 1988; Ellingsen, et al. 1993; Letz, et al. 2000; Levine, et al. 1982). The prevalence of symptomatic peripheral nerve impairment as a result of low-level mercury exposure, however, may be low in this population. In addition, this is not a clinical population. Thus, the results of this study may differ from those that use a clinical population, if any. The results of this study point to a need for further development and evaluation of the methods used to screen for neuropathy in the feet.

Figures and Tables

Table 5.1: Demographic Charateristics of Subjects

	Dentist				
	N	Overall (100%)	Diabetic(4.10%)	Non-diabetic (95.90%)	Men (75.41%)
Age	242	56.14	60.50	55.96	59.24
BMI		26.38	28.02	26.31	26.83
	Non-dentist				
	N	Overall (100%)	Diabetic (4.46%)	Non-diabetic (95.54%)	Men (5.20%)
Age	269	48.20	53.67	47.94	59.86
BMI		26.44	30.09	26.27	27.98
	Overall				
	N	Overall (100%)	Diabetic (4.29%)	Non-diabetic (95.71%)	Men (38.52%)
Age	512	52.00	56.77 ƚ	51.79 ƚ	59.28
BMI		26.41	29.15 ƚƚ	26.29 ƚƚ	26.91

ƚ *p* <0.05; ƚƚ p<0.005

Table 5.2: Neuropathy Definitions from Results of Body Diagram Scores and Symptom Questionnaire

Symptom Consistent with Neuropathy	Abberviation	Definition
Definition a: Body diagram score	BDS	Probable or possible body diagram score for neuropathy
Definition b: Body diagram score & numbness and/or tingling in feet lasting no more than 12 weeks	BDS & Sx (<=12)	Probable or possible body diagram score for neuropathy and numbness and/or tingling in feet lasting no more than 12 weeks
Definition c: Body diagram score & numbness and/or tingling in feet lasting more than 12 weeks	BDS & Sx (>12)	Probable or possible body diagram score for neuropathy and numbness and/or tingling in feet lasting more than 12 weeks
Definition d: Body diagram score & numbness and/or tingling in feet	BDS & Sx	Probable or possible body diagram score for neuropathy and numbness and/or tingling in feet
Definition e: Body diagram score or numbness and/or tingling in feet	BDS or Sx	Probable or possible body diagram score for neuropathy or numbness and/or tingling in feet

Table 5.3: Prevalence of Subjects with Findings among All Subjects,Diabetic and Non-diabetic Subjects

	Subjects tested	Diabetic subjects	Subjects with findings	%	Subjects w/ findings among Diabetic	%	Subjects w/ findings among Non-diabetic	%
Covariate-adjusted Nerve Conduction Test								
Peak latency>4.1ms (or 5ms)	453	19	85	18.76	3	15.79	82	18.89
Amplitude<=6μV (or 5μV)	491	20	50	10.18	4	20.00	46	9.77
Peak latency>4.1ms (or 5ms) & amplitude<=6μV (or 5μV)	453	19	16	3.53	2	10.53	14	3.23
Peak latency>4.1ms (or 5ms) or amplitude<=6μV (or 5μV)	491	20	119	24.24	5	25.00	114	24.20
Symptom Consistent with Neuropathy in Feet								
BDS	515	23	35	6.80	4	17.39	31	6.30
BDS & Sx (<=12)	515	23	8	1.55	1	4.35	7	1.42
BDS & Sx (>12)	515	23	19	3.69	2	8.70	17	3.46
BDS & Sx	515	23	27	5.24	3	13.04	24	4.88
BDS or Sx	515	23	75	14.56	10	43.48	65	13.21
Monofilament test								
Monofilament test	501	21	17	3.39	3	14.29	14	2.92

Table 5.4: Agreement of Age- and temperature- adjusted Neuropathy in Feet (Peak Latency>4.1 or 5ms and amplitude<=6µV or 5µV) with Symptoms and Monofilament Test, and Combinations of Symptoms and Monofilament Test, Respectively for All Subjects, Diabetic and Non-

	Overall					Diabetic subjects					Non-diabetic subjects				
Symptom Consistent with Neuropathy in Feet	Simple Kappa coefficient (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Pearson χ2	χ2 *p* value	Simple Kappa coefficient (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Fisher's exact (one-tail) *p*	Fisher's exact (two-tail) *p*	Simple Kappa coefficient (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Fisher's exact (one-tail) *p*	Fisher's exact (two-tail) *p*
BDS	0.10 (-0.05, 0.24)	18.75% (0, 0.38)	94.28% (0.92, 0.96)	4.52	**0.03**	0.44 (-0.21, 1)	50.00% (0, 1)	94.12% (0.83, 1)	0.20	0.20	0.06 (-0.07, 0.19)	14.29% (0, 0.33)	94.29% (0.92, 0.96)	0.16	0.20
BDS & Sx (<=12)	-0.02 (-0.03, 0.01)	NA	98.63% (0.98, 1)	-	1 [1]	-	NA	NA	-	-	-0.02 (-0.03, -0.01)	NA	98.57% (0.97, 1)	0.82	1
BDS & Sx (>12)	0.15 (-0.03, 0.33)	18.75% (0, 38)	96.80% (0.95, 0.98)	-	**0.02** [1]	0.44 (-0.21, 1)	50.00% (0, 1)	94.12% (0.83, 1)	0.20	0.20	0.11 (-0.07, 0.28)	14.29% (0, 0.33)	96.90% (0.95, 0.99)	0.07	0.08
BDS & Sx	0.12 (-0.04, 0.27)	18.75% (0, 0.38)	95.42% (0.93, 0.97)	-	**0.04** [1]	0.44 (-0.21, 1)	50.00% (0, 1)	94.12% (0.83, 1)	0.20	0.20	0.08 (-0.07, 0.23)	14.29% (0, 0.33)	95.48% (0.94, 0.97)	0.12	0.14
BDS or Sx	0.04 (-0.04, 0.13)	25.00% (0.04, 0.46)	85.81% (0.82, 0.89)	-	0.27 [1]	0.07 (-0.28, 0.42)	50.00% (0, 1)	64.71% (0.42, 0.87)	0.49	1	0.03 (-0.05, 0.12)	21.43% (0, 0.43)	86.67% (0.84, 0.90)	0.19	0.41
Monofilament test (MF)	0.16 (-0.03, 0.34)	18.75% (0, 0.38)	97.02% (0.95, 0.99)	-	**0.02** [1]	-0.12 (-0.23, -0.003)	NA	88.24% (0.73, 1)	0.79	1	0.19 (-0.02, 0.30)	21.43% (0, 0.43)	97.37% (0.96, 0.99)	**0.007**	**0.008**
BDS & MF	0.09 (-0.09, 0.27)	6.25% (0, 0.18)	99.31% (0.98, 1)	-	0.13 [1]	-	NA	NA	-	-	0.10 (-0.10, 0.30)	7.14% (0, 0.21)	99.29% (0.98, 1)	0.12	0.12
BDS & Sx (<=12) & MF	-0.004 (-0.01, 0.004)	NA	99.77% (0.99, 1)	-	1 [1]	-	NA	NA	-	-	-0.004 (-0.01, 0.004)	NA	99.76% (0.99, 1)	0.96	1
BDS & Sx (>12) & MF	0.10 (-0.09, 0.30)	6.25% (0, 0.18)	99.77% (0.99, 1)	-	0.17 [1]	-	NA	NA	-	-	0.12 (-0.10, 0.34)	7.14% (0, 0.21)	99.76% (0.99, 1)	0.06	0.06
BDS & Sx & MF	0.10 (-0.09, 0.28)	6.25% (0, 0.18)	99.54% (0.99, 1)	-	0.10 [1]	-	NA	NA	-	-	0.11 (-0.10, 0.32)	5.56% (0, 0.16)	99.52% (0.99, 1)	0.09	0.09
BDS or Sx & MF	0.16 (-0.05, 0.37)	12.50% (0, 0.29)	98.86% (0.98, 1)	-	**0.02** [1]	-0.08 (-0.18, 0.03)	NA	94.12% (0.83, 1)	0.89	1	0.18 (-0.05, 0.42)	14.29% (0, 0.33)	99.05% (0.98, 1)	0.01	**0.01**

[1] Fisher's exact p value is reported instead because the expected number of subjects in at least one of Chi-square 2x2 table cells is smaller than 5.

[2] Pearson Chi-square is reported instead because the expected number of subjects in all Chi-square 2x2 table cells is greater than 5.

'-' denotes the expected number of subjects in at least one of Chi-square 2x2 table cells is zero.

'NA' denotes that the expected number of subjects needed to caculate sensitivity or specificity is zero.

