The Influence of Genetic Polymorphisms on Mercury Toxicokinetics: Evidence from Epidemiological and *In Vitro* Studies

by

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To

My mother for daily inspiration,

My family for constant support in all of my endeavors, and

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List of Abbreviations

ADME	Absorption, Distribution, Metabolism and Elimination
ATSDR	Agency for Toxic Substances and Disease Registry
Blood:Urine	Blood Hg (µg/L) to Urine Hg (µg/L) Ratio
CDC	Centers for Disease Control and Prevention
CDNB	1-chloro-2,4-dinitrobenzene
CRM	Certified Reference Material
DBP	Diastolic Blood Pressure
db SNP	Database Reference Number for Single Nucleotide Polymorphism
ELEMENT	Early Life Exposures in Mexico to Environmental Toxicants
EPA	Environmental Protection Agency
GCL	Glutamate-Cysteine Ligase
GPX	Glutathione Peroxidase
GSH	Glutathione
GSR	Glutathione Reductase
GSS	Glutathione Synthetase
GST	Glutathione s-Transferase
GSTP1 IA	GST pi 1, 105 Isoleucine 114 Alanine
GSTP1 VA	GST pi 1, 105 Valine 114 Alanine
GSTP1 IV	GST pi 1, 105 Isoleucine 114Valine
GSTP1 VV	GST pi 1, 105 Valine 114Valine
Hair:Blood	Hair Hg (ng/g) to Blood Hg (µg/L) Ratio
Hg	Mercury
$HgCl_2$	Mercuric Chloride
HWE	Hardy-Weinberg Equilibrium
IC_{50}	Concentration Inhibiting 50%
K_m	Michaelis-Menten Constant
MAF	Minor Allele Frequency
MDA	Michigan Dental Association
MXL	Mexican Ancestry from Los Angeles, HapMap Population
MeHg	Methylmercury
NCBI	National Center for Biotechnology Information
NHANES	National Health and Nutrition Examination Survey
NRC	National Research Council
RfD	Reference Dose

SBP	Systolic Blood Pressure
SD	Standard Deviation
SEM	Standard Error of the Mean
SEPP1	Selenoprotein P1
SES	Socioeconomic Status
SNP	Single Nucleotide Polymorphism
UTR	Untranslated Region
V_{max}	Maximum Velocity

Abstract

Recent studies suggest that genetic polymorphisms may influence inter-individual differences observed in toxicokinetics of mercury (Hg), a potent toxicant of concern to the general population and occupationally exposed workers (e.g., dentists). This work hypothesized that polymorphisms in glutathione pathway and selenoprotein genes would associate with Hg biomarker levels, modify the impact of Hg on blood pressure, and alter enzyme activity of encoded proteins. We utilized a multi-dimensional approach by considering two forms of Hg (methylmercury from fish consumption; elemental Hg from dental amalgams) through measurement in biomarkers and 23 polymorphisms in two populations- the Michigan Dental Association cohort (MDA, n=511) and the Early Life Exposures in Mexico to Environmental Toxicants cohort (ELEMENT, n=353 mothers, 368 children). In the MDA cohort, mean (±SD) urine (1.04±1.18 μg/L) and hair Hg levels (0.49±0.63 μg/g), indicative of elemental Hg and methylmercury exposure, respectively, mirrored US population averages, with dentists exhibiting the highest concentrations of both. While elevated compared to the US population, biomarker Hg levels among ELEMENT mothers (hair) and children (hair, blood, urine) reflected low-level exposures.

Simple and multivariate linear regression models investigated the association of polymorphisms in glutathione pathway and selenoprotein genes with Hg biomarker levels in both cohorts. Statistical modeling in the MDA cohort tested for significant interactions between genotype and exposure sources (Hg intake from fish, dental amalgams) on biomarker (hair, urine) levels, though the ELEMENT cohort lacked exposure data.

Several polymorphisms were found to be associated with Hg accumulation and elimination as measured in hair (lower hair Hg: *GSTP1*: rs1695, rs1138272; *SEPP1*: rs7579; higher hair Hg: *GSS*: rs3761144) and urine (lower urine Hg: *GSTT1* deletion; *SEPP1*: rs7579). All significant associations ($p<0.05$) were observed in the MDA cohort, with the exception of the rs7579 'T' allele (*SEPP1*) which correlated with lower urine Hg concentrations in both cohorts. No associations were significant after correction for multiple testing

We further assessed the impact of Hg and polymorphism-Hg interactions on systolic and diastolic blood pressure (SBP, DBP) in the MDA cohort. In multivariate models, hair Hg levels were significantly associated with increasing DBP ($\beta=2.76$ mmHg, $p=0.02$). Contrariwise, urine Hg levels were associated with decreasing SBP ($\beta=-1.80$ mmHg; $p=0.04$), a relationship driven by the male subgroup. Significant interactions between polymorphisms and Hg biomarkers were not observed in SBP or DBP models.

Mechanisms underlying epidemiological associations were explored through *in vitro* characterization of enzymatic activity and heavy metal inhibition of four GSTP1 variants encoded by two nonsynonymous polymorphisms (rs1695: 105Ile/Val, rs1138272: 114Ala/Val). Kinetic parameters varied significantly ($p<0.01$) depending on genotype. Allozymes with 105Ile had better catalytic efficiency and greater electrophilic substrate affinity (105Ile $K_m=0.33$ mM; 105Val $K_m=1.15$ mM). Inorganic Hg and methylmercury inhibited all GSTP1 variants. Genotype influenced the extent of inhibition, and GSTP1 105Val 114Ala was the most sensitive to inhibition by both Hg species.

Overall, evidence from two epidemiological cohorts and *in vitro* enzymatic assays relate genetic polymorphisms in selected selenoprotein and glutathione pathway genes to variability in Hg toxicokinetics. Exploration of such genetic factors will help ascertain sources of biomarker variability and narrow the gap between perceived and true health risks accompanying methylmercury and inorganic Hg exposure.

Chapter 1

Introduction

1.1 Background

1.1.1 Mercury

Mercury (Hg), a potent toxicant, is deemed by the Environmental Protection Agency (US EPA, 1997) and the ATSDR to be a top three priority pollutant of concern (ATSDR, 1999). Mercury exists as several inorganic and organic species, and the speciation of Hg dictates targeted organs and toxicity (Clarkson and Magos, 2006). Inorganic Hg exposure is mainly to the elemental (Hg^0) or mercuric (Hg^{2+}) forms while exposure to organic Hg is primarily via the methylated ($MeHg^+$) form. The chemical forms of Hg absorb and distribute differently in the body and often cause toxicity through divergent mechanisms. In the end, humans are exposed to Hg from multiple sources, including dental amalgams, fish, vaccines, thermostats, compact fluorescent light bulbs (CFL), mining practices, and thermometers (ATSDR, 1999; Clarkson and Magos, 2006).

1.1.1.1 Methylmercury

Humans are predominantly exposed to methylmercury via fish consumption (Clarkson and Magos, 2006). Due to the prevalence of coal-fired power plants, which release Hg upon coal combustion, and the increasing need for energy, especially in the developing world, release of Hg is increasing worldwide (Millman et al., 2008; Swain et

al., 2007). This is evident in fish and fish-eating animal species across the globe (Scheuhammer et al., 2007). After chemical transformation in the atmosphere, inorganic Hg deposits in water systems, is transformed into methylmercury by bacteria, accumulates in fish and biomagnifies up the food chain. Due to its affinity for thiol groups and ability to cross the blood brain barrier, methylmercury has a long half-life in the body and resides in the brain and proteinaceous tissue of fish-eating species (ATSDR, 1999).

The nervous system is a well-establish target of methylmercury, and recent studies link methylmercury exposure to cardiovascular problems and immunotoxicity (Clarkson and Magos, 2006; Roman et al., 2011; Sweet and Zelikoff, 2001). Methylmercury poisoning following high dose exposure, as observed in Minamata Bay in the 1950s, results in a slew of neurological symptoms including sensory disturbances in the extremities, ataxia, and disequilibrium. *In utero* methylmercury exposure precedes limb deformations, coordination problems, dysarthria and mental retardation (ATSDR, 1999; Eto, 2000).

The adverse health effects of chronic, low dose exposure to methylmercury are less well characterized, though evidence suggests several outcomes (e.g., neurological, cardiovascular) may be affected by low dose exposure in both prenatally exposed children and adults. Methylmercury impairs cognitive development (learning, memory, attention) among children exposed *in utero* from mothers' consumption of pilot whale meat (Grandjean et al., 1997). Likewise, prenatal methylmercury exposure has been linked to decreased IQ (Trasande et al., 2005), increased blood pressure in 7 year old children (Sørensen et al., 1999) and altered autonomic modulation of heart rate variability

in 14 year old children (Grandjean et al., 2004). Despite potential effects on developing children, 8% of American, 89% of Taiwanese, and 13% of South Korean women of childbearing age exceed the EPA recommended blood Hg level (a biomarker for primarily methylmercury exposure) of 5.8 μg/L while Hg levels remain relatively unknown in many countries such as Mexico (Hsu et al., 2007; Lee et al., 2009; Trasande et al., 2005). Regulatory guidelines for methylmercury intake typically target women of child-bearing age as prenatal development is deemed the most vulnerable exposure time point (NRC, 2000). However, fish contain nutrients beneficial for neurodevelopment, and as a result, the benefit-risk analysis surrounding fish consumption is constantly reevaluated (Cohen et al., 2005a, 2005b).

Associations between methylmercury and adverse health effects have been documented in adult populations with low to moderately high exposures from fish consumption. Carta et al. (2003) found males with median blood Hg of 44 μg/L from frequent fresh tuna consumption performed worse on neurobehavioral tests compared to controls (median blood Hg: 3.9 μg/L). Methylmercury exposure may be linked to acute myocardial infarction and increased blood pressure, and the evidence for the association with the former was compelling enough for an US EPA committee to recommend inclusion of this health outcome in analyses impacting Hg emission regulations (Roman et al., 2011). The relationship between methylmercury and increased blood pressure is inconsistent though several studies found significant associations among adults between increasing systolic and/or diastolic blood pressure (SBP, DBP) and hair (Fillion et al., 2006) or blood (Choi et al., 2009; Valera et al., 2009) Hg levels in primarily subsistence fish or marine mammal consuming populations.

1.1.1.2 Inorganic Mercury

Dental amalgams comprise the main source of exposure to inorganic Hg in the general population and certain occupational groups (dentists, miners, manufacturers of chloralkali) experience elevated inorganic Hg exposures (ATSDR, 1999, Martin et al., 1995). Elemental Hg constitutes 50% of the composition of dental amalgam fillings. Mercury vapor (Hg^0) is inhaled when released from amalgams in the mouth, and this exposure influences urine Hg levels (Dye et al., 2005). Upon absorption in the lungs, Hg^0 is oxidized to inorganic Hg^{2+} which binds to thiol groups of proteins, and this may lead to toxicity. The primary target for inorganic Hg is the kidney, though it can also affect the nervous system (Clarkson and Magos, 2006)

Toxic effects from high dose inorganic Hg exposure have been characterized. Construction workers with acute elemental Hg exposure resulting in urine levels above 100 μg/L had multiple neuropsychological symptoms including increased temper, anxiety, and forgetfulness (Bluhm et al., 1992). High doses of inorganic Hg further led to renal tube damage and proteinuria (ATSDR, 1999). Various studies of occupationally exposed workers have also found associations between chronic, lower dose exposures to inorganic Hg and declines in cognitive and motor function (Echeverria et al., 2005, 2006). Echeverria et al. (2005) found dentists exposed to Hg vapor had adverse effects on hand steadiness, memory and cognitive flexibility. Thirty percent of dentists with elevated Hg levels (20 μg/g in tissues) experienced polyneuropathies (Shapiro et al., 1982). Little is known about the effect of inorganic Hg exposure on the cardiovascular system in humans, though animal studies suggest that inorganic Hg may depress heart

rate and blood pressure (Massaroni et al., 1995; Rhee and Choi, 1989; Rossoni et al., 1999).

1.1.2 Absorption, Distribution, Metabolism and Elimination (ADME) of Mercury

The differential health effects observed with methylmercury and elemental (inorganic) Hg can be partially attributed to divergent absorption, distribution, metabolism, and elimination (ADME) of the two Hg species in the body. An estimated 95% of methylmercury consumed from fish is absorbed into the bloodstream from the gastrointestinal tract (for reviews see Clarkson et al., 2007; Mergler et al., 2007). A large proportion of methylmercury binds to hemoglobin in the blood while the freely movable fraction binds to low molecular weight thiol groups such as cysteine (Doi et al., 1983). Methylmercury-cysteine complexes resemble methionine and enter cells via large neutral amino acid carriers, enabling this toxicant to cross both blood brain and placental barriers (Simmons-Willis et al., 2002). Methylmercury is primarily eliminated through the biliary system following conjugation with glutathione (Ballatori and Clarkson, 1985) though some urinary excretion has been observed (Carta et al., 2003). Binding to hair follicles accounts for <10% of elimination (Magos and Clarkson, 2008). Methylmercury accumulation in various organs and tissues is not equivalent and typically follows the pattern: hair > brain > red blood cells > plasma (Clarkson et al., 2007).

The general population is exposed to inorganic Hg primarily as elemental Hg vapor (Hg^0) released from dental amalgams. Approximately 80% of Hg^0 is absorbed in the lungs into the bloodstream and can initially cross cell membranes and the blood brain barrier (Clarkson et al., 2007; Hursh et al., 1976). However, Hg^0 is quickly oxidized via

the catalase pathway to the mercuric form, Hg^{2+}, a transformation that diminishes the ability to cross lipophilic barriers (Clarkson et al., 2007). Over time, the majority of the inorganic Hg body burden is found in the kidney (Cherian et al., 1978; Hursh et al., 1976). Glutathione conjugation with Hg^{2+} promotes excretion through the biliary system, and urinary excretion is a major elimination pathway for inorganic Hg (Ballatori and Clarkson, 1985; Berglund et al., 2005; Cherian et al., 1978).

1.1.3 Inter-Individual Variability

1.1.3.1 Inter-individual Variability and Mercury Biomarkers

Given the multifaceted ADME mechanisms for Hg, it is not surprising that inter-individual variability exists in accumulation of both methylmercury and inorganic Hg as assessed by common biomarkers of exposure (blood, hair, urine). Hair, whole blood, and erythrocytes are biomarkers of methylmercury exposure from fish consumption while urine, whole blood, and plasma have been used to measure inorganic Hg exposure (Berglund et al., 2005; Cherian et al., 1978; Clarkson et al., 2007). Total Hg in hair is assumed to be 80% organic, though this proportion ranges from 79 to 99%. Correlation of both organic and inorganic Hg fractions in hair with fish consumption suggest that the inorganic portion may result from demethylation of methylmercury (Berglund et al., 2005), though some exposure to Hg vapor can be reflected in the hair of occupationally exposed individuals (Morton et al., 2004). In general, methylmercury bound to cysteine is incorporated into the growing hair follicle and as such, this biomarker reflects average body burden over time with each centimeter of hair approximating one month of exposure (Mergler et al., 2007).

Blood Hg reflects methylmercury exposure from recently consumed fish before distribution to other tissues. Inorganic Hg can also be found in the blood, particularly in the plasma. Fish consumption correlates with total Hg in whole blood and erythrocytes and with organic Hg in whole blood, erythrocytes, and plasma while dental amalgams correlate with the inorganic fraction in plasma and whole blood (Berglund et al., 2005). Variability exists in the proportion of organic Hg that localizes to erythrocytes (76-100%) versus plasma (5-20%) and the proportion of blood inorganic Hg found in erythrocytes (15-54%) versus plasma (30-81%) according to Berglund et al. (2005). Substantial variability has been observed in the half-life of methylmercury in various tissues or biomarkers. For example, Birke et al. (1972) observed methylmercury elimination rates following fish consumption in a handful of individuals and calculated half-lives ranging from 33-120 days in hair (n=5), 99-120 days in blood cells (n=2) and 47-130 days in plasma (n=2).

Urine Hg, the common biomarker for elemental or inorganic Hg exposure, is typically >98% inorganic (Berglund et al., 2005; Cherian et al., 1978). Urine Hg associates with dental amalgams in the general population (Berglund et al., 2005; Dye et al., 2005) and occupational exposure to inorganic Hg (Gibb et al., 2008; Morton et al., 2004; Paruchuri et al., 2010). Variability in urinary Hg excretion may correlate with different retention rates in the kidney as observed in humans (Hursh et al., 1976) and mice (Ekstrand et al., 2009), the latter of which are strain and gender dependent.

Mercury biomarker ratios, specifically the hair to blood ratio, provide further indication of the immense inter-individual variability in Hg toxicokinetics. The hair to blood ratio is generally assumed to be 250:1 though average values have ranged from 140

to 370 in studied populations and the range is even wider (maximum >600) among individuals (Bartell et al., 2000; Berglund et al., 2005; Budtz-Jørgensen et al., 2004). Multiple factors underlie the observed differences including age (Budtz-Jørgensen et al., 2004), fraction of blood Hg that is organic (Berglund et al., 2005), and fish consumption patterns as infrequent fish consumers will not achieve a steady state Hg level in their blood (Bartell et al., 2000). Variability in factors involved in the ADME of Hg may additionally contribute to inter-individual differences in distribution of Hg to the hair and the blood, and these same factors may influence Hg distribution to the brain or other target organs.

1.1.3.2 Inter-individual Variability and Mercury Intake

Current risk assessment often fails to account for the inter-individual differences in body burden that arise from variability in Hg metabolism and detoxification, and this variability may influence susceptibility to Hg accumulation and subsequent toxicity. Differential Hg accumulation was evident when Canuel et al. (2006) predicted hair Hg levels based on average parameters (e.g., methylmercury half-life, hair growth rate, hair to blood ratio), fish consumption data, and fish Hg levels in several subsistence fish eating populations and compared them to actual hair Hg measurements. Wide variability between predicted and measured hair Hg levels was observed with a maximum 14-fold difference in one population. Similarly, Lipfert (1997) calculated the ratio of hair Hg to Hg intake estimated from fish consumption using data from 11 studies and observed substantial variation in the hair-to-intake ratios between studies (range 2.3 to 18). Variability in genes involved in Hg metabolism and elimination may partially underlie

observed differences in Hg intake and biomarkers as well as variability in half-life and biomarker distribution (section 1.1.3.1).

1.1.4 Genetic Polymorphisms and Mercury

Gene-environment interaction studies are of increasing importance to the risk assessment of chemical exposures (Omenn, 2001). In the normative risk assessment process, mean values of effect are often used to characterize the hazard of a given drug or exposure to protect public health, while outliers, which may represent subpopulations susceptible to certain exposures, are largely ignored. While pharmacology (de Leon et al., 2005; Ingelman-Sundberg, 2004) and cancer research (Ravn-Haren et al., 2006; Rothman et al., 2001) studies have invested in this field, gene-environment considerations have only recently become standard practice in toxicology.

Single nucleotide polymorphisms (SNPs) are prevalent forms of variation that involve change of one base pair in DNA and may alter expression levels or function of encoded proteins. Deletion and insertion polymorphisms (ranging from one base pair to an entire gene) are found in some genes. Genetic polymorphisms in biotransformation enzymes such as glutathione s-transferases (GSTs) and cytochrome P450s modify the effects of environmental exposures on cancer development and drug metabolism (see reviews: Ingelman-Sundberg, 2004; Rothman et al., 2001).

Toxicological characterization of metals has recently considered genetic susceptibility. For example, a SNP in arsenic (+3 oxidation state) methyltransferase (*AS3MT*) is associated with increased formation of the toxic metabolite monomethylated arsenic (Hernández et al., 2008). Likewise, other SNPs in the arsenic metabolic pathway

have been shown to alter metabolite proportions (Engström et al., 2007; Lindberg et al., 2007; Marcos et al., 2006). Risk for chronic beryllium disease in occupationally exposed workers is influenced by genotype of a major histocompatibility complex class II marker (Richeldi et al., 1993, 1997). Polymorphisms in the vitamin D receptor (*VDR*) and δ-aminolevulinic acid dehydrogenase (*ALAD*) influence lead concentrations in urine (Gundacker et al., 2009).

Gene-environment studies have great potential to enhance the risk assessment of Hg and improve the use of Hg biomarkers through better understanding of the mechanisms underlying inter-individual variability. Recent epidemiological studies link polymorphisms in GSH pathway genes to altered hair, blood, and urine Hg levels (Custodio et al., 2004, 2005; Engström et al., 2008; Gundacker et al., 2007, 2009). Table 1.1 details the study populations, polymorphisms genotyped, biomarkers analyzed, and significant results ($p<0.05$) observed in these studies. Overall, seven polymorphisms in six GSH pathway genes were genotyped and each polymorphism was significantly associated with at least one biomarker in one or more statistical test (e.g., association with median biomarker levels, effect modification on relationship between exposure source and biomarker). Deletion polymorphisms for GST theta 1 (*GSTT1*) and GST mu 1 (*GSTM1*), known to decrease catalytic efficiency of the GST system, were significantly more frequent among Austrian students with hair Hg levels above the median (Gundacker et al., 2007). Minor alleles of SNPs in subunits of glutamyl-cysteine ligase (rs17883901 in *GCLC*; rs41303970 in *GCLM*), the rate limiting enzyme for GSH synthesis are known to decrease promoter activity and gene expression (Koide et al., 2003; Nakamura et al., 2002). These detrimental alleles were associated with increased Hg biomarker levels

following methylmercury exposure (Custodio et al., 2004; Engström et al., 2008; Gundacker et al., 2009) or inorganic Hg exposure (Custodio et al., 2005). Many of the significant results in these studies involved polymorphism combinations (comparing individuals with minor alleles for two different loci to the rest of the population). Gundacker et al. (2009) additionally observed a significant relationship between a metallothionein SNP and higher hair Hg levels (not reported in Table 1.1).

Collectively, these early studies call for increased attention to genetics in Hg risk assessment. However, the small number of polymorphisms studied (n=7) from primarily one pathway and the existing discrepancies among studies point to the need for additional research characterizing gene-environment interactions pertaining to Hg exposure and biomarkers. For example, Custodio et al. (2004) found higher erythrocyte Hg levels in individuals with the minor allele of a nonsynonymous SNP in GST pi 1 (*GSTP1*, rs1138272) compared to polyunsaturated fatty acid levels, an indicator of fish consumption, while Engström et al. (2008) observed the opposite relationship. A variety of factors may underlie discrepancies among studies including sources of exposure, genotype frequencies in the study populations, biomarkers analyzed, statistical methodology, and power to detect an association. Polymorphisms studied to date may not adequately describe variability in the ADME of inorganic Hg. Only one study of occupationally exposed gold miners observed a significant association between genotype (*GCLM*, rs41303970) and inorganic Hg exposure biomarkers (Custodio et al., 2005).

Beyond the influence of genetic polymorphisms on Hg retention and distribution to hair, blood, and urine, there is evidence that polymorphisms may impact the relationship between Hg exposure and adverse health effects. A series of studies

involving occupationally exposed male dentists and female dental assistants found significant additive or modifying effects of polymorphisms on the association between urine Hg levels and atypical urinary porphyrin excretion (Woods et al., 2005) or between urine Hg and performance on various neurobehavioral tests (Echeverria et al., 2005, 2006, 2010). Interactions between blood Hg concentrations and polymorphisms influencing adverse health outcomes were also observed. The offspring of mothers with *GSTM1* and *GSTT1* deletions and higher blood Hg levels had increased risk for low birth weight (Lee et al., 2009). Genotype of matrix metalloproteinase, *MMP-9*, impacted MMP-9 protein levels among Brazilians with low blood and plasma Hg levels, and increased expression of this protein is associated with multiple adverse cardiovascular effects (Jacob-Ferreira et al., 2010). The impact of genetic factors on Hg-associated health effects such as the aforementioned overt and sub-clinical outcomes calls for incorporation of genetic variability in studies of Hg and its toxic effects on the cardiovascular and nervous systems of adults and developing children.

1.1.5 Gene Classes of Interest

Alterations to expression or enzyme activity of proteins and enzymes involved in the ADME of methylmercury and inorganic Hg due to genetic polymorphisms have the potential to modify Hg accumulation in the body and the ensuing toxic effects. Important protein classes include those that bind and transport Hg (e.g., selenoproteins, hemoglobin), enzymes involved in GSH and cysteine conjugation of Hg (e.g., GSTs, GCLC, GCLM), and enzymes protecting against reactive oxygen species created by Hg (glutathione peroxidases, GPX). This dissertation focuses specifically on GSH pathway

and selenoprotein genes due to the prevalence of polymorphisms in these classes as well as their importance in Hg distribution, detoxification, and elimination. Polymorphisms are ubiquitous among the genes encoding GSTs, GSH synthesizing enzymes and selenoproteins. For example, GST pi 1 (*GSTP1*) alone has 158 SNPs with minor allele frequencies (MAFs) ranging from <1% to 50% in various ethnic populations, including 14 nonsynonymous (amino acid changing) SNPs according to the NCBI database. Many GST isozymes have overlapping substrate specificities and can compensate for one another if necessary. However, individuals with several detrimental polymorphisms resulting in low expression or activity of multiple GSTs could have compromised antioxidant systems less capable of conjugating toxicants with GSH (Ginsberg et al., 2009).

Glutathione binds inorganic Hg and methylmercury enabling cellular efflux via transporters (e.g., multi-drug resistance proteins) and ultimately elimination via fecal excretion, though entero-hepatic cycling of methylmercury-GSH conjugates also occurs (Ballatori and Clarkson, 1985). Polymorphisms in genes of the GSH synthesis pathway (*GCLM*, *GCLC*, *GSS*, *GSR*) could impact the concentration of GSH available for this process. Though not absolutely necessary for GSH conjugation of Hg to occur, GSTs (*GSTP1*, *GSTM1*, *GSTM3*, *GSTT1*, *GSTO1*) may catalyze the conjugation reaction or may act as transporters of Hg-GSH conjugates (Ballatori and Clarkson, 1985). Genetic variation in γ-glutamyltransferase 1 (*GGT1*) could affect methylmercury and inorganic Hg metabolism. GGT1 cleaves Hg-GSH conjugates, a process that is essential to uptake of methylmercury or inorganic Hg (as cysteine conjugates) by multiple cell types including enterocytes of the gastrointestinal tract and proximal tubules cells of the kidney

(Bridges and Zallups, 2005). In mice, GGT knockouts excrete more methylmercury and accumulate less inorganic Hg in the kidneys following treatment with the respective Hg species (Ballatori et al., 1998). Hence, polymorphisms drastically altering expression or activity of GGT1 could impact the elimination rates and distribution of either Hg form in the body. The role of a given gene in the Hg metabolism and elimination pathway must be considered when contemplating the possible impact (positive or negative) of a polymorphism that diminishes gene expression or enzyme activity on Hg toxicokinetics.

Selenoproteins are dually employed in Hg toxicokinetics by combatting the oxidative stress created by Hg and by binding the toxicant directly (Chen et al., 2006). Selenoproteins contain a unique amino acid, selenocysteine, that can bind and transport Hg-selenium or $MeHg^{+}$. With up to ten selenocysteine residues per protein depending on the isoform, selenoprotein P1 (SEPP1) is particularly equipped for this task (Suzuki et al., 1998; for review: Khan and Wang, 2009). Genotype of *SEPP1* influences isoform prevalence and gene expression, and variation in these parameters may reduce protective binding of Hg and/or alter Hg distribution to various tissues such as the kidney or brain (Jureša et al., 2005; Méplan et al., 2007, 2009). Another class of selenoproteins, glutathione peroxidases (e.g., *GPX1*, *GPX2, GPX4*), typically contain one selenocysteine residue each and have potent antioxidant properties. Both Hg exposure (Chen et al., 2006) and polymorphisms in the encoding genes (Méplan et al., 2008; Ravn-Haren et al., 2006) can influence expression and activity levels of these proteins.

1.1.6 In Vitro Characterization of Polymorphic Gene Products

Associations between genetic polymorphisms and Hg biomarker levels or Hg associated health effects are evident in several epidemiological cohorts (section 1.1.4). However, as in many gene-environment cohort studies, the underlying mechanisms are often unknown. A significantly associated polymorphism could truly impact gene expression or protein structure and function, or that polymorphism could merely be a marker for a different locus of functional importance. For example, *in vitro* studies of *GSTP1* have discovered several SNPs that alter enzyme activity (substrate affinity, catalytic efficiency) or gene expression and have also identified SNPs in linkage disequilibrium that lack functional significance (Hu et al., 1997; Kitteringham et al., 2007; Moyer et al., 2008; Zimniak et al., 1994). Laboratory based, *in vitro* methods discovered a dependence of GPX1 activity in human erythrocytes on genotype of a nonsynonymous SNP and selenium status (Jablonska et al., 2009). Li and Woods (2009) found a nonsynonymous SNP in coproporphrinogen oxidase (*CPOX*) decreased catalytic efficiency, especially in the presence of inorganic Hg. Such *in vitro* tests complement associative findings in epidemiological cohorts.

1.2 Knowledge Gaps

Despite decades of Hg research, knowledge gaps remain in Hg exposure assessment, cardiotoxic effects of Hg, and genetic factors related to Hg ADME and subsequent toxicity. Mercury body burdens of dental professionals require monitoring due to their dual exposure- occupational exposure to Hg vapor via dental amalgams and to methylmercury from fish consumption, the latter of which may be higher than the

average population according to socioeconomic status (Hightower and Moore, 2003). Dentists historically exhibited relatively high Hg levels (head and wrist Hg >20 μg/g; Shapiro et al., 1982), but in recent years, restoration with Hg-containing amalgam has declined (Eklund, 2010) and safety precautions have increased. Therefore, Hg body burden among dental professionals has decreased (Echeverria et al., 2005). While general population exposures are well characterized in many regions of the world, population data in countries such as Mexico remain unknown despite the targeting of Hg pollution and exposure as a prominent environmental issue in North America (Pilgrim et al., 2000). Neurodevelopmental problems associated with prenatal lead exposure have been observed in the Mexican population, though methylmercury exposure, another potential contributor to developmental effects, has not been approximated (Surkan et al., 2008).

