

APPLICATION OF URINARY ARSENIC METABOLITES TO ASSESS ARSENIC EXPOSURE
IN SOUTHEASTERN MICHIGAN: ADVANCING EXPOSURE ASSESSMENT FOR
EPIDEMIOLOGY RESEARCH

by

Zorimar Rivera

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Environmental Health Sciences)
in The University of Michigan
2009

Doctoral Committee:

Professor Jerome O. Nriagu, Chair
Associate Professor Amr S. Soliman
Assistant Professor John D. Meeker
Assistant Professor Marie S. O'Neill
Assistant Professor Jaymie R. Meliker, Stony Brook University

© Zorimar Rivera

All Rights Reserved
2009

Acknowledgements

This dissertation is only a small part of an extraordinary amount of effort guided and supported by an excellent team of professionals. It is clear to me that without them my work would not be possible. My advisor, Dr. Jerome Nriagu, has taught me patience and commitment with my career. I am incredible grateful to Dr. Jaymie Meliker, for his mentoring, assistance, and direction during my writing processes. His passion for this work inspires and challenges me to do my best as a scientist and to continue my learning process with dedication. I am very thankful to Dr. Marie O’Neill for her support and genuine camaraderie. I am also thankful for the guidance provided by Dr. John Meeker and Dr. Arm Soliman who offered me support and assistance throughout my education.

My time in Michigan has been an assortment of experiences, academic and personal. During tougher times, distinctive faculty members were there to listen and support me. I will always be grateful to Dr. Robert Gray, Dr. Craig Harris, and Dr. Rita Loch-Caruso. My work in this dissertation would not be possible without the assistance and guidance of Ms. Lingling Zhang from the University of Michigan Center for Statistical Consultation and Research (CSCAR). Lingling has been an amazing teacher with

abundant compassion for students. Likewise, the English Language Institute (ELI) instructors were essential keys in helping to improve my English skills.

I spent many hours driving around Michigan, working in the lab, or simply, contemplating life with a wonderful group of people during four years of sample collection and analysis. Nick Mank, Caitlyn Meservey, and Angela Hungerink accompanied me during my field trips. Gillian AvRuskin and Stacey Fedewa contributed in the field and in every step of the research work. My fellow doctoral student during that time, Dr. Jaymie Meliker and Dr. Melissa Slotnick, who offered me guidance as senior doctoral students. I was very fortunate to work with Aaron Linder, an extraordinary chemist, who provided me with assistance and advice in the laboratory. Dr. Bin Chen, a postdoctoral fellow, helped me with the fundamentals of the analytical protocols. Both of them are co-authors in the publishable version of Chapter 3 submitted to the Science of the Total Environment. In addition to all team members, this research could not have taken place without the participation of more than 900 individual who donated their time to this study. Many of these individuals welcomed us sincerely into their homes, showed true interest in the study and offered encouragement and appreciation for our work.

This research was funded by the National Cancer Institute, grant RO-1 CA96002-10. My graduate student research assistantship was a supplement to this main grant (O5S1). I was also grateful to receive support from the University of Michigan Residential College (RC) for the last year of my degree. Over there, I learned what the

“Michigan Difference” is. The RC has a group of dedicated and special group of professors that wake up every day with the goal of making each of their students a better human being.

Lastly, one of the most difficult things during all these years has been the fact that I am away from home. It is really hard to describe how difficult and challenging is not to have my family and friends close to me everyday. Fortunately, Michigan provided me with a group of extraordinary new friends who became my extended family here. Lorena, Vivian, Juan, Alexis, Elena, and Rosario have been my family in Michigan and good friends for years to come. My adored friends back home Moraima, Sandra, and Carmen for each of those encouraging calls during the good and the bad times. I can not imagine trying to conquer all my goals in life without the continuous and unconditional support of my family. Families in Puerto Rico are warm, close and noisy. I have no words to thank my Mom who has been my standard and guide all my life. Her husband, Luis, who gave me two amazing brothers. My three brothers, Juan Ernesto, Luis Antonio, and Luis Daniel who always keep me balanced and remind me of my possibilities. My uncle Angel, who has been my father and a vertical line to follow all my life. My cousins Danny, Alejandro, Eunice, Angel, and Yaniel because they are my brothers too. My grandma Margot, who has a blind faith on me. My aunt Raquel, who left me during my time in Michigan, cultivated in me my desire for knowledge and the love for my culture. My two baby cousins, Andrea and Brianaliz because they represent my new responsibilities in life. Michigan also gave me a best friend and partner in life, Luis, who believes in me when I don't. Finally, I am a product of the public education system of

Puerto Rico. To all those teachers back home, which are dedicated to their students and have faith on them, I am extremely thankful. To my loved Puerto Rico, for a future of freedom, peace, opportunities, and the dream of going back.