Table 5.5: Agreement of Age- and temperature- adjusted Neuropathy in Feet (Peak Latency>4.1 or 5ms _or_ amplitude<=6µV or 5 µV) with Symptoms and Monofilament Test, Respectively for All Subjects, Diabetic and Non-diabetic Subjects

Symptom Consistent with Neuropathy in Feet	Overall					Diabetic subjects					Non-diabetic subjects				
	Simple Kappa coefficient (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Pearson χ2	χ2 *p* value	Simple Kappa coefficient (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Fisher's exact (one-tail) *p*	Fisher's exact (two-tail) *p*	Simple Kappa coefficient (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Fisher's exact (one-tail) *p*	Fisher's exact (two-tail) *p*
BDS	0.04 (-0.04, 0.12)	9.24% (0.04, 0.14)	93.82% (0.92, 0.97)	1.31	0.25	0.08 (-0.37, 0.53)	20.00% (0, 0.55)	86.67% (0.70, 1)	0.46	1	0.04 (-0.04, 0.11)	8.77% (0.04, 0.14)	94.12% (0.92, 0.97)	1.17 [2]	0.28
BDS & Sx (<=12)	0.001 (-0.04, 0.04)	1.68% (0, 0.04)	98.39% (0.97, 0.99)	-	1 [1]	-0.09 (-0.25, 0.07)	NA	93.33% (0.81, 1)	0.75	1	0.01 (-0.03, 0.05)	1.75% (0, 0.04)	98.60% (0.97, 1)	0.31	0.68
BDS & Sx (>12)	0.04 (-0.03, 0.10)	5.88% (0.02, 0.10)	96.77% (0.95, 0.99)	1.71	0.19	0.17 (-0.29, 0.62)	20.00% (0, 0.71)	93.33% (0.81, 1)	0.39	0.45	0.03 (-0.03, 0.09)	5.26% (0.01, 0.09)	96.92% (0.95, 0.99)	1.18 [2]	0.28
BDS & Sx	0.04 (-0.03, 0.11)	7.56% (0.03, 0.12)	95.16% (0.93, 0.97)	1.29	0.26	0.08 (-0.37, 0.53)	20.00% (0, 0.55)	86.67% (0.70, 1)	0.46	1	0.03 (-0.03, 0.11)	7.02% (0.02, 0.12)	95.52% (0.94, 0.98)	1.15 [2]	0.28
BDS or Sx	0.07 (-0.02, 0.16)	19.33% (0.12, 0.26)	86.83% (0.83, 0.90)	2.73	0.10	0 (-0.41, 0.41)	40.00% (0.10, 0.83)	60.00% (0.15, 0.65)	0.40	1	0.07 (-0.02, 0.17)	18.42% (0.11, 0.26)	87.96% (0.84, 0.91)	2.99 [2]	0.08
Monofilament test	0.06 (-0.01, 0.13)	6.72% (0.02, 0.11)	97.84% (0.96, 0.99)	5.95	**0.01**	0.17 (-0.28, 0.62)	20.00% (0, 0.55)	93.33% (0.81, 1)	0.39	0.45	0.06 (-0.01, 0.12)	6.14% (0.02, 0.11)	98.03% (0.96, 0.99)	5.21 [2]	**0.02**
BDS & MF	0.001 (-0.03, 0.03)	0.84% (0, 0.02)	99.19% (0.98, 1)	-	1 [1]	-	NA	NA	-	-	0.001 (-0.03, 0.03)	0.88% (0, 0.03)	99.16% (0.98, 1)	0.42	1
BDS & Sx (<=12) & MF	-0.004 (-0.01, 0.004)	NA	99.73% (0.99, 1)	-	1 [1]	-	NA	NA	-	-	-0.004 (-0.01, 0.004)	NA	99.72% (0.99, 1)	0.75	1
BDS & Sx (>12) & MF	0.01 (-0.02, 0.03)	0.84% (0, 0.03)	99.73% (0.99, 1)	-	0.43	-	NA	NA	-	-	0.01 (-0.02, 0.04)	0.88% (0, 0.03)	99.72% (0.99, 1)	0.37	0.42
BDS & Sx & MF	0.004 (-0.02, 0.03)	0.84% (0, 0.03)	99.46% (0.99, 1)	-	0.57 [1]	-	NA	NA	-	-	0.005 (-0.02, 0.03)	0.88% (0, 0.03)	99.44% (0.99, 1)	0.42	0.57
BDS or Sx & MF	0.04 (-0.01, 0.10)	3.36% (0.001, 0.07)	99.19% (0.98, 1)	-	**0.06** [1]	0.27 (-0.16, 0.71)	20.00% (0, 0.55)	NA	0.25	0.25	0.03 (-0.02, 0.07)	2.63% (0, 0.06)	99.16% (0.98, 1)	0.12	0.16

[1] Fisher's exact p value is reported instead because the expected number of subjects in at least one of Chi-square 2x2 table cells is smaller than 5.

[2] Pearson Chi-square is reported instead because the expected number of subjects in all Chi-square 2x2 table cells is greater than 5.

'-' denotes the expected number of subjects in at least one of Chi-square 2x2 table cells is zero.

'NA' denotes that the expected number of subjects needed to caculate sensitivity or specificity is zero.

Table 5.6: Agreement of Age- and temperature-adjusted Neuropathy in Feet (Peak Latency>4.1 or 5ms) with Symptoms and Monofilament Test, Respectively for All Subjects, Diabetic and Non-diabetic Subjects

	Overall					Diabetic subjects					Non-diabetic subjects				
Symptom Consistent with Neuropathy in Feet	Simple Kappa coefficient (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Pearson χ2	χ2 *p* value	Simple Kappa coefficient (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Fisher's exact (one-tail) *p*	Fisher's exact (two-tail) *p*	Simple Kappa coefficient (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Fisher's exact (one-tail) *p*	Fisher's exact (two-tail) *p*
BDS	0.03 (-0.05, 0.12)	8.24% (0.02, 0.14)	94.29% (0.91, 0.96)	0.76	0.38	0.31 (-0.27, 0.90)	33% (0, 0.87)	93.75% (0.81, 1)	0.28	0.30	0.02 (-0.06, 0.10)	7.32% (0.02, 0.13)	94.32% (0.91, 0.96)	0.32 [2]	0.57
BDS & Sx (<=12)	-0.002 (-0.04, 0.03)	1.18% (0, 0.03)	98.64% (0.97, 0.99)	-	0.40 [1]	-	NA	NA	-	-	-	1.22% (0, 0.04)	98.58% (0.97, 0.99)	-	-
BDS & Sx (>12)	0.04 (-0.04, 0.11)	5.88% (0.008, 0.11)	96.74% (0.95, 0.99)	1.31	0.25	0.31 (-0.27, 0.90)	33% (0, 0.87)	93.75% (0.81, 1)	0.28	0.29	0.03 (-0.05, 0.10)	4.88% (0.01, 0.10)	96.88% (0.95, 0.99)	0.61 [2]	0.43
BDS & Sx	0.03 (-0.05, 0.12)	7.06% (0.02, 0.12)	95.38% (0.93, 0.98)	0.85	0.36	0.31 (-0.27, 0.90)	33% (0, 0.87)	93.75% (0.81, 1)	0.28	0.29	0.02 (-0.06, 0.10)	6.10% (0.01, 0.11)	95.45% (0.93, 0.98)	0.35 [2]	0.56
BDS or Sx	0.03 (-0.07, 0.12)	16.47% (0.09, 0.24)	85.87% (0.83, 0.89)	0.3	0.58	-0.03 (-0.41, 0.35)	33% (0, 0.87)	62.50% (0.38, 0.86)	0.48	1	0.03 (-0.07, 0.13)	15.85% (0.08, 0.24)	86.93% (0.83, 0.90)	0.44 [2]	0.51
Monofilament test	0.04 (-0.03, 0.12)	5.88% (0.009, 0.11)	97.00% (0.95, 0.99)	1.68	0.19	-0.14 (-0.28, -0.007)	NA	87.50% (0.71, 1)	0.70	1	0.05 (-0.03, 0.13)	6.10% (0.009, 0.11)	97.44% (0.96, 0.99)	2.65 [2]	0.1
BDS & MF	0.01 (-0.03, 0.04)	1.18% (0,0.03)	99.18% (0.98, 1)	-	0.40 [1]	-	NA	NA	-	-	0.01 (-0.04, 0.05)	1.22% (0, 0.04)	99.15% (0.98, 1)	0.40	0.57
BDS & Sx (<=12) & MF	-0.004 (-0.01, 0.004)	NA	99.73% (0.99, 1)	-	1 [1]	-	NA	NA	-	-	-0.004 (-0.01, 0.004)	NA	99.72% (0.99, 1)	0.81	1
BDS & Sx (>12) & MF	0.01 (-0.02, 0.05)	1.18% (0,0.03)	99.73% (0.99, 1)	-	0.30 [1]	-	NA	NA	-	-	0.01 (-0.02, 0.05)	1.22% (0, 0.04)	99.72% (0.99, 1)	0.31	0.34
BDS & Sx & MF	0.01 (-0.03, 0.05)	1.18% (0,0.03)	99.46% (0.99, 1)	-	0.37 [1]	-	NA	NA	-	-	0.01 (-0.03, 0.05)	1.22% (0, 0.04)	99.43% (0.99, 1)	0.37	0.47
BDS or Sx & MF	0.02 (-0.04, 0.07)	2.35% (0, 0.06)	98.64% (0.98, 1)	-	0.26 [1]	-0.09 (-0.22, 0.05)	NA	93.75% (0.82, 1)	0.84	1	0.02 (-0.03, 0.07)	2.44% (0, 0.07)	98.86% (0.98, 1)	0.23	0.32

[1] Fisher's exact p value is reported instead because the expected number of subjects in at least one of Chi-square 2x2 table cells is smaller than 5.