The impact of methylmercury exposure on cardiovascular health has recently been realized (Roman et al., 2011). Evidence linking methylmercury to increased blood pressure is somewhat inconsistent and was observed primarily among subsistence fish and marine mammal consumers with relatively high exposures (Choi et al., 2009; Fillion et al., 2006; Valera et al., 2009). The influence of low level methylmercury exposure, observed in most populations, on blood pressure is unknown. Furthermore, while animal studies link acute, high dose inorganic Hg exposure to decreased blood pressure and heart rate (Massaroni et al., 1995; Rhee and Choi, 1989; Rossoni et al., 1999), cardiovascular health effects associated with elemental/inorganic Hg exposure have not been evaluated in humans.

Genetic factors are hypothesized to underlie a portion of the inter-individual variability observed in Hg half-life, biomarker distribution, and toxicity, though only

recently have gene-Hg relationships been explored. To date, several cohort studies analyzed seven polymorphisms in GSH pathway genes with respect to their associations with Hg biomarker concentrations (Table 1.1). Significant relationships observed were often inconsistent between studies and disproportionately favored associations with methylmercury biomarkers. A comprehensive assessment of more polymorphisms in GSH pathway genes using exposure information pertaining to both methylmercury and elemental Hg is necessary to further explore the contribution of this pathway to Hg toxicokinetic variability. Additionally, the influence of polymorphisms in other gene classes implicated in the ADME of Hg (e.g., selenoproteins) remains undetermined and merits investigation. Genetic polymorphisms influencing Hg accumulation and distribution may furthermore modify susceptibility to health effects following elemental Hg and/or methylmercury exposure such as altered blood pressure.

Beyond epidemiological studies assessing gene-Hg interactions, laboratory-based mechanistic analyses are needed to explore the biological plausibility of population-based associative findings. *In vitro* assays provide quick and informative assessment of the functional impact of a given polymorphism on gene expression or enzyme activity, and additionally allow for isolated analysis of the interaction between toxicants and specific proteins. A U.S. National Research Council (NRC) recommendation calls for development of these high throughput *in vitro* assays in an effort to improve toxicity testing and risk assessment (NRC, 2007). Ideally, a multitude of polymorphisms should be assessed in this manner to better understand the potential systemic impacts of multi-loci genotype combinations of interest.

1.3 Objective

This dissertation aims to further our understanding of the association of genetic polymorphisms with Hg body burden and toxicity by utilizing epidemiological and *in vitro* approaches. **Polymorphisms in genes implicated in the distribution, metabolism, and elimination of Hg are hypothesized to affect individual susceptibility to Hg accumulation in biomarkers, to modify the relationship between Hg and blood pressure, and to alter enzyme activity of encoded protein products**. This work builds upon previous studies by using a holistic approach considering two forms of Hg (through analytical measurement of urine, hair, and blood biomarkers) and various sources of exposure (e.g. fish, dental amalgam) in two populations (Michigan dental professionals and Mexican mother-child pairs). Furthermore, this study reevaluates the associations of previously studied functional polymorphisms (n=6), and assesses novel SNPs (n=17) in GSH-related and selenoprotein genes which may modify Hg metabolism. Possible mechanisms underlying epidemiological associations are explored by means of *in vitro* tests on four genetically engineered GSTP1 allozymes that compare enzymatic activity and Hg inhibition among the variant proteins. Overall, these studies investigate the impact of genetic factors on Hg toxicokinetics and toxicodynamics in humans. Research on the relationship between genetic variation and Hg metabolism and elimination will help ascertain sources of biomarker variability and narrow the gap between perceived and true health risks accompanying methylmercury and inorganic Hg exposure.

1.4 Specific Aims

The following specific aims explore the aforementioned hypotheses in two epidemiological cohorts and *in vitro*:

AIM 1: Epidemiological Biomarker Studies.

With gene-environment modeling approaches, couple the genotype of 23 polymorphisms in fourteen genes involved in Hg metabolism with Hg biomarkers (urine, hair, blood) to elucidate polymorphisms statistically linked to higher body burden in two genetically dissimilar populations.

AIM 1.1: In the Michigan Dental Association (MDA) cohort.

Sample 515 dental professionals with occupational exposure to inorganic Hg from work with dental amalgams and methylmercury exposure from fish consumption, and determine if fifteen polymorphisms in glutathione enzymes and selenoproteins associate with urine and hair biomarker levels.

AIM 1.2: In the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) Cohort.

Sample 400 mother-child pairs from Mexico City and investigate associations between 17 polymorphisms and blood, hair and urine Hg biomarker levels and ratios.

AIM 2: Epidemiological Study: Cardiovascular Outcomes, Hg and Polymorphisms.

Measure blood pressure in a subset of the MDA cohort (n=262), and assess the impact of Hg exposure and mercury-polymorphism interactions on this outcome.

AIM 3: *In Vitro* Studies: Enzyme Activity Assessment.

Genetically engineer two key polymorphic genes associated with Hg toxicokinetics *(GSTP1*-105, *GSTP1*-114), assess enzymatic activity of the protein products in the presence of and absence of methylmercury, inorganic Hg and several other heavy metals, and determine whether *in vitro* results provide mechanistic understanding of associations found in previous studies and evaluated in Aims #1 and 2.

Table 1.1 Significant Associations Between Genetic Polymorphisms and Mercury Biomarker Levels in Recent Epidemiological Studies.

Reference	Population	n	Hg Biomarkers	Exposure Sources	Polymorphisms Assessed	Polymorphisms Significantly Associated with Higher Biomarker Hg Levels	Polymorphisms Significantly Associated with Lower Biomarker Hg Levels
Custodio et al., 2004	Swedish adults	365	erythrocytes	fish	*GCLC* (rs17883901), *GCLM* (rs41303970), *GSTP1*-105, -114 (rs1695, rs1138272), *GSTA1* (rs3957396), *GSTT1* and *GSTM1* deletion	*GCLC* T and/or *GSTP1*-114 T carriers had steeper regression of erythrocyte Hg on polyunsaturated fatty acids	
Custodio et al., 2005	Ecuadorian gold miners and buyers	309	whole blood, plasma, urine	gold mining practices	*GCLC, GCLM, GSTP1*-105, *GSTP1*-114, *GSTA1*, *GSTT1* and *GSTM1* deletion	*GCLM* T and elevated blood, plasma, urine Hg in moderately exposed miners	
Gundacker et al., 2007	Austrian students	174 to 222	whole blood, urine, hair	fish	*GSTT1* and *GSTM1* deletion	*GSTT1/M1* deletions and hair Hg	
Engström et al., 2008	Swedish adults	292	erythrocytes	fish	*GCLC* , *GCLM, GSTP1*-105, *GSTP1*-114	*GCLM* TT and erythrocyte Hg (adjusted for polyunsaturated fatty acids)	*GSTP1*-105 G and/or *GSTP1*-114 T have less steep regression of erythrocyte Hg on polyunsaturated fatty acids
Gundacker et al., 2009	Austrian students	184 to 304	whole blood, hair, urine	dental amalgam, fish	*GCLC, GSTP1*-105, *GSTP1*-114, *GSTA1*, *GSTT1* and *GSTM1* deletion, metallothionein SNPs	*GSTP1*-105 G and unadjusted median blood and hair Hg; in CATREG analysis on dichotomized Hg levels: *GSTP1*-114 T and/or *GCLC* T and hair Hg; *GSTP1*-114 T and/or *GCLC* T on blood Hg, near sig; *GSTM1* deletion and/or *GSTP1*-105 G on hair Hg; *GSTT1* deletion and *GSTP1*-114 T on Hair Hg	*GSTA1* A and unadjusted median blood Hg

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Chapter 2

Glutathione Enzyme and Selenoprotein Polymorphisms Associate with Mercury Biomarker Levels in Michigan Dental Professionals[1]

2.1 Introduction

Mercury toxicokinetics and toxicodynamics are largely influenced by the chemical form of the metal. Though humans are exposed to mercury through a variety of sources (e.g. vaccines, thermostats, compact fluorescent lights), the main sources of elemental mercury (inorganic) and methylmercury (organic) exposure come from dental amalgams and fish consumption, respectively (ATSDR, 1999; Clarkson and Magos, 2006). Urine is commonly used to measure elemental mercury exposure and blood or hair to measure methylmercury exposure (Berglund et al., 2005; Clarkson et al., 2007). However, each biomarker shows tremendous inter-individual variation, and this complicates risk assessment. For example, mercury typically accumulates 250 times more in the hair than it does in the blood, though this ratio ranges widely among individuals (ATSDR, 1999; Berglund et al., 2005). The half-life of methylmercury in hair ranges from 33 to 120 days (Birke et al., 1972), and the half-life of inhaled elemental mercury ranges from 35-90 days in the whole body (Hursh et al., 1976). Failure to understand such inter-individual variation in the accumulation of mercury, its distribution to various biomarkers and its

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half-life in the body may lead to risk assessment decisions that fail to protect the most susceptible individuals.

Inter-individual variation in mercury biomarkers may be partly explained by genetic factors. For example, mercury is eliminated following conjugation with glutathione, a process that may involve glutathione s-transferases (GSTs; GST theta 1, GSTT1; GST mu 1, GSTM1; GST pi 1, GSTP1) and indirectly depends on enzymes of the glutathione synthesis and degradation pathways (glutamate-cysteine ligase catalytic and modifier submits, GCLC, GCLM; glutathione synthetase, GSS; glutathione reductase, GSR; γ-glutamyltransferase 1, GGT1) (Ballatori and Clarkson, 1985; Rooney 2007). Selenoproteins (e.g. selenoprotein pi 1, SEPP1; glutathione peroxidases, GPX) combat the oxidative stress created by mercury and bind the toxicant directly via selenocysteine residues (Chen et al., 2006). Genetic polymorphisms are ubiquitous among these glutathione and selenoprotein genes that facilitate the distribution, metabolism and elimination of mercury. As documented in recent epidemiological studies, such polymorphisms may influence mercury biomarker levels, which may ultimately improve understanding of inter-individual variability following exposure to methylmercury (Custodio et al., 2004; Engström et al., 2008; Gundacker et al., 2007; Gundacker et al., 2009) and elemental mercury (Custodio et al. 2005; Gundacker et al., 2009).

The aforementioned studies provide early evidence that genetic polymorphisms in key mercury handling genes may underlie differences in mercury distribution and accumulation in exposure biomarkers. However, discrepancies exist among the studies (see Table 1.1 for more details). For example, Custodio et al. (2004) found an association between the minor allele (T) of the *GSTP1*-114 polymorphism (rs1138272) and increased

blood mercury levels while Engström et al. (2008) found the opposite in subjects with higher fish consumption. Disparate findings may be related to differences between the studies in terms of biomarkers examined, statistical power and methodologies utilized, sources of mercury exposure, and polymorphism frequency. Alternatively, lack of replication of a polymorphism-Hg association may be due to a false positive.

Here we aim to address these limitations and help improve understanding of gene-environment interactions influencing mercury biomarker levels by studying both forms of mercury (through analytical measurement of biomarkers in urine and hair), various sources of exposure (dental amalgams and fish as determined from surveys), and additional polymorphisms in glutathione-related (*GSTT1, GSTM1, GSTP1, GSTM3, GGT1, GSS, GSR, GCLC, GCLM*) and selenoprotein (*SEPP1, GPX1, GPX4*) genes in a group of dental professionals that are exposed to mercury via occupational and environmental routes. Polymorphisms in the listed genes, which are important in the metabolism and elimination of mercury, are hypothesized to affect individual susceptibility to elemental mercury and/or methylmercury accumulation in the body as assessed by urine and hair biomarkers.

2.2 Materials and Methods

2.2.1 Study Population

A convenience sample of 515 dental professionals (dentists, hygienists, and other related professions) was recruited during the 2009 (n=231) and 2010 (n=284) Michigan Dental Association (MDA) Annual Conventions. Institutional Review Board (IRB) approval was obtained for this study from the University of Michigan (HUM00027621).

2.2.2 Survey Data

For each participant a self-administered survey provided information on subject demographics (e.g., age, gender, weight), occupational practices (e.g., hours worked per week, number of amalgams removed and placed per week), and personal exposures to elemental mercury (amalgams in subject's mouth) and methylmercury (fish consumption). Subjects provided detailed information on fish consumption (e.g., portion size, type of fish, monthly consumption). Fish data were used to estimate a mercury intake variable (ug per kg body weight per day) using mercury concentration data from the U.S. Food and Drug Administration for each fish species (Bahnick et al., 1994; Mierzykowski et al., 2001; US FDA).

2.2.3 Mercury Exposure Assessment

Spot urine samples, biomarkers of elemental mercury exposure, (>30 mL) were collected from each participant and stored frozen. Hair samples, biomarkers for methylmercury, (>10 mg) were cut from the occipital region of the scalp, and the first 2 cm of hair closest to the scalp (proximal end) were used for measurement. Total mercury levels in urine and hair were measured using a direct mercury analyzer (DMA-80, Milestone Inc., CT) according to U.S. EPA Method 7473 as previously described (Basu et al., 2010; Paruchuri et al., 2010). Quality control measures included incorporating one blank, one replicate sample, and a certified reference material (CRM; hair: NIES Japan CRM#13; two urine CRMs: QMEQAS08U-01 and ClinChek; dogfish liver: DOLT, National Research Council Canada) in every batch of 10-15 samples. All samples

exceeded the theoretical method detection limits, defined as 3x SD of blanks, (0.03 ug/L mercury for urine and 0.01 ug/g mercury for hair). Mean (±SD) recovery of mercury from CRMs was 94.4 ± 15.4%. Mean within day (3.1 ± 1.7%) and between day (6.7 ± 6.8%) variability of CRMs were similar to that of duplicated subject samples.

2.2.4 Genotype Analysis

To obtain DNA from buccal cells, subjects swabbed the inside of their cheeks with four cotton swabs for fifteen seconds each. Cotton swabs were stored at room temperature in 0.5 mL of buffer (100 mM sodium chloride, 10 mM Tris base, 10 mM EDTA, 0.5% sodium dodecyl sulfate with 0.1 mg/mL of Proteinase K, according to Min et al., 2006). DNA was purified from buccal cells using Promega SV Wizard Genomic DNA purification kits (Promega Corporation, Madison, WI), and DNA quantification was performed using PicoGreen reagent with relative fluorescence measured by a Perkin Elmer HTS 7000 Plus Bioassay Reader (Shelton, CT).

Over the two study years, fifteen polymorphisms were genotyped in glutathione s-transferases, glutathione synthesizing enzymes, and selenoproteins, but in a given study year ten or eleven polymorphisms were genotyped. Eleven polymorphisms were selected based on functional impacts on gene expression or enzyme activity associated with genotype in previous research (summarized in Table 2.2), while the remaining polymorphisms were selected as tag SNPs in genes of interest. Fourteen polymorphisms (rs1695, rs1138272, rs3877899, rs713041, rs7579, rs17883901, rs41303970, rs1050450, rs5751901, rs7483, rs3761144, rs1002149, rs2911678, *GSTT1* deletion) were genotyped using Taqman Allelic Discrimination Assays (Applied Biosystems; Livak et al., 1995).

Results were read and interpreted using an ABI 7700 (Carlsbad, CA). All primers and probes were pre-designed by Applied Biosystems except for the *GSTT1* deletion genotyping which utilized primers and probes designed by Mordukhovich et al. (2009). The *GSTM1* deletion polymorphism was genotyped in the 2009 samples using the PCR-based method of Lee et al. (2009). *GSTT1* and *GSTM1* genotyping resulted in two distinct groups: deletion homozygotes and individuals with at least one intact copy of the gene.

2.2.5 Statistical Analyses

All statistical analyses were performed using PASW Statistics v18 (Chicago, IL). The 2009 and 2010 data were grouped together given that demographics, mercury biomarker levels and exposures had similar distributions both years. In all analyses, mercury values were natural log-transformed to achieve normality. The association of genetic polymorphisms with mercury biomarker levels (hair or urine) was studied via simple linear regression models with one ordinal variable per polymorphism (coded 1=major homozygote, 2=heterozygote, 3=minor homozygote) in each model. Genotype coding assumed an additive impact of each additional variant allele on any potential association between genotype and biomarker levels, regardless of direction. The association of genetics with urine mercury and hair mercury levels adjusted for predictors was assessed by multiple linear regression. Base models without genetic variables were first created. All variables (e.g., demographics, fish consumption, occupational exposures) were included in the base model, and insignificant variables ($p>0.05$) were removed via a manual backward selection process. Final base models consisted of significant predictors of hair or urine mercury ($p<0.05$ for parameter estimate) unless

otherwise noted. The effect of genotype (main effect and interaction with each exposure predictor variable) was then assessed with separate models for each polymorphism. Models with separate dummy variables for heterozygotes and minor homozygotes and interaction terms for each were also run (data not shown). The effect estimates (magnitude, direction, significance) for genotype obtained from this method, which did not assume genetic additivity, were similar to results reported here.

According to the method of Levine and Fahy (1945), which has been shown to reduce variability of metal excretion associated with spot urine samples, urine mercury levels were adjusted for the mean specific gravity (1.017) of the study population (Lee et al., 1996; Mason and Calder, 1994). The linear regression models described were run with unadjusted and adjusted urine. Parameter estimates did not notably change and significance only changed in a few instances in specific gravity adjusted models. Unadjusted models are reported here unless otherwise noted. In all urine mercury models, eight subjects were excluded due to a history of kidney disease (lithiasis, pyelonephritis, and/or orthostatic proteinuria) that may have influenced urinary excretion of mercury.

Statistical significance was defined as $p<0.05$. Correction for multiple testing would necessitate a p value <0.002 to attain significance after accounting for 30 independent tests (two Hg biomarkers with fifteen polymorphisms). No estimates for polymorphism-Hg biomarker relationships in this study had significance of $p<0.002$. As such, nominally significant results ($p<0.05$) are reported and discussed as interesting trends to consider in future research.

2.3 Results

2.3.1 Descriptive Statistics

The majority of the 515 participants provided urine (n=502) and hair (n=505). Subjects lacking both biomarker samples and demographic information were excluded (n=4). Sixty-one percent of all subjects were female, and the majority of the population (>90%) self-identified racially as Caucasian. Table 2.1 provides average (±SD) demographic and occupational variables for the total population and occupation-stratified population (dentists, n=243 vs. non-dentists, n=268). Genders were unequally distributed in the occupational groups as 24% of dentists were female compared to 95% of non-dentists (e.g., dental hygienists, dental assistants). Stratification of subjects into two occupational groups yielded significant differences ($p<0.05$) between the groups. On average, dentists were older, removed and placed more dental amalgams in the office, and had more amalgams in their own mouths compared with non-dentists.

2.3.2 Mercury Biomarkers

Average mercury biomarker levels are reported in Table 2.1. Urine mercury (mean ±SD, 1.04±1.18 ug/L; median 0.66 ug/L) ranged from 0.03 to 9.26 ug/L with a distribution resembling that of the U.S. population according to the National Health and Nutrition Examination Survey (NHANES 2003-2004, median 0.48 ug/L; CDC, 2009). Dentists had significantly higher urine mercury levels compared to non-dentists (1.37±1.3 ug/L vs. 0.75±0.97 ug/L; $p<0.001$ for ANOVA comparing natural log-transformed values). The distribution of hair mercury (0.49±0.63 ug/g; range 0.02 to 6.15 ug/g) also resembled NHANES data from 1999-2000 (mean 0.47 ug/g; McDowell et al., 2004).

Dentists had significantly higher hair mercury levels compared to non-dentists (0.69±0.81 vs. 0.31±0.33 ug/g; $p<0.001$) even though the estimated mercury intake from fish consumption was not significantly different between the two groups.

2.3.3 Genotype and Mercury Biomarkers

All genotyped polymorphisms achieved Hardy-Weinburg Equilibrium (HWE). Minor allele frequencies (MAF) for each polymorphism were generally similar to HapMap reference Caucasian population MAFs (Table 2.2). In statistical models comparing mercury levels among genotype groups, all polymorphisms were divided into major homozygote, heterozygote, and minor homozygote groups with the exception of *GSTM1* and *GSTT1* deletion polymorphisms which were divided into two genotype groups (double deletion vs. at least one intact copy).

Simple linear models were used to test the differences in mean natural log-transformed urine (ug/L) or hair (ug/g) mercury levels among genotype groups for each polymorphism (Table 2.3). Three statistically significant associations emerged. Urine levels were lower among individuals with double deletion of *GSTT1* compared to those with the intact gene (means: 0.82 vs. 1.06 ug/L; p-value=0.03). Likewise, individuals with the T allele for *SEPP1* 3'UTR (rs7579) had lower urine mercury levels (means: CC=1.08, CT=1.04, TT=0.91 ug/L; p=0.03). Hair mercury levels differed by *GSTP1*-105 (rs1695) genotype with minor homozygotes (GG) exhibiting the lowest hair mercury measurements (means: AA=0.52, AG=0.51, GG=0.29 ug/g; p-value=0.004).

2.3.4 Linear Regression Modeling of Urine Biomarker

Linear regression was used to model natural log-transformed urine mercury levels with significant exposure variables. In the final base model, significant predictors were number of dental amalgams in the subjects' mouth ('amalgam'), amalgams removed or placed per week in the dental office ('amalgam handled', an ordinal variable), and occupation (dentist vs. non-dentist; Table 2.4). The categories for 'amalgam handled' were defined as follows: reference (0 amalgams handled), low (1 to 20 per week), medium (21 to 45), and high (>45). The effect of genotype for each polymorphism was tested (main effect, interactions between genotype and the three exposure variables). For all models, only the interactions between genotype and 'amalgam' are reported here as amalgams were the dominant predictor of urine Hg levels. The number of subjects in each model ranged from 204 to 466 depending on the polymorphism; not all polymorphisms were genotyped both years, and some subjects did not provide adequate DNA to complete the genotyping.

Urine mercury models with genotype (main effect or interaction) are reported in Table 2.4. Nominally significant ($p<0.05$) main effects of genotype were observed for *SEPP1* 3'UTR (β estimate: -0.26 ug/L change to ln(urine Hg) per T allele) and *GSTT1* deletion (-0.25 ug/L ln(urine Hg) with double deletion), though the latter was only observed when urine was first adjusted for specific gravity (data not shown). A significant interaction was observed with *SEPP1* 3'UTR T alleles and personal amalgams (β=0.04 ug/L change to ln(urine Hg) per T allele per amalgam), though the interactions with amalgam handled and occupation were not significant (data not shown).

2.3.5 Linear Regression Modeling of Hair Biomarker

Hair mercury levels were modeled using statistical methods similar to urine mercury modeling. The final base model included the most significant predictor of hair Hg, 'fish Hg' (calculated ug Hg intake per kg body weight per day from fish consumption, $p<0.001$ for parameter estimate; see Table 2.5). Addition of genotype into the model resulted in two genotypes with significant main effects (*GSTP1*-105 β=-0.22 ug/g change to ln(hair Hg) per minor allele; *GSTP1*-114, rs1138272, β=-0.31 ug/g). Significant interactions between genotype and fish Hg were also observed in models with *SEPP1* 3'UTR and *GSS* 5' (rs3761144). Individuals with the minor allele (T) of *SEPP1* 3'UTR accumulated less mercury in the hair per intake from fish consumption (β=-1.61 ug/g change to ln(hair Hg) per T allele per 1 ug fish Hg intake per kg body weight per day). The opposite relationship was observed with the minor allele of *GSS* 5' (G) and fish Hg (β=1.88 ug/g). To highlight the nature of these results, two scatterplots of hair mercury by mercury intake from fish (both natural log-transformed) are shown stratified by *SEPP1* 3'UTR genotype (Figure 2.1A) and *GSS* 5' genotype (Figure 2.1B). Significant results (main effect or genotype-by-exposure variable interactions) were not observed for any other polymorphism in the models of urine or hair mercury.

2.4 Discussion

Recent epidemiological studies suggest that seven genetic polymorphisms in GSTs and glutathione synthesizing enzymes may influence mercury accumulation in common biomarkers (hair, blood, urine) following exposure to elemental mercury or methylmercury (Custodio et al., 2004, 2005; Engström et al., 2008; Gundacker et al.,

2007, 2009). Here, we build upon these studies and assess the association of fifteen polymorphisms in GSTs, glutathione synthesizing enzymes and selenoproteins with the accumulation of both elemental mercury and methylmercury in urine and hair biomarkers, respectively, in a population of occupationally and environmentally exposed dental professionals. Our results suggest that five polymorphisms associate with accumulation of mercury in urine (*GSTT1* deletion), hair (*GSTP1*-105, *GSTP1*-114, *GSS* 5') or both (*SEPP1* 3'UTR).

Dental professionals provide a convenient sample to assess mercury-gene interactions as they share common exposure routes with the general population (e.g. fish consumption, personal amalgams) along with an additional occupational exposure. While dentists' occupational exposures are typically higher than the average population, a shift towards use of composite resin fillings and safer handling of mercury may result in dentists having urine biomarker levels comparable to that of the general U.S. population (Eklund et al., 2010). Here, participating dentists and other related professionals (e.g., dental hygienists, dental assistants) exhibited urine and hair mercury levels similar to that of the general U.S. population according to NHANES (CDC, 2009; McDowell et al., 2004). The best predictors of urine mercury levels were personal dental amalgams and occupational exposure to amalgams with dentists having the greatest exposure and biomarker levels. Study subjects displayed a wide range of methylmercury exposure from fish consumption, enabling the study of both mercury forms.

Immense variability in mercury biomarker measurements and half-lives has been observed in human populations, complicating risk assessment of both elemental and methylmercury (Berglund et al., 2005; Birke et al., 1972; Hursh et al., 1976). Differential

metabolism of mercury in the body could affect its half-life and reactivity, as conjugated mercury is less able to induce oxidative stress, bind to thiol groups, and/or inhibit proteins in the body. Functional GSTs, adequate glutathione and selenoproteins are essential for protection against mercury (Ballatori and Clarkson, 1985; Chen et al., 2006; Clarkson and Magos, 2006). Polymorphisms are ubiquitous among the genes encoding these mercury-handling proteins and have the potential to modify mercury toxicokinetics, reflected partially in biomarker levels, via altered enzyme activity or gene expression.

Previous studies assessing the association of genetic polymorphisms with mercury biomarkers focused on seven variants in glutathione synthesizing enzymes and glutathione s-transferases, six of which were genotyped here (see Table 1.1 for a summary of key results from these studies). Our results follow several trends previously reported, and this study further explores genotype-mercury relationships that were inconsistent in past research. Custodio et al. (2005) found the T allele of *GCLM* rs41303970 to be associated with increased blood, plasma and urine mercury in gold miners occupationally exposed to elemental mercury. Our study did not find associations between urine mercury levels and *GCLM* genotype. Genotype associations may differ based on the level of exposure as the miners had almost seven-fold higher urinary mercury compared to the dental population (miners: median=4.5 ug/L, maximum=230 ug/L; dental population: median=0.7 ug/L, maximum=9.3 ug/L). Other studies have explored the influence of glutathione-related polymorphisms on urine mercury levels, and a lack of significant outcomes indicate that the polymorphisms studied to date may not influence variability observed in urinary excretion of elemental mercury. Alternatively, given that the impact of an individual polymorphism on mercury accumulation is

expected to be small, studies to date, including the present, may have insufficient statistical power to detect some associations.

Two previous epidemiological studies have assessed the relationship between glutathione-related polymorphisms and hair mercury levels (Gundacker et al., 2007, 2009), and four explored the modification of polymorphisms on methylmercury accumulation from fish consumption using blood as an exposure biomarker (Custodio et al., 2004; Engström et al., 2008; Gundacker et al. 2007, 2009). Several associative findings linking polymorphisms to hair or blood biomarker levels were inconsistent among studies (Table 1.1). Gundacker et al. found *GSTM1* and *GSTT1* deletions together (2007) or in combination with other SNPs (2009) to associate with higher hair mercury levels. The present study did not observe significant associations between *GSTM1* or *GSTT1* deletion polymorphisms with hair mercury levels when modeling the effects of single polymorphisms or combined genotype (both intact vs. one or more deletions).

Conflicting relationships between two *GSTP1* SNPs (105, rs1695 A>G and 114, rs1138272 C>T) and hair or blood biomarkers were observed among past studies. Given that both SNPs encode amino acid changes in GSTP1 that decrease enzyme activity (Ali-Osman et al., 1997), these SNPs may alter the ability of GSTP1 to conjugate glutathione to mercury or transport mercury-glutathione conjugates. This study suggests that the *GSTP1*-105 G allele is linked to decreased hair mercury levels (Table 2.3 and Table 2.5), though it has no significant effect modification on the relationship between mercury intake from fish consumption and hair levels. Likewise, the *GSTP1*-114 T allele has a negative main effect in the adjusted model but no significant interaction with mercury intake from fish. Engström et al. (2008) found that carriers of *GSTP1*-105 G and/or

GSTP1-114 T accumulated less mercury in erythrocytes per measured polyunsaturated fatty acids (an indicator of fish consumption), suggesting a similar trend as the findings of this study for *GSTP1* variant alleles. In contrast, other studies found associations between *GSTP1*-105 G or *GSTP1*-114 T and higher hair or blood biomarker measurements, after adjustment for fish consumption in many cases and often in combination with other polymorphisms (Custodio et al., 2004; Gundacker et al., 2009). Several factors may influence these incongruent results. While hair and blood, especially erythrocytes, are both biomarkers for methylmercury exposure, two centimeters of scalp hair represent the average exposure over two months with a lag period of approximately one month while blood mercury levels reach a steady-state among consistent fish consumers but fluctuate among infrequent consumers (Berglund et al., 2005). Thus, while similar gene-mercury relationships could be observed in models of hair and blood mercury, they may be influenced by the frequency, duration, and dose of the exposure. Several significant findings in previous studies for *GSTP1*-105 and *GSTP1*-114 involved polymorphism combinations. Our sample size was not large enough to confidently test combinations of all fifteen polymorphisms studied.