Table of Contents

Acknowledgements.....	ii
List of Figures	viii
List of Tables	x
List of Appendices	xiii
List of Abbreviations	xiv
Abstract.....	xvii
Chapter	
I. Improving Arsenic Exposure Assessment in Southeastern Michigan:	
Background and Dissertation Goals.....	1
References	13
II. Urine as a Medium to Study Arsenic Metabolites.....	16
Urine Composition and Function	16
Biomarkers of Exposure	18
Advantages of Urine over other Biomarkers	20
Arsenic in Human Urine	21
Arsenic Metabolism	23
Urinary Arsenic Metabolites as Biomarkers of Arsenic Exposure	28
Urine Analysis.....	31
References	42
III. Low-level Determination of Six Arsenic Species in Urine by High Performance Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometry....	52
Introduction	52
Methodology.....	54
Results and Discussion	59
References	77
IV. Reliability of Spot Urine Samples in Assessing Arsenic Exposure.....	81

	Introduction	81
	Methods	83
	Results.....	88
	Discussion.....	91
	References	106
V.	Urinary Arsenic Species, Toenail Arsenic, and Estimates of Arsenic Intake in a Southeastern Michigan Population With Low-to-Moderate Exposure to Arsenic in Drinking Water	110
	Introduction	110
	Methods.....	113
	Results.....	120
	Discussion.....	124
	References	150
VI.	Arsenic Methylation in Southeastern Michigan	154
	Introduction	154
	Methods.....	158
	Results.....	163
	Discussion.....	165
	References	185
VII.	Conclusions	191
	References.....	198
	Appendices.....	199

List of Figures

Figure 1-1. Arsenic concentrations and distribution of cases and controls in the study area in Southeastern Michigan (Meliker 2009).	12
Figure 2-1. Classical inorganic arsenic biotransformation pathway (Cullen and Reimer 1989).	38
Figure 2-2 New Inorganic Arsenic Biotransformation Pathway (Hayakawa et al. 2005) 39	
Figure 3-1. Change in retention time of five arsenic species with increasing mobile phase pH using the Gemini, 5µm, 150 X 4.6 mm column with 5 mM tetrabutylammonium hydroxide, 3mM malonic acid and 5% (v/v) methanol.....	72
Figure 3-2. Chromatogram of a urine sample using the Gemini, 5 µm, 150 X 4.6 mm column with 10 mM ammonium phosphate, 5 mM tetrabutylammonium hydroxide and 4% (v/v) methanol at pH 9.2.	73
Figure 3-3. Chromatogram of a urine sample, spiked with 10 µg L ⁻¹ of AsB, As[III], DMA[V], MMA[V], As[V] using the Gemini, 5 µm, 250 X 4.6 mm column with 10 mM ammonium phosphate, 5 mM tetrabutylammonium hydroxide and 4% (v/v) methanol at pH 9.5.....	74
Figure 3-4. Chromatogram of aqueous solution of AsB, As[III], DMA[V], MMA[V], As[V], 10 µg L ⁻¹ , and MMA[III], unknown concentration using the Gemini, 5 µm, 250 X 4.6 mm column with 10 mM ammonium phosphate, 5 mM tetrabutylammonium hydroxide and 4% (v/v) methanol at pH 9.5.	75
Figure 3-5. Urine sample showing two unknown arsenic species, one co-eluting with DMA[V], the other near 12.7 minutes.....	76
Figure 4-1 Bland-Altman plot of difference between FMV and spot TotAs(µg/L) urine samples (n=131).....	104

Figure 4-2 Correlation between FMV and spot samples for Log ₁₀ -TotAs.....	105
Figure 5-1. Urinary arsenic vs. arsenic in drinking water (n=343).....	147
Figure 5-2. Urinary Arsenic vs. Arsenic in drinking water (≥1ug/L) (n=137).....	148
Figure 5-3. Urinary arsenic vs. arsenic in drinking water (≥1ug/L) and among those with water intake above median (n=80).....	149
Figure 6-1 Proposed mode of action for arsenic-induced urinary bladder carcinogenesis (Cohen et al. 2007).....	174