[2] Pearson Chi-square is reported instead because the expected number of subjects in all Chi-square 2x2 table cells is greater than 5.

'-' denotes the expected number of subjects in at least one of Chi-square 2x2 table cells is zero.

'NA' denotes that the expected number of subjects needed to caculate sensitivity or specificity is zero.

Table 5.7: Agreement of Age-adjusted Neuropathy in Feet (amplitude<=6μV or 5μV) with Symptoms and Monofilament Test, Respectively for All Subjects, Diabetic and Non-diabetic Subjects

	Overall					Diabetic subjects					Non-diabetic subjects				
Symptom Consistent with Neuropathy in Feet	Simple Kappa coefficient (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Pearson χ2	χ2 *p* value	Simple Kappa coefficient (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Fisher's exact (one-tail) *p*	Fisher's exact (two-tail) *p*	Simple Kappa coefficient (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Fisher's exact (one-tail) *p*	Fisher's exact (two-tail) *p*
BDS	0.09 (-0.02, 0.21)	14% (0.04, 0.24)	93.88% (0.92, 0.96)	4.32	**0.04**	0.14 (-0.36, 0.63)	25% (0, 0.67)	87.50% (0.71, 1)	0.42	0.51	0.08 (-0.03, 0.20)	13.04% (0.03, 0.23)	94.12% (0.92, 0.96)	3.46 [2]	0.06
BDS & Sx (<=12)	0.01 (-0.06, 0.07)	2% (0, 0.06)	98.41% (0.97, 1)	-	0.58 [1]	-0.09 (-0.23, 0.06)	NA	93.75% (0.82, 1)	0.8	1	0.01 (-0.06, 0.08)	2.17% (0, 0.06)	98.59% (0.97, 1)	0.37	0.52
BDS & Sx (>12)	0.09 (-0.02, 0.21)	10% (0.02, 0.18)	96.83% (0.95, 0.98)	5.62	**0.02**	0.23 (-0.29, 0.75)	25% (0, 0.67)	93.75% (0.81, 1)	0.34	0.37	0.08 (-0.04, 0.19)	8.70% (0.006, 0.17)	96.94% (0.95, 0.98)	0.06	0.07
BDS & Sx	0.09 (-0.02, 0.20)	12% (0.03, 0.21)	95.24% (0.93, 0.97)	4.53	**0.03**	0.14 (-0.36, 0.63)	25% (0, 0.67)	87.50% (0.71, 1)	0.42	0.51	0.08 (-0.03, 0.20)	10.87% (0.02, 0.20)	95.53% (0.94, 0.97)	0.05	0.07
BDS or Sx	0.11 (0.001, 0.21)	26% (0.14, 0.38)	86.62% (0.83, 0.90)	5.72	**0.02**	0.09 (-0.31, 0.49)	50% (0.01, 0.99)	37.50% (0.14, 0.61)	0.38	1	0.10 (-0.01, 0.20)	23.91% (0.12, 0.36)	87.53% (0.84, 0.91)	4.63 [2]	**0.03**
Monofilament test	0.14 (0.01, 0.26)	12% (0.03, 0.21)	97.73% (0.96, 0.99)	13.45	**0.0002**	0.23 (-0.29, 0.75)	25% (0, 0.67)	93.75% (0.82, 1)	0.34	0.37	0.13 (0.0001, 0.25)	10.87% (0.02, 0.20)	97.88% (0.97, 0.99)	**0.006**	**0.007**
BDS & MF	0.002 (-0.04, 0.09)	2% (0, 0.06)	99.32% (0.99, 1)	-	0.35 [1]	-	NA	NA	-	-	0.02 (-0.05, 0.10)	2.17% (0, 0.06)	99.29% (0.98, 1)	0.29	0.34
BDS & Sx (<=12) & MF	-0.004 (-0.01, 0.004)	NA	99.77% (0.99, 1)	-	1 [1]	-	NA	NA	-	-	-0.004 (-0.01, 0.004)	NA	99.76% (0.99, 1)	0.90	1
BDS & Sx (>12) & MF	0.03 (-0.04, 0.10)	2% (0, 0.06)	99.77% (0.99, 1)	-	0.19 [1]	-	NA	NA	-	-	0.03 (-0.04, 0.11)	2.17% (0, 0.06)	99.76% (0.99, 1)	0.18	0.19
BDS & Sx & MF	0.03 (-0.04, 0.09)	2% (0, 0.06)	99.55% (0.99, 1)	-	0.28 [1]	-	NA	NA	-	-	0.03 (-0.04, 0.10)	2.17% (0, 0.06)	99.53% (0.99, 1)	0.24	0.27
BDS or Sx & MF	0.12 (0.002, 0.23)	8% (0.005, 0.16)	99.32% (0.99, 1)	-	**0.003 [1]**	0.35 (-0.17, 0.86)	25% (0, 0.67)	NA	0.20	0.2	0.1 (-0.02, 0.21)	6.52% (0, 0.14)	99.29% (0.99, 1)	**0.01**	**0.01**

[1] Fisher's exact p value is reported instead because the expected number of subjects in at least one of Chi-square 2x2 table cells is smaller than 5.

[2] Pearson Chi-square is reported instead because the expected number of subjects in all Chi-square 2x2 table cells is greater than 5.

'-' denotes the expected number of subjects in at least one of Chi-square 2x2 table cells is zero.

'NA' denotes that the expected number of subjects needed to caculate sensitivity or specificity is zero.

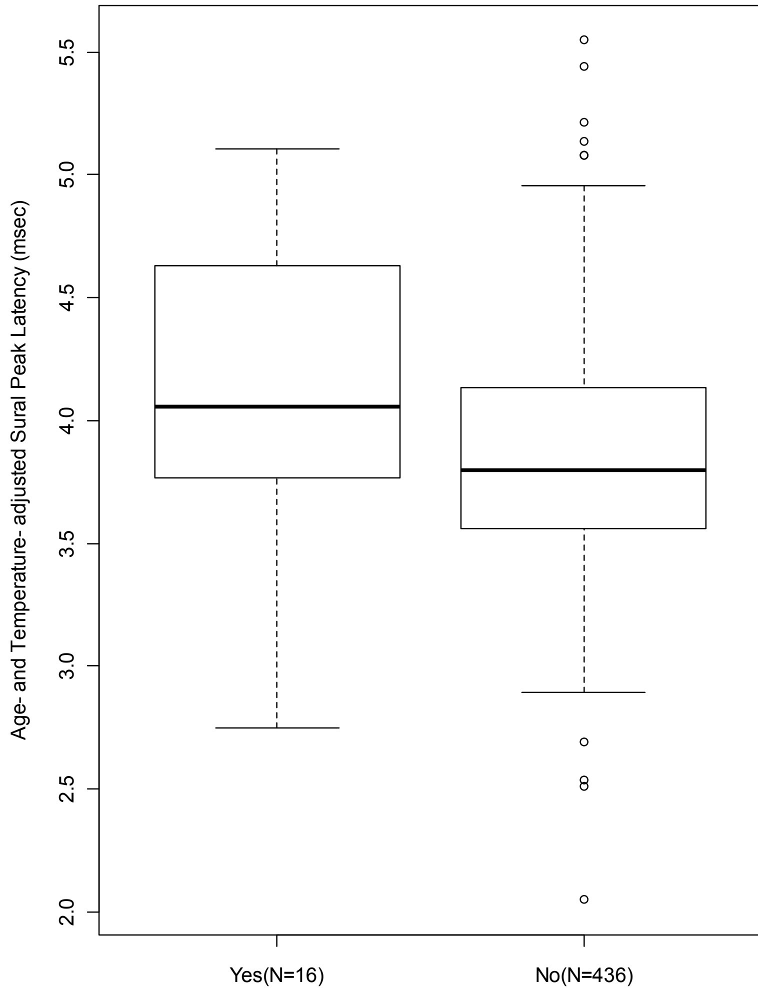
Table 5.8 Age- and temperature- adjusted Neuropathy in Feet (Abnormal Peak Latency>4.1 or 5ms; normal: <=4.1 or 5ms) Stratified by Various Combinations of Symptoms and Monofilament Test for All Subjects, Diabetic and Non-diabetic Subjects