This study expanded upon previous work by exploring gene-mercury biomarker relationships with nine additional SNPs in glutathione-related enzymes (n=5) and selenoproteins (n=4). Two SNPs (*SEPP1* 3'UTR, and *GSS* 5') had significant main effects and/or interactions with exposure sources in regression models of urine and/or hair mercury levels. While these SNPs displayed nominally significant associations ($p<0.05$), none had p-values that maintained significance after multiple test correction

($p<0.002$). As such, the results should be interpreted with caution and viewed as potential trends worthy of future exploration.

Genotype of the *SEPP1* 3'UTR SNP was significantly associated with both urine (positive interaction with amalgam) and hair Hg (negative interaction with fish mercury) in linear regression models. This SNP is found in the 3' untranslated region (UTR), a gene region crucial to the regulation of selenocysteine incorporation. Selenocysteine is an amino acid unique to selenoproteins that can bind mercury-selenium (HgSe) conjugates or methylmercury (Khan and Wang, 2009). Expression of *SEPP1* depends on genotype and selenium availability and can also be influenced by gender and BMI (Méplan et al., 2007, 2009). The 3'UTR T allele is linked to greater SEPP1 expression among people supplemented with selenium and higher prevalence of the 60 kDa isoform of SEPP1 which has more selenocysteine residues and greater mercury binding capacity (Méplan et al., 2007, 2009). Elevated elemental mercury exposure increases *SEPP1* expression and the ability of SEPP1 to bind mercury as evident in highly exposed miners (Chen et al., 2006). In the dental population, individuals with CT or TT genotype had lower urine mercury levels on average (Table 2.3). However, the T allele modified the relationship between the source of exposure, dental amalgams, and urine mercury levels. At higher exposure levels, CT/TT individuals excrete more mercury in the urine per amalgam compared to those with the CC genotype (Table 2.4; β=0.04 ug/L change to ln(Urine Hg) per T allele per amalgam). The opposite relationship is observed for the hair biomarker whereby each additional T allele is associated with less accumulation of mercury in the hair given methylmercury intake from fish (Figure 2.1A; β=-1.61 ug/g change to ln(hair Hg) per T allele per unit Hg intake from fish). Expression changes of *SEPP1* linked to the

T genotype may explain the complex relationship observed between *SEPP1* genotype, mercury biomarker levels, and exposures to mercury that may be complicated further by micronutrient status (e.g. selenium) and chemical speciation of mercury. Higher expression of *SEPP1* from the CT or TT genotypes could affect mercury binding and subsequent distribution to various tissues. Excretion of elemental mercury and methylmercury via urine and hair, respectively, may be differentially modified by this SNP which warrants further investigation and incorporation of selenium biomarker levels into multivariate analysis.

Glutathione synthetase (GSS) is involved in glutathione synthesis, and a SNP upstream of the coding region (rs3761144, C>G) may modify the relationship between mercury intake from fish consumption and hair mercury levels. With each variant allele (G), more mercury is accumulated in the hair given intake from fish consumption (Figure 2.1B, Table 2.5; 1.88 ug/g change to ln(hair Hg) per G allele per unit intake from fish). This SNP, which is associated with sporadic amyotrophic lateral sclerosis among individuals with past heavy metal exposure, may influence expression of *GSS* directly or may be a marker for an expression-altering SNP (Morahan et al., 2007). Decreased expression of *GSS* leading to decreased glutathione synthesis could impact the body's ability to eliminate methylmercury as a glutathione conjugate with the higher body burden reflected in hair mercury levels.

We found several nominally significant associations between polymorphisms in glutathione-related enzymes and *SEPP1* and elemental mercury or methylmercury biomarker levels in a population of dental professionals despite several study limitations. This cross-sectional study was dependent upon self-reporting of mercury exposures (fish

consumption, number of amalgams handled). Though the voluntary nature of subject participation could lead to selection bias, our subjects did not know their mercury levels or genotype prior to participation in the study, and we obtained a range of mercury biomarker levels similar to that of the general US population (CDC, 2009; McDowell et al., 2004).

In the dually exposed MDA cohort, total Hg in urine was predicted by amalgam exposures and occupation, and hair Hg levels correlated with estimated mercury intake from fish. As such, total Hg in urine and hair were assumed to reflect elemental mercury and methylmercury exposures, respectively. While urine and hair are often used as biomarkers of different mercury species (Berglund et al., 2005), cohorts occupationally exposed to elemental mercury attribute a fraction of hair Hg to the elemental mercury exposure source (Wranová et al., 2008). Likewise, in populations with negligible elemental mercury exposure, urine Hg concentrations correlate with fish consumption (Ohno et al., 2007). While the possibility of such biomarker crossover cannot be eliminated, urine and hair Hg concentrations in the MDA cohort primarily reflect elemental mercury and methylmercury exposures, respectively.

In genotype analyses, the sample size was decreased due to inadequate DNA from a subset of subjects, and not all polymorphisms were genotyped each collection year resulting in sample sizes as small as 204 in some models. Previous gene-mercury biomarker studies found significant associations of dual polymorphism combinations with mercury biomarker levels. However, due to the sample size and number of loci genotyped, polymorphism combinations could not be tested confidently in this study without encountering probable errors of multiple testing or insufficient statistical power

due to small numbers of cases with some genotype combinations. Without studying polymorphism combinations, we may have missed several significant relationships with mercury biomarkers that would mirror findings in previous studies. Overall, while several significant genotype-mercury biomarker relationships were observed ($p<0.05$), none remain significant after correction for multiple testing ($p<0.002$). As such, the nominally significant results reported in the MDA cohort should be considered trends and require further exploration in future cohort studies with greater power.

Inconsistencies among polymorphism-mercury biomarker studies and the lack of significant associations ($p<0.002$) observed after correcting for multiple testing in the MDA cohort could reflect complications inherent to studies of complex genetic traits. If polymorphisms in glutathione-related and selenoprotein genes have any true impact on Hg toxicokinetics, the influence of one individual polymorphism is expected to be quite small (e.g., explain <1% of toxicokinetic variability) and would be difficult to detect in a small cohort or replicate in another cohort. Further complications arise when studying ubiquitous gene families such as GSTs that upregulate expression following mercury exposure (Brambila et al., 2002) and are able to compensate for dysfunctional isozymes due to overlapping substrate specificity. Thus, the combination of a multitude of polymorphisms impacting gene expression and enzyme activity in various genes is expected to underlie the true variability observed in human mercury toxicokinetics.

In conclusion, this study adds to growing knowledge about genetic associations with mercury biomarker levels and inter-individual variability in the toxicokinetics of elemental mercury and methylmercury. Results suggest that five polymorphisms in *GSTP1*, *GSTT1*, *GSS* and *SEPP1* may influence urine and hair mercury levels, though

results should be interpreted with caution and further investigation is warranted. Future research on genetic factors influencing mercury toxicokinetics should assess both major mercury exposure sources (amalgam, fish consumption) using all three biomarkers (urine, blood, hair) in larger cohorts and more polymorphisms in glutathione related enzymes, selenoproteins, and proteins involved in mercury transport to help ascertain sources of biomarker variability and to narrow the gap between the perceived and true health risks accompanying mercury exposure.

Figure and Tables

Figure 2.1 Hair Hg vs. Hg Intake from Fish Stratified by A) *SEPP1* 3'UTR (C>T) Genotype or B) *GSS* 5' (C>G) Genotype. Note: hair Hg and Hg intake are natural log-transformed, though axes are labeled with untransformed units for ease of interpretation.

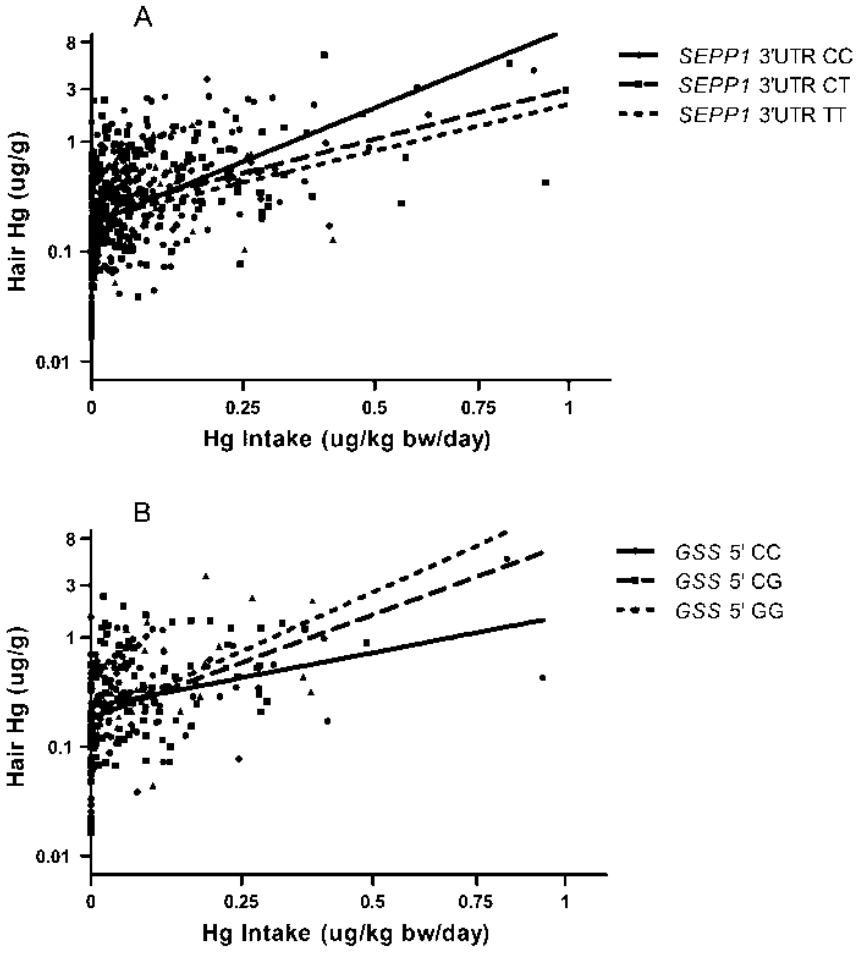

Table 2.1 Demographic and Exposure Variables. Mean (SD) for the total population and occupation stratified populations.

	Total Population	**Occupation Stratified**	
		Dentists	**Non-Dentists**
N	511	243	268
% Female	61.3	24.3	94.8
BMI (kg/m^2)	26.4 (4.7)	26.4 (4.0)	26.5 (5.3)
Age (years)	52.1 (12.1)	56.1 (11.6)	48.3 (11.2)[b]
Hours worked/ week (in past 6 months)	27.9 (11.6)	29.1 (11.1)	27.0 (11.9)[a]
# Amalgam (personal)	4.1 (4.1)	4.6 (4.2)	3.7 (3.9)[a]
# Amalgam placed/ week	12.1 (25.8)	20.4 (31.1)	4.7 (16.7)[b]
# Amalgam removed/ week	13.3 (23.1)	23.2 (27.2)	4.4 (13.4)[b]
Hg Intake (ug/kg body weight/day)	0.08 (0.13)	0.09 (0.14)	0.08 (0.12)
Urine Hg (ug/L)	1.04 (1.18)	1.37 (1.3)	0.75 (0.97)[b]
Hair Hg (ug/g)	0.49 (0.63)	0.69 (0.81)	0.31 (0.33)[b]

[a,b] denote p-value <0.05 and <0.001, respectively, for ANOVA test comparing dentists and non-dentists. ANOVA comparing urine and hair Hg used natural log-transformed values.

Table 2.2 Genotype Frequencies. Genotype data for 15 polymorphisms in glutathione S-transferases, glutathione synthesizing enzymes, and selenoproteins. Most polymorphisms were selected due to known functional impacts. Associations between polymorphisms and alterations to gene expression, protein structure, or enzyme activity are summarized in the table with references. SNPs without known functional impacts were selected as tag SNPs.

Gene	db SNP ID	Alleles	SNP Details	N	Minor allele frequency	Functional Impact[d]	Reference
GSTP1	rs1695	A>G	105 Ile>Val	485[a]	0.32	105Val ↓ catalytic efficiency	Ali-Osman et al. 1997
GSTP1	rs1138272	C>T	114 Ala>Val	475[a]	0.08	114Val ↓ catalytic efficiency?	Ali-Osman et al. 1997
GCLM	rs41303970	C>T	5' near gene	481[a]	0.19	T ↓ promoter activity	Nakamura et al. 2002
SEPP1	rs3877899	C>T	234 Ala>Thr	485[a]	0.24	Thr ↓ 60kDa isoform	Méplan et al. 2009
SEPP1	rs7579	C>T	3' UTR	479[a]	0.28	C ↓expression, ↓60 kDa isoform	Méplan et al. 2007, 2009
GSTT1	N/A	+ > -	Deletion	437[a]	0.41	-- ↓ expression	Moyer et al. 2007
GPX1	rs1050450	C>T	200 Pro>Leu	223[b]	0.28	200Leu ↓activity, Se-dependent	Jablonska et al. 2009
GPX4	rs713041	C>T	3' UTR	226[b]	0.45	T ↓ expression/protein levels	Méplan et al. 2008
GCLC	rs17883901	G>A	5' near gene	230[b]	0.08	A ↓ promoter activity	Koide et al. 2003
GSTM1	N/A	- > +	Deletion	209[b]	0.32	-- ↓ activity	Bell et al. 1992
GGT1	rs5751901	T>C	Intron	251[c]	0.36		
GSTM3	rs7483	G>A	224 Val>Ile	243[c]	0.26	224Val ↓catalytic efficiency	Tetlow et al. 2004
GSS	rs3761144	C>G	5' near gene	255[c]	0.40		
GSR	rs1002149	G>T	5' near gene	240[c]	0.18		
GSR	rs2911678	T>A	Intron	250[c]	0.24		

[a] Number genotyped including subjects from 2009 and 2010.
[b] Number genotyped including subjects from 2009.
[c] Number genotyped including subjects from 2010.
[d] Activity refers to enzymatic activity unless otherwise indicated, and '?' indicates an unclear association. The 60 kDa isoform of SEPP1 is the isoform with the most selenocysteine residues.

Table 2.3 Simple Linear Regression: Genotype and Mean Urine and Hair Hg. Beta estimates (p-value, bolded if p<0.05) are reported for correlations between genotype and natural-log transformed urine Hg or hair Hg.

Polymorphism	Urine Hg Model: β ug/L (p-value)	Hair Hg Model: β ug/g (p-value)
GSTP1-105	-0.05 (0.52)	**-0.22 (0.004)**
GSTP1-114	0.01 (0.96)	-0.19 (0.16)
GCLM 5'	-0.08 (0.32)	0.02 (0.86)
SEPP1-234	0.07 (0.34)	0.01 (0.92)
SEPP1 3'UTR	**-0.16 (0.03)**	0.03 (0.67)
GSTT1 deletion	**-0.27 (0.03)**	0.03 (0.84)
GPX1-200	-0.11 (0.28)	0.12 (0.29)
GPX4 3'UTR	-0.03 (0.77)	-0.1 (0.31)
GCLC 5'	0.05 (0.78)	0.01 (0.98)
GSTM1 deletion	-0.02 (0.91)	-0.04 (0.79)
GGT1 intron	-0.05 (0.62)	-0.16 (0.12)
GSTM3-224	-0.07 (0.47)	-0.08 (0.45)
GSS 5'	-0.05 (0.53)	-0.02 (0.82)
GSR 5'	-0.01 (0.93)	-0.07 (0.55)
GSR intron	0.1 (0.33)	0.14 (0.22)

Table 2. 4 Predictors of Urine Hg and Influence of Genotype. Parameter estimates (β) are reported for models of natural log-transformed urine Hg levels with exposure predictor variables, genotype, and genotype-by-amalgam interactions. Estimates indicate change to ln-transformed urine Hg (ug/L) per unit predictor.

Polymorphism	n[a]	Adj. r^2	Amalgam	Amalgam Handled	Non-Dentist	Genotype	Amalgam* Genotype
Base Model	492	0.233	0.08[d]	0.12[c]	-0.45[d]		
GSTP1-105	206, 214, 46	0.231	0.09[c]	0.11[b]	-0.45[d]	0.01	-0.006
GSTP1-114	388, 66, 2	0.225	0.06	0.11[b]	-0.45[d]	-0.002	0.01
GCLM 5'	310, 126, 27	0.244	0.11[d]	0.11[b]	-0.46[d]	0.03	-0.02
SEPP1-234	267, 173, 25	0.228	0.1[d]	0.1[b]	-0.48[d]	0.05	-0.02
SEPP1 3'UTR	240, 186, 35	0.254	0.03	0.11[b]	-0.44[d]	-0.26[c]	0.04[b]
GSTT1 deletion	347, 71	0.237	0.08[b]	0.12[b]	-0.4[d]	-0.18	0
GPX1-200	114, 82, 21	0.211	0.11[c]	0.15	-0.39[b]	0.08	-0.03
GPX4 3'UTR	67, 107, 46	0.208	0.09[b]	0.16[b]	-0.39[b]	0.07	-0.01
GCLC 5'	193, 28, 3	0.208	0.08	0.15	-0.39[b]	0.11	-0.004
GSTM1 deletion	93, 111	0.217	0.09[b]	0.16	-0.42[b]	0.17	-0.01
GGT1 intron	93, 122, 23	0.251	0.1[b]	0.07	-0.52[d]	-0.01	0
GSTM3-224	134, 77, 19	0.241	0.02	0.06	-0.48[d]	-0.14	0.05
GSS 5'	91, 110, 41	0.245	0.14[c]	0.07	-0.52[d]	0.01	-0.02
GSR 5'	155, 64, 7	0.228	0.12[b]	0.05	-0.53[d]	0.05	-0.02
GSR intron	140, 82, 15	0.238	0.09[b]	0.07	-0.54[d]	0.02	0.01

[a] Number of subjects in model in each genotype group. Genotype groups are major homozygote, heterozygote, minor homozygote except in the case of *GSTT1* (gene present vs. double deletion) and *GSTM1* (double deletion vs. gene present)
[b,c,d] denote p-value ≤0.05, <0.01, and <0.001, respectively, for parameter estimate.

Table 2.5 Predictors of Hair Hg and Influence of Genotype. Parameter estimates (β) are reported for models of natural log-transformed hair Hg levels with exposure variable (estimated Hg intake from fish consumption, ug per kg body weight per day), genotype, and genotype-by-fish Hg interactions. Estimates indicate change to ln-transformed hair Hg (ug/g) per unit predictor.

Polymorphism	n[a]	Adj. r^2	Fish Hg	Genotype	Genotype*Fish Hg
Base Model		0.186	3.62[d]		
GSTP1-105	214, 216, 44	0.197	2.87[c]	-0.22[c]	0.42
GSTP1-114	393, 69, 2	0.195	2.91[c]	-0.31[b]	0.67
GCLM 5'	315, 127, 28	0.184	4.49[d]	0.06	-0.63
SEPP1-234	272, 176, 26	0.187	2.41[b]	-0.04	0.78
SEPP1 3'UTR	245, 188, 36	0.194	6.22[d]	0.15	-1.61[c]
GSTT1 deletion	356, 70	0.179	2.38	-0.01	1.07
GPX1-200	113, 84, 21	0.224	3.65[c]	0.04	0.06
GPX4 3'UTR	70, 104, 47	0.231	2.69	-0.12	0.55
GCLC 5'	192, 30, 3	0.228	4.53[c]	0.07	-0.6
GSTM1 deletion	92, 112	0.247	5.51[d]	0.04	-1.04
GGT1 intron	96, 124, 25	0.161	6.05[d]	-0.09	-1.32
GSTM3-224	137, 80, 21	0.131	3.58[c]	0.02	-0.29
GSS 5'	94, 111, 44	0.147	0.34	-0.15	1.88[b]
GSR 5'	159, 67, 8	0.12	1.4	-0.21	1.1
GSR intron	143, 86, 15	0.139	1.7	0.06	1.18

[a] Number of subjects in model in each genotype group. Genotype groups are major homozygote, heterozygote, minor homozygote except in the case of *GSTT1* (gene present vs. double deletion) and *GSTM1* (double deletion vs. gene present)
[b,c,d] denote p-value ≤0.05, <0.01 and <0.001, respectively, for parameter estimate.

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Chapter 3

Genetic Polymorphisms and Mercury Biomarker Distribution in Mexican Mother-Child Pairs

3.1 Introduction

Mercury (Hg) is a potent toxicant that exists in multiple forms and has toxic effects on the nervous, cardiovascular, and immune systems, among others (ATSDR and CDC, 2009; NRC, 2000; Roman et al., 2011). Humans are mainly exposed to organic Hg as methylmercury from fish consumption and to elemental Hg via dental amalgams (Clarkson et al., 2007). Prenatal development is a particularly sensitive period for methylmercury exposure, due to its known effects on neurodevelopment, and as such, risk assessment decisions on safe exposure levels are often geared towards women of child-bearing age (NRC, 2000). In 2000, Mexico, Canada and the U.S. deemed Hg pollution and the associated human health risks a large enough issue to form the North American Regional Action Plan on Mercury (Pilgrim et al., 2000).

Despite the toxicity of Hg and its elevation to a priority pollutant in North America, little data exists on sources of exposure and Hg biomarker levels in Mexicans. The few studies measuring Hg in Mexican populations focused on small samples of fish-eating populations living near bodies of water (Guentzel et al., 2007; Trasande et al., 2010) or workers and residents of mining and waste disposal sites contaminated with inorganic Hg and other heavy metals (Costilla-Salazar et al., 2011; de Lourdes Sotos-

Ríos et al., 2010; Diaz-Barriga et al., 1993). Mercury exposure assessment of fish caught in waters in and around Mexico have revealed commonly consumed fish species to have a wide range of Hg contamination, some of which exceed maximum permissible levels set by various regulatory bodies (Guentzel et al., 2007; Ruelas-Inzunza et al., 2011a, 2011b; Soto-Jiménez et al., 2010; Trasande et al., 2010). Given information on fish consumption patterns of the general population (Soto-Jiménez et al., 2010), some Mexicans may be at risk from exposures to methylmercury and possibly to inorganic Hg (from dental amalgams and other sources). Women of child bearing age and their children are a potentially susceptible group, but to our knowledge, their Hg exposures have not been characterized in the general Mexican population.

Exposure assessment is an important part of Hg risk assessment though it is complicated by variability in common biomarkers. Hair and blood (whole blood or erythrocytes) are mostly indicators of methylmercury exposure while urine and plasma reflect exposure to elemental/inorganic Hg (Berglund et al., 2005; for review see Clarkson et al., 2007). Immense inter-individual and inter-population variability has been observed among these biomarkers with respect to Hg half-life, biomarker Hg concentration to Hg intake ratios, and proportion inorganic Hg (Berglund et al., 2005; Birke et al., 1972). Furthermore, the hair Hg to blood Hg ratio (hair:blood), often used in risk assessment to extrapolate blood Hg levels from hair or vice versa (NRC, 2000), is usually assumed to be 250:1 though this ratio ranges widely (Berglund et al., 2005; Budtz-Jørgensen et al., 2004). Recent studies link genetic polymorphisms in genes of the glutathione pathway to variability in methylmercury (Custodio et al., 2004; Engström et al., 2008; Gundacker et al., 2009) and inorganic Hg biomarkers (Custodio et al., 2005;

Gundacker et al., 2009). Glutathione conjugation is one mechanism that detoxifies and aids in elimination of Hg from the body (Ballatori and Clarkson, 1985). Glutathione s-transferase (GST) and glutathione synthesizing genes are highly polymorphic, and genetic variation in these genes along with other gene classes involved in Hg toxicokinetics (e.g., selenoproteins) may be important to individual accumulation of Hg in the body and subsequent health risks.

This study seeks to address several knowledge gaps in epidemiological Hg research using the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) cohort, a sample of mother-child pairs recruited from Mexico City. Mercury body burden was assessed using common biomarkers in children (hair, blood, urine) and mothers (hair). Additionally, 17 single nucleotide polymorphisms (SNPs) in glutathione (GSH) pathway and selenoprotein genes implicated in Hg metabolism and elimination were genotyped in ELEMENT subjects. Finally, genotype was related to Hg biomarker levels to test the hypothesis that SNPs in GSH pathway and selenoprotein genes will impact Hg body burden and Hg distribution as reflected in biomarker concentrations.

3.2 Materials and Methods

3.2.1 Study Population

This study included participants from the ELEMENT birth cohort study, a collection of three mother-child cohorts recruited before or during pregnancy between 1994 and 2002 with the intention of assessing the impact of prenatal lead exposure on development. Subjects of all three cohorts are Mexico City residents originally recruited

from three maternity hospitals serving low to moderate income individuals in Mexico City. For further information on subject recruitment and study design, see Cantonwine et al. (2010) and Surkan et al. (2008). This study utilizes data collected from a subset of ELEMENT mother-child pairs attending a recent follow-up visit (2007-2009) when the children were 6-15 years old. At the follow-up visit, biomarkers for Hg measurement were collected from the children and mothers (hair, blood, urine) along with demographic information, and anthropometric measurements. DNA was purified from blood samples for genotyping. The final sample was limited to subjects with genotype data (n=355 mothers, 369 children). Sample size was further reduced in individual models as many mother-child pairs had missing data points (e.g., urine Hg biomarkers, mother's education, successful genotype of certain SNPs). Subjects with missing data points were excluded on an analysis by analysis basis. Mothers signed a letter of informed consent before participating, and research protocols were approved by Ethics and Research Committees of all participating organizations including the University of Michigan and the National Institute of Public Health of Mexico.

3.2.2 Mercury Exposure Assessment

Analytical measurement of total Hg in children's blood and urine samples and hair from both mothers and children was performed using the Direct Mercury Analyzer 80 (DMA-80, Milestone Inc., CT) according to U.S. EPA Method 7473 as previously described (Basu et al., 2010; Paruchuri et al., 2010). Eight hundred μL of urine, 250 μL of blood, or 5-10 mg of hair from the 2 cm closet to the scalp were used for analysis. Prior to analysis, hair samples were washed with acetone, rinsed three times with Milli-Q

water and dried overnight. Following placement of quartz or nickel sampling boats containing the human biomarkers into the DMA-80, decomposition at 800°C liberated Hg from the sample as a vapor, which was subsequently carried to an absorbance cell (253.65 nm) by oxygen for Hg quantification.

Accuracy and precision were assessed by measuring duplicate samples and five certified reference materials (CRM) including NIES CRM #13 (human hair; Japanese National Institute for Environment Studies) and DOLT-4 (dogfish liver; Canadian National Research Council). Mean recovery of five different CRMs was 90.8% (mean of each ranged from 81.5 to 102.2%). Within day and between day variability of CRMs (mean 4.1% and 8%, respectively) was similar to that of duplicated samples. The analytical detection limit, calculated as three times the standard deviation of blank values, was 0.01 µg/g for hair samples, 0.08 µg/L for urine, and 0.17 µg/L for blood. Four urine samples fell below the detection limit. The specific gravity of urine samples was measured using a refractometer (PAL-10S, Atago U.S.A., Inc., WA). Urine Hg values were adjusted to the average specific gravity (1.017) to reduce variability in excretion associated with spot urine samples (Levine and Fahy 1945; Mason and Calder 1994).

3.2.3 Genotype Analysis

DNA was purified from whole blood using DNeasy Blood and Tissue Kits according to the manufacturer's protocol (Qiagen, Valencia, CA). Single nucleotide polymorphisms (SNPs) in glutathione synthesizing enzymes (n=2), glutathione s-transferases (GST, n=10), and selenoproteins (n=5) were genotyped in 355 mothers and 369 children (see Table 3.1 for list of SNPs). Ten SNPs were selected based on known

functional impacts (e.g., altered enzyme activity, gene expression; summarized in Table 3.1) of the genotype. The remaining seven SNPs were tag SNPs in glutathione-related and selenoprotein genes of interest. Genotyping was performed using the Sequenom iPLEX Gold platform according to a published protocol (Gabriel et al., 2009).

3.2.4 Statistical Analysis

Statistical operations were performed with PASW® v. 18 (Chicago, IL). Descriptive statistics for all variables were first computed for mothers and children. Mercury biomarker levels and ratios were natural log-transformed to achieve normality in all analyses. To minimize variability among children associated with age and gender, growth parameters (e.g., BMI, waist circumference) were converted into percentiles based on the distributions of these parameters in US children of the same age (by year) and gender according to the US Centers for Disease Control and Prevention database (http://www.cdc.gov/growthcharts/percentile_data_files.htm). Bivariate analysis (Pearson correlation) determined the association of demographic, anthropometric and Hg biomarker variables with one another.