List of Tables

Table 1-1. Chemical Properties and Other Facts of Arsenic	11
Table 2-1 Most common chromatography columns and conditions used in arsenic speciation	40
Table 2-2 Studies reporting individual level exposure-biomarker association for arsenic	41
Table 3-1. Final HPLC operating conditions.	68
Table 3-2. Final ICP-MS operating conditions.	69
Table 3-3. Quantitative results for arsenic speciation method in spiked sample and CRM	70
Table 3-4. Summary of arsenic species in urine samples.	71
Table 4-1 Arsenic concentrations below detection limit for each arsenic species	97
Table 4-2. Univariate analysis for total arsenic concentration (TotAs) of FMV, spot urine samples and total arsenic concentration toenails.....	98
Table 4-3. Table arsenic species concentration in FMV and spot urine samples.....	99
Table 4-4. Pearson correlation coefficients and ICC between FMV and Spot samples for each arsenic species.....	100
Table 4-5. Proportion of arsenic species in FMV and spot urine samples.	101
Table 4-6. Pearson correlations coefficients (r) between FMV and spot urine samples for each arsenic species between FMV and Spot samples by groups.....	102

Table 4-7 Association between TotAs in urine and total arsenic concentration in toenails	103
Table 5-1. Arsenic exposure metrics.....	132
Table 5-2. Inorganic arsenic concentration estimates.	133
Table 5-3. Percent of participants self-reported food consumptions during three days prior to the urine sample collection.	134
Table 5-4. Amount of samples below detection limits by individual species.....	136
Table 5-5. Average concentrations of arsenic in urine (TotAs), arsenic in water and arsenic in toenail for the study population.	137
Table 5-6. Average arsenic concentrations in urine ($\mu\text{g/L}$), toenails ($\mu\text{g/g}$) and water samples ($\mu\text{g/L}$) from Michigan samples and NHANES (Caldwell et al. 2009).	138
Table 5-7. Study population characteristics as predictors of TotAs ($\mu\text{g/L}$).	139
Table 5-8. Relationship between urinary arsenic concentrations (TotAs $\mu\text{g/L}$) and estimated arsenic exposure, stratified by arsenic drinking-water concentration and water intake.	140
Table 5-9. Relationship between InAs arsenic concentrations in urine ($\mu\text{g/L}$) and estimated arsenic exposure, stratified by arsenic drinking-water concentration and water intake.	141
Table 5-10. Relationship between MMA arsenic concentrations ($\mu\text{g/L}$) in urine and estimated arsenic exposure, stratified by arsenic drinking-water concentration and water intake.	142
Table 5-11. Relationship between DMA arsenic concentrations ($\mu\text{g/L}$) in urine and estimated arsenic exposure, stratified by arsenic drinking-water concentration and water intake.	143
Table 5-12. Estimates of arsenic in food as predictors of TotAs ($\mu\text{g/L}$).....	144

Table 5-13. Correlations of urinary arsenic metabolites with arsenic in water and toenail.	145
Table 5-14. Arsenic in drinking water as a predictor of urinary arsenic and toenail arsenic concentration.....	146
Table 6-1. Comparison of demographic characteristics between the total sample (parent project) and the subsample evaluated in this research	175
Table 6-2. Amount of samples below detection limits by individual species.....	176
Table 6-3. Sociodemographic characteristics of 167 bladder cancer cases and 219 non-cancer controls.....	177
Table 6-4. Bladder cancer odds ratio for smoking status stratified by lifetime arsenic exposure.....	178
Table 6-5. Univariate analysis of the absolute values of each arsenic value.	179
Table 6-6. Univariate analysis of the proportions of each arsenic species.	180
Table 6-7. Bladder cancer odds ratio for median levels of %MMA stratified by lifetime exposure including all individuals.	181
Table 6-8. Bladder cancer odds ratio for for median levels of %MMA stratified by lifetime exposure in the non-smokers group.	182
Table 6-9. Bladder cancer odds ratios for median levels of %MMA stratified by lifetime exposure in the smokers group	181
Table 6-10. Studies in populations exposed to low-moderate levels of arsenic in drinking water (<150µg/L).	184

List of Appendices

Appendix A Questionnaire for Computer-Aided Telephone Interview	200
Appendix B Food Frequency Questionnaire	251