		Age- and Temperature- adjusted Peak Latency (msec)								
		All subjects			Diabetic subjects			Non-diabetic subjects		
Overall		3.86 (453)			3.95 (19)			3.86 (434)		
		Abnormal (N)	Normal (N)	Total (N)	Abnormal (N)	Normal (N)	Total (N)	Abnormal (N)	Normal (N)	Total (N)
Symptom Consistent with Neuropathy in Feet										
BDS	Positive	4.59(7)	3.72 (21)	3.94(28)	4.66 (1)	3.69 (1)	4.18 (2)	4.58 (6)	3.72 (20)	3.92 (26)
	Negative	4.42 (78)	3.73 (347)	3.86 (425)	4.84 (2)	3.80 (15)	3.92 (17)	4.42 (76)	3.72 (332)	3.85 (408)
	p			0.36			65.00			0.46
BDS & Sx (<=12)	Positive	4.22 (1)	3.72 (5)	3.80 (6)	-	-	-	4.22 (1)	3.72 (5)	3.80 (6)
	Negative	4.45 (84)	3.73 (363)	3.86 (447)	4.78 (3)	3.79 (16)	3.95 (19)	4.43 (81)	3.72 (347)	3.86 (428)
	p			0.76			-			0.76
BDS & Sx (>12)	Positive	4.60 (5)	3.80 (12)	4.03 (17)	-	-	-	4.58 (4)	3.81 (11)	4.01 (15)
	Negative	4.43 (80)	3.72 (356)	3.85 (436)	4.78 (3)	3.79 (16)	3.95 (19)	4.42 (78)	3.72 (341)	3.85 (419)
	p			0.11			-			0.16
BDS & Sx	Positive	4.52 (6)	3.78 (17)	3.97 (23)	4.66 (1)	3.69 (1)	4.18 (2)	4.51 (5)	3.78 (16)	3.96 (21)
	Negative	4.44 (79)	3.72 (351)	3.85 (430)	4.84 (2)	3.80 (15)	3.92 (17)	4.43 (77)	3.72 (336)	3.85 (413)
	p			0.23			0.65			0.31
BDS or Sx	Positive	4.46 (14)	3.72 (52)	3.86 (66)	4.66 (1)	3.54 (6)	3.70 (7)	4.44 (13)	3.74 (46)	3.90 (59)
	Negative	4.44 (71)	3.73 (316)	3.86 (387)	4.84 (2)	3.94 (10)	4.09 (12)	4.43 (69)	3.72 (306)	3.85 (375)
	p			0.79			0.27			0.48
Symptom Consistent with Neuropathy in Feet and Monofilament test										
MF	Positive	4.85 (5)	3.80 (11)	4.13 (16)	-	3.41 (2)	3.41 (2)	4.85 (5)	3.89 (9)	4.23 (14)
	Negative	4.42 (80)	3.72 (356)	3.51 (436)	4.78 (3)	3.85 (14)	4.01 (17)	4.40 (77)	3.72 (342)	3.84 (419)
	p			**0.01**			0.28			**0.001**
BDS & MF	Positive	5.11 (1)	3.98 (3)	4.27 (4)	-	-	-	5.11 (1)	3.98 (3)	4.27 (4)
	Negative	4.43 (84)	3.72 (365)	3.85 (449)	4.78 (3)	3.79 (16)	3.95 (19)	4.42 (81)	3.72 (349)	3.85 (430)
	p			0.08			-			0.06
BDS & Sx (<=12) & MF	Positive	-	4.47 (1)	4.47 (1)	-	-	-	-	4.47 (1)	4.47 (1)
	Negative	4.44 (85)	3.72 (367)	3.86 (452)	4.78 (3)	3.79 (16)	3.95 (19)	4.43 (82)	3.72 (351)	3.86 (433)
	p			0.18			-			0.17
BDS & Sx (>12) & MF	Positive	5.11 (1)	3.66 (1)	4.39 (2)	-	-	-	5.11 (1)	3.66 (1)	4.39 (2)
	Negative	4.44 (84)	3.73 (367)	3.86 (451)	4.78 (3)	3.79 (16)	4.01 (20)	4.42 (81)	3.72 (351)	3.85 (432)
	p			0.10			-			0.09
BDS & Sx & MF	Positive	5.11 (1)	4.06 (2)	4.41 (3)	-	-	-	5.11 (1)	4.07 (2)	4.41 (3)
	Negative	4.44 (84)	3.72 (366)	3.86 (450)	4.78 (3)	3.79 (16)	4.01 (20)	4.42 (81)	3.72 (350)	3.85 (431)
	p			**0.04**			-			**0.03**
BDS or Sx & MF	Positive	5.11 (2)	3.74 (5)	4.13 (7)	-	-	2.75 (1)	5.11 (2)	3.98 (4)	4.36 (6)
	Negative	4.43 (83)	3.73 (363)	3.86 (446)	4.67 (5)	3.79 (15)	4.01 (18)	4.41 (80)	3.72 (348)	3.85 (428)
	p			0.12			0.09			**0.005**

Table 5.9 Age-adjusted Neuropathy in Feet (abnormal amplitude<=6µV or 5µV; normal: >6µV or 5µV) Stratified by Various Combinations of Symptoms and Monofilament Test for All Subjects, Diabetic and Non-diabetic Subjects