Linear regression further explored predictors of Hg biomarker levels and ratios and the association of genotype with these outcomes in both simple and multivariate models. The association of genotype (of the respective children or mothers) with six Hg biomarker variables (children's hair, urine, blood Hg levels, hair to blood and blood to urine ratios; mother's hair Hg) was assessed in linear regression with each SNP coded as one variable assuming additivity of variant alleles (1= major homozygote, 2=heterozygote, 3=minor homozygote). In multivariate models, available variables (age,

mother's education, gender, BMI and waist circumference- percentile for children) were tested as predictors of Hg biomarkers in linear regression models without genetic factors. While none of the variables were consistently significant predictors of all Hg biomarkers or biomarker ratios, BMI, age, mother's education, and gender (in children's models) were selected for adjustment in models with genotype. Hair to blood ratio models additionally controlled for urine Hg as an indicator of inorganic exposure which can influence whole blood Hg. Genetic variables were added into the final models (one SNP per model). Though statistical tests were run with both unadjusted and specific gravity adjusted urine Hg, tests reported here used specific gravity adjusted urine unless otherwise indicated.

3.3 Results

3.3.1 Descriptive Statistics

Current demographic information and anthropometric measurements were collected from ELEMENT cohort mother-child pairs participating in a follow-up visit between 2007 and 2009. Table 3.2 describes characteristics of the mothers and children with genotype data used in this study. Demographic variables and anthropometric measurements (e.g., age, BMI, education) of the subjects reported in Table 3.2 were similar to that of all ELEMENT subjects attending a follow-up visit during the same time frame (n=697, data not shown). Mercury exposure source data (e.g., fish consumption frequency, number of dental amalgams) was not available for ELEMENT participants.

3.3.2 Mercury Biomarkers

Mercury levels were quantified in hair, blood, and urine samples from the children and hair from the mothers (Table 3.3A). Not all mother-child pairs provided each biomarker of interest (n=238 with four). As such, analyses for each biomarker or biomarker ratio include all subjects with that specific biomarker and other model variables. The distribution of hair Hg concentrations of mothers (median, range: 0.39, 0.03-4.19 µg/g) was similar to that of the children (0.38, 0.03-5.1 µg/g). Blood Hg levels in children ranged from 0.18-11.2 µg/L while urine Hg ranged from 0.06-9.34 µg/L. Age stratification revealed significant differences ($p<0.05$, ANOVA comparing natural log-transformed data) between two age groups of ELEMENT children for hair, blood, and urine Hg with younger children (ages 6-11 years) having higher biomarker levels (Table 3.3B). Children from each of the three combined ELEMENT cohorts were treated the same with respect to initial recruitment and follow-up visits. Even so, a cohort effect could underlie the Hg biomarker differences observed between the younger and older children who were originally from different ELEMENT sampling groups, though this is unlikely since cohort was not a significant predictor in any multivariate Hg biomarker model (data not shown). Stratification by gender did not reveal differences in mean or median biomarker levels.

Biomarker ratios (hair:blood=ng Hg/ g hair divided by µg Hg/ L blood; blood:urine=µg Hg/ L blood divided by µg Hg/ L urine) were calculated for the children (Tables 3.3A, B) as these ratios are influenced by type of Hg exposure but also reflect inter-individual variability in the distribution and handling of Hg. The blood:urine was more variable than the hair:blood (10 fold difference between the 90^{th} and 10^{th} percentiles

versus 3 fold difference), and this variability decreased (7 fold difference) when urine was adjusted for specific gravity.

3.3.3 Bivariate Analyses

Correlations between Hg biomarkers, biomarker ratios, and select demographic variables were assessed using natural log-transformed biomarker values (Table 3.4). Children's age was negatively associated with biomarker levels (hair, blood, specific gravity adjusted urine) and mother's education was positively correlated with children's hair and blood (and mothers' hair, data not shown). As observed by others (Berglund et al., 2005), blood and hair Hg levels of the same individuals were highly correlated ($r=0.77$, $p<0.001$). Although the strongest mother-child biomarker correlation was between hair Hg levels ($r=0.47$, $p<0.001$), mothers' hair Hg was also correlated with children's blood ($r=0.37$, $p<0.001$) and urine Hg (0.19, $p<0.01$).

3.3.4 Genotype Data

Seventeen SNPs were genotyped in mother-child pairs of the ELEMENT cohort using the Sequenom iPLEX Gold Platform (Table 3.1). Four SNPs had minor allele frequencies (MAF) below 5% (*GCLC* rs17883901, *GSTM1* rs1065411, *GSTO1* rs11509438, *GSTP1* rs1138272) in this population. All SNPs were in Hardy-Weinberg Equilibrium except one; *GPX4* rs713041 had a higher prevalence of heterozygotes than expected ($p=0.002$). Two SNPs were genotyped for the first time, to our knowledge, in a Mexican population (*GCLC* rs17883901, *GCLM* rs41303970). Comparing the genotype data of the remaining fourteen SNPs to that of populations with Mexican ancestry

previously genotyped (e.g., HapMap MXL, Table 3.1) reveal similar MAFs among most SNPs. MAF differences observed (i.e., *GSTP1* rs749174) may be influenced by the ancestry of the populations. HapMap MXL consists of individuals with Mexican descent living in the United States (Los Angeles, CA), and here we have genotyped residents of Mexico City with unknown proportions of Indigenous, Spanish and African ancestry.

3.3.5 Genotype and Mercury

The influence 17 SNPs in genes involved in Hg distribution, metabolism, and elimination on Hg accumulation was assessed via the association with Hg biomarker concentrations. Table 3.6 summarizes significant ($p<0.05$) or near significant ($p<0.10$) relationships observed between SNPs and biomarkers as observed in simple linear regression models or multivariate models adjusting for age, BMI (percentile in children's models, kg/m^2 in mothers' models), gender and education (years completed by the mother, an indicator of socioeconomic status). Multivariate models for hair:blood additionally controlled for urine Hg to account for the inorganic fraction of exposure. All urine Hg levels were first adjusted to average specific gravity to reduce variability in the reported analyses (Mason and Calder, 1994). Table 5.5 reports parameter estimates of the base multivariate models before addition of genotype.

Eight SNPs displayed near significant ($0.05<p<0.1$) parameter estimates for simple and/or multivariate regression models of Hg biomarkers or biomarker ratios, though only one SNP (*SEPP1*, rs7579) attained statistical significance in the multivariate urine Hg model (β= -0.14 ug/L change to ln(urine Hg) per minor allele, $p=0.03$). Apart from the relationship between rs7579 and urine Hg, no SNP significantly influenced Hg

biomarker levels. Furthermore, the borderline significant ($0.05<p<0.1$) relationships observed between SNPs and biomarker levels did not replicate in both simple and multivariate models with two exceptions: the aforementioned *SEPP1* (rs7579) with urine Hg and *GSTP1* (rs1695) with blood:urine. *GSTP1* rs1695 trended towards a negative relationship between minor alleles and blood:urine in simple ($\beta=-0.11$, $p=0.08$) and multivariate ($\beta=-0.1$, $p=0.099$) linear regression models. No polymorphism-Hg biomarker relationship was significant after correcting for multiple testing.

3.4 Discussion

This study reports Hg biomarker levels (hair, blood, urine) and genotype frequency of 17 SNPs in key antioxidant and detoxification genes of the glutathione and selenoprotein families in mothers (n=355) and children (n=369) from the ELEMENT cohort. To our knowledge, this is the first Hg biomarker assessment performed in a cohort representing the general Mexican population and the first to report genotype frequencies for 17 SNPs in a population residing in Mexico. Furthermore, we explored the relationship between Hg biomarkers and SNPs and found one significant relationship between the minor allele of *SEPP1* rs7579 and decreasing urine Hg levels, though due to multiple testing this association could have occurred by chance alone.

Mothers and children living in Mexico City have higher Hg levels in hair, blood, and urine biomarkers than that of similarly aged subgroups of the U.S. population (comparing medians) according to NHANES (National Health and Nutrition Examination Survey). These differences are the most substantial when comparing Mexican and American children's hair and blood Hg levels, especially among 6-11 year old children.

Hair Hg levels of Mexican mothers are approximately two-fold higher than that of American women of child-bearing age (medians 0.39 vs. 0.19 µg/g; McDowell et al., 2004). ELEMENT children's hair Hg levels are three times greater than U.S. children aged 1-5 years (medians 0.38 vs. 0.11 µg/g; McDowell et al., 2004). While this difference may be partially due to exposure (e.g., fish consumption) and toxicokinetic differences existing between the age groups, comparison of blood Hg levels between ELEMENT and NHANES children of similar age suggest that Mexican children may have greater exposure (Caldwell et al., 2009). Blood Hg differences are the most pronounced when comparing younger (6-11 years) Mexican and American children (medians 1.36 vs. 0.40 µg/L) as opposed to Mexican (13-15 years) and American (12-19 years) adolescents (1.14 vs. 0.50 µg/L). Since hair and blood Hg are typically biomarkers of methylmercury exposure (Berglund et al., 2005; Clarkson et al., 2007), this data suggests that ELEMENT children may have greater exposure to methylmercury compared to their American counterparts. Variation is also observed, albeit to a lesser extent, among urine Hg levels from the two populations, signifying the presence of elemental or inorganic Hg exposures among Mexican children (ELEMENT median: 6-11 year olds- 0.53 µg/L, 13-15 year olds- 0.44 µg/L vs. NHANES median: 6-11 year olds- 0.19 µg/L, 12-19 year olds- 0.32 µg/L; ATSDR and CDC, 2009).

This is the first cohort study, to our knowledge, to quantify Hg biomarker levels in a general Mexican population. Previous studies assessing methylmercury exposure in Mexico focused on populations with heavy fish consumption living near bodies of water. In these highly exposed populations, 58% (Guentzel et al., 2007) and 26% (Trasande et al., 2010) had hair Hg greater than 1 µg/g compared to approximately 10% of ELEMENT

mothers and children. While information on fish consumption patterns was not available for the ELEMENT cohort, national fish consumption surveys in Mexico reveal a majority of the population (85%) to consume fish with 35% consuming fish at least once per week (Soto-Jiménez et al., 2010). While fish intake estimates in Mexico imply much lower consumption compared with many other regions of the world, Hg levels in popular fish species are particularly elevated in certain regions of Mexico (Ruelas-Inzunza et al., 2011a, 2011b; Soto-Jiménez et al., 2010; Trasande et al., 2010). For example, 65-90% of fish species collected in the Southern Gulf of California coast had Hg levels exceeding regulatory standards set by the WHO, the US or Mexican governments (Soto-Jiménez et al., 2010). Thus, fish consumption of contaminated species may underlie the hair and blood Hg levels observed here.

Previous studies analyzing elemental or inorganic Hg exposures in Mexico using urine as a biomarker focused on populations living or working near contaminated regions such as former Hg mining sites or hazardous waste landfills (Costilla-Salazar et al., 2011; Díaz-Barriga et al., 1993). To our knowledge, this is the first study to quantify urine Hg levels in Mexican children from the general population. While not reaching levels of concern, urine Hg in Mexican children was slightly higher than that of American children (ATSDR and CDC, 2009). Information on exposures from dental amalgams and other inorganic Hg sources was not available for this cohort. Dental caries are prevalent among Mexican children (>75%), especially in families with lower SES and/or less parental education (Casanova-Rosado et al., 2005; Medina-Solis et al., 2009). A large proportion of the children may therefore be exposed to elemental Hg via dental amalgams. However, while SES is negatively associated with preventative dental care, it is also negatively

associated with curative care, and as such children with low SES may have fewer Hg-containing dental amalgams compared with the wealthy (Medina-Solis et al., 2009).

The hair:blood is used in risk assessment to extrapolate blood Hg levels from hair Hg and vice versa. Calculations of important parameters such as the reference dose for daily methylmercury intake often use a widely accepted hair:blood average of 250 (NRC, 2000) even though variability in this ratio has been observed on both population and individual levels. This variability depends on many factors including age, Hg exposure level, and genetic factors (Berglund et al., 2005; Budtz-Jørgensen et al., 2004). Younger ELEMENT children (ages 6-11 years) had a significantly higher hair:blood median compared to older children (315 vs. 273). This difference is consistent with a Faroe Islands study that found median hair:blood of 369 and 248 for 7 and 14 year olds, respectively (Budtz-Jørgensen et al., 2004), and suggests that young children may incorporate Hg into the hair at dissimilar rates compared to adolescents and adults as observed with other metals such as aluminum and selenium (Paschal et al., 1989). The blood:urine is less well studied, and here we found Hg levels in blood to be 2.63 times more concentrated than in urine (Table 3.3), and the ratio was not dependent on age. The blood:urine may fluctuate based on the extent of inorganic Hg exposure, and this is reflected in the variability of the blood:urine.

We assess the association of seventeen SNPs in glutathione synthesizing (n=2), GST (n=10), and selenoprotein (n=5) genes with hair, blood, and urine Hg biomarker levels, hair:blood and blood:urine in the ELEMENT cohort. Two SNPs (*GCLC* rs17883901, *GCLM* rs41303970) had not been previously genotyped in a population with Mexican ancestry. Minor allele frequencies (MAFs) of many of the remaining SNPs were

similar to MAFs in a HapMap population (MXL) of individuals with Mexican ancestry living in Los Angeles, California (e.g., *GSTP1* rs1695, *SEPP1* rs7579) while MAFs of other SNPs differed (e.g., *GSTP1* rs6591256, rs1138272). Divergent MAFs are not surprising as MXL members and residents of Mexico City could have dissimilar proportions of European, Native American and African ancestry as observed in other Latino populations in Mexico and Columbia (Florez et al., 2009; Martinez-Fierro et al., 2009; Martinez-Marignac et al., 2007).

Genetic polymorphisms in GSTs and glutathione synthesizing genes may impact metabolism and elimination of methylmercury (Custodio et al., 2004; Engström et al., 2008; Gundacker et al., 2009) and inorganic Hg (Custodio et al., 2005) according to previous studies including our work with the Michigan Dental Association (MDA) cohort (see Chapter 2). A SNP in the regulatory 3' untranslated region (UTR) of selenoprotein p 1 (*SEPP1*) additionally associated with hair and urine Hg biomarker levels with respect to known exposure in the MDA cohort. Exploration of the relationships between 17 SNPs and 6 Hg biomarker variables in the ELEMENT cohort was limited by the lack of available exposure data (e.g., fish consumption frequency, number of dental amalgams). As such, analyses were restricted to simple linear regression models testing the additive impact of variant alleles on each Hg biomarker or biomarker ratio (Table 3.6). Multivariate regression models additionally adjusted for mother's education- an indicator of socioeconomic status (SES), BMI, gender, and age. Higher SES could be linked to fish consumption and subsequently higher hair and blood Hg levels as observed in other populations (Hightower and Moore, 2003) and supported by bivariate analyses revealing a significant positive correlation between mother's education and children's hair Hg,

blood Hg, and mother's hair Hg (Table 3.4). Furthermore, controlling for SES may partially account for confounding effects of ancestry as SES has been inversely associated with proportion of Native American ancestry in other Latino populations (Florez et al., 2009).

A solitary SNP- rs7579 in *SEPP1*- attained statistical significance ($p<0.05$) in the multivariate model for urine Hg (Table 3.6). Given the number of independent models tested (>100) and the observed p-value (0.03 for multivariate model with rs7579), such a result could be obtained by chance alone. Even so, experimental findings exist in support of the possibility that SEPP1 could influence Hg body burden. SEPP1 is a unique protein that contains multiple selenocysteine residues which are able to bind both methylmercury and inorganic Hg-selenium conjugates (Khan and Wang, 2009). Interestingly, SNP rs7579 resides in the 3'UTR, a regulatory region crucial for selenocysteine incorporation, and this SNP is positively associated with SEPP1 expression and increased proportion of the 60 kDa isoform, the isoform with the most selenocysteine residues, specifically among individuals receiving selenium supplementation (Méplan et al., 2007, 2009). In the MDA cohort, the T allele of rs7579 was significantly associated with lower urine Hg. However, incorporation of exposure data revealed a significant interaction between T alleles and number of dental amalgams among MDA subjects. At higher exposure levels, rs7579 T was associated with higher urine Hg levels even though the opposite was observed at lower exposures (see Table 2.4, Figure 6.2). While the exposure-SNP interaction could not be assessed in the ELEMENT population, a similar main effect of rs7579 was observed with decreasing children's urine Hg levels and the T allele. Therefore, this SNP should be considered in future work on genetic factors and Hg

toxicokinetics. Presently, the nominally significant associations between SEPP1 rs7579 T and decreased urine Hg in the ELEMENT and MDA cohorts should be merely considered trends as neither remains statistically significant after correction for multiple testing.

The explanation for the paucity of significant SNP-Hg biomarker associations in the ELEMENT cohort may be multifaceted. While some studies have found nominally significant associations between unadjusted mean or median biomarker levels and SNPs (Gundacker et al., 2009; Chapter 2, this dissertation), most significant relationships with Hg biomarkers involved interactions between SNPs and exposure sources (Custodio et al., 2004; Engström et al., 2008; Gundacker et al., 2009). SNPs genotyped in the ELEMENT cohort were selected for their involvement in Hg distribution, metabolism and elimination. Therefore, influence of SNPs on Hg biomarker levels would depend on the dose of methylmercury or elemental/inorganic Hg exposure from sources such as fish consumption and dental amalgams, respectively. Exposure source-SNP interactions were not computable in this cohort. Consequently, we tested merely for detectable changes in mean biomarker Hg levels, with and without adjustment for age, BMI, education, and gender. Significant modification by SNPs genotyped here on Hg uptake and distribution to hair, blood, or urine following exposures to methylmercury or elemental Hg could not be examined.

In addition to the lack of exposure source data, overall power to detect significant associations was limited by sample size (smallest sample size of 254 in mothers' hair Hg model with *GSTM3* rs1332018) and minor allele frequency in some models (MAF<10% for *GCLC* rs17883901, *GCLM* rs41303970; *GSTP1* rs1138272, *GSTO1* rs11509438,

GSTM1 rs1065411, *SEPP1* rs3877899). Many genotyped SNPs including *GSTP1* rs1695 and rs1138272 (Hu et al., 1997; Chapter 5, this dissertation), *SEPP1* rs7579 and rs3877899 (Méplan et al., 2009), and *GPX4* rs713041 (Méplan et al., 2008) were evaluated due to previous research relating these SNPs to changes in protein function or gene expression. However, some SNPs (e.g., *GSTP1* rs749174, *GPX2* rs4902346) are tag SNPs, and their impact on gene expression or protein function could be nonexistent or might be dependent on another SNP in linkage disequilibrium. Future gene-Hg work incorporating tag SNPs may benefit from analyzing haplotypes instead of single SNPs (Lake et al., 2003).

The limited significance of SNP-Hg relationships observed in this study may be further reduced by absence of Hg exposure source data, confounding inherent in studies of admixed populations, the limited cross-sectional study design, or errors associated with multiple testing. Out of all SNP-Hg biomarker combinations tested, only one SNP (*SEPP1* rs7579) significantly associated with a biomarker (urine Hg). We cannot rule out the possibility that this association occurred by chance or was due to widely different Hg exposure levels between *SEPP1* genotype groups in this cohort.

In recently admixed populations such as the Mexican population, ancestry proportion (Native American vs. European vs. African) can confound gene-environment relationships due to influential SNPs in linkage disequilibrium with the SNP of interest and/or disproportionate exposure to toxicants related to race (Gee and Payne-Sturges, 2004). While we did not perform admixture mapping with ancestry-informative markers in this cohort, we controlled for mothers' education as SES is often associated with percent ancestry (Florez et al., 2009) and toxicant exposure levels. While controlling for

SES may partially account for percent ancestry and its potential relationship with exposure levels, SES was not significantly correlated with the only biomarker (urine Hg) associated with a SNP (SEPP1 rs7579). In the event that *SEPP1* rs7579 T was found more frequently among individuals of a given race (e.g., Caucasian) and Mexicans with higher percent Caucasian ancestry had lower exposure to elemental Hg and/or higher prevalence of other SNPs influencing quicker urinary Hg elimination compared with Mexicans with predominantly Native American or African descent, rs7579 T would appear associated with decreased urine Hg due to these confounding factors. We did not fully adjust for percent admixture (merely for SES) and cannot rule out the possibility that the relationship between *SEPP1* genotype and urine Hg is a spurious association brought on by confounding from population stratification.

The aforementioned limitations negate our ability to provide conclusive evidence about the *SEPP1* rs7579 SNP and its impact on urine Hg levels and/or inorganic Hg metabolism and elimination. Nevertheless, the known impact of this SNP on gene expression and isoform prevalence (Méplan et al., 2009) and the similar finding in our Michigan Dental Association cohort (see Chapter 2) make rs7579 an intriguing SNP worthy of future study pertaining to Hg toxicokinetics.

Despite several study limitations, this study contributes to Hg exposure assessment, and genomics pertaining to a Mexican population. This is the first study, to our knowledge, to analyze Hg levels in Mexican mothers and children from a general urban population. Furthermore, we report genotype frequencies of SNPs in genes crucial to both antioxidant defense and metabolism and elimination of toxicants including Hg in a population living in Mexico. Since no outstanding associations between Hg biomarkers

and SNPs were observed, the need to assess SNP-exposure source interactions in future work is evident.

Table 3.1 SNPs Genotyped in ELEMENT Cohort. Descriptions of 17 SNPs genotyped in mother-child pairs (n=355 mothers, 369 children) compared with reference population with Mexican ancestry. Several SNPs were selected due to known impacts on gene expression, protein structure, or enzyme activity summarized in the table (with references). SNPs without listed functional impacts were selected as tag SNPs.

	Gene	dbSNP ID	Alleles	SNP Details	MAF[a], ELEMENT	MAF[b], MXL ref.	Functional Impact[e]	Reference
GSH Synth.	GCLC	rs17883901	G>A	5' near gene	0.04	N/A	A ↓ promoter activity	Koide et al. 2003
	GCLM	rs41303970	C>T	5' near gene	0.08	N/A	T ↓ promoter activity	Nakamura et al. 2002
Glutathione S-Transferase	GSTP1	rs1695	G>A	Missense (Val105Ile)	0.45	0.45	Val ↓ activity	Ali-Osman et al. 1997
	GSTP1	rs749174	C>T	Intron	0.13	0.24		
	GSTP1	rs1138272	C>T	Missense (Ala114Val)	0.01	0.08	Val ↓ activity?	Ali-Osman et al. 1997
	GSTP1	rs6591256	A>G	5' near gene	0.16	0.31		
	GSTP1	rs947895	C>A	3' near gene	0.13	0.24		
	GSTO1	rs4925	C>A	Missense (Ala140Asp)	0.16	0.19	Asp ↓ activity	Tanaka-Kagawa et al. 2003
	GSTO1	rs11509438	G>A	Missense (Glu208Lys)	0.003	0.02		
	GSTM1	rs1065411	C>G	Missense(Lys173Asn)	0.04	0.49[c]	Lys ↓ [GSTM1]	Moyer et al. 2007
	GSTM3	rs1332018	G>T	5' UTR	0.34	0.37		
	GSTM3	rs7483	G>A	Missense (Val224Ile)	0.47	0.37	Val ↓ activity	Tetlow et al. 2004
Selenoprotein	SEPP1	rs3877899	C>T	Missense (Ala234Thr)	0.09	0.11	Thr ↓60 kDa	Méplan et al. 2009
	SEPP1	rs7579	C>T	3' UTR	0.43	0.43	C ↓ [SEPP1], ↓ 60 kDa	Méplan et al. 2007, 2009
	GPX4	rs713041	C>T	3' UTR	0.29	0.37	T ↓ [GPX4]	Méplan et al. 2008
	GPX2	rs4902346	A>G	Intron	0.21	0.19[d]		
	GPX2	rs2737844	C>T	Intron	0.4	0.4		

[a] MAF= minor allele frequency
[b] MXL is a HapMap population consisting of individuals of Mexican ancestry living in Los Angeles, CA
[c] Data not available for HapMap MXL. MAF from Mexican Americans in Moyer et al. (2007; rs1065411) or Environmental Genome Project, Hispanic population (rs4902346).
[d] The allele associated with the 'detrimental' effect is listed followed by a brief description. Activity refers to enzymatic activity unless otherwise specified. [Protein name] refers to protein concentration, and 60 kDa is an isoform of SEPP1 with the most selenocysteine residues.

Table 3.2 Characteristics of Mother-Child Pairs of the ELEMENT Cohort.

	Children	**Age Stratified Children**		**Mothers**
	(n=368)[a]	6-11 years (n=212)	13 -15 years (n=156)	(n=353)
% Female	46.3	48.6	43.3	100
Age (years)	11.1 (2.4)	9.1 (1.0)	13.7 (0.6)[d]	36.0 (5.8)
BMI (kg/m^2)	20.4 (4.1)	19.0 (3.5)	22.3 (4.2)[d]	28.3 (5.1)
BMI (percentile)[b]	67.5 (27.9)	67.7 (27.8)	67.3 (28.0)	
Education (years)[c]	10.3 (3.1)	10.8 (3.0)	9.5 (3.1)[d]	10.3 (2.9)

[a] Children and mothers with genotype data included here. Some subjects do not have all of the demographic data (n=340 children and 329 mothers with education; n=331 mothers with age).
[b] BMI percentile compares child's BMI to the distribution of same age and gender U.S. children.
[c] Mothers' education.
[d] $p<0.001$ for ANOVA comparing two age-stratified groups.

Table 3.3 Mercury Biomarker Levels and Ratios of A) All ELEMENT Samples and B) Children Stratified by Age.

A	Biomarker	N	Mean	SD	25th%	50th%	75th%	90th%	95th%
Children	Blood Hg (μg/L)	320	1.69	1.34	0.8	1.28	2.21	3.21	4.44
	Hair Hg (μg/g)	345	0.49	0.44	0.22	0.38	0.63	0.93	1.31
	Urine Hg (μg/L)	346	0.78	1.01	0.28	0.48	0.82	1.75	2.44
	Hair:Blood	304	324	151	218	299	406	518	597
	Blood:Urine	303	3.69	3.31	1.55	2.63	4.93	8.33	10.7
Mothers	Hair Hg (μg/g)	302	0.53	0.47	0.23	0.39	0.7	1.02	1.3

B	Biomarker	n	Mean	SD	25th%	50th%	75th%	90th%	95th%
6-11 yrs	Blood Hg (μg/L)	188	1.80	1.42	0.79	1.36	2.39	3.48	4.62
13-15 yrs		132	1.53[a]	1.22	0.80	1.14	1.81	2.97	4.42
6-11 yrs	Hair Hg (μg/g)	198	0.55	0.49	0.22	0.44	0.77	1.05	1.35
13-15 yrs		147	0.42[b]	0.35	0.22	0.33[c]	0.52	0.77	0.92
6-11 yrs	Urine Hg (μg/L)	203	0.87	1.13	0.30	0.53	0.98	1.85	2.76
13-15 yrs		143	0.65[a]	0.80	0.24	0.44	0.73	1.16	1.92
6-11 yrs	Hair:Blood	180	335	153	226	315	409	561	645
13-15 yrs		124	309	147	207	273[c]	393	504	565
6-11 yrs	Blood:Urine	184	3.64	3.38	1.50	2.49	4.82	7.28	10.7
13-15 yrs		119	3.77	3.23	1.58	2.68	5.23	9.01	10.7

[a,b] $p<0.05$, $p<0.01$ ANOVA comparing natural-log transformed biomarker across two age groups.
[c] $p<0.05$ for Median test comparing biomarker medians across two age groups.

Table 3.4 Correlations Between Hg Biomarkers and Demographic Variables.
Pearson correlation coefficients are reported. All biomarker variables were natural log-transformed before analysis, and urine Hg was adjusted to the mean specific gravity (1.017). Biomarkers are children's unless otherwise indicated.

	Blood Hg	Hair Hg	Urine Hg	Hair:Blood	Blood:Urine
BMI, percentile	-0.01	-0.09	-0.06	-0.10	0.05
Age	-0.15[b]	-0.17[b]	-0.13[a]	-0.04	0.02
Education	0.13[a]	0.15[b]	0.06	0.01	0.07
Mother's Hair Hg	0.37[c]	0.47[c]	0.19[b]	0.1	0.17[a]
Blood Hg	1	0.77[c]	0.47[c]	-0.27[c]	0.45[c]
Hair Hg		1	0.37[c]	0.40[c]	0.30[c]
Urine Hg			1	-0.08	-0.58[c]
Hair:blood				1	-0.18[b]

[a,b,c] Denote p-value: $p<0.05$, $p<0.01$ and $p<0.001$, for correlation.

Table 3.5 Base Models for Hg Biomarkers. Parameter estimates are reported from multivariate linear regression before addition of genotype. P-value is indicated by: *p<0.05, †p<0.01. All biomarker dependent variables are first natural-log transformed and urine is adjusted to mean specific gravity. Biomarkers are for children unless otherwise indicated.

Dependent Variable	Adj. r^2	n	Age	Gender	BMI[a]	Mother's Education	Urine Hg[b]
Blood Hg	0.02	295	-0.04*	0.06	0	0.02	
Hair Hg	0.04	316	-0.05*	0.03	-0.003	0.03*	
Urine Hg	0.01	317	-0.03	0.14	-0.002	0.01	
Hair:Blood	0.03	265	-0.01	-0.04	-0.002	0.002	-0.12†
Blood:Urine	0	279	0.01	-0.08	0.001	0.02	
Mother's Hair Hg	0.04	277	0.03†		-0.007	0.04*	

[a] In children's models, BMI is a percentile based on U.S. children of the same age and gender. BMI is not transformed (kg/m^2) in mothers' models.
[b] Hair:blood model contains urine Hg (specific gravity adjusted) to control for inorganic Hg exposure.

Table 3.6 Linear Regression Modeling of Hg Biomarker: Influence of Genotype. Biomarker variables are natural-log transformed and from the children unless otherwise indicated. Urine Hg is adjusted for mean specific gravity. Beta coefficients for genotype with p<0.10 are reported and one significant coefficient (p<0.05) is shaded.