List of Abbreviations

As[III]	Arsenite
As[V]	Arsenate
AsB	Arsenobetaine
ATG	Arsenic Triglutathione
AFS	Atomic Fluorescence Spectrometry
BLD	Below Detection Limit
CI	Confidence Interval
CDC	Center for Disease Control and Prevention
CRM	Certified Reference Material
DMA[III]	Dimethylarsinous acid
DMA[V]	Dimethylarsinic acid
EPA	Environmental Protection Agency
FFQ	Food Frequency Questionnaire
FMV	First Morning Void
HG	Hydride Generation
HGAAS	Hydride Generation Atomic Absorption Spectrometry
HGAFS	Hydride Generation Atomic Fluorescence Spectrometry
HPLC	High Performance Liquid Chromatograph

IARC	International Agency of Research on Cancer
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectrometry
ICP-MS	Inductively Coupled Plasma Mass Spectrometer
InAs	Inorganic Arsenic (As[III]+As[V])
LDPE	Low-density Polyethylene
MAGD	Monomethylarsenic Diglutathione
MeOH	Methanol
MCL	Minimum Contaminant Level
MMA[III]	Methylarsonous acid
MMA[V]	Methylarsonic acid
NCI	National Cancer Institute
NIH	National Institute of Health
NIES	National Institute of Environmental Studies
NRC	National Research Council
PAH	Polycyclic Aromatic Hydrocarbon
PTFE	Polytetrafluoroethylene
ROS	Reactive Oxygen Species
RSD	Relative Standard Deviation
SAS	Statistical Analysis System
SNP's	Single Nucleotic Polymorphism
TBAH	Tetrabutylammonium Hydroxide
TMAO	Trimethylarsine Oxide

TotAs Total Toxic Arsenic Concentration (As[III]+As[V]+MMA[V]+DMA[V])

WHO World Health Organization

Abstract

Urinary arsenic metabolites are used in epidemiological studies to assess arsenic exposure. However, to ensure reliable application of the biomarker, further development is needed. This research contributes to the validation of urinary arsenic metabolites as arsenic exposure biomarkers in a subsample of 167 bladder cancer cases and 254 controls enrolled in a large case-control study in Southeastern Michigan. This work aims to develop an analytical protocol to speciate arsenic metabolites in urine, assess the reliability of sampling strategies, the exposure-biomarker relationship, and the relationship between the biomarker and disease.

Information on demographics, smoking, dietary intake and drinking water intake was collected from participants living in an eleven-county region of Michigan with historically high groundwater arsenic concentrations. Drinking water and toenails samples were collected and analyzed for total arsenic by ICP-MS. Urine samples were speciated using an HPLC-ICPMS coupled-system. Six species were measured As[III], As[V], DMA[V], MMA[V], MMA[III], and Arsenobetaine. The sum of As[III], As[V], MMA[V], and DMA[V] was designated total arsenic (*TotAs*). A subpopulation ($n=131$) provided additional urine samples to compare the arsenic levels in spot and first morning void (FMV) samples.

The interclass correlation coefficient between *TotAs* in FMV and *TotAs* in spot samples showed that 90% of variation comes from between individuals and not within individuals. Drinking water arsenic concentration was a significant predictor ($p < 0.0001$) of *TotAs* ($R^2 = 0.17$). The correlation increased to 0.24 ($p < 0.0001$) and 0.39 ($p < 0.0001$) when the exposure was categorized by arsenic concentrations in water ($\geq 1 \mu\text{g/L}$) and water intake (above and below median), respectively. Significant associations between *TotAs* and bladder cancer may suggest that individuals who ingest elevated levels of arsenic may retain the metalloid in their bodies. However, sample size limitations and study design have to be considered when evaluating these associations.

These results indicate that urinary arsenic metabolites can be used as biomarkers to assess recent arsenic intake via drinking water. Categorical estimates of water consumption better characterize intake in this population. Selection of adequate exposure measures may reduce misclassification in self-reported water consumption. FMV and spot samples can be used without preference when evaluating arsenic exposure in epidemiological studies. These results advance the validation process of urinary arsenic metabolites as biomarkers of arsenic exposure which are essential tools in risk assessment and epidemiological studies.

Chapter I

Improving Arsenic Exposure Assessment in Southeastern Michigan: Background and Dissertation Goals

I. Arsenic in Water and Health

Chronic exposure to inorganic arsenic in drinking water is a concern for regulatory