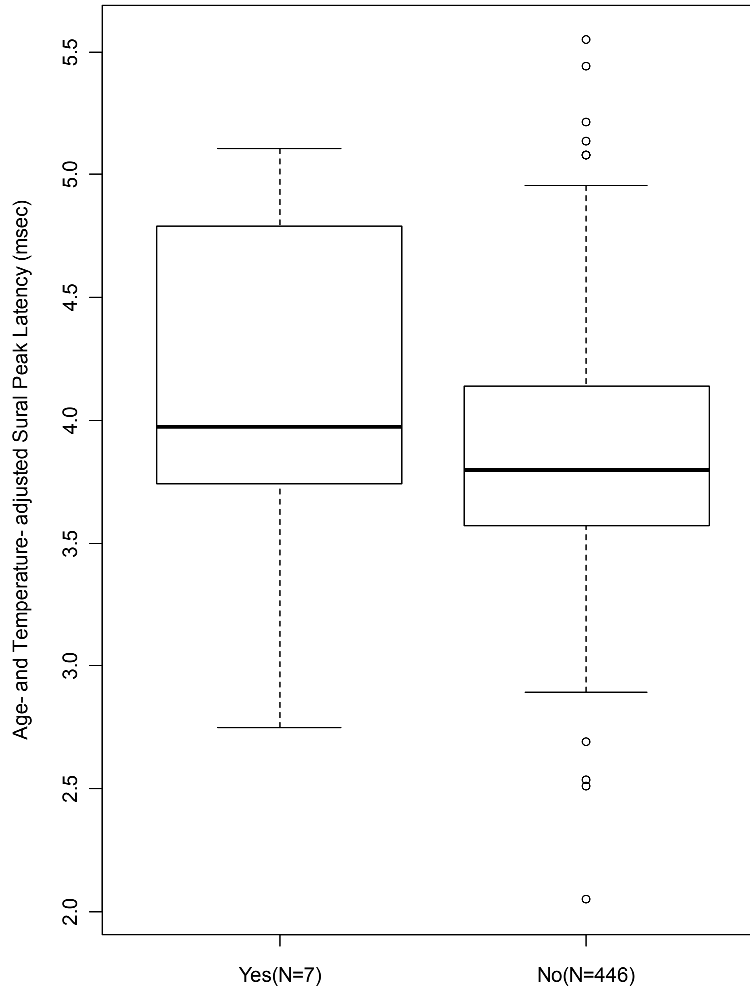
		Amplitude (µV)								
		All subjects			Diabetic subjects			Non-diabetic subjects		
Overall		12.92 (491)			8.67 (20)			13.10 (471)		
		Abnormal (N)	Normal (N)	Total (N)	Abnormal (N)	Normal (N)	Total (N)	Abnormal (N)	Normal (N)	Total (N)
Symptom Consistent with Neuropathy in Feet										
BDS	Positive	3.21(7)	12.27 (27)	10.40 (34)	3.54 (1)	16.31 (2)	12.06 (3)	3.15 (6)	11.94 (25)	10.24 (31)
	Negative	4.13 (43)	14.04 (414)	13.11 (457)	3.11 (3)	9.13 (14)	8.07 (17)	4.21 (40)	14.21 (400)	13.30 (440)
	p			**0.02**			0.18			**0.02**
BDS & Sx (<=12)	Positive	1.50 (1)	13.54 (7)	12.03 (8)	-	10.96 (1)	10.96 (1)	1.50 (1)	13.97 (6)	12.18 (7)
	Negative	4.05 (49)	13.94 (434)	12.93 (483)	3.22 (4)	9.97 (15)	8.55 (19)	4.13 (45)	14.08 (419)	13.11 (464)
	p			0.71			0.63			0.72
BDS & Sx (>12)	Positive	3.55 (5)	11.99 (14)	9.77 (19)	3.54 (1)	21.67 (1)	12.60 (2)	3.55 (4)	11.25 (13)	9.44 (17)
	Negative	4.05 (45)	13.99 (427)	13.04 (472)	3.11 (3)	9.26 (15)	8.23 (18)	4.12 (42)	14.16 (412)	13.23 (454)
	p			**0.04**			0.22			**0.02**
BDS & Sx	Positive	3.21 (6)	12.51 (21)	10.44 (27)	3.54 (1)	16.31 (2)	12.06 (3)	3.14 (5)	12.11 (19)	10.24 (24)
	Negative	4.11 (44)	14.00 (420)	13.06 (464)	3.11 (3)	9.13 (14)	8.07 (17)	4.18 (41)	14.17 (406)	13.25 (447)
	p			**0.05**			0.18			**0.03**
BDS or Sx	Positive	3.21 (13)	12.30 (59)	10.66 (72)	3.40 (2)	11.60 (6)	9.55 (8)	3.19 (11)	12.38 (53)	10.80 (64)
	Negative	4.28 (37)	14.18 (382)	13.31 (419)	3.04 (2)	9.09 (10)	8.08 (12)	4.35 (35)	14.32 (372)	13.45 (407)
	p			**0.002**			0.51			**0.004**
Symptom Consistent with Neuropathy in Feet and Monofilament test										
MF	Positive	2.85 (6)	9.02 (10)	6.71 (16)	3.25 (1)	8.13 (1)	5.69 (2)	2.78 (5)	9.12 (9)	6.86 (14)
	Negative	4.15 (44)	14.04 (430)	13.12 (474)	3.21 (3)	10.16 (15)	9.00 (18)	4.22 (41)	14.18 (415)	13.29 (456)
	p			**0.0002**			0.35			**0.0005**
BDS & MF	Positive	3.04 (1)	9.72 (3)	8.05 (4)	-	-	-	3.04 (1)	9.72 (3)	8.05 (4)
	Negative	4.02 (49)	13.96 (438)	12.96 (487)	3.22 (4)	10.03 (16)	8.67 (20)	4.09 (45)	14.11 (422)	13.14 (467)
	p			0.15			-			0.14
BDS & Sx (<=12) & MF	Positive	-	5.33 (1)	5.33 (1)	-	-	-	-	5.33 (1)	5.33 (1)
	Negative	4.00 (50)	13.95 (440)	12.93 (490)	3.22 (4)	10.03 (16)	8.67 (20)	4.07 (46)	14.10 (424)	13.11 (470)
	p			0.26			-			0.25
BDS & Sx (>12) & MF	Positive	3.04 (1)	13.96 (1)	8.50 (2)	-	-	-	3.04 (1)	13.96 (1)	8.5 (2)
	Negative	4.02 (49)	13.93 (440)	12.94 (489)	3.22 (4)	10.03 (16)	8.67 (20)	4.09 (45)	14.08 (424)	13.12 (469)
	p			0.36			-			0.34
BDS & Sx & MF	Positive	3.04 (1)	9.65 (2)	7.44 (3)	-	-	-	3.04 (1)	9.65 (2)	7.44 (3)
	Negative	4.02 (49)	13.95 (439)	12.95 (488)	3.22 (4)	10.03 (16)	8.67 (20)	4.09 (45)	14.10 (423)	13.13 (4680
	p			0.16			-			0.15
BDS or Sx & MF	Positive	3.15 (4)	9.72 (3)	5.96 (7)	3.25 (1)	-	3.25 (1)	3.11 (3)	9.72 (3)	6.42 (6)
	Negative	4.07 (46)	13.96 (438)	13.02 (484)	3.21 (3)	10.03 (16)	8.95 (19)	4.13 (43)	14.11 (422)	13.18 (465)
	p			**0.006**			0.24			**0.02**

Figure 5.1a Peak Latency Stratified by MF



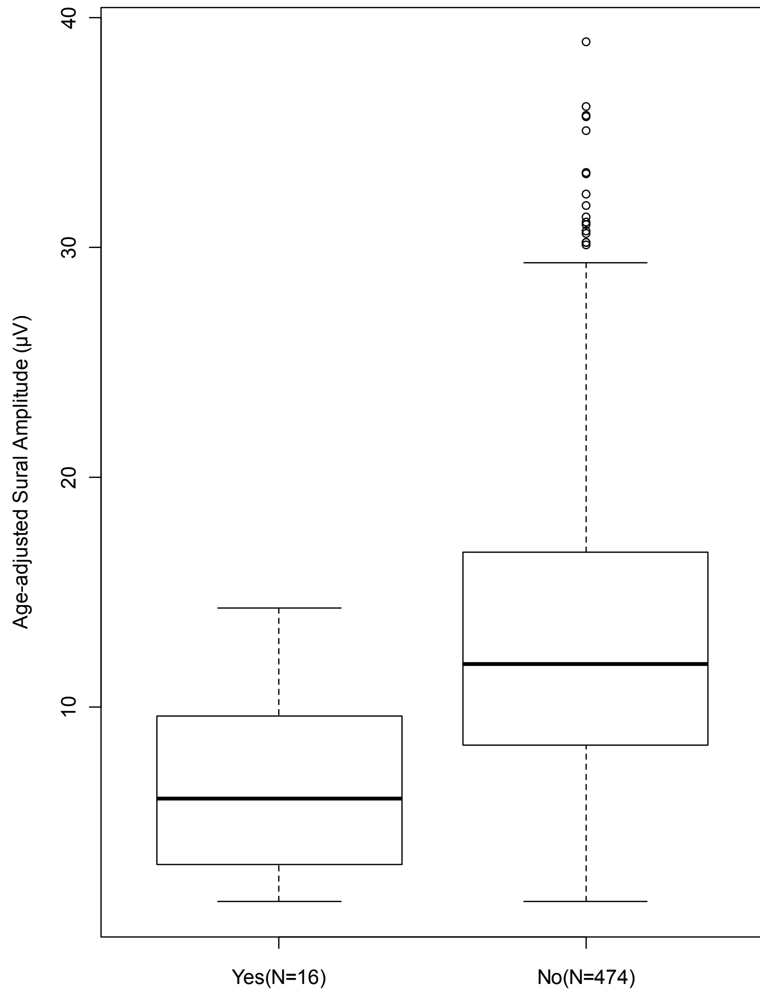
Monofilament result
Horizontal lines(from top): 95,75,50,25,&5 percentiles, respectively

Figure 5.1b Peak Latency Stratified by BDS or Sx &MF



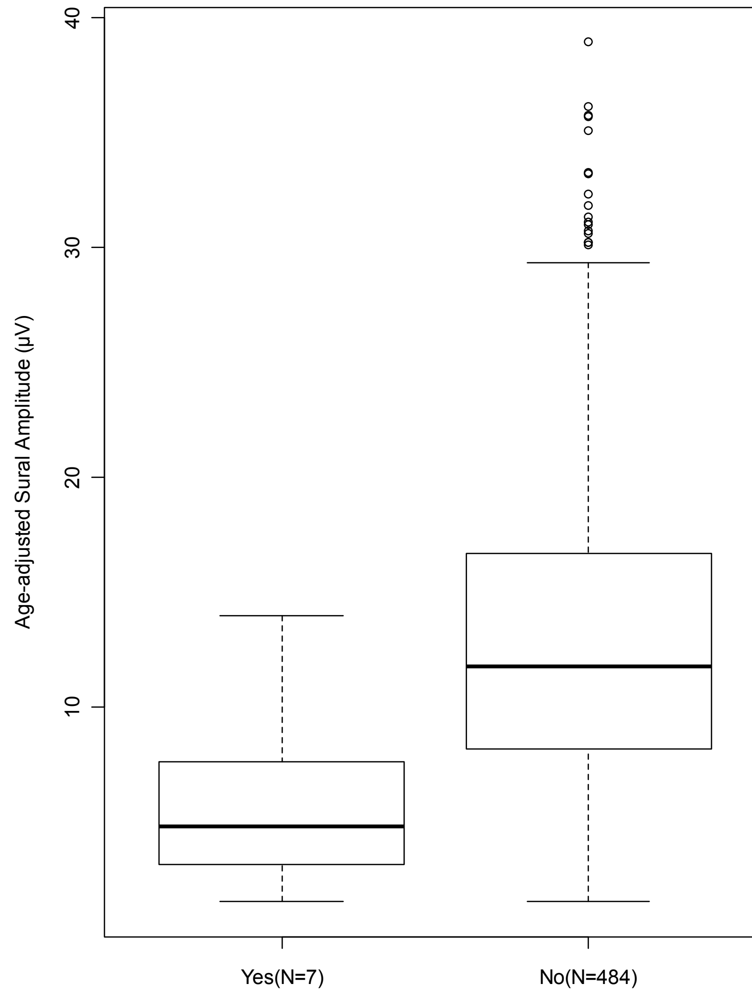
BDS or Sx & MF
Horizontal lines(from top): 95,75,50,25,&5 percentiles, respectively