		Simple Linear Model[a]						Multivariate Model[a,b]					
Gene	**dbSNP ID**	**Blood**	**Hair**	**Mother's Hair**	**Urine**	**Hair:Blood**	**Blood:Urine**	**Blood**	**Hair**	**Mother's Hair**	**Urine**	**Hair:Blood[c]**	**Blood:Urine**
GCLC	rs17883901									-0.28			
GCLM	rs41303970												
GSTP1	rs1695						-0.11						-0.10
GSTP1	rs749174		0.17										
GSTP1	rs1138272												
GSTP1	rs6591256												
GSTP1	rs947895		0.16							-0.19			
GSTO1	rs4925												
GSTO1	rs11509438												
GSTM1	rs1065411												
GSTM3	rs1332018												
GSTM3	rs7483												
SEPP1	rs3877899					-0.12	0.20						
SEPP1	rs7579				-0.10						-0.14		
GPX4	rs713041									-0.15			
GPX2	rs4902346			-0.14									
GPX2	rs2737844												

[a] Number of subjects in simple regression models range from 254 to 345 and sample size in multivariate models range from 233 to 317. Mothers' hair models have the least subjects and children's hair models have the most subjects. *GSTM3* (rs7483) genotype models have the smallest sample size.
[b] Models adjusted for age, BMI (percentile for children or kg/m^2 for mothers), gender, and mother's education.
[c] Hair:blood multivariate model additionally adjusted for urine Hg levels (non-transformed but specific gravity adjusted) to control for inorganic Hg exposure.

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Chapter 4

Methylmercury and Elemental Mercury Differentially Associate with Blood Pressure among Dental Professionals[2]

4.1 Introduction

Mercury is ranked a top three priority pollutant by the U.S. Environmental Protection Agency (EPA; US EPA, 1997) and the Centers for Disease Control (ATSDR, 2007). The chemical speciation of mercury is complex and dictates its environmental fate, human exposure pathways, and toxic impacts (Clarkson and Magos, 2006). The general population is largely exposed to methylmercury ($MeHg^{+}$) through fish consumption and to elemental mercury (Hg^{0}) through dental amalgams. Approximately 6,600 tons of mercury is released into the atmosphere annually and concentrations continue to rise in many regions of the world (Swain et al., 2007). Accordingly, mercury will remain of public health concern for the foreseeable future.

Health concerns associated with methylmercury and elemental mercury exposure are primarily focused on the nervous system (Clarkson and Magos, 2006; US EPA, 1997). However, in recent years epidemiological studies have suggested a negative impact of methylmercury on the cardiovascular system. Methylmercury exposure has been linked to acute myocardial infarction, and a US EPA-sponsored committee deemed

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the evidence compelling to include this outcome in its regulatory risk assessment of mercury (Roman et al., 2011). Though discrepancies exist, many studies have also found methylmercury-associated increases in diastolic (DBP) and systolic blood pressure (SBP). In a study of 251 fish-consumers in the Brazilian Amazon, Fillion et al. (2006) found that participants with higher hair mercury, a biomarker for methylmercury exposure, had an increased risk of elevated SBP. In a study of 42 male Faroese whalers, Choi et al. (2009) found a positive association between blood total mercury levels, also reflective primarily of methylmercury exposure, and both SBP and DBP. In another study from the Faroe Islands, Sørensen et al. (1999) found increased SBP and DBP in 7-year-old children in relation to prenatal methylmercury exposure, though this association was not observed when children were re-evaluated at 14 years old (Grandjean et al., 2004). Likewise, Valera et al. (2009) found a positive association with blood mercury and SBP in an Inuit population. From the 1999-2000 US National Health and Nutrition Examination Survey (NHANES), Vupputuri et al. (2005) found a negative association between blood total mercury and SBP but only in women that did not consume fish.

The notion that methylmercury may be associated with increased risk of hypertension poses several health dilemmas. Hypertension may affect one billion people worldwide (including 65 million in the US) and rates continue to rise (Egan et al., 2010; Lawes et al., 2008). Methylmercury is mainly derived from fish consumption, but fish are promoted as an excellent source of nutrients (e.g. omega-3 fatty acids) and protein. Scientific reviews have concluded that the heart-protective benefits of fish consumption outweigh health risks (Mozaffarian and Rimm, 2006), but when faced with the decision many consumers chose to avoid consuming fish (Oken et al., 2003).

In addition to methylmercury exposure, the general public is also exposed to elemental mercury largely through dental amalgams. Though several animal studies have documented that elemental mercury may decrease myocardial mechanical activity, depress heart rate, promote heart arrhythmias, and cause hypotension (Massaroni et al., 1995; Rhee and Choi, 1989; Rossoni et al., 1999), to our knowledge these relationships have not been investigated in an epidemiological study.

Hypertension is a multifactorial health trait influenced by environmental and genetic factors, many of which remain undiscovered. Genome wide association studies in several populations have linked potential single nucleotide polymorphisms (SNPs), primarily in genes involved in some aspect of cardiovascular function or regulation, to increased blood pressure. However, results are inconsistent across studies, and the associated SNPs explain only a fraction (<5%) of blood pressure variability (Adeyemo et al., 2009; Levy et al., 2007; for review: Kraja et al., 2011). Oxidative stress is a potential mechanism underlying hypertension, and several studies have linked polymorphisms in antioxidant enzymes to hypertension (Bessa et al., 2009; Mansego et al., 2011). Recent evidence suggests that polymorphisms in antioxidant genes of the glutathione pathway may modify metabolism and elimination of methylmercury (Custodio et al., 2004; Engström et al., 2008; Gundacker et al., 2007, 2009) and elemental mercury exposure (Custodio et al., 2005) as assessed with common biomarkers. Gene-environment interactions influencing hypertension are not well studied, and may contribute to the onset of this prevalent disease state. Polymorphisms in glutathione pathway genes and selenoproteins, two antioxidant gene classes that are involved in the toxicokinetics of

mercury, may interact with methylmercury or elemental mercury to modify the toxicants' impact on blood pressure.

The goal of this study was to increase understanding of the association between mercury exposure (both methylmercury and elemental mercury) and blood pressure in a cohort of dental professionals and the modifying effect of polymorphisms in mercury metabolizing genes on these relationships. This work extends upon previous studies that focused solely on methylmercury exposure by also considering exposures to elemental mercury and gene-environment interactions. Further, mercury exposures in this study are more relevant to the general population than the aforementioned studies focused on susceptible groups (e.g., indigenous peoples, fish-consumers) with moderate to high methylmercury intakes.

4.2 Materials and Methods

4.2.1 Study Population

A convenience sample of 284 dental professionals (dentists, hygienists, dental assistants) was recruited during the 2010 Michigan Dental Association (MDA) Annual Convention as part of a larger cohort designed to study the influence of genetic variability on mercury body burden. Institutional Review Board (IRB) approval for this work was obtained from the University of Michigan (HUM00027621). A self-administered survey was used to collect information on demographics (e.g., age, height), occupational practices, medical history, and alcohol consumption. Subjects also provided detailed information on fish consumption patterns (e.g., portion size, frequency of consumption, fish species) which was used to calculate a mercury intake value (μg mercury/kg body

weight/day) based on the most recent mercury levels measured in common fish species in the US (Bahnick et al., 1994; Mierzykowski et al., 2001; US FDA). Total polyunsaturated fatty acid (PUFA; mg/kg body weight/day) and selenium (μg/kg body weight/day) intake values from fish consumption were also calculated using the US Department of Agriculture Nutrient Database. Subjects reported the number of mercury-containing dental amalgams in their own mouths along with the average number per week that they remove and place in their dental practice (amalgams handled). Subjects with missing data points (e.g., urine mercury, SBP, age) were excluded. Four additional subjects reporting kidney disease were excluded due to the potential effects on mercury excretion, resulting in a sample size of 262.

4.2.2 Mercury Exposure Assessment

Urine is used to assess elemental mercury exposure and hair is used to assess methylmercury exposure (Berglund et al., 2005; Clarkson and Magos, 2006). From each participant, spot urine samples (~30-50mL) were collected and stored frozen. Hair was collected by cutting 20-50 strands from the occipital region of the head as close to the scalp as possible, wrapping in paper, and then storing at room temperature.

Total mercury levels were measured using a direct mercury analyzer (DMA-80, Milestone Inc., CT) according to US EPA Method 7473. Briefly, 800 μL of urine or 4-9 mg of hair from the two cm closest to the scalp were analyzed according to methods we have previously described (Basu et al., 2010; Paruchuri et al., 2010). In every batch of 10-15 samples, one blank, one replicate sample, and a certified reference material (hair: NIES Japan CRM #13; urine: Institut National de Sante Publique Quebec standard

QMEQAS08U-01; dogfish liver: DOLT4, National Research Council Canada) were included. Specific gravity was measured using a refractometer (PAL-10S, Atago U.S.A., Inc., WA). Urine mercury levels were adjusted to reflect the average specific gravity in all samples (1.017) according to the method of Levine and Fahy (1945) as this has been shown to reduce variability in metal analysis of spot urine samples (Lee et al., 1996; Mason and Calder, 1994). All final values reported here are unadjusted.

The average theoretical method detection limit (3x standard deviation of blanks) was 0.003 µg/g mercury for hair and 0.014 µg/L mercury for urine. The average recovery of mercury was 88.9±1.1% for the hair CRM, 71.5±3.9% for the urine CRM, and 91.8±6.6% for DOLT4. The mercury value in the urine CRM has a range of expected values, and our percent recovery was judged according to the reported mean. Machine accuracy is deemed high given that recovery of other reference materials (e.g., DOLT4) measured alongside the urine CRM had excellent recovery (>90%). Within-day (0.7% for hair, 4.2% for urine, 2.8% for DOLT4) and between day (1.0% for hair, 5.4% for urine, 6.1% for DOLT4) variability of CRMs were calculated, and these values corresponded well to replicate analysis of actual samples provided by participants (data not shown).

4.2.3 Blood Pressure and Pulse Assessment

Participants were seated for at least five minutes before blood pressure was measured. A commercially available blood pressure device (Omron HEM 432-C) was placed over the right brachial artery and used to measure SBP, DBP, and pulse. From

each participant, three readings were averaged. Variability within replicates of individuals averaged 4.2% (SBP), 4.8% (DBP), and 3.3% (pulse).

4.2.4 Genotype Analysis

The majority of subjects provided buccal cell samples that were subsequently stored at room temperature in buffer described by Min et al. (2006). SV Wizard Genomic DNA purification kits (Promega Corporation, Madison, WI) and PicoGreen reagent (Invitrogen, Eugene, OR) were used to purify and quantify DNA, respectively, from buccal samples. Ten SNPs in glutathione s-transferase (GST), glutathione synthesizing, and selenoprotein genes (rs1138272, rs1695, rs3877899, rs41303970, rs7579, rs5751901, rs7483, rs3761144, rs1002149, rs2911678) were genotyped using pre-designed Taqman Allelic Discrimination Assays (Applied Biosystems, Foster City, CA; Livak et al., 1995), and results were interpreted with an ABI 7700 (Carlsbad, CA). One deletion polymorphism in GST theta 1 (*GSTT1*) was additionally genotyped using the method of Mordukhovich et al. (2009). All polymorphisms were selected in genes of the mercury toxicokinetic pathway and were initially tested for association with mercury biomarker levels (see Chapter 2). Several polymorphisms are tag SNPs while others have known impacts on gene expression or enzyme function (summarized in Table 2.2). Genotype data resulted in three distinct groups (major homozygotes, heterozygotes, minor homozygotes) for the ten SNPs, and two groups (gene present or deleted) for the *GSTT1* polymorphism.

4.2.5 Statistical Analyses

All statistical operations were performed using PASW® Statistics Software (v. 18; Chicago, IL). Preliminary data analysis included tabulation of descriptive statistics for all measurements. Bivariate (Pearson correlations) and multivariate analyses were used to identify factors that influenced SBP and DBP. Blood pressure measurements of individuals using hypertension controlling medications were imputed 15 mmHg higher (SBP) and 10 mmHg higher (DBP) before linear regression as this has been shown to reduce bias and improve statistical power (Tobin et al., 2005). All bivariate and multivariate analyses were performed with adjusted and unadjusted SBP and DBP; analyses with the latter excluded subjects using anti-hypertensive medication (n=39).

The backward elimination method was used to determine predictors of SBP and DBP (adjusted for medication use) with an initial cut-off significance value of $p>0.10$. Variables considered in the multivariate models were age, BMI, gender, race, occupation (dentist vs. non-dentist), alcohol (drinks/day), fish nutrients/toxicants (PUFA, selenium, mercury), personal amalgams, and occupational exposures (hours worked/week, categorical variable for number of amalgams handled/week). The final model for SBP included the only significant predictors ($p<0.05$): BMI, age, and gender. Significant predictors of DBP were BMI and age, though gender was also included in the final model to control for gender differences observed in our population. Hair and urine mercury (together and in separate models, with unadjusted or specific gravity adjusted urine mercury) were added into SBP and DBP base models to assess the association between mercury biomarkers and blood pressure after controlling for confounders. Multivariable linear regression models were run for the total population and for subgroups (males,

females, dentists, non-dentists). Potentially influential subjects were identified using statistical diagnostics (e.g. Cook's distance, dfbeta) on total population models, and removed individually to assess the impact of the subject on the relationships between mercury biomarkers and blood pressure.

Genotype data was added into the final models for adjusted SBP and DBP with decreased sample size as not all subjects provided adequate DNA for successful genotyping (n=205 to 239 depending on the polymorphism). One ordinal variable per polymorphism, assuming additivity of variant alleles, was added into SBP or DBP models containing hair or urine mercury. An interaction term with the included mercury biomarker was also tested in each model.

4.3 Results

4.3.1 Descriptive Statistics

Table 4.1 outlines demographics, cardiovascular parameters, and major sources of mercury exposure in study participants, and is stratified according to genotype (includes subjects successfully genotyped for the *SEPP1* 3'UTR locus), gender, occupation (dentists versus non-dentists), and anti-hypertensive medication usage. Of all participants, 38% were males and 44% were dentists. Overall, males were significantly older, had greater BMIs and alcohol consumption compared with females while also having higher blood pressure and lower pulse. Dentists, of which 80% are males, likewise had similar differences compared to non-dentists (dental hygienists, dental assistants and other professionals, of whom 94% were female). A significantly larger proportion of individuals taking blood pressure medication were males and dentists (χ^2 test, p-value

<0.05, data not shown). The influence of race-ethnicity on blood pressure could not be adequately assessed in this population as 92% of the subjects identified as non-Hispanic and Caucasian. Not all individuals provided adequate DNA for successful genotyping of all polymorphisms, limiting genetic model sample sizes (n=205 to 239). ANOVA tests comparing demographic characteristics, blood pressure, and mercury biomarker levels for subjects with genotype (n=232) and those without (n=30) revealed no significant differences.

4.3.2 Mercury Exposure Assessment

Table 4.2 reports total mercury levels in hair and urine. In this population, estimated mercury intake from fish consumption was the best predictor of hair mercury levels in linear regression modeling, though personal dental amalgams contributed to a lesser extent. Occupation and amalgams (personal and handled in the dental practice) were the predictors of urine mercury levels (data not shown) indicating hair and urine as biomarkers of primarily methylmercury and elemental mercury, respectively, as others have previously established (Berglund et al., 2005; Clarkson and Magos, 2006) with some crossover in the hair biomarker. All subjects had mercury levels above the method detection limit. Mean hair mercury (±SD) was 0.45±0.53 μg/g (range: 0.02-5.18) and mean urine mercury was 0.94±0.99 μg/L (range: 0.03-5.54). While median hair and urine mercury values were 47% and 31% higher than U.S. population medians reported by NHANES (CDC, 2009; McDowell et al., 2004), there is considerable overlap of the distributions for both biomarkers between the dental cohort and NHANES (Table 4.2). Mean hair and urine mercury levels were significantly higher in males and dentists, the

latter of which correspond with greater occupational exposure to amalgams (ANOVA $p<0.05$).

4.3.3 Genotype Data

Minor allele frequencies of the polymorphisms were similar to that of Caucasian populations (HapMap, data not shown). Table 4.3 displays p-values from ANOVA tests comparing linear trends among unadjusted means of study parameters for each additional variant allele. Cardiovascular parameters and mercury biomarker levels were not significantly different among genotype groups for any of the eleven polymorphisms though the difference in DBP (adjusted for blood pressure medication use) among *GSTM3*-224 genotype groups was borderline significant ($p=0.05$) and may have occurred by chance alone due to multiple testing.

4.3.4 Blood Pressure Measurement

Seventy-three participants (28% of study population) displayed hypertension (SBP $\geq$ 140 mmHg and/or DBP $\geq$ 90 mmHg as defined by the US Department of Health and Human Services, 2004) and/or were using blood pressure medication at the time of measurement. Blood pressure measurements performed by us were in the hypertension range for 47 individuals (18%). When mercury exposures were related to blood pressure outcomes, several significant associations were found ($p<0.05$). Bivariate analyses estimated that SBP and DBP (adjusted for anti-hypertensive medication use) were significantly correlated with hair mercury levels ($r=0.22$, 0.19, respectively). There were no significant bivariate correlations between urine mercury and adjusted SBP ($r=0.05$) or

DBP (r=0.06). BMI and age were significantly positively correlated with adjusted SBP (r=0.33, 0.58) and DBP (r=0.38, 0.31)). Hair and urine biomarker measurements were also significantly correlated with one another (r=0.29).

4.3.5 Blood Pressure and Mercury Biomarkers

Multivariate linear regression modeling of SBP and DBP was used to assess associations with urine or hair mercury levels after adjusting for BMI, age, and gender. Parameter estimates for total, gender stratified, and dentist only populations in models of SBP and DBP adjusted for hypertension-controlling medication use are reported in Table 4.4. In the majority of models, BMI, age, and gender were significant predictors of these outcomes. There was a trend towards positive association of hair mercury with SBP and DBP in all models, though this association was only significant when modeling adjusted DBP (β=2.76 mmHg DBP increase per 1 ug/g Hg in hair). Further, the parameter estimates were consistently larger in males versus females (e.g., hair Hg parameter estimate in DBP model: β=2.94 mmHg among males, 1.87 mmHg among females). Alcohol consumption (drinks/day) and dental amalgams were near significant predictors ($p<0.10$) of adjusted DBP. However, inclusion of these parameters in the DBP model did not change parameter estimates (significance, magnitude) of mercury biomarkers (data not shown).

The urine mercury and blood pressure relationship differed from hair mercury results. Urine mercury levels were associated with decreased SBP (in total population model: β= -1.8 mmHg SBP per 1 ug/L Hg in urine), though this was only significant in models adjusting for anti-hypertensive medication use and appeared to be driven by the

males and the dentists. Urine mercury was not associated with DBP, though negative trends were also observed among males and dentists. Even though several model parameters were significantly correlated with one another (e.g. BMI and age, hair and urine mercury), multicollinearity is not expected to be problematic as variance inflation factors were less than 1.5 for all aforementioned regression models.

The significance levels of parameter estimates for mercury biomarkers in blood pressure models were sensitive to several influential subjects discovered via standard diagnostic tests. The exclusion of one subject partially diminished the association between hair mercury and adjusted DBP (β=2.29 mmHg, p=0.07). The magnitude and significance of the association between urine mercury and decreased SBP were slightly diminished when excluding several influential subjects, most of whom had urine mercury levels above the 95th percentile ($0.06<p<0.13$ for new parameter estimates). Adjusting urine mercury for specific gravity altered its significance in the total population model of SBP (β= -1.71 mmHg, p=0.14), though adjusted urine mercury remained significant in models of SBP with males or dentists alone.

4.3.6 Blood Pressure, Mercury Biomarkers, and Genetic Polymorphisms

The impact of polymorphisms on blood pressure and their interaction with hair or urine mercury were examined in the linear regression models of adjusted SBP and DBP (Table 4.5 and 4.6, models with hair and urine mercury, respectively). In linear regression models with hair mercury, no gene-biomarker interaction terms or genetic main effects were significant ($p<0.05$), though several interaction terms were borderline significant ($p<0.10$), and these polymorphisms warrant further investigation in a larger sample

(*SEPP1*-234, *GCLM* 5', *GSTT1* deletion). Likewise, genetic variables in SBP and DBP models including urine mercury failed to attain statistical significance apart from one main effect (*GCLM* 5', SBP model). Two interaction terms (*GSTM3*-224 and *GSTT1* deletion with urine mercury) were near significant ($p<0.10$). The observed nominally significant/near significant polymorphism-blood pressure relationships (main effect or interaction with mercury) could have occurred by chance alone given the number of tests (44 models).

4.4 Discussion

There are a growing number of studies documenting associations between methylmercury exposure and elevated blood pressure but discrepancies exist. Despite the fact that our cohort was not initially designed to study cardiovascular effects of mercury exposure and lacks information on one important potential confounder- smoking status, our study contributes to data on mercury exposure and blood pressure in several ways. Here we report that exposures relevant to the general population to both elemental mercury and methylmercury may be associated with altered blood pressure measures, though the significance of these results is partially dependent on several subjects with higher exposure (>95th percentile for biomarker levels). Interestingly, divergent blood pressure results were found for mercury type and may be influenced by gender. Hair mercury levels were associated with increased DBP (after adjustment for anti-hypertensive medication use according to the method of Tobin et al., 2005). For urine mercury, the results from linear regression models suggest that elemental mercury exposure is associated with decreased SBP in the total population, and this appears to be driven by the male subgroup. While Kobal et al. (2004) previously found an association

between extremely high past exposures to elemental mercury (>800 μg/L urinary mercury) and increased SBP, to our knowledge this is the first human study to investigate elemental mercury exposures relevant to the general population in relation to blood pressure.

Previous studies have reported associations between methylmercury exposure biomarkers and increased blood pressure (Choi et al., 2009; Fillion et al., 2006; Sørensen et al., 1999; Valera et al., 2009), but these have largely been conducted in populations of subsistence fish and marine mammal consumers that experience moderate to high methylmercury exposures. Here, we find a similar trend between elevated blood pressure and hair mercury levels in a population that is exposed to methylmercury at concentrations that better reflect exposures of the general US population (McDowell et al., 2004) and other countries (Díez et al., 2008; Gundacker et al., 2007). As expected, the male gender, age, and BMI were significant predictors of increased SBP. Likewise, age and BMI predicted DBP in multivariate linear regression, factors which are often associated with increased risk for hypertension (Greenlund et al., 2009; Kim et al., 2007). In addition, we found a trend towards a methylmercury exposure dependent increase in SBP and DBP across all sub-groups in our study (e.g., males, females, dentists, excluding medication users), though this relationship only attained statistical significance in models of adjusted DBP and was partially dependent on one influential subject.

The prevalence of hypertension in our study population (28% of total had SBP ≥ 140 mmHg, DBP ≥ 90 mmHg, and/or reported using anti-hypertensive medication) is similar to the US average of 28.9% which continues to increase (Cutler et al., 2008). The fact that we found a weak association between "background" methylmercury exposure

and increased DBP within this cohort suggests that the magnitude of effect may be more pronounced in sensitive or greater exposed groups. These findings are of public health concern given that nearly 30% of adults in the US and ~1 billion worldwide may suffer from hypertension, and that elevated blood pressure accounts for 54% of strokes, 47% of heart disease, and 14% of all deaths (Lawes et al., 2008).

This is the first study, to our knowledge, to directly assess the relationship between relevant elemental mercury exposure and blood pressure outcomes in a human population that experiences background exposures. Dental amalgams typically consist of 50% mercury by weight (Clarkson and Magos, 2006). Accordingly, urine mercury levels among dental practitioners are strongly predicted by the number of amalgams they remove or place (Martin et al., 1995). In the 1970s and 1980s, urine mercury levels in dentists regularly exceeded 10 µg/L but values have dropped significantly in recent years owing to educational campaigns and a shift towards composite resin fillings (Eklund, 2010; Shapiro et al., 1982). This decrease is supported by the current study where urine biomarkers of elemental mercury exposure among dental professionals in Michigan mirrored the general US population (CDC, 2009; Table 4.2), suggesting that our findings may have broad relevance to public health. Despite low-level elemental mercury exposure (maximum=5.5 µg/L), associations with SBP were found. Unlike the hair mercury associations, a urine mercury-associated decrease in adjusted SBP was observed in the total population and was driven by the males and dentists. The significance of this association was influenced by several subjects with higher urine mercury levels, and as such this relationship should be further explored in a population with a wider range of exposure (maximum >10 µg/L). These findings suggest that levels of urine mercury

found in the general adult US population, which average 20-100 times less than exposure limits set by the World Health Organization (50 μg/L), may be associated with alterations in blood pressure and that these may be gender-specific. Elemental mercury exposures have decreased tremendously for the general population though certain groups still remain at great risk of elemental mercury exposure, such as small-scale gold miners (Paruchuri et al., 2010).

For hair and urine mercury, gender influenced the observed trends. At this moment it is not clear why elemental mercury-associated decreases in SBP are observed in males only, or why methylmercury-associated increases in blood pressure are stronger in males, though increasing toxicological and epidemiological studies are stressing the importance of considering gender-specific differences in chemical exposures, toxicokinetics, and health impacts (Institute of Medicine, 2001; Vahter et al., 2007). Experimental rodent studies have documented gender differences in the distribution, metabolism, and elimination of methylmercury and inorganic mercury (Ekstrand et al., 2010; Thomas et al., 1986, 1987). With respect to hypertension, gender-specific differences have been reported in women in terms of age-related onset and metal sensitivity (Reckelhoff, 2001; Vahter et al., 2007). The differences observed in this study may reflect true gender differences in the relationship between mercury and blood pressure, or they may have resulted from random variation due to small sample sizes.

In addition to disparate gender results, elemental mercury results differed from the methylmercury results in many cases. The effect of elemental mercury on cardiovascular function in humans is not well characterized, but there are laboratory animal studies that may shed light on our findings. The general trends observed in our elemental mercury-

exposed male population are consistent with animal studies reporting associations between high doses of inorganic mercury and depressed arterial systolic pressure (Massaroni et al., 1995; Rhee and Choi, 1989; Rossoni et al., 1999). Differences between elemental mercury and methylmercury effects may be realized at the cellular level. One purported mechanism by which mercury affects blood pressure is through disruption of calcium homeostasis, and there are reported differences between methylmercury and elemental mercury in terms of potency, sensitivity towards certain calcium channel subtypes, the nature of inhibition, and alteration of channel function (Atchinson, 2003; Sakamoto et al., 1996). Evidence in animals and humans suggests that methylmercury-induced oxidative stress can inhibit production of nitric oxide, a vasodilator, and lead to vascular endothelial dysfunction, mechanisms related to hypertension (Dharmashankar and Widlansky, 2010; de Marco et al., 2010; Grotto et al., 2009; Mazerik et al., 2010). Several differential mechanisms may underlie the opposite association observed between elemental mercury and SBP. Massaroni et al. (1995) found mercuric chloride increased autonomic neurotransmitter release in rats experiencing hypotension following treatment. Inorganic mercury may furthermore impact blood pressure indirectly via interaction with the kidney, an organ specifically targeted by inorganic species of mercury (Clarkson and Magos, 2006). Mercurial drugs such as calomel inhibited sodium and chloride reabsorption in the kidney and were formerly prescribed as diuretics and anti-hypertensive medication until the mid-1900s (Norn et al., 2008; Wolf et al., 1966). Interactions between elemental mercury, kidneys and decreased SBP merit further exploration.

This study examined the impact of polymorphisms in GSTs, glutathione synthesizing enzymes, and selenoproteins, all implicated in the distribution, metabolism and elimination of both elemental mercury and methylmercury in the body, on the relationship between biomarkers for elemental mercury and methylmercury exposure and hypertension, though no significant and compelling genetic main effects or gene-mercury interactions were observed. Genetic factors are known to contribute to etiology of hypertension. Polymorphisms in many genes involved with cardiovascular function (Adeyemo et al., 2009; reviewed by Kraja et al., 2011) and antioxidant pathways (Bessa et al., 2009; Mansego et al., 2011) are associated with increased blood pressure. Antioxidant gene polymorphisms furthermore modify the relationship between toxicants and biomarkers of oxidative stress, a mechanism linked to hypertension (Ren et al., 2010), and the accumulation of potentially cardiotoxic chemicals such as elemental mercury (Custodio et al., 2005) and methylmercury in the human body (Custodio et al., 2004; Engström et al., 2008; Gundacker et al., 2007, 2009). The polymorphisms genotyped in this study were selected primarily for their involvement in mercury toxicokinetics. The glutathione pathway and selenoprotein genes assessed also have the potential to impact cardiovascular health through their antioxidant roles as evident by the association between hypertension and *GSTT1/GSTM1* deletion polymorphisms according to Bessa et al. (2009). Even so, this study failed to find significant genetic main effects or polymorphism-mercury biomarker interactions in models of adjusted SBP or DBP beyond what is expected by chance given the number of models tested. Increasing the sample size and studying a population with a wider range of mercury exposures may allow for a better assessment of mercury-gene interactions and blood pressure. Several

polymorphisms (*SEPP1*-234, *GCLM* 5', *GSTT1* deletion, *GSTM3*-224) had near significant ($p<0.10$) main effects and/or interaction terms with hair and/or urine mercury in SBP and DBP models and should be explored in future studies.

Even though this study had several limitations, associations were found between low-level mercury exposures and blood pressure alterations. Associations and trends observed here corroborate several epidemiological (for methylmercury) and animal (for elemental mercury) studies, and thus diminish concern of chance-related significant outcomes stemming from multiple statistical tests. The voluntary nature of subject participation could have led to selection bias, but this is unlikely since subjects did not know their urine or hair mercury levels before participating in the study, and we observed mercury distributions that were in agreement with the NHANES survey. Due to the cross-sectional design, we were unable to assess the impact of past exposures or lifestyle changes on blood pressure. Gender stratification was performed on all analyses due to the age, BMI, mercury exposure and occupational differences observed between our male and female participants, but this may have limited our statistical power due to smaller sample size. Significant associations between hair mercury and DBP and urine mercury and SBP were still observed in the male population even with the decreased power. Gene-mercury interaction analyses were limited in statistical power due to small sample sizes in each model (n=205 to 239), and multiple testing (n=44 genetic models).

Our analyses did not include one major potential confounder- smoking status- as this information was not collected from our subjects. While smoking is often considered a risk factor for hypertension and has been shown to influence cadmium and lead biomarker levels, smoking has not been shown to affect mercury biomarker levels in

most studies (Dewailly et al., 2001; Levy et al., 2007), with exceptions (Freire et al., 2010). Another limitation of this study may be the lack of mercury speciation in biomarker samples. While hair and urine are typically deemed biomarkers of methylmercury and inorganic mercury exposure, respectively, (Berglund et al., 2005), evidence in occupational cohorts with exposure to elemental mercury suggests that a fraction of hair mercury may reflect inorganic mercury exposure (Morton et al., 2004; Wranová et al., 2008). In the MDA cohort, amalgams were weakly associated with hair mercury even though fish consumption was the main predictor. However, mercury speciation of the MDA biomarker samples would be predicted to increase the significance of the relationships observed (elemental mercury with decreased SBP and methylmercury with increased DBP) if the two mercury forms truly have opposing associations with blood pressure.

This study reports significant, albeit borderline significant ($0.01<p<0.05$) and partially outlier influenced, associations between elevated DBP and hair mercury and between decreased SBP and urine mercury at exposure levels relevant to the general population. Even though these differential relationships were observed in face of many study limitations, comparable significant associations were observed (blood mercury with increased DBP, $p<0.05$, and urine mercury with decreased SBP, $p<0.0001$) using NHANES data ($n>4,000$) after controlling for seven confounders including smoking status and race (data not published). Significant interactions between hair or urine mercury and genotype of eleven polymorphisms were not observed in this limited study of the MDA cohort. Future work on mercury and cardiovascular health should consider both elemental mercury and methylmercury at wide ranges of exposure in males and

females along with genetic factors to gain a better understanding of how these toxicants influence blood pressure and ultimately cardiovascular disease.

Tables

Table 4.1 Characteristics of Total and Stratified Study Population (mean ± SD).

	Total Population	Population with Genotype[a]	Gender		Occupation		Blood Pressure Medication	
			Males	Females	Dentists	Non-Dentists	No	Yes
n	262	232	99	163	114	148	223	39
BMI (kg/m^2)	26.4 (4.5)	26.5 (4.6)	27.2 (3.7)	25.9 (4.9)[c]	26.6 (3.9)	26.2 (4.9)	26.2 (4.5)	27.8 (4.3)[c]
Age (years)	52.3 (12.3)	52.3 (12.1)	60.2 (10.8)	47.5 (10.6)[e]	57.8 (11.4)	48.0 (11.3)[e]	50.7 (12.0)	61.4 (9.8)[e]
SBP (mm Hg)	124 (15.3)	125 (15.4)	133 (13.3)	119 (14.1)[e]	130 (15.2)	120 (13.9)[e]	123 (14.6)	135 (15.1)[e]
DBP (mm Hg)	73.5 (9.3)	74.0 (9.3)	75.9 (8.2)	72.0 (9.7)[d]	75.0 (8.7)	72.3 (9.7)[c]	72.9 (9.2)	76.8 (9.6)[c]
Pulse (beats/min)	72.7 (11.8)	73.3 (11.8)	69.1 (12.9)	74.9 (10.5)[e]	69.8 (12.6)	75.0 (10.6)[e]	73.3 (11.5)	69.4 (13.1)
Alcohol (drinks/day)	0.42 (0.55)	0.42 (0.55)	0.54 (0.65)	0.34 (0.47)[d]	0.55 (0.66)	0.31 (0.43)[d]	0.38 (0.52)	0.64 (0.65)[d]
Amalgam	3.58 (3.42)	3.67 (3.44)	4.01 (3.44)	3.33 (3.39)	4.15 (3.59)	3.15 (3.24)[c]	3.25 (3.12)	5.49 (4.40)[e]
Amalgams handled	27.9 (47.3)	28.2 (47.0)	43.6 (57.1)	18.4 (37.4)[e]	48.0 (57.1)	12.4 (30.2)[e]	26.9 (46.9)	33.2 (50.0)
Hg intake[b] (µg/kg bw/day)	0.08 (0.12)	0.08 (0.12)	0.09 (0.13)	0.07 (0.12)	0.09 (0.13)	0.07 (0.12)	0.07 (0.12)	0.10 (0.14)

[a]Subjects with genotype for *SEPP1* 3'UTR (rs7579) and all other parameters of interest. This polymorphism had approximately the average number of subjects with genotype compared to all eleven polymorphisms.
[b]Hg intake estimated from reported fish consumption (type, portion size, consumption frequency).
[c] $p<0.05$, ANOVA tests comparing paired categories (male vs. female, dentists vs. non-dentists, blood pressure medication users vs. non-users)
[d] $p<0.01$
[e] $p<0.001$

Table 4.2 Mercury Biomarker Levels in Total and Stratified Population.

		n	Mean	SD	25th %	50th %	75th %	90th %	95th %
HAIR MERCURY (µg/g)									
	Total	262	0.45	0.53	0.14	0.28	0.55	1.06	1.31
	NHANES[a]	1726	0.47		0.09	0.19	0.42	1.11	1.73
Genotyped	With genotype	232	0.45	0.55	0.14	0.29	0.55	1.00	1.34
Gender	Males	99	0.65	0.71	0.24	0.50	0.83	1.33	1.43
	Females	163	0.33[c]	0.34	0.11	0.21	0.43	0.82	1.06
Occupation	Dentists	114	0.64	0.69	0.25	0.48	0.83	1.31	1.69
	Non-Dentists	148	0.30[c]	0.29	0.11	0.19	0.39	0.72	1.00
Medication	No BP Meds	223	0.41	0.51	0.13	0.26	0.51	0.95	1.31
	BP Meds	39	0.66[b]	0.63	0.21	0.56	0.90	1.22	1.39
URINE MERCURY (µg/L)									
	Total	262	0.94	0.99	0.31	0.63	1.18	2.09	2.76
	NHANES[a]	1529				0.48	1.12	2.20	3.33
Genotyped	With genotype	232	0.95	1.00	0.32	0.61	1.19	2.14	2.96
Gender	Males	99	1.27	1.22	0.51	0.85	1.50	2.66	4.87
	Females	163	0.74[c]	0.75	0.26	0.47	0.98	1.76	2.20
Occupation	Dentists	114	1.26	1.19	0.49	0.85	1.53	2.56	4.47
	Non-Dentists	148	0.69[c]	0.70	0.25	0.44	0.92	1.61	2.00
Medication	No BP Meds	223	0.93	1.02	0.29	0.60	1.13	1.94	3.54
	BP Meds	39	1.01	0.79	0.38	0.66	1.60	2.35	2.46

[a]NHANES- National Health and Nutrition Examination Survey. Urine data from CDC 2009; hair data from McDowell et al. 2004.
[b] $p<0.01$ and [c] $p<0.001$ for ANOVA comparing natural log-transformed values for paired categories (male vs. female, dentists vs. non-dentists, BP medication users vs. non-users).

Table 4.3 Genetic Polymorphisms and Relationship with Select Variables. Results of weighted linear term ANOVA comparing genotype groups for each polymorphism are reported.

Polymorphisms			ANOVA p-value						
Gene-Polymorphism	**db SNP ID**	**n[a]**	**SBP[b]**	**DBP[b]**	**Pulse**	**BMI**	**Age**	**Hair Hg[c]**	**Urine Hg[c]**
GSTP1-114	rs1138272	190, 38, 1	0.84	0.70	0.64	0.27	0.17	0.39	0.73
GSTP1-105	rs1695	109, 107, 23	0.12	0.41	0.08	0.55	0.05	0.32	0.96
SEPP1-234	rs3877899	138, 83, 15	0.94	0.45	0.59	0.30	0.40	0.34	0.67
GCLM 5'	rs41303970	159, 62, 17	0.46	0.87	0.64	0.02	0.91	0.82	0.55
SEPP1 3'UTR	rs7579	122, 95, 15	0.09	0.08	0.85	0.05	0.50	0.39	0.14
GGT1 intron	rs5751901	93, 118, 22	0.28	0.90	0.38	0.81	0.61	0.17	0.36
GSTM3- 224	rs7483	130, 78, 19	0.10	0.05	0.54	0.88	0.56	0.16	0.46
GSS 5'	rs3761144	89, 108, 40	0.38	0.10	0.90	0.29	0.58	0.58	0.37
GSR 5'	rs1002149	153, 61, 7	0.79	0.72	0.55	0.75	0.16	0.98	0.96
GSR intron	rs2911678	137, 80, 15	0.93	0.58	0.83	0.73	0.38	0.19	0.17
GSTT1 deletion	N/A	166, 39	0.18	1.00	0.26	0.44	0.43	0.62	0.39

[a]Number of subjects with major homozygote, heterozygote, and minor homozygote genotype, respectively.
[b]Adjusted for blood pressure medication use according to the method of Tobin et al. (2005).
[c]Natural log-transformed before comparison.

Table 4.4 Parameter Estimates for Blood Pressure Models. Blood pressure measurements of individuals using hypertension controlling medications were imputed 15 mmHg higher (SBP) or 10 mmHg higher (DBP) according to the method of Tobin et al. (2005).

Dependent Variable	Population	n	Adj. r^2	BMI	Age	Female	Hair Hg	Urine Hg
SBP	Total	262	0.425	0.97[c]	0.67[c]	-5.74[b]	2.67	-1.80[a]
	Males	99	0.244	1.17[b]	0.59[c]		3.15	-3.26[b]
	Females	163	0.346	0.86[c]	0.74[c]		1.54	0.71
	Dentists	114	0.440	1.15[b]	0.72[c]	-7.89[a]	2.07	-3.35[b]
DBP	Total	262	0.224	0.83[c]	0.18[b]	-1.26	2.76[a]	-0.32
	Males	99	0.058	0.54[a]	0.05		2.94[a]	-1.13
	Females	163	0.260	0.89[c]	0.27[c]		1.87	1.10
	Dentists	114	0.152	0.71[b]	0.12	-3.31	2.11	-0.88

[a] $p \leq 0.05$ for β estimate
[b] $p < 0.01$
[c] $p < 0.001$

Table 4.5 Parameter Estimates for Linear Regression Models of SBP, DBP with Hair Hg and Genotype. SBP and DBP values are adjusted for anti-hypertensive medication use.

Polymorphism	Dependent Variable	Adj. r^2	BMI	Age	Female	Hair Hg	Genotype	Genotype* Hair Hg
Base model[a]	SBP	0.428	0.93[d]	0.71[d]	-5.91[c]	1.68		
(n=232)	DBP	0.208	0.77[d]	0.18[c]	-1.76	2.19[e]		
***GSTP1*-114**	SBP	0.428	0.89[d]	0.72[d]	-5.88[c]	6.64	2.84	-3.56
(n=229)	DBP	0.213	0.78[d]	0.17[c]	-2.18	2.39	-1.18	-0.05
***GSTP1*-105**	SBP	0.427	0.98[d]	0.69[d]	-5.37[b]	-2.68	-2.37	2.78
(n=239)	DBP	0.209	0.80[d]	0.17[c]	-1.18	-1.82	-1.65	2.51
***SEPP1*-234**	SBP	0.433	0.96[d]	0.67[d]	-6.80[c]	-5.41	-3.51[e]	5.19[e]
(n=236)	DBP	0.215	0.79[d]	0.16[c]	-2.10	-0.51	-2.32[e]	2.08
***GCLM* 5'**	SBP	0.432	1.03[d]	0.69[d]	-5.85[c]	-3.26	0.79	3.47[e]
(n=238)	DBP	0.204	0.82[d]	0.17[c]	-1.55	0.16	0.22	1.53
***SEPP1* 3'UTR**	SBP	0.430	0.91[d]	0.69[d]	-6.38[c]	7.53	-0.15	-3.55
(n=232)	DBP	0.206	0.75[d]	0.17[c]	-1.87	3.64	-0.77	-0.86
***GGT1* intron**	SBP	0.433	0.96[d]	0.73[d]	-5.43[b]	8.45	-0.24	-3.92
(n=233)	DBP	0.217	0.79[d]	0.19[c]	-1.89	4.37	0.90	-1.13
***GSTM3*-224**	SBP	0.425	0.93[d]	0.71[d]	-5.44[b]	5.21	-1.18	-2.46
(n=227)	DBP	0.234	0.80[d]	0.19[c]	-1.80	6.26[e]	-0.54	-3.25
***GSS* 5'**	SBP	0.426	0.97[d]	0.68[d]	-5.49[c]	-0.86	-0.87	1.79
(n=237)	DBP	0.212	0.79[d]	0.17[c]	-1.09	1.56	-1.38	0.69
***GSR* 5'**	SBP	0.431	1.00[d]	0.70[d]	-5.45[b]	7.02[e]	2.38	-2.50
(n=221)	DBP	0.216	0.79[d]	0.17[c]	-1.71	3.52	0.99	-0.38
***GSR* intron**	SBP	0.429	0.96[d]	0.70[d]	-5.07[b]	2.57	0.44	0.24
(n=232)	DBP	0.213	0.80[d]	0.17[c]	-1.42	3.32	-0.39	-0.37
***GSTT1* del**	SBP	0.454	1.04[d]	0.71[d]	-4.67[b]	-9.88	-1.82	10.1[e]
(n=205)	DBP	0.217	0.82[d]	0.19[c]	-0.69	-5.22	-3.45	6.00

[a]Individuals with *SEPP1* 3'UTR genotype used to compute the base model.
[b,c,d]$p<0.05$, <0.01, <0.001 for parameter estimate.
[e]Near significant parameter estimate ($p<0.10$).

Table 4.6 Parameter Estimates for Linear Regression Models of SBP, DBP with Urine Hg and Genotype. SBP and DBP are adjusted for anti-hypertensive medication use.

Polymorphism	Dependent Variable	Adj. r^2	BMI	Age	Female	Urine Hg	Genotype	Genotype* Urine Hg
Base model[a]	SBP	0.435	0.89[d]	0.73[d]	-7.12[c]	-1.77[e]		
(n=232)	DBP	0.196	0.74[d]	0.20[c]	-2.32	-0.06		
***GSTP1*-114**	SBP	0.437	0.85[d]	0.75[d]	-7.40[c]	-2.02	1.48	-0.08
(n=229)	DBP	0.200	0.75[d]	0.18[c]	-2.79[e]	0.07	-0.91	-0.14
***GSTP1*-105**	SBP	0.428	0.93[d]	0.71[d]	-6.76[c]	-2.84	-1.78	0.70
(n=239)	DBP	0.186	0.76[d]	0.19[c]	-1.99	0.08	-0.56	-0.04
***SEPP1*-234**	SBP	0.437	0.90[d]	0.71[d]	-7.77[d]	-4.15	-2.78	1.48
(n=236)	DBP	0.199	0.75[d]	0.18[c]	-2.59[e]	-0.77	-1.90	0.44
***GCLM* 5'**	SBP	0.435	1.00[d]	0.73[d]	-6.82[c]	1.23	4.40[b]	-2.12
(n=238)	DBP	0.189	0.79[d]	0.20[c]	-2.04	1.37	1.99	-1.02
***SEPP1* 3'UTR**	SBP	0.435	0.86[d]	0.72[d]	-7.32[c]	0.11	-0.61	-1.32
(n=232)	DBP	0.195	0.72[d]	0.19[c]	-2.45[e]	1.17	-0.29	-0.86
***GGT1* intron**	SBP	0.430	0.93[d]	0.75[d]	-6.66[c]	-0.76	-1.74	-0.30
(n=233)	DBP	0.203	0.77[d]	0.21[d]	-2.34	1.78	0.95	-0.83
***GSTM3*-224**	SBP	0.426	0.89[d]	0.73[d]	-6.46[c]	1.46	-0.75	-1.68
(n=227)	DBP	0.227	0.76[d]	0.20[d]	-2.23	3.40[e]	-0.27	-1.92[e]
***GSS* 5'**	SBP	0.431	0.93[d]	0.72[d]	-6.91[c]	-0.50	0.67	-0.85
(n=237)	DBP	0.192	0.76[d]	0.19[c]	-1.89	-0.31	-1.02	0.08
***GSR* 5'**	SBP	0.426	0.95[d]	0.73[d]	-6.75[c]	-2.62	0.26	1.03
(n=221)	DBP	0.205	0.75[d]	0.19[c]	-2.41[e]	-1.91	-0.50	1.69
***GSR* intron**	SBP	0.434	0.92[d]	0.74[d]	-6.57[c]	-0.23	1.99	-1.03
(n=232)	DBP	0.193	0.77[d]	0.19[c]	-2.20	0.39	-0.14	-0.33
***GSTT1* del**	SBP	0.449	0.99[d]	0.72[d]	-5.39[b]	-5.78	-1.00	5.35
(n=205)	DBP	0.219	0.80[d]	0.19[c]	-1.14	-5.09	-4.27	5.25[e]

[a] Base model computed including subjects with *SEPP1* 3'UTR genotype.
[b,c,d] $p<0.05$, <0.01, <0.001 for parameter estimate.
[e] Near significant parameter estimate ($p<0.10$).

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Chapter 5

Variants of Glutathione *S*-Transferase Pi 1 Exhibit Differential Enzymatic Activity and Inhibition by Heavy Metals[3]

5.1 Introduction

The influence of gene-environment interactions on the toxicokinetics of chemicals and susceptibility to toxicity has recently been considered an important aspect of risk assessment (Omenn, 2001). Pharmacological studies have established that genetic factors may underlie the immense variability in an individual's ability to absorb, distribute, metabolize, and eliminate drugs (de Leon et al., 2005; Ingelman-Sundberg, 2004). Similarly, in environmental health sciences, inter-individual differences in the metabolism and toxicodynamics of hazardous chemicals may be influenced by genetic variation. For example, in human epidemiological studies focused on heavy metals, polymorphisms in key metabolic and antioxidant genes have been associated with altered metabolism and elimination of methylmercury (MeHg; Custodio et al., 2004; Engström et al., 2008; Gundacker et al., 2009), elemental mercury (Custodio et al., 2005), lead (Gundacker et al., 2009), and arsenic (Engström et al., 2007; Marcos et al., 2006) using common biomarkers of exposure

Many of the polymorphisms associated with metal biomarker levels are in the glutathione s-transferase (GST) family. GSTs are implicated in phase II detoxification of various endogenous metabolic byproducts and environmental chemicals including heavy

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metals (e.g. inorganic arsenic, inorganic mercury-$HgCl_2$, MeHg) (Adamis et al., 2004; Ballatori and Clarkson, 1985; Hayes et al., 2005; Wang and Lee, 1993). GSTs catalyze the conjugation of glutathione (GSH) to electrophilic substrates, resulting in less reactive products ready for elimination (Hayes et al., 2005). Conjugation of GSH to $HgCl_2$ and MeHg promotes elimination through the biliary system. While GSH is able to conjugate spontaneously with Hg, GSTs have also been implicated in the process. The exact role of GSTs in Hg conjugation is debated and may involve reaction catalysis, transport of the conjugated product, or both (Ballatori and Clarkson, 1985). *In vitro* results suggest that GSH conjugation of arsenic and cadmium and subsequent elimination from cells are GST-dependent (Adamis et al., 2004; Wang and Lee, 1993). The aforementioned studies highlight the important role that GSTs play in the detoxification of heavy metals. However, the interactions between GSTs and heavy metals are further complicated by the ability of some metals (e.g., $HgCl_2$, MeHg) to bind and inhibit GSTs (Almar and Dierickx, 1990; Dierickx, 1982; Poon and Chu, 2000; Reddy et al., 1981).

The catalytic efficiency of GSTs varies tremendously among individuals. Seven classes of cytosolic GSTs exist in humans, and polymorphisms are ubiquitous among the genes encoding these enzymes (Strange et al., 2000). A search in the NCBI database revealed 158 single nucleotide polymorphisms (SNPs) in GSTP1 pi 1 (*GSTP1*) with minor allele frequencies as high as 50% in some populations. Polymorphisms in GSTs resulting in altered catalytic activity or expression have been linked to increased risk for cancer (Cote et al., 2005; Strange et al., 2000) and differential metabolism of many toxicants (Ishimoto and Ali-Osman, 2002; Srivastava et al., 1999) including heavy metals (Custodio et al., 2004; Engström et al., 2007, 2008; Gundacker et al., 2009; Marcos et al.,

2006). GSTP1 is an enzyme of particular interest as it is the most widely expressed GST (found in erythrocytes, placenta, lung, brain, muscle, and liver, among other tissues) and has two prevalent nonsynonymous SNPs that directly influence enzyme activity (Strange et al., 2000; Suzuki et al., 1987). The nonsynonymous SNPs change the 105th amino acid from isoleucine to valine (Ile/Val 105) and 114th amino acid from alanine to valine (Ala/Val 114). Altering the 105th position affects the geometry of the substrate binding site of GSTP1. Consequently, this modification results in an approximately three-fold difference in substrate affinity *in vitro* (Ali-Osman et al., 1997; Hu et al., 1997; Zimniak et al., 1994). The 114th amino acid is positioned outside the active site but may still influence GSTP1 activity and substrate access to the site (Ali-Osman et al., 1997; Hu et al., 1997; Parker et al., 2008).

These structural changes may affect the interaction of heavy metals with GSTP1, but to our knowledge this has not been tested. Epidemiological studies found associations between GST polymorphisms (including *GSTP1* 105 and 114) and inter-individual differences in metabolism and elimination of Hg and arsenic as assessed by biomarkers (Custodio et al., 2004; Gundacker et al., 2009; Marcos et al., 2006), but effects of genotype on GSTP1 interaction with metals at the protein level are unknown. Here, we genetically engineered *E. coli* to express two key *GSTP1* polymorphisms (Ile/Val 105 and Ala/Val 114- four combinations). The goal was to use an *in vitro* platform to assess the functional effects of these polymorphisms on enzyme kinetics and to characterize the impact of heavy metal exposure ($HgCl_2$, MeHg, selenium, lead, arsenic, cadmium, and manganese) on enzyme function. Such a screening approach has previously been used in cancer biology to supplement epidemiological results. We propose a similar scheme to

improve mechanistic understanding of environmental epidemiological and toxicological studies. Our work is in line with a recent US National Research Council (NRC, 2007) recommendation to develop and validate high throughput *in vitro* screening assays for toxicity testing purposes that may improve understanding of inter-individual variability and risk.

5.2 Materials and Methods

5.2.1 Synthesis of GSTP1 Variant Proteins

Four variants of the human *GSTP1* gene were genetically engineered using commonly employed methods (Chang et al., 1999; Kitteringham et al., 2007). The most prevalent version of *GSTP1* ('IA' with genotype Ile105 Ala114) was obtained from Harvard University (Plasmid ID HSCD00000618). Two PCR reactions (referred to as Steps 1 and 2) using primers detailed in Table 5.1 mutated GSTP1 IA into GSTP1 VA (Val105 Ala114). The QuikChange II Site-directed Mutagenesis Kit (Stratagene, CA, USA) was used to mutate *GSTP1* to IV (lle105 Val114) and VV (Val105 Val114) using primers listed in Table 5.1. Reactions were carried out with a Mastercycler Gradient thermocycler (Eppendorf, NY, USA). The four *GSTP1* genes were cloned into pET100/D-TOPO vectors with N-terminal polyhistidine tags according to the manufacturer's protocol (Invitrogen, CA, USA). DNA sequencing was performed with the 3730 XL Sequencer (Applied Biosystems) to ensure proper sequence of each mutant. Constructs were propagated in TOP10 *E. coli*, and expressed in BL21 Star (DE3) *E. coli* following induction by isopropyl β-D-1-thiogalactopyranoside (IPTG). GSTP1 proteins were purified with nickel-affinity chromatography using PerfectPro Ni-NTA resin

following manufacturer's instructions (5Prime, MD, USA) and were stored in 0.1 M potassium phosphate buffer (pH 6.6) at -20 °C until use. The Bradford method determined protein concentration (Bradford, 1976).

5.2.2 GST Assays

Enzyme activity was assessed according to the method of Habig et al. (1974) by measuring GSH conjugation with 1-chloro-2,4-dinitrobenzene (CDNB). The HTS 7000 Plus Bioassay Reader (Perkin Elmer, MA, USA) measured absorption at 340 nm signifying product formation. Briefly, all reactions took place in 0.1 M potassium phosphate buffer containing 5 µg/mL protein, 0.01-10 mM GSH, and 0.01-3 mM CDNB. Each trial contained triplicates for every substrate concentration. Enzyme kinetics (V_{max}, K_m) were calculated for each trial by holding GSH (10 mM) or CDNB (2 mM) constant and varying the concentration of the other substrate. Final kinetic parameters are based on the mean of four to six kinetic trials. Several failed trials, mainly due to microplate reader malfunction, were excluded.

5.2.3 Metal Inhibition

Enzyme activity of GSTP1 allozymes was measured after incubation with 1 nM-1 mM of methylmercury (II) chloride (MeHg), mercury (II) chloride ($HgCl_2$, 10 pM-320 µM), manganese (II) chloride, lead (II) acetate, cadmium (II) chloride (100 pM-1 mM), sodium selenate, sodium selenite, or sodium arsenate (1 nM-10 mM). Metal doses, specifically for MeHg and $HgCl_2$, were selected as ranges encompassing inhibitory concentrations of soluble GSTs observed in previous experiments performed by us (data

not published) and others (Almar and Dierickx, 1990). Assays contained 5 µg/mL purified protein and fixed GSH and CDNB concentrations set to approximately the K_m values for a given GSTP1 allozyme. These substrate concentrations enable comparison of metal inhibition across allozymes as each variant is exposed to substrate concentrations associated with 50% maximum enzyme activity for that particular variant. All metals, purchased from Sigma Aldrich or Fisher Scientific, were administered from 50-200 mM stock solutions in distilled water with the exception of MeHg which was dissolved in 100% dimethylsulfoxide. MeHg reactions and controls contained a final concentration of 3% dimethylsulfoxide for dissolving purposes. Enzymes were incubated with metal for ten minutes prior to addition of the substrates, GSH and CDNB, and subsequent measurement of product formation. Six to eighteen replicates of each Hg treatment were analyzed depending on the allozyme. GSTP1 IA and VA were used for method development and as such had the most replicates for $HgCl_2$ and MeHg treatment. Three to nine replicates of all other heavy metal treatments were analyzed. All failed replicates were excluded.

5.2.4 Statistical Analyses

Enzyme activity data was curve fitted using a non-linear regression program (GraphPad Prism Version 3.02, GraphPad Software Inc., CA, USA) to calculate the maximum velocity of substrate formation (V_{max}) and the Michaelis constant (K_m) for substrate affinity according to the following equation:

$$Y = (V_{max})(S)/K_m + S$$

Y represents enzyme activity (μmol/min/mg) and S represents substrate concentration (mM). The concentrations of metal inhibiting 50% of enzyme activity (IC_{50}) and inhibition constants (K_i) were calculated using GraphPad Prism.

All statistical analyses were performed with PASW Statistics v. 18 (SPSS, Chicago, IL). Mean kinetic and inhibition parameters were compared among GSTP1 allozymes using ANOVA tests followed by pairwise comparisons with the Tukey method. For variables with unequal variances according to Levene's test, Welch ANOVA and Dunnett's T3 test were used instead. In all statistical tests, p-value <0.05 was considered statistically significant. All data are presented as mean ± SEM.

5.3 Results

5.3.1 GSTP1 Gene Mutation and Protein Synthesis

The human *GSTP1* gene (IA) was mutated to encode *GSTP1* VA, IV, and VV. DNA sequencing confirmed the desired sequence in all four variants before expression via pet-TOPO-d-100 plasmids in BL21 Star *E. coli* cells. Proteins were purified with nickel-resin affinity chromatography, which yielded approximately 1 mg purified allozyme per batch. SDS-PAGE 2D gel electrophoresis and Coomassie Blue staining confirmed single bands around 25 kDa in the purified fractions used for enzyme activity assays.

5.3.2 Enzyme Activity of GSTP1 Allozymes

Enzyme activity with varying levels of the substrates CDNB and GSH revealed the four allozymes to have different kinetic properties (Table 5.2, Figure 5.1). GSTP1 IA

(K_m ±SEM=0.33±0.07 mM) and IV (K_m=0.28±0.02 mM) had significantly greater affinity for CDNB compared with GSTP1 VA and VV (K_m=1.15±0.07, 0.63±0.1 mM, respectively). VA had the least affinity for CDNB, but the highest V_{max} suggesting it requires more available substrate to catalyze efficiently. Overall, IA had significantly better catalytic efficiency compared to the three less frequent allozymes as assessed by k_{cat}/K_m (IA: 98.2±14 $mM^{-1}s^{-1}$ vs. VA: 35.9±6.8, IV 55±9.8, VV 43.4±9.3 $mM^{-1}s^{-1}$). Kinetic parameters with varying levels of GSH were similar among the four GSTP1 allozymes. ANOVA revealed a significant difference among the allozymes' K_m values for GSH, though the sole significant pairwise difference was between GSTP1 VA (0.93±0.11 mM) and IV (0.48±0.15 mM; p=0.03 for comparison).