Figure 5.2a Amplitude Stratified by MF



Monofilament result
Horizontal lines(from top): 95,75,50,25,&5 percentiles, respectively

Figure 5.2b Amplitude Stratified by BDS or Sx &MF



BDS or Sx & MF
Horizontal lines(from top): 95,75,50,25,&5 percentiles, respectively

Appendix

Appendix 5.1:

The criteria for defining neuropathy in the feet:

2 Probable

- If both feet are entirely shaded
- If a large portion of both feet, including all toes, is shaded
- If all toes are shaded in both feet

1 Possible

- If shaded areas include one or more but not all of the toes
- If shaded areas include anywhere in the foot but not toes
- If shaded areas include anywhere in the foot including toes
- If one foot is fully shaded but the other foot has only partial shading

0 Unlikely

- If no shading anywhere on feet below ankle
- If other non-lateral parts of foot are shaded; toes are not shaded
- No shading anywhere on toes regardless of shading elsewhere
- Shading present on only one foot

Bibliography

Albers, J.W., Cavender, G.D., Levine, S.P., Langolf, G.D., 1982. Asymptomatic sensorimotor polyneuropathy in workers exposed to elemental mercury. Neurology 32, 1168.

Albers, J.W., Kallenbach, L.R., Fine, L.J., Langolf, G.D., Wolfe, R.A., Donofrio, P.D., Alessi, A.G., Stolp-Smith, K.A., Bromberg, M.B., 1988. Neurological abnormalities associated with remote occupational elemental mercury exposure. Ann. Neurol. 24, 651.

American Association of Electrodiagnostic Medicine, 2002. Practice parameter for electrodiagnostic studies in carpal tunnel syndrome: summary statement. Muscle Nerve 25, 918.

American Diabetes Association, 2004. Standards of medical care in diabetes (Position Statement). Diab. Care 27 (suppl. 1),15-35.

Blando, A.V., 1998. Lower extremity sensory nerve conduction studies. Phys. Med. Rehabil. Clin. N. Am. 9, 853-70, vii.

Boulton, A.J.M., Vinik, A., Arezzo, J., Bril, V., Feldman, E., Freeman, R., Malik, R., Maser, R., Sosenko, J., Ziegler, D., 2005. Diabetic neuropathies: a statement by the American Diabetes Association. Diabetes Care 28, 956-962.

Buschbacher, R., 2003. Sural and saphenous 14-cm antidromic sensory nerve conduction studies. American journal of physical medicine rehabilitation 82, 421-426.

Ellingsen, D.G., Mrland, T., Andersen, A., Kjuus, H., 1993. Relation between exposure related indices and neurological and neurophysiological effects in workers previously exposed to mercury vapour. Br. J. Ind. Med. 50, 736-744.

Feng, Y., Schlsser, F., Sumpio, B., 2009. The Semmes Weinstein monofilament examination as a screening tool for diabetic peripheral neuropathy. Journal of vascular surgery 50, 675-82, 682.e1.

Herman, W., Kennedy, L., 2005. Underdiagnosis of peripheral neuropathy in type 2 diabetes. Diabetes Care 28, 1480-1481.

Homan, M.M., Franzblau, A., Werner, R.A., Albers, J.W., Armstrong, T.J., Bromberg, M.B., 1999. Agreement between symptom surveys, physical examination procedures and electrodiagnostic findings for the carpal tunnel syndrome. Scandinavian journal of work, environment health 25, 115-124.

Jirkovsk, A., Boucek, P., Woskov, V., Bartos, V., Skibov, J., 2001. Identification of patients at risk for diabetic foot: a comparison of standardized noninvasive testing with routine practice at community diabetes clinics. J. Diabetes Complications. 15, 63-68.

Kimura, J., 1984. Principles and pitfalls of nerve conduction studies. Ann. Neurol. 16, 415.

Leonard, G., Wright, M., Quinn, M., Fioravanti, S., Harold, N., Schuler, B., Thomas, R., Grem, J., 2005. Survey of oxaliplatin-associated neurotoxicity using an interview-based questionnaire in patients with metastatic colorectal cancer. BMC Cancer 5, 116-116.

Letz, R., Gerr, F., Cragle, D., Green, R.C., Watkins, J., Fidler, A.T., 2000. Residual neurologic deficits 30 years after occupational exposure to elemental mercury. Neurotoxicology 21, 459-474.

Levine, S.P., Cavender, G.D., Langolf, G.D., Albers, J.W., 1982. Elemental mercury exposure: peripheral neurotoxicity. Br. J. Ind. Med. 39, 136.

Margolis, R.B., Chibnall, J.T., Tait, R.C., 1988. Test-retest reliability of the pain drawing instrument. Pain 33, 49-51.

Rahman, M., Griffin, S.J., Rathmann, W., Wareham, N.J., 2003. How should peripheral neuropathy be assessed in people with diabetes in primary care? A population-based comparison of four measures. Diabetic Med. 20, 368-374.

Schuchmann, J.A., 1977. Sural nerve conduction: a standardized technique. Arch. Phys. Med. Rehabil. 58, 166-168.

Streiner, D., Norman, G., 1994. Health measurement scale, 4th ed. .

Tong, H., Werner, R., Franzblau, A., 2004. Effect of aging on sensory nerve conduction study parameters. Muscle Nerve 29, 716-720.

Truong, X.T., Russo, F.I., Vagi, I., Rippel, D.V., 1979. Conduction velocity in the proximal sural nerve. Arch. Phys. Med. Rehabil. 60, 304-308.

Wainapel, S.F., Kim, D.J., Ebel, A., 1978. Conduction studies of the saphenous nerve in healthy subjects. Arch. Phys. Med. Rehabil. 59, 316-319.

Chapter 6

Conclusions

1. Objectives

The overarching hypothesis of this study is that single nucleotide polymorphisms in genes such as metallothionein may underlie the inter-individual differences in mercury susceptibility in association with peripheral nerve function and changes in biomarker levels. Building on the hypothesis, this study has four main objectives:

1. To investigate whether SNPs in metallothionein and related genes modify the relationships of elemental mercury exposure and methylmercury exposure with urinary Hg levels and hair Hg levels, respectively.

Large inter-individual variations have been seen in urinary mercury levels in the general population and workers following exposures to elemental mercury of similar magnitude (Tsuji, et al. 2003), and in hair mercury levels in association with dietary fish consumption (Canuel, et al. 2006; Haxton, et al. 1979). Five prior studies have shown that single nucleotide polymorphisms (SNPs) in glutamate cysteine ligase (GCL) and glutathione-S-transferase (GST) may lead to variations of mercury levels in biomarkers (Custodio, et al. 2004; Custodio, et al. 2005; Gundacker, et al. 2007; Gundacker, et al. 2009; Schläwicke Engström, et al. 2008). Little is known about the effects of SNPs in the family of metallothionein genes. No study has investigated the potential effect modification of metallothionein SNPs on the relationship of urinary Hg levels with elemental Hg exposure. The effects on the association of methylmercury exposure with hair Hg levels were investigated in only one prior study and were found to be significant in subjects with MT4 (rs11643815) (Gundacker, et al. 2009).

2. To assess the associations of the sensory nerve function measurements of onset latency, peak latency and amplitude with urinary Hg levels (a marker of elemental Hg exposure) and hair Hg levels (a marker of methylmercury Hg exposure).

Much of the work on the peripheral nervous system impact of mercury has been conducted using clinical neurological examinations at high levels of exposure (Albers, et al. 1982; Albers, et al. 1988; Ellingsen, et al. 1993; Letz, et al. 2000; Levine, et al. 1982). However, clinical neurological examinations may not be adequate to detect subclinical effects of low-level exposure to methylmercury and elemental mercury. Nerve conduction tests, widely used in clinical practice and research, can serve as an objective measure of peripheral nerve function in relation to low levels of mercury exposure. There are four prior studies of low-level elemental Hg exposure in relation to the peripheral nervous system (DeRouen, et al. 2006; Echeverria, et al. 2005; Echeverria, et al. 2006; Kingman, et al. 2005). Two out of these studies that involved nerve function tests focused solely on motor nerve conduction. Motor nerve conduction is generally considered a less sensitive indicator of peripheral nerve impairment than sensory nerve conduction. Furthermore, ulnar nerve conduction in the hand was the only measurement used in those two studies. Inclusion of the sural nerve is likely to provide a more sensitive indicator to the effects of peripheral neurotoxicants than the ulnar or median nerves in the hand. There is no prior study of low-level methylmercury Hg exposure in relation to the peripheral nervous system.