5.3.3 GSTP1 and Mercury Inhibition

The four GSTP1 variants were incubated with a range of $HgCl_2$ (10 pM- 320 µM) and MeHg (1 nM- 1mM) doses prior to addition of substrates, and genotype influenced sensitivity towards both Hg forms (Table 5.3, Figure 5.2). $HgCl_2$ was the most potent inhibitor of all GSTP1 allozymes ($p<0.001$, test not shown) compared with MeHg treatment. Sensitivity of the GSTP1 allozymes followed the same trend for $HgCl_2$ and MeHg inhibition (most sensitive first: VA > IA > VV > IV). Pairwise comparisons revealed significant differences between IC_{50s} and inhibitory constants (K_i), accounting for substrate concentration and allozyme substrate affinity, of all three variant enzymes treated with $HgCl_2$ and the respective IC_{50} and K_i values for GSTP1 IA (Dunnett's T3 test, $p<0.05$). Furthermore according to Dunnett's T3 test, IC_{50s} and K_i values for $HgCl_2$ were significantly different from one another for all pairwise combinations of GSTP1

allozymes excluding GSTP1 IV with VV. MeHg inhibition varied among allozymes to a lesser extent as only IC_{50} values for GSTP1 IV and VV were significantly larger than that of the common form, GSTP1 IA (Dunnett's T3 test, $p<0.05$). GSTP1 IV was the most resistant allozyme to MeHg inhibition (IC_{50} ±SEM=480 ±54.1 μM) compared with three other variants and was five times less sensitive than GSTP1 VA (IC_{50}=93.9 ±11.3 μM).

5.3.4 GSTP1 and Other Heavy Metals

The inhibitory effects of two inorganic selenium compounds (sodium selenate, sodium selenite) were tested on four GSTP1 allozymes. GSTP1 was not inhibited by sodium selenate concentrations as high as 1 mM, while sodium selenite had similar potency to $HgCl_2$ (IC_{50} range for four allozymes: 43.7-62.8 μM; see Table 5.4, Figure 5.2), and the potency did not vary significantly by allozyme. GSTP1 enzyme activity, regardless of amino acid sequence, did not reach 50% inhibition by lead acetate, manganese chloride, or cadmium chloride at concentrations up to 1 mM or by sodium arsenate at concentrations up to 10 mM.

5.4 Discussion

Genetic polymorphisms in *GSTP1*, which encodes an important detoxification enzyme that catalyzes the conjugation of GSH with many endogenous and exogenous electrophilic substrates, have been linked in epidemiological studies to altered metabolism and biomarker levels of toxicants such as arsenic (Marcos et al., 2006) and methylmercury (Custodio et al. 2004; Engström et al., 2008; Gundacker et al. 2009). We assessed the kinetic properties of four GSTP1 variants encoded by two key

nonsynonymous SNPs in *GSTP1*, Ile/Val 105 and Ala/Val 114, on protein function with common substrates *in vitro* in the presence and absence of mercury ($HgCl_2$, MeHg), selenium, arsenic, lead, manganese and cadmium. Kinetic parameters and inhibition by $HgCl_2$ and MeHg significantly differed ($p<0.05$) among the four recombinant allozymes suggesting a role for both polymorphisms in altering enzyme function.

In the majority of human populations, the major *GSTP1* alleles are Ile105 (frequency >50%) and Ala114 (>90%; NCBI). The two most common allozymes of GSTP1 in human populations are GSTP1 IA and VA, though GSTP1 IV and VV exist to a limited extent (estimated ≤5% based on allele frequencies in HapMap populations). The 105 and 114 loci are in linkage disequilibrium in many populations (Moyer et al., 2008) including the Michigan Dental Association cohort. For example, in a gene-environment study we conducted on 515 Michigan dental professionals, *GSTP1* 105 and 114 loci were in linkage disequilibrium with the Val alleles found together more frequently.

Previous biochemical characterization of GSTP1 allozymes have focused on GSTP1 IA and VA and the few studies incorporating GSTP1 IV and VV had inconsistent results (Ali-Osman et al., 1997; Hu et al., 1998; Johansson et al., 1998; Moyer et al., 2008; Zimniak et al., 1994). In this study, GSTP1 IA and IV had the greatest affinity for the electrophilic substrate, CDNB (K_m= 0.33 mM (IA), 0.28 mM (IV)), and their affinities were 3-4 fold greater than that of GSTP1 VA (K_m=1.15 mM). Previous biochemical studies of GSTP1 allozymes found similar trends with GSTP1 IA having a greater affinity for CDNB (1.6-3.7 fold lower K_m) compared with GSTP1 VA (Ali-Osman et al., 1997; Hu et al., 1997; Johansson et al., 1998; Moyer et al., 2008, Zimniak et al., 1994). GSTP1 IA was the most efficient at catalyzing the conjugation of GSH with

CDNB (see k_{cat}/K_m, Table 5.2). Similar to previous studies, the affinity of GSTP1 IA, VA, and VV for GSH were near identical (Ali-Osman et al., 1997; Zimniak et al., 1994). Interestingly, GSTP1 IV had a significantly greater affinity for GSH compared to GSTP1 VA (Km= 0.48 mM (IV) vs. 0.93 mM (VA)). Overall, allozymes with Ile105 (GSTP1 IA, IV) performed better in kinetic assays with varying CDNB concentrations compared to allozymes with Val105 as noted when comparing K_m and k_{cat}/K_m between GSTP1 IA and VA or IV and VV.

Crystallography and structural 3D modeling have shown that residue 105 is found in the H-site of GSTP1, the site of electrophilic substrate binding (Oakley et al., 1997; Parker et al., 2008). Substituting valine for isoleucine in this position alters the hydrophobicity and shape of the H-site (Ali-Osman et al., 1997; Zimniak et al., 1994) and interaction with the catalytically important residue, Tyr109 (Johansson et al., 1998; Parker et al., 2008). This substitution decreases substrate (CDNB) affinity and catalytic activity of GSTP1 as observed in the present study. The impact on substrate affinity and catalytic efficiency from substituting valine for alanine at residue 114 was much less pronounced. While residue 114 is found outside the H-site, it has the potential to influence the secondary and tertiary structure of GSTP1 as it partakes in a superhelical structure impacting the H-site shape (Ali-Osman et al., 1997; Parker et al., 2008). Furthermore, 114 may be involved in a hydrophobic clamp at the opening of the solvent channel that leads to both the G- (GSH binding) and H-sites (Hu et al., 1997). Therefore, while residue 105 directly impacts the H-site, amino acid substitution at position 114 may also indirectly influence active site shape and substrate access.

Along with comparing the enzymatic activity of four GSTP1 variants, this study assessed the impact of genotype and heavy metal exposure on GSTP1. The four versions of GSTP1 were significantly inhibited by $HgCl_2$ and MeHg (Table 5.3). $HgCl_2$ was the more potent inhibitor, and this is consistent with other *in vitro* studies that found enzymes and neurochemical receptors were more sensitive to $HgCl_2$ compared with MeHg (Allen et al., 2001; Basu et al., 2005). The observed IC_{50s} for $HgCl_2$ (range across allozymes: 24.1 to 172 μM) and MeHg (93.9 to 480 μM) were within the range (10 to 300 μm) of IC_{50s} observed for human, rat, and calf GSTs following $HgCl_2$ (Almar and Dierickx, 1990; Dierickx, 1982; Poon and Chu, 2000; Reddy et al., 1981) and MeHg treatment (Reddy et al., 1981).

While Dierickx (1982) observed differential inhibition of rat GSTs depending on GST isozyme, this is the first study to show that genotype of a specific GST, GSTP1, may also influence mercury sensitivity. For both $HgCl_2$ and MeHg treatments, allozymes ranked according to sensitivity with the most sensitive first were as follows: GSTP1 VA>IA>VV>IV. This trend suggests that the Ile105 and Val114 residues may confer some protection against inhibition by Hg. Inhibition of GSTP1 activity by Hg likely occurs via two mechanisms: direct binding to GSTP1 thiol groups and indirectly by conjugation with available GSH. GSTP1 contains four cysteine residues, including one residue (Cys48) known to inhibit enzyme function when bound by compounds with high thiol affinity and one (Cys102) near the H-site (Oakley et al., 1997). Studies on mercury inhibition of GSTs, including the present, have observed that increasing GSH concentration partially decreases inhibition, irrespective of CDNB concentration (Almar and Dierickx, 1990; Dierickx 1982). Addition of cysteine with $HgCl_2$ also rescued GST

activity in previous studies (Almar and Dierickx, 1990; Dierickx 1982). Amino acid substitutions at residues 105 and 114 could influence both purposed inhibitory mechanisms. Structural alterations accompanying the sequence change may affect the positioning of the cysteine residues, and thus the ability of Hg to bind and inhibit the enzyme. As previously discussed, substitution of Val for Ile105 changes the structure of the H-site and subsequently affinity for CDNB and catalytic efficiency. The impaired enzymatic activity of GSTP1 VA and VV compared to GSTP1 IA and IV, respectively, may hinder their ability to utilize available GSH efficiently when Hg binds to GSH and/or binds and inhibits the enzyme itself.

Sodium selenite inhibited GSTP1 activity with similar potency to $HgCl_2$ while sodium selenate was not inhibitory (up to 1 mM). Selenium exhibits hormesis as some selenium is needed in the body (e.g., for selenoprotein synthesis) but high levels are toxic (Chiang et al., 2010). Unlike sodium selenate, selenite is known to bind thiols, including GSH, and GSH binding may constitute the main mechanism of GST inhibition observed here (Żbikowska et al., 1997). Selenium treatment has been shown to increase GST expression and activity levels in several animal species *in vivo* (El-Sayed et al., 2006; Sidhu et al., 1993), an effect which may be organ dependent (Sidhu et al., 1993). *In vitro* treatment of pig GSTs found selenite inhibited activity while selenate had no effect as observed in the present study (Żbikowska et al., 1997). *GSTP1* genotype did not affect sensitivity to selenite inhibition.

GSTP1 activity, regardless of genotype, was not significantly inhibited (>50%) after incubation with arsenic, lead, manganese, or cadmium. Previous studies revealed arsenic treatment to have no effect on GST activity in human erythrocytes (Poon and

Chu, 2000) or to increase GST activity in rat hepatocytes treated *in vitro* (Kojima et al., 2006). GSTP1 specifically has been shown to play an important role in increased arsenic excretion in a strain of Chinese Hamster Ovarian cells, stemming from increased expression (Wang et al., 1993). The lack of inhibition of recombinant GSTP1 in the present study is consistent with previous studies suggesting no direct interaction between arsenic and GSTs. Though rat studies have implicated lead in increasing GST expression and subsequent activity (Daggett et al., 1998), lead has not been shown to directly inhibit GST enzyme activity (Dierckx, 1982) as seen here with human GSTP1. Consistent with GSTs isolated from rats and calves, recombinant human GSTP1, regardless of genotype, was not inhibited by manganese chloride (Dierckx, 1982; Reddy et al., 1981).

Cadmium inhibited 50% of GST activity *in vitro* at concentrations between 10 and 750 µM in calf liver (Reddy et al. 1981), human erythrocytes (Poon and Chu, 2000) and of GSTP1 isolated from a hepatoma cell line (Almar and Dierckx, 1990) while 200 µM decreased the activity of GSTs isolated from rat liver by 37% (Dierckx, 1982). In the present study, the IC_{50} for all GSTP1 allozymes was greater than 1 mM, though significant inhibition at 1 mM compared to untreated controls was observed for all variants (data not shown, 81-88% of specific activity of control, $p<0.05$). Species differences among GSTs and experimental conditions (substrate concentrations, source and concentration of GSTs, GST isoforms) may contribute to the differences observed.

GSTP1 105 and 114 polymorphisms, which are linked to differences in enzyme function and Hg sensitivity *in vitro*, are significantly associated with altered mercury biomarker levels (hair and blood), representing primarily MeHg exposure, at the population level. Several studies found significant associations between Val105 and/or

Val114 alleles and lower biomarker levels (Engström et al., 2008; Chapter 2, this dissertation) while other studies linked higher biomarker levels to Val alleles (Custodio et al., 2004; Gundacker et al., 2009). Multiple factors may underlie the discrepant results including Hg exposure level, specific biomarkers analyzed, statistical methodologies employed (e.g., ANOVA vs. linear regression; modeling effect of single SNP vs. SNP combinations), frequency of *GSTP1* 105 and 114 SNPs in the study populations, and linkage with other SNPs influencing *GSTP1* expression. Alternatively, given multiple testing in many of these epidemiological studies and the opposite direction of relationships between *GSTP1* genotype and Hg biomarker levels observed across studies, the significant associations could be false positives. In arsenic exposed populations, deletion polymorphisms in *GSTT1* and *GSTM1* have been shown to alter the proportion of methylated arsenic metabolites in urine. For example, *GSTM1* deletion genotype was associated with a higher proportion of monomethylated arsenic (MMA), a particularly toxic metabolite (Engström et al., 2007). However, *GSTP1* Val105 was only associated with a near-significant shift in the urinary metabolic profile (Marcos et al., 2006).

Limited epidemiological associations and *in vitro* results suggest that *GSTP1* 105 and *GSTP1* 114 genotypes have the potential to influence Hg toxicokinetics. While the relationships between *GSTP1* genotype, Hg biomarker levels, and ensuing toxicity merit future study, several considerations must be employed when interpreting this gene-environment data. Glutathione-Hg conjugation may be influenced by many factors including glutathione availability and oxidation state, Hg exposure (species, dose, duration), exposures to other thiol-reactive compounds, and overall functionality of GSTs. While Hg spontaneously reacts with GSH intracellularly, GSTs can also aid this

important Hg detoxification process by way of reaction catalysis and conjugate transport (Ballatori and Clarkson, 1985). Furthermore, GST inhibition by Hg at high doses, as observed in this study, may act as a temporary detoxification mechanism, preventing Hg from inhibiting other key intracellular proteins. Expression of GST increases following Hg exposure, and in rats, the *GSTP1* equivalent experiences the most pronounced upregulation (11-fold; Brambila et al., 2002). While *GSTP1* may be of particular importance to the Hg toxicokinetic pathway, it is one of several GSTs which can interact with Hg. Likewise, *GSTP1* 105 and 114 are two of a multitude of polymorphisms in *GSTP1*, many of which are suspected to alter enzyme activity or gene expression (Moyer et al., 2008). Thus, while *GSTP1* Ile/Val 105 and Ala/Val 114 may significantly influence enzyme activity and sensitivity to Hg inhibition *in vitro*, genotype at two loci in one GST isozyme would be expected to have a very minor impact, if any, on Hg toxicokinetics *in vivo*.

In a broader context, the combined genotype across dozens of key polymorphic sites that affect expression or enzymatic activity of several GST isozymes (and of other key proteins in the Hg toxicokinetics pathway) has the potential to impact the wide inter-individual variability observed in Hg metabolism. *In vitro* characterization of protein products encoded by polymorphisms may provide a better understanding of epidemiological links between polymorphisms and interaction with metals or other toxic exposures (Li and Woods, 2009; Moyer et al., 2008). Nevertheless, careful consideration of a multitude of factors (e.g., toxicant exposure source and dose, protein expression, SNP prevalence) and synthesis of *in vitro* and epidemiological data related to all

functional SNPs in the pathway must be employed when interpreting gene-environment data and incorporating it into risk assessment.

Figures and Tables

Figure 5.1. Specific Activity of Four GSTP1 Variants with Increasing Concentration of the Electrophilic Substrate CDNB. Data points represent mean ± SEM specific activity from four or five trials.

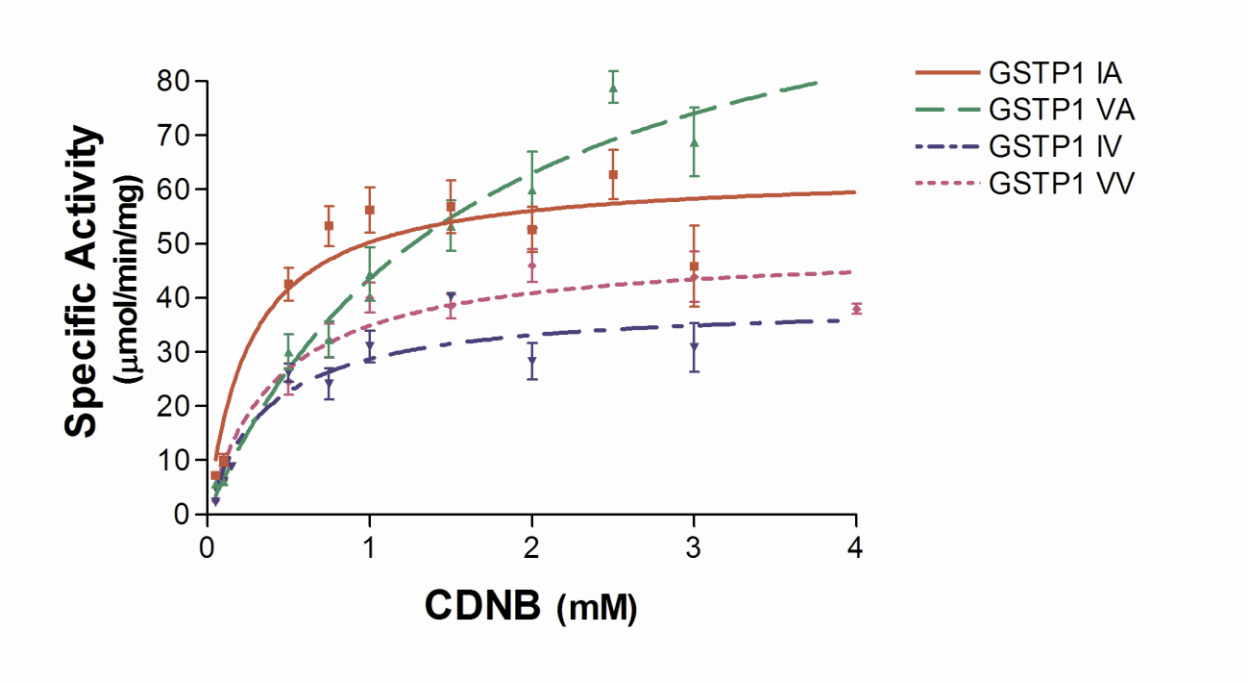

Figure 5.2. Inhibition of Four GSTP1 Variants by Heavy Metals: A) GSTP1 Ile105 Ala114; B) GSTP1 Val Ala; C) GSTP1 Ile Val; D) GSTP1 Val Val. GSTP1 allozymes were treated with a range of concentrations of $HgCl_2$, MeHg, sodium arsenate, lead acetate, cadmium chloride, sodium selenite, or manganese chloride. Specific activities of metal-treated enzymes were normalized to specific activity of control for each allozyme. Points represent mean ±SEM of 3-18 replicates depending on the metal. Inhibition curves for $HgCl_2$, MeHg, and selenium, the only significant inhibitors of GSTP1 activity, are emphasized.

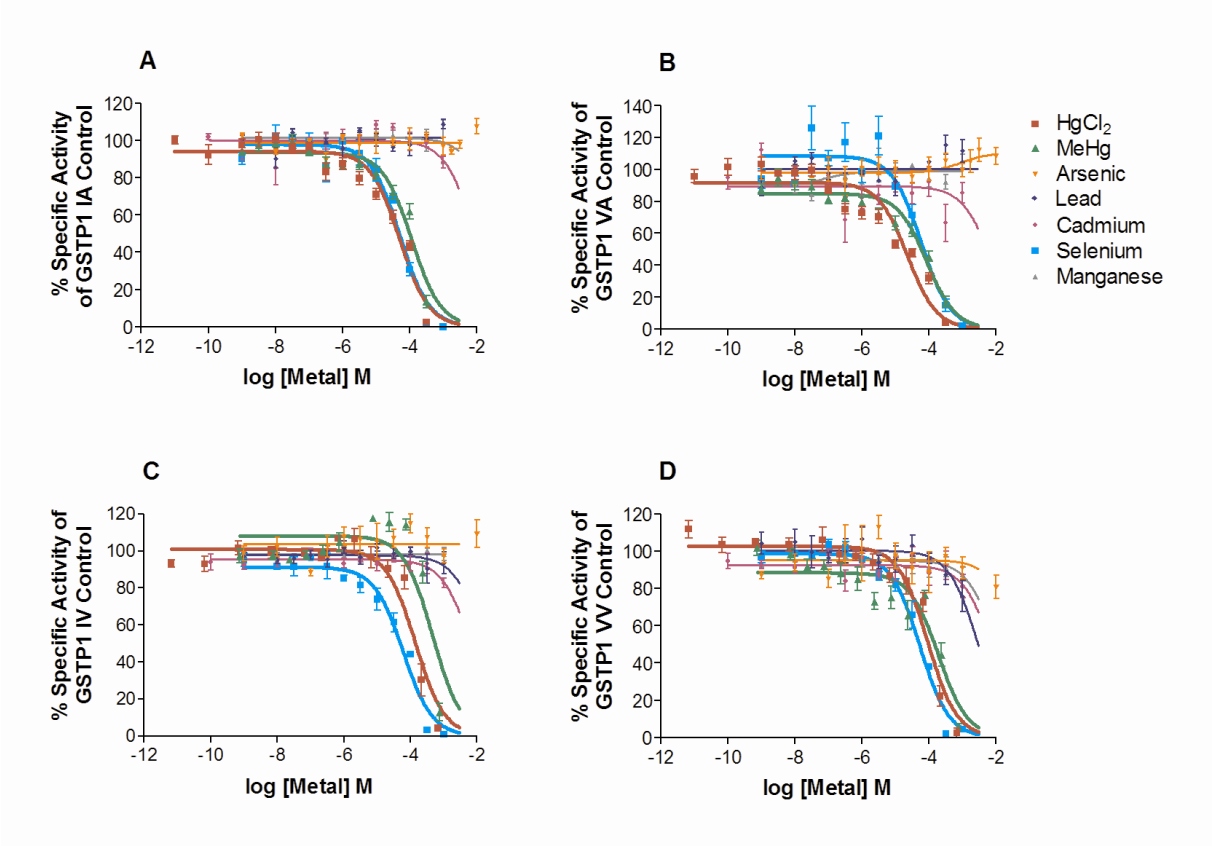

Table 5.1. Primers Used to Mutate *GSTP1*.

Primer Purpose	Primer Sequence (5' → 3')	Direction
Mutate Val105, Step 1	CACCATGCCGCCCTACAC	Forward
Mutate Val105, Step 1	GATGAGGGAGACGTATTTGCAGC	Reverse
Mutate Val105, Step 2	Megaprimer- product from two previous primers	Forward
Mutate Val105, Step 2	TCACTGTTTCCCGTTGCCATTGATG	Reverse
Mutate Val114	CTACACCAACTATGAGGTGGGCAAGGATGACTATG	Forward
Mutate Val114	CATAGTCATCCTTGCCCACCTCATAGTTGGTGTAG	Reverse

Table 5.2. Kinetic Parameters for Conjugation of CDNB and GSH by Four GSTP1 Variants. Data represent mean ± SEM based on four to six trials. For a given row, the p-value is reported for the ANOVA test[a] comparing the kinetic parameter among GSTP1 allozymes. Pairwise comparisons with the reference GSTP1 (IA) were made in each row using the Tukey method[a], and significance is indicated: *p<0.05, †p<0.01, ‡p<0.001.

		GSTP1 IA	GSTP1 VA	GSTP1 IV	GSTP1 VV	p value
Substrate	**Amino Acid**	Ile105 Ala114	Val105 Ala114	Ile105 Val114	Val105 Val114	
CDNB	**K_m (mM)**	0.33 ± 0.07	1.15 ± 0.07‡	0.28 ± 0.02	0.63 ± 0.1*	<0.001
	V_{max} (μmol/min/mg)	68.1 ± 8.2	93.4 ± 16.8	33.0 ± 3.7*	57.0 ± 5.0	0.006
	K_{cat}/K_m ($mM^{-1}s^{-1}$)	98.2 ± 14	35.9 ± 6.8†	55.0 ± 9.8*	43.4 ± 9.3*	0.003
GSH	**K_m (mM)**	0.90 ± 0.02	0.93 ± 0.11	0.48 ± 0.15	0.81 ± 0.08	0.04
	V_{max} (μmol/min/mg)	70.2 ± 14.1	59.1 ± 11.1	47.6 ± 5.1	42.6 ± 7.2	0.41
	K_{cat}/K_m ($mM^{-1}s^{-1}$)	33.6 ± 6.1	28.3 ± 4.8	52.5 ± 11.2	23.4 ± 4.1	0.05

[a] For V_{max} (both CDNB and GSH), p-value reported from Welch ANOVA due to unequal variances. For these parameters, pairwise comparison used Dunnett's T3 test instead of the Tukey method.

Table 5.3. Inhibitory Concentrations (IC_{50}) and Constants (K_i) of Two Hg species on Four GSTP1 Variants (mean ± SEM based on at least six replicates). The p-value for the Welch ANOVA test, used to compare means in each row, is indicated. For all variables, comparisons of each allozyme to the reference, GSTP1 IA, were made with Dunnett's T3 test and significance is labeled as follows: *p<0.05, †p<0.01, ‡p<0.001.

		GSTP1 IA	GSTP1 VA	GSTP1 IV	GSTP1 VV	p value
$HgCl_2$	IC_{50} (μM)	53.7 ± 4.4	24.1 ± 2.7‡	172 ± 22.8*	96.8 ± 9.9*	<0.001
	K_i [CDNB] (μM)	24.7 ± 2.0	12.6 ± 1.6‡	81.5 ± 10.8*	48.4 ± 5.0‡	<0.001
	K_i [GSH] (μM)	29.3 ± 2.4	12.8 ± 1.3‡	61.4 ± 8.2*	45.6 ± 4.7*	<0.001
MeHg	IC_{50} (μM)	135 ± 16.4	93.9 ± 11.3	480 ± 54.1†	196 ± 7.4*	<0.001
	K_i [CDNB] (μM)	61.9 ± 7.5	48.8 ± 5.9	227 ± 25.6†	98.1 ± 3.7†	<0.001
	K_i [GSH] (μM)	73.5 ± 8.9	51.2 ± 6.2	171 ± 19.3*	92.4 ± 3.5	<0.001

Table 5.4. Inhibitory Concentrations (IC_{50}) of Five Heavy Metals on Four GSTP1 Variants (mean ± SEM based on at least three replicates)**.**

Metal	Form	IC_{50} (µM) GSTP1 IA	IC_{50} (µM) GSTP1 VA	IC_{50} (µM) GSTP1 IV	IC_{50} (µM) GSTP1 VV
As	sodium arsenate	>10,000	>10,000	>10,000	>10,000
Pb	lead (II) acetate	>1000	>1000	>1000	>1000
Mn	manganese (II) chloride	>1000	>1000	>1000	>1000
Se	sodium selenite	52.6 ± 3.4	62.8 ± 10.5	60.7 ± 4.9	43.7 ± 4.1
Cd	cadmium (II) chloride	>1000	>1000	>1000	>1000

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Chapter 6

Conclusion

6.1 Objectives

This dissertation aims to further the growing understanding of genetic impact on Hg accumulation and toxicity using epidemiological and *in vitro* methodologies. Polymorphisms in selenoprotein and GSH pathway genes, two families implicated in Hg distribution, metabolism and elimination, are hypothesized to influence inter-individual variability observed in methylmercury and/or inorganic Hg accumulation in exposure biomarkers and to modify the interaction between Hg and blood pressure. This project expands upon a limited body of literature on genetics and Hg accumulation as it evaluates several exposure routes (fish consumption, dental amalgams), Hg biomarkers (urine, hair, and blood), and 23 polymorphisms (selenoprotein and GSH pathway genes) in two genetically distinct populations (Michigan dental professionals of the MDA cohort and Mexican mother-child pairs of the ELEMENT cohort). Knowledge gaps in Hg biomarker assessment are addressed in the aforementioned cohorts, and the influence of low level methylmercury and inorganic Hg exposure on blood pressure is explored in the MDA group. This work further investigates possible mechanisms underlying epidemiological genetic associations by means of *in vitro* enzymatic tests on four genetically engineered variants of GSTP1 that assess the way Hg interacts with variant proteins to influence activity.

6.2 Major Results and Discussion

6.2.1 Mercury Exposure Assessment

Validated methods for Hg exposure assessment exist, and data on Hg body burden estimated from biomarkers for methylmercury (blood, hair) and inorganic Hg exposure (urine) has been collected for many populations. However, general population data on Hg levels remains unknown in some countries (e.g., Mexico), and exposures of certain occupational groups (dentists) merit monitoring due to the potential susceptibility to Hg-related health effects from elevated exposures. Table 6.1 summarizes Hg levels measured in biomarker samples collected from Michigan dental professionals (hair, urine) of the MDA cohort, and Mexican mothers (hair) and children (hair, blood, urine) from the ELEMENT cohort. As expected, estimated Hg intake from reported fish consumption was the main predictor of MDA hair Hg concentrations (Table 2.5) while amalgams (in subjects' mouths and handled in the dental office) and occupation correlated with urine Hg levels (Table 2.4). Overall, dentists had higher hair and urine Hg levels compared with non-dentists (dental hygienists, assistants, etc.), and this corresponds with greater exposures through fish consumption and dental amalgams, though only the latter difference was statistically significant when comparing the two occupational groups (Table 2.1). Eleven percent of the MDA cohort exceeded 1.1 μg/g hair Hg, a concentration that approximately corresponds to the US EPA reference dose for methylmercury exposure (NRC, 2000), and most of the individuals with elevated hair Hg were male dentists. Though the median urine Hg concentration of dentists was 80% higher than that of the US adult population according to NHANES (CDC, 2009), no

MDA subjects had urine Hg >10 µg/L, possibly reflecting reduction of amalgam use for dental restorations and/or safer occupational handling of Hg (Eklund, 2010).