3. To investigate whether SNPs in metallothionein and other genes (Glutathione-related genes and selenoprotein) modify the relationships between nerve function and Hg levels in urine and hair, respectively.

Among the four prior studies of low-level elemental Hg exposure in relation to the peripheral nervous system, none of them assessed the potential effect modification of SNPs in genes important for Hg metabolism in relation to the association investigated in Objective #2.

4. To assess the agreement between electrodiagnostic testing, monofilament testing and a lower extremity symptom survey in identification of possible peripheral neuropathy in the feet.

Quick and accurate screening and clinical diagnosis of peripheral neuropathy in the lower extremity relies on high sensitivity and specificity of the methods employed. The sensitivity and specificity of the techniques employed significantly affects the outcomes of clinical screening and are also important in epidemiological research of peripheral neuropathy in the lower extremity.

2. Methods

We tested the hypothesis and addressed these objectives in a convenience sample of dental professionals (n=515) recruited from two MDA conventions in 2009 and 2010. All statistical analyses were performed in SAS 9.2 (SAS Institute, Inc. Cary, NC). Each subject completed a self-administered questionnaire to collect variables that were used as surrogates for recent mercury exposures from different sources. The questionnaire included items for occupational exposure to elemental mercury (how many amalgams placed/removed per week), personal mercury exposure from dental amalgam (how many dental amalgam restorations in mouth), methylmercury exposure from dietary fish consumption, pre-existing diseases, current symptoms in the extremities, demographics (race, gender, height, weight, age, occupation) and other factors that may influence their exposures to mercury such as gum-chewing, teeth-grinding, alcohol consumption and recent chelation activities. Each subject was asked to provide a spot urine sample in a mercury-free container and a scalp hair sample. Total mercury content in urine and hair samples was determined using atomic absorption spectroscopy in the Direct Mercury Analyzer-80 (DMA-80, Milestone Inc., Shelton, CT) based on US Environmental Protection Agency Method 7473 as previously described by our laboratory (Paruchuri, et al. 2010). Buccal swabs were used to collect DNA samples from the subjects based on a published protocol (Min, et al. 2006).

Sensory nerve conduction was performed on each subject and included measurements of amplitude, onset latency and peak latency of the median and ulnar sensory nerves in the right wrist and the sural nerve in the right ankle, respectively. The temperature of the right midpalm and midfoot at the time of measurements were also recorded. Monofilament testing for loss of sensation in the lower extremity was conducted on the plantar surface of the great toe in the right foot using a 5.07-gauge Semmes-Weinstein nylon monofilament (Wound Central, Aurora, IL) (Herman and Kennedy 2005). A standard self-administrated hand diagram (Katz and Stirrat 1990; Katz, et al. 1990) and also a body diagram was given to each participate to shade the areas where numbness, tingling, burning or pain had occurred more than three times, or lasting more than one week in the six months prior to the measurement.

3. Main Results

The mean Hg levels in urine (1.06μg/L) and hair (0.51μg/g) were not significantly different from the US population (0.95 μg/L and 0.47μg/g, respectively) (NHANES 1999-2000, 2003-2004). Multivariate regression analysis found subjects with MT1M (rs2270836) AA genotype (n=10) had lower urinary Hg levels than GG after controlling for exposure and potential cofounders. After controlling for methylmercury intake from fish, subjects with MT1A (rs8052394) GA and GG (n=24), MT1E (rs708274) GT and TT (n=51), MT1M (rs9936741) TT had lower hair Hg levels compared to AA, GG, and TC and CC (n=15), respectively.

There was a consistent relationship of improved nerve function with increased hair Hg levels for all nerve measurements. No consistent relationship was observed for urine Hg levels and nerve function. Only 3 out of a total of 504 multivariate models that investigated effect modification of SNPs on nerve-function-biomarker relationships had stable and statistically significant interaction terms. Compared to those with CC alleles, subjects with CT alleles of the GPX1 (rs1050450) SNP had lower ulnar peak latency for the same increment of urine

Hg levels. Subjects with GA alleles of the GCLC 5'UTR (rs17883901) SNP had higher sural amplitude compared to those with GG alleles for the same increment of hair Hg levels. Subjects with GT or TT alleles of the MT1E 3'UTR (rs708274) had higher ulnar onset latency compared to those with GG alleles for the same increment of hair Hg levels.

Three screening procedures (electrodiagnostic testing, monofilament testing and survey of body diagram and symptoms consistent with neuropathy in the feet) were employed for detecting neuropathy or symptoms of neuropathy in the lower extremities. Overall, the kappa coefficients assessing the agreement between the methods were poor. The agreement among diabetic subjects was consistently better than the non-diabetic subjects, but due to the small numbers of subjects, these differences are not significant. Unlike the differences in nerve function means, the relative relationships of sensitivity (mostly <30%) and specificity (mostly >90%) followed the same patterns as seen in kappa values. There were significant overlaps in both peak latency and amplitude between the strata of combinations. The results demonstrated a low discriminatory power between the screening procedures and the need and challenges for further development and evaluation of methods for screening for neuropathy in the feet in a population based studies.

4. Discussion/Conclusions

In reviewing the existing literature regarding adverse effects of mercury, one would likely notice the abundance of evidence for neurological deficits in adults and children at high levels of Hg exposure compared to that for low levels of exposure. The body of literature (n=4) regarding the peripheral nervous system impairment following low-level exposures is smaller than that for low levels of exposure. By utilizing electrodiagnostic testing, a more sensitive and objective measure of the peripheral nervous system impairment, this study contributes to the field with the unexpected finding that higher hair Hg levels may be associated with better nerve function (shorter onset and peak latencies and larger

amplitudes of the median, ulnar and sural nerves) after accounting for covariates. There was no consistent association in either direction between urine Hg levels and nerve function. One possible explanation of this finding would be that the inverse association of nerve function with hair mercury reflects the association between better nerve function with higher omega-3 fatty acid intake co-occurring with methylmercury as a result of dietary fish consumption. In the follow-up analysis, we tested this hypothesis in models with estimated daily intake of polyunsaturated fatty acid (PUFA), as crude surrogates of omega-3 fatty acid intake after accounting for hair Hg levels. We were able to observe the same associations of higher intake of PUFA with better nerve function in all nerve measurements except median onset and peak latencies. Overall, the results suggested a possible beneficial effect of PUFA intake via fish consumption on peripheral nerve function. These findings certainly call for more sophisticated study design to further explore these unanticipated associations.

A major part of this study was designed to tackle Objective #1. It contributes to a small body of previous research on MT SNPs modifying the mercury exposure-biomarker relationship in humans in a number of ways. One study reported findings of effect modifications of MT4 (rs11643815), and MT1A (rs11640851) on exposure-biomarker relationship in relation to mercury in hair but not in urine (Gundacker, et al. 2009). Our study is the first to report potential effect modification of selected MT SNPs on the relationship of urinary Hg with occupational and non-occupational exposures. It also extends beyond the work of Gundacker et al. 2009 and utilizes a more precise survey methodology for estimating individual daily methylmercury intake from fish based on the NHANES Food Frequency Questionnaire (1999-2000) and species-specific Hg levels. Unlike Gundacker et al. 2009, the assumption that all fish has the same level of Hg was not made in our study. We found evidence of effect modifications of MT1A (rs8052394), MT1M (rs9936471) and MT1E (rs708274) SNPs on the relationship of hair Hg level and estimated daily methylmercury intake from fish after adjusting for methylmercury intake. We did not find any effect modification

of MT4, which differs from the finding of Gundacker et al.2009. This may be the result of using fish species-specific Hg levels in our calculation of estimated daily methylmercury intake, which is likely a better estimation compared to the total fish meals that were used in their study. There was another study that used a relatively reliable and refined exposure surrogate to account for methylmercury exposure from fish, albeit in the investigation of GST related SNPs (Schläwicke Engström, et al. 2008). In our study, neither MT4 (rs11643815) nor MT1A (rs11640851) were found to modify elemental mercury exposure-biomarker relationship.

In addition, in our study, ANOVA results on mean Hg level of subjects with homozygote compared to the pooled mean Hg level of those with heterozygotes and homozygote variants did not show any significant difference in most MT SNPs. We saw insignificant changes for MT1A (rs11640851) similar to Gundacker et al. 2009. Furthermore, Gundacker et al., 2009 found unadjusted higher levels of hair Hg to be associated with MT1A (rs11640851) CC genotype and MT4 (rs11643815) GG and GA genotypes. We then calculated the Mantel-Haenszel statistics in our study for the effects of these two SNPs on dichotomized mercury hair levels as described in Gundacker et al. 2009 (data not shown). There was no significant difference in associations of unadjusted hair Hg levels with MT1A (rs11640851) (Chi-square $p=0.73$) and MT4 (Chi-square $p=0.26$). It is not clear why there were such inconsistent results between the studies considering that methylmercury exposure levels in the two study groups are similar as suggested by respective median hair Hg levels. One possible explanation would be that the number of subjects with heterozygotes and/or homozygote variants was small in both studies and subject to random errors.

For Objective #3, it is the first attempt to link SNPs in metallothionein, glutathione-related genes and selenoprotein with sensory nerve function and Hg biomarkers. The mostly insignificant results of the interaction between Hg biomarkers and these SNPs were not surprising considering the fact that these

SNPs were selected based on their relationships with Hg metabolism rather than their potential links to nerve function. It was unexpected, though, that in Objective #1 there was no significant effect modification on Hg exposure-biomarker relationships observed in the SNPs that are located in the MRE sequences of the upstream promoter region. The low agreement between the screening procedures investigated in Objective #4 is not surprising for a non-clinical population. It takes a large study population to have enough subjects classified into the 'overlaps' of different case definitions. The small numbers in the current study likely led to unstable and low kappa values which were observed.

Overall, the findings suggested that some MT genetic polymorphisms may influence mercury biomarker levels. There might be a beneficial effect of fish consumption on nerve function. There was little evidence for effect modification of the studied SNPs on nerve-function-biomarker relationships. Poor between-procedure agreement (Kappa: -0.14 ~ 0.44) and poor sensitivity and specificity demonstrated the challenges for further development and evaluation of methods for detecting neuropathy in a non-clinical population.

5. Future Research

To get a complete picture of the potential effect modification of MT SNPs on Hg exposure-biomarker relationships, we suggest to genotype MT SNPs with microarray techniques. While having a larger sample would certainly increase the power of a study, it would be ideal to conduct this study in a randomly sampled non-occupational population, preferably in the US general population. With a large sample size and a complete list of MT SNPs, gene-gene interactions between MT SNPs and SNPs of other genes (e.g. MT with glutathione-related genes and selenoprotein) could be explored. In addition, studies that run epigenetic profiles of MT genes are warranted in association with exposure-biomarker relationships. One would also need to genotype SNPs related to genes that have been hypothesized or proved to affect nerve function in humans so that the effect modification of these SNPs could be investigated properly on

the relationships between biomarkers and nerve function. Regarding the unanticipated association between increased hair Hg biomarker and improved nerve function, it would be better to measure the content of fatty acids in blood to correlate with nerve function. Lastly, one could investigate the agreement between these procedures in a clinical population, which would increase the power of a study given the sample size being equal.

Bibliography

Albers, J.W., Cavender, G.D., Levine, S.P., Langolf, G.D., 1982. Asymptomatic sensorimotor polyneuropathy in workers exposed to elemental mercury. Neurology 32, 1168.

Albers, J.W., Kallenbach, L.R., Fine, L.J., Langolf, G.D., Wolfe, R.A., Donofrio, P.D., Alessi, A.G., Stolp-Smith, K.A., Bromberg, M.B., 1988. Neurological abnormalities associated with remote occupational elemental mercury exposure. Ann. Neurol. 24, 651.

Canuel, R., de Grosbois, S., Atikess, L., Lucotte, M., Arp, P., Ritchie, C., Mergler, D., Chan, H., Amyot, M., Anderson, R., 2006. New evidence on variations of human body burden of methylmercury from fish consumption. Environ. Health Perspect. 114, 302-306.

Custodio, H.M., Broberg, K., Wennberg, M., Jansson, J., Vessby, B., Hallmans, G., Stegmayr, B., Skerfving, S., 2004. Polymorphisms in glutathione-related genes affect methylmercury retention. Arch. Environ. Health 59, 588.

Custodio, H.M., Harari, R., Gerhardsson, L., Skerfving, S., Broberg, K., 2005. Genetic influences on the retention of inorganic mercury. Archives of environmental occupational health 60, 17.

DeRouen, T., Martin, M., Leroux, B., Townes, B., Woods, J., Leito, J., Castro-Caldas, A., Luis, H., Bernardo, M., Rosenbaum, G., Martins, I., 2006. Neurobehavioral effects of dental amalgam in children: a randomized clinical trial. JAMA 295, 1784-1792.

Echeverria, D., Woods, J.S., Heyer, N.J., Rohlman, D.S., Farin, F.M., Bittner, A.C., Li, T., Garabedian, C., 2005. Chronic low-level mercury exposure, BDNF polymorphism, and associations with cognitive and motor function. Neurotoxicol. Teratol. 27, 781.

Echeverria, D., Woods, J.S., Heyer, N.J., Rohlman, D., Farin, F.M., Li, T., Garabedian, C.E., 2006. The association between a genetic polymorphism of coproporphyrinogen oxidase, dental mercury exposure and neurobehavioral response in humans. Neurotoxicol. Teratol. 28, 39.

Ellingsen, D.G., Mrland, T., Andersen, A., Kjuus, H., 1993. Relation between exposure related indices and neurological and neurophysiological effects in workers previously exposed to mercury vapour. Br. J. Ind. Med. 50, 736-744.

Gundacker, C., Gencik, M., Hikkel, I., Komarnicki, G., Kukuckova, M., Wittmann, K., 2009. Genetic background of lead and mercury metabolism in a group of medical students in Austria. Environ. Res. 109, 786.

Gundacker, C., Komarnicki, G., Jagiello, P., Gencikova, A., Dahmen, N., Wittmann, K.J., Gencik, M., 2007. Glutathione-S-transferase polymorphism, metallothionein expression, and mercury levels among students in Austria. Sci. Total Environ. 385, 37.

Haxton, J., Lindsay, D.G., Hislop, J.S., Salmon, L., Dixon, E.J., Evans, W.H., Reid, J.R., Hewitt, C.J., Jeffries, D.F., 1979. Duplicate diet study on fishing communities in the United Kingdom: mercury exposure in a "critical group". Environ. Res. 18, 351-368.

Herman, W., Kennedy, L., 2005. Underdiagnosis of peripheral neuropathy in type 2 diabetes. Diabetes Care 28, 1480-1481.

Katz, J.N., Stirrat, C.R., 1990. A self-administered hand diagram for the diagnosis of carpal tunnel syndrome. J. Hand Surg. 15, 360-363.

Katz, J.N., Stirrat, C.R., Larson, M.G., Fossel, A.H., Eaton, H.M., Liang, M.H., 1990. A self-administered hand symptom diagram for the diagnosis and epidemiologic study of carpal tunnel syndrome. J. Rheumatol. 17, 1495-1498.

Kingman, A., Albers, J.W., Arezzo, J.C., Garabrant, D.H., Michalek, J.E., 2005. Amalgam exposure and neurological function. Neurotoxicology 26, 241.

Letz, R., Gerr, F., Cragle, D., Green, R.C., Watkins, J., Fidler, A.T., 2000. Residual neurologic deficits 30 years after occupational exposure to elemental mercury. Neurotoxicology 21, 459-474.

Levine, S.P., Cavender, G.D., Langolf, G.D., Albers, J.W., 1982. Elemental mercury exposure: peripheral neurotoxicity. Br. J. Ind. Med. 39, 136.

Min, J.L., Lakenberg, N., Bakker-Verweij, M., Suchiman, E., Boomsma, D.I., Slagboom, P.E., Meulenbelt, I., 2006. High microsatellite and SNP genotyping success rates established in a large number of genomic DNA samples extracted from mouth swabs and genotypes. Twin Research and Human Genetics 9, 501.

Paruchuri, Y., Siuniak, A., Johnson, N., Levin, E., Mitchell, K., Goodrich, J., Renne, E., Basu, N., 2010. Occupational and environmental mercury exposure among small-scale gold miners in the Talensi-Nabdam District of Ghana's Upper East region. Sci. Total Environ. 408, 6079-6085.

Schläwicke Engström, K., Strömberg, U., Lundh, T., Johansson, I., Vessby, B., Hallmans, G., Skerfving, S., Broberg, K., 2008. Genetic variation in glutathione-related genes and body burden of methylmercury. Environ. Health Perspect. 116, 734.

Tsuji, J., Williams, P.R.D., Edwards, M., Allamneni, K., Kelsh, M., Paustenbach, D., Sheehan, P., 2003. Evaluation of mercury in urine as an indicator of exposure to low levels of mercury vapor. Environ. Health Perspect. 111, 623-630.