The ELEMENT cohort data provides the first estimate, to our knowledge, of Hg exposures via biomarker measurements in a sample of the general Mexican population. While Mexican mothers had higher median hair Hg values compared to American women of child-bearing age (McDowell et al., 2004), a similar proportion (8%) exceed the recommended hair Hg limit (1.1 µg/g) and children born to these mothers could be at risk for neurodevelopmental toxicity (NRC, 2000). Mexican children exhibited higher Hg biomarker levels compared with American children, and this was particularly pronounced in hair and blood- biomarkers of methylmercury exposure (ATSDR and CDC, 2009; Caldwell et al., 2009; McDowell et al., 2004). Although Mexican children may experience higher Hg exposures than American children, exposures are relatively low as few exceeded biomarker concentrations of concern set by various regulatory bodies (<10% with hair Hg >1.1 µg/g, <5% with blood Hg >5.8 µg/L, 0% with urine Hg >10 µg/L). Data on sources of exposure (e.g., fish consumption frequency, number of dental amalgams) was not available from ELEMENT cohort participants. Fish consumption is expected to be the major contributor to hair and blood Hg levels observed in the ELEMENT cohort as analytical Hg quantification in fish samples from Mexico reveal elevated Hg levels in commonly consumed species (Soto-Jiménez et al., 2010; Trasande et al., 2010).

6.2.2 Cardiovascular Health Impacts

Methylmercury has been linked to cardiotoxic effects including increased blood pressure, though these associations were observed in populations with relatively high exposures from fish or marine mammal consumption (see review: Roman et al., 2011). Animal studies suggest inorganic Hg may have the opposite effect on blood pressure, though epidemiological studies assessing this relationship are non-existent (Massaroni et al., 1995; Rhee and Choi, 1989). In the MDA cohort (2010 subjects, n=262), we provide the first evidence for associations between low level methylmercury and elemental Hg exposures and systolic and diastolic blood pressure (SBP, DBP) alterations (Table 4.4). In SBP and DBP models, after adjustment for several covariates (age, BMI, gender, anti-hypertensive medication), hair Hg levels trended towards a positive association with blood pressure regardless of population stratification (total, by gender, by occupation), though this relationship only attained statistical significance in models of DBP with the total population or males alone. This result, obtained from a group with general population-level methylmercury body burden, correlates well with associations between blood Hg and SBP/DBP observed among highly exposed individuals (Choi et al., 2009).

Elemental Hg exposure, represented by urine Hg, displayed the opposite relationship with blood pressure (Table 4.4). In adjusted models, urine Hg levels were significantly associated with decreasing SBP, and this relationship seemed to be driven by the males/dentists. Hypotensive tendencies of high doses of inorganic Hg have been observed in animal models (Massaroni et al., 1995; Rhee and Choi, 1989). Intriguingly, mercurial compounds were prescribed as anti-hypertensive medications due to their diuretic properties in the early 1900s before their toxicity was discovered (Norn et al.,

2008). This is the first study to suggest a hypotensive association between low level elemental Hg exposure and blood pressure in humans. Due to several study limitations (e.g., small sample size, several influential data points, lack of smoking data), the results should be interpreted cautiously, and the relationship between elemental Hg and blood pressure should be analyzed in other epidemiological cohorts. We observed a similar relationship between urine Hg levels and decreasing SBP using 2003 to 2008 NHANES data collected from adults (>19 years), pointing to a greater need for future study of this outcome (unpublished data).

6.2.3 Genetic Variability and Mercury

Exposure assessment and health hazard characterization are two key components to the risk assessment of toxicants such as methylmercury and elemental Hg. Due to the immense inter-individual variability observed in Hg ADME and toxicity, elucidation of genetic factors contributing to this variability is also vital to the risk assessment process. In the past decade, research exploring genetic influence on Hg accumulation (Custodio et al., 2004, 2005; Engström et al., 2008; Gundacker et al., 2007, 2009) and modification of Hg-related health effects (Echeverria et al., 2005, 2006, 2010) has commenced, though few polymorphisms were analyzed and significant associations were inconsistent across studies (see Table 1.1). This dissertation examined the association of 23 polymorphisms in 14 GSH synthesizing, GST and selenoprotein genes implicated in Hg distribution, metabolism and elimination with Hg biomarker levels in two epidemiological cohorts.

Given the number of Hg biomarker models including genetic polymorphisms in both the MDA and ELEMENT cohorts (n>200), few significant ($p<0.05$) genetic terms

were observed. Furthermore, none of the significant polymorphism-Hg biomarker relationships observed remained statistically significant after correcting for multiple testing. The nominally significant findings should thus be viewed as trends and potentially important polymorphisms, even though no conclusive evidence can be garnered from these genetic associations. Tables 6.2 and 6.3 summarize the significant ($p<0.05$) associations observed between polymorphisms and hair or urine Hg biomarker levels. Models addressing ELEMENT children's blood Hg, hair:blood, and blood:urine are not reported due to a lack of nominally significant ($p<0.05$) genetic associations. Multivariate analyses using the MDA data are better suited to detect genetic associations because they control for exposures (fish consumption or dental amalgams) and test for interaction between exposure source and genotype on hair or urine Hg levels. Statistical analyses were limited in the ELEMENT cohort since exposure data was not available, and this may be reflected in the number of significant genetic terms observed in MDA models (n=9) compared with ELEMENT models (n=1).

Overall, five polymorphisms were associated ($p<0.05$) with hair Hg levels (*GSS* rs3761144; *GSTP1* rs1695, rs1138272), urine Hg levels (*GSTT1* deletion*)* or both (*SEPP1* rs7579) in various statistical analyses, primarily in the MDA cohort. The *GSTP1* SNPs have been implicated in Hg toxicokinetics in previous studies (Custodio et al., 2004; Engström et al., 2008; Gundacker et al., 2009), though the direction of the relationships we observed (decreasing biomarker levels associated with minor alleles of rs1695 and rs1138272) only correlate with the results of one study (Engström et al., 2008; see Figure 6.1). Polymorphisms in *GSS*, a gene important to GSH synthesis, have not been related to Hg exposure biomarkers in past studies, and the relationship we observed

(higher hair Hg per intake from fish consumption with each variant allele) should be analyzed in other cohorts.

Results from both the MDA and ELEMENT cohorts suggest that a 3'UTR polymorphism in *SEPP1* (rs7579, C>T) may influence inter-individual variability in Hg body burden, and this relationship should be further investigated in future studies. Figure 6.2 simplifies the ADME pathway for methylmercury and elemental Hg with SEPP1 implicated primarily in the distribution phase. The findings of several studies, including the MDA and ELEMENT cohort work, pertaining to *SEPP1* genotype are also summarized in Figure 6.2. SEPP1 binds both inorganic Hg (with selenium, in the form Hg-Se) and methylmercury via its selenocysteine and cysteine residues (Khan and Wang, 2009). In rats, co-exposure to selenite and $HgCl_2$ results in less urinary excretion of Hg, and this may be due to SEPP1 binding of Hg-Se and subsequently less Hg distribution to the kidneys (Jureša et al., 2005). Previous epidemiological studies designate the 3'UTR SNP (rs7579) as a modifier of gene expression and protein synthesis. The T allele is associated with increased plasma SEPP1, though this relationship is partially dependent on selenium levels (Méplan et al., 2007). Furthermore, *SEPP1* 3'UTR T led to a higher proportion of the SEPP1 isoform containing more selenocysteine residues (Méplan et al., 2009).

In the MDA and ELEMENT cohorts, *SEPP1* 3'UTR T significantly associated with lower urine Hg concentrations. Coupling our associative findings with that of previous toxicological studies suggests that decreased urinary Hg could result from increased Hg retention via binding to SEPP1 and less urinary excretion. The relationship between Hg and *SEPP1* genotype may be further complicated by selenium status (not

quantified here), Hg exposure level, and Hg species. In MDA cohort models of urine Hg, a significant positive interaction was observed between *SEPP1* genotype and amalgams (Table 6.3). At lower exposure levels (<5 dental amalgams, few handled in the dental office), individuals with CT/TT genotypes had lower urine Hg compared with CC while the opposite was observed at higher exposures. Furthermore, SEPP1 may impact methylmercury distribution and retention as reflected in hair Hg levels. The 3'UTR T allele is associated with lower hair Hg per unit of Hg intake from fish consumption (Table 6.2). Results must be interpreted cautiously due to limitations in both the MDA and ELEMENT cohorts. Due to multiple testing, the *SEPP1* SNP-Hg associations could have occurred by chance alone in either cohort. Furthermore, confounding related to percent ancestry in the ELEMENT cohort may have influenced the result. Nevertheless, *SEPP1* genotype potentially impacts retention of inorganic Hg and methylmercury and should be considered in future gene-Hg studies.

In addition to the association between genetic polymorphisms and Hg biomarker levels, we examined the modification of polymorphisms on the relationship between blood pressure and hair Hg or urine Hg. Out of 44 models of SBP and DBP including hair or urine Hg and one polymorphism per model, one significant genetic main effect was observed (Table 4.6, *GCLM* rs41303970 in SBP model), and this finding may have occurred by chance. The lack of significant ($p<0.05$) interactions (between genotype and biomarker) or other significant genotype main effects in multivariate linear regression models for SBP or DBP could be due to polymorphism selection. The polymorphisms assessed were chosen because of their involvement in Hg ADME. As such, these genetic variants might not impact the mechanisms through which elemental Hg and

methylmercury affect cardiovascular function. Furthermore, we may have lacked the statistical power to detect weak associations due to small sample size (n=205 to 239 depending on the polymorphism).

6.2.4 GSTP1 In Vitro Characterization

We explored the impact of GSTP1 genotype of two nonsynonymous SNPs (rs1695, Ile/Val 105; rs1138272, Ala/Val 114) on enzyme function *in vitro*, following several significant, albeit inconsistent, epidemiological associations observed between these SNPs and Hg biomarker levels in the MDA cohort and other studies (Custodio et al., 2004; Engström et al., 2008; Gundacker et al., 2009). Figure 6.1 summarizes both *in vitro* and epidemiological associations pertaining to *GSTP1* 105 and 114 SNPs. Four allozymes of GSTP1 (IA, VA, IV, VV) displayed significantly different kinetic parameters and sensitivity to $HgCl_2$ and MeHg inhibition (Tables 5.2 and 5.3). Enzymes with the more common Ile105 had the greatest affinity for the electrophilic substrate, CDNB and the best catalytic efficiency (k_{cat}/K_m). This residue is found in the electrophilic substrate binding site, and valine substitution may alter the shape and hydrophobicity of the site (Zimniak et al., 1994). All GSTP1 allozymes were inhibited by $HgCl_2$, MeHg, and sodium selenite but resistant to arsenic, lead, manganese, and cadmium treatment. Genotype influenced potency of Hg inhibition with allozymes ordered according to sensitivity to both Hg species as follows: GSTP1 VA>IA>VV>IV. Overall, GSTP1 VA had the poorest performance in enzyme activity assays and was the most sensitive to $HgCl_2$ and MeHg inhibition.

Laboratory based assessments as described with GSTP1 provide quick and high-throughput screening for the functional impact of polymorphisms, and such methods are in line with goals of twenty-first century toxicity testing (NRC, 2007). However, in light of inconsistent associations observed between Hg biomarkers and *GSTP1* genotype in the MDA cohort and previous epidemiological studies (see Figure 6.1), it is clear that gene-Hg biomarker relationships are complex with contributing factors beyond biochemical differences apparent at the *in vitro* level.

6.3 Future Research

The known dangers of methylmercury and inorganic Hg and the uncertainty about safe exposure levels due to inter-individual differences in Hg accumulation and toxicity necessitate consideration of genetics in the Hg risk assessment process. The work presented in this dissertation and a handful of other studies constitute the infancy of gene-Hg work, and there is a need for future research. Future studies assessing genetics and Hg accumulation would benefit from larger cohorts (>1000) of various genetic backgrounds as this would increase statistical power to detect significant relationships, allow for the analysis of more polymorphisms, and allow for multiple testing correction to decrease the chance of false positive discovery. Epidemiological studies should collect detailed information on exposures to both methylmercury (e.g., reported fish consumption or biomarker measurement of polyunsaturated fatty acids) and elemental Hg (e.g., number of amalgam surfaces) and relate exposures to Hg levels (total or speciated) in hair, blood, and urine. Statistical analyses accounting for estimated Hg exposures when assessing polymorphism-Hg biomarker relationships will produce more meaningful results.

Furthermore, the influence of selenium status, measured in biomarker samples, on Hg-SNP associations should be examined, especially when analyzing selenoprotein SNPs. While future Hg research can continue to explore the influence of glutathione pathway and selenoprotein SNPs, polymorphisms in other gene families involved in Hg metabolism (e.g., metallothioneins; transporters such as *MRP1, MRP2, OAT1*) merit investigation. Analysis of polymorphism combinations or haplotypes might aid the discovery of influential genes. If large cohorts are available, genome-wide association studies (GWAS) could be employed to scan for undiscovered polymorphic regions that potentially influence Hg toxicokinetics. Given the paucity of GWAS or large cohort (n>1000) candidate gene studies in the fields of toxicology and environmental health, an alternative approach would be to perform a meta-analysis comparing trends and significant associations observed across all small-scale, epidemiological studies focusing on candidate gene-Hg relationships.

Coupling epidemiological results with *in vitro* assays may provide insight into possible mechanisms (e.g., alterations in protein structure and function or gene expression) underlying associations. In the case of SEPP1, *in vitro* determination of gene expression and Hg binding capacity according to rs7579 genotype, oxidation state, various concentrations of Hg (inorganic or methylmercury) and selenium treatment would increase our understanding of Hg biomarker associations observed in the MDA and ELEMENT cohorts.

While the toxic effects of methylmercury and inorganic Hg have been studied for decades, their involvement with cardiovascular health, specifically blood pressure, has only recently been addressed and requires further research. Future epidemiological

studies should consider the relationships between SBP, DBP, pulse pressure, and other markers of cardiovascular health and both forms of Hg using hair and urine biomarkers in populations with a range of exposure levels. To better control for confounding that may have underscored the divergent associations observed in the MDA cohort with hair and urine Hg and blood pressure, future studies should control for a multitude of factors including gender, race, smoking status, physical activity, and nutrient status (e.g., polyunsaturated fatty acids, selenium). *In vitro* and *in vivo* toxicological studies are needed to understand the mechanisms through which methylmercury and inorganic Hg might influence cardiovascular activity and blood pressure. Mechanistic and epidemiological data relating inorganic Hg to cardiovascular outcomes are particularly scarce, and require future evaluation given our preliminary findings in a limited cohort with low-level exposure. In general, studies assessing Hg toxicity may also investigate the modification of genetic polymorphisms involved in Hg toxicokinetics or specifically with the system of interest (e.g., nervous, cardiovascular) on Hg-health outcome relationships to better comprehend variability observed in Hg toxicity.

Gene-environment research has the potential to increase our understanding of the immense variability in the normative risk assessment of Hg and to enable risk managers to make objective and informed decisions about the true health risks of Hg. However, more gene-environment studies pertaining to Hg, both epidemiological and *in vitro*, are necessary. No single polymorphism-Hg association or study is meaningful on its own as causality cannot be determined from epidemiological association studies or *in vitro* assays. Furthermore, a given polymorphism is expected to have a minor, if any, impact on the actual variability observed in Hg toxicokinetics (<1%). In future research, the

synthesis of association data obtained from dozens of key polymorphisms in the Hg toxicokinetic pathway explored in multiple epidemiological cohorts with polymorphism functionality data (e.g., alterations to gene expression or enzyme activity) may provide a better picture of the multi-loci genetic contribution to Hg toxicokinetic variability.

Figures and Tables

Figure 6.1. *GSTP1* Genotype and Hg: Inconclusive Epidemiological and *In Vitro* Data. The simplified diagram below places biomarkers analyzed in this dissertation in the ADME pathways for Hg vapor and MeHg. GSTs are expected to primarily influence the metabolism and elimination of Hg. Significant associations observed between *GSTP1* genotype and Hg biomarkers from various epidemiological studies (e.g., differences between unadjusted means or medians, effect modification of fish consumption-biomarker relationship) are listed from the following studies: [1]Gundacker et al., 2009; [2]Engström et al., 2008; [3]Custodio et al., 2004; [4]MDA Cohort study. Enzymatic property differences observed based on genotype at the *in vitro* level are also reported. While the Ile/Val 105 and Ala/Val 114 SNPs appear to influence enzyme activity and interaction with Hg, epidemiological findings pertaining to Hg body burden and genotype remain inconsistent.

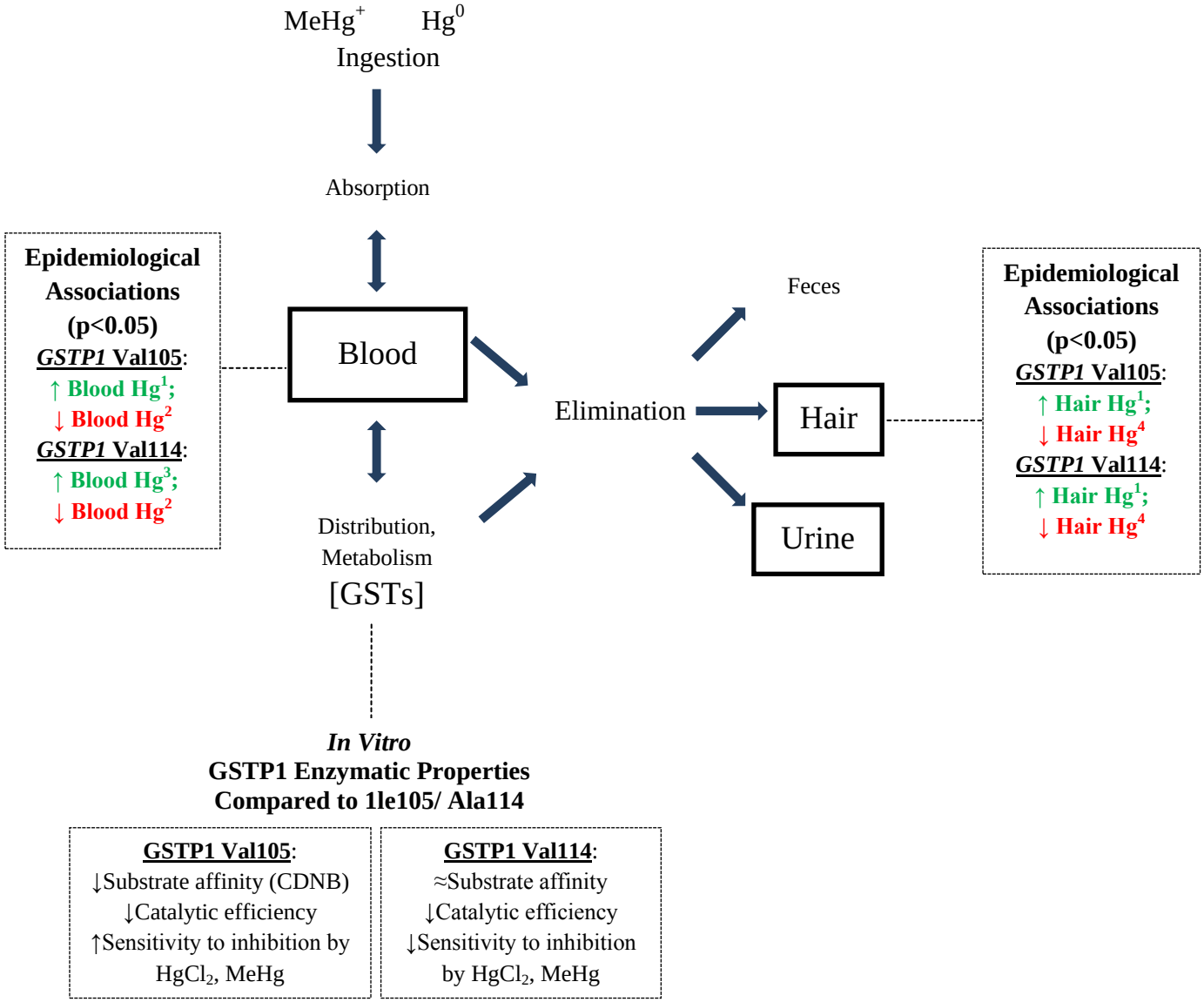

Figure 6.2. *SEPP1* Genotype and Hg Body Burden. An overview of the ADME pathway for $MeHg^{+}$ and Hg^{0} exposure is depicted below with a focus on Hg biomarkers analyzed in the MDA and ELEMENT cohorts. SEPP1 is proposed to influence this pathway primarily through binding of Hg-selenium or MeHg via its selenocysteine and cysteine residues. Statistically significant ($p<0.05$) associations between SEPP1 3'UTR genotype (rs7579, C>T) and biomarkers observed in the MDA and ELEMENT cohorts are reported along with impacts of this SNP on protein expression and synthesis as observed in by others ([1]Méplan et al., 2007; [2]Méplan et al., 2009).

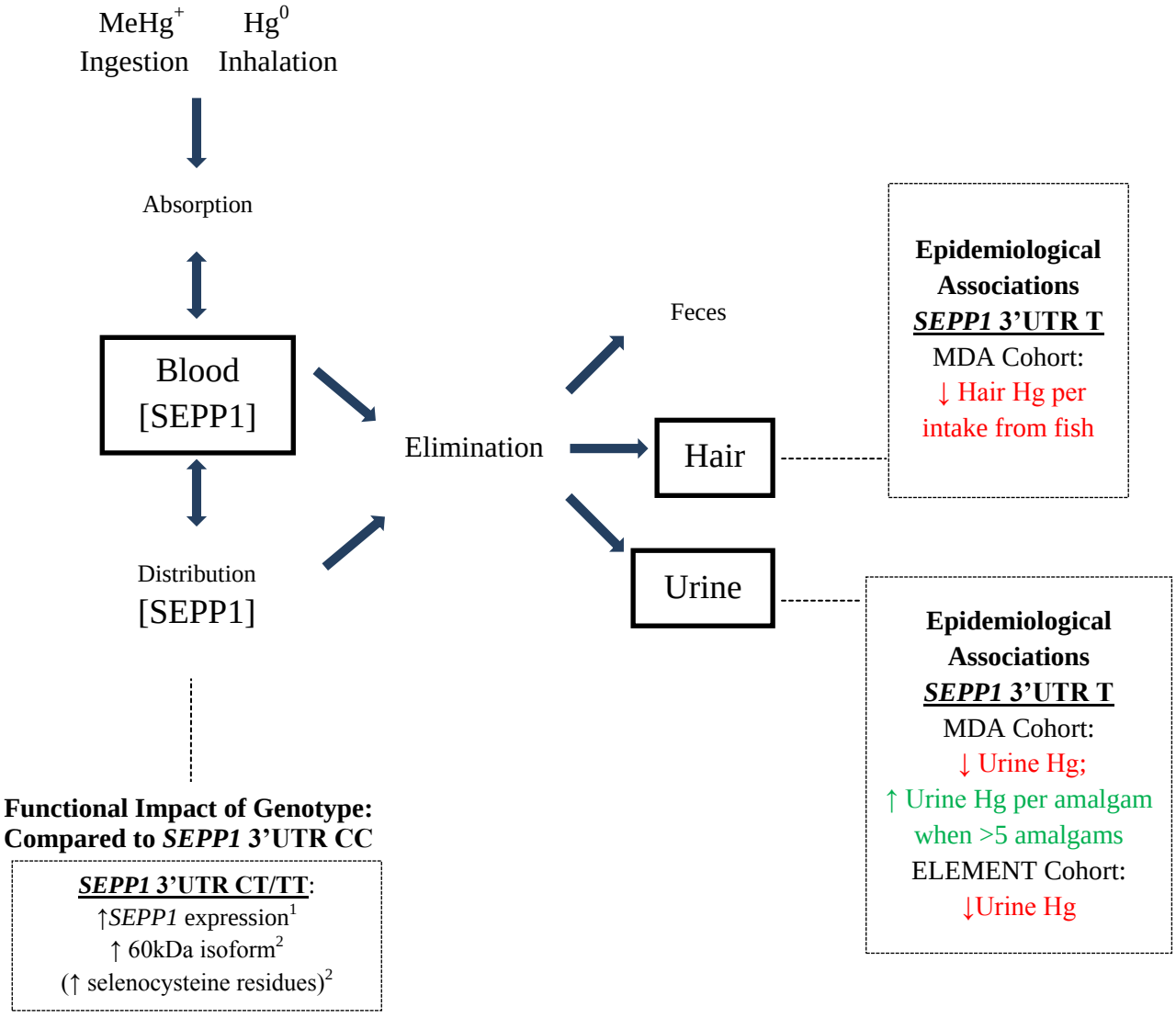

Table 6.1. Mercury Biomarker Levels: MDA and ELEMENT Cohorts. Mean (SD) are reported with selected stratifications of both cohorts.

	Hair Hg (µg/g)		Blood Hg (µg/L)	Urine Hg (µg/L)
Population	**Mean (SD)**	**% Over RfD[a]**	**Mean (SD)**	**Mean (SD)**
MDA, Total	0.49 (0.63)	10.9%	--	1.04 (1.18)
MDA, Dentists	0.69 (0.81)	19.7%	--	1.37 (1.30)
MDA, Non-dentists	0.31 (0.33)	3.0%	--	0.75 (0.97)
ELEMENT, Mothers	0.53 (0.47)	7.9%	--	--
ELEMENT, children 6-11 yrs	0.55 (0.49)	9.4%	1.80 (1.42)	0.87 (1.13)
ELEMENT, children 13-15 yrs	0.42 (0.35)	4.1%	1.53 (1.22)	0.65 (0.80)

[a] Individuals with hair Hg greater than 1.1 µg/g. This hair Hg level approximately corresponds with the US EPA reference dose (RfD) for methylmercury exposure (NRC, 2000). Reference value comparisons are not reported for blood or urine Hg as <5% of individuals exceeded limits suggested by various regulatory bodies.

Table 6.2. Genotype and Hair Hg: Compare Models from MDA and ELEMENT Cohorts. Parameter estimates for nominally significant (p<0.05) genotype variables are reported from various models of hair Hg (described in Sections 2.3.5 and 3.3.5). Shading corresponds to p-value of estimate: p>0.05, p<0.05, p<0.01. Not all polymorphisms were analyzed in both cohorts (white boxes).

Gene	db SNP ID	Minor Allele Frequency		MDA			ELEMENT Children		ELEMENT Mothers	
		MDA	ELEMENT	Simple	Main Effect, Multivariate	Genotype *Fish Hg	Simple	Multivariate	Simple	Multivariate
GCLC	rs17883901	0.08	0.04							
GCLM	rs41303970	0.19	0.08							
GSTP1	rs1695[a]	0.32	0.45	-0.22	-0.22					
GSTP1	rs1138272	0.08	0.01		-0.31					
GSTM3	rs7483	0.26	0.47							
SEPP1	rs3877899	0.24	0.09							
SEPP1	rs7579	0.28	0.43			-1.61				
GPX4	rs713041	0.45	0.29							
GSTT1	deletion	0.41								
GSTM1	deletion	0.32								
GGT1	rs5751901	0.36								
GSS	rs3761144	0.4				1.88				
GSR	rs1002149	0.18								
GSR	rs2911678	0.24								
GPX1	rs1050450	0.28								
GSTP1	rs749174		0.13							
GSTP1	rs6591256		0.16							
GSTP1	rs947895		0.13							
GSTO1	rs4925		0.16							
GSTO1	rs11509438		0.003							
GSTM1	rs1065411		0.04							
GSTM3	rs1332018		0.34							
GPX2	rs4902346		0.21							
GPX2	rs2737844		0.4							

[a]Minor allele encodes Val105 in the MDA cohort but Ile105 in the ELEMENT cohort.

Table 6.3. Genotype and Urine Hg: Compare Models from MDA and ELEMENT Cohorts. Parameter estimates for nominally significant (p<0.05) genotype variables are reported from various models of urine Hg (described in Sections 2.3.4 and 3.3.5). Shading corresponds to p-value of estimate: p>0.05, p<0.05, p<0.01. White boxes denote polymorphism was not analyzed in that cohort.

Gene	db SNP ID	Minor Allele Frequency		MDA			ELEMENT Children	
		MDA	ELEMENT	Simple	Main Effect, Multivariate	Genotype *Amalgam	Simple	Multivariate
GCLC	rs17883901	0.08	0.04					
GCLM	rs41303970	0.19	0.08					
GSTP1	rs1695[a]	0.32	0.45					
GSTP1	rs1138272	0.08	0.01					
GSTM3	rs7483	0.26	0.47					
SEPP1	rs3877899	0.24	0.09					
SEPP1	rs7579	0.28	0.43	-0.16	-0.26	0.04		-0.14
GPX4	rs713041	0.45	0.29					
GSTT1	N/A	0.41		-0.27				
GSTM1	N/A	0.32						
GGT1	rs5751901	0.36						
GSS	rs3761144	0.4						
GSR	rs1002149	0.18						
GSR	rs2911678	0.24						
GPX1	rs1050450	0.28						
GSTP1	rs749174		0.13					
GSTP1	rs6591256		0.16					
GSTP1	rs947895		0.13					
GSTO1	rs4925		0.16					
GSTO1	rs11509438		0.003					
GSTM1	rs1065411		0.04					
GSTM3	rs1332018		0.34					
GPX2	rs4902346		0.21					
GPX2	rs2737844		0.4					

[a]MDA and ELEMENT cohorts do not have the same minor allele for rs1695 (Val105 for MDA, Ile105 for ELEMENT).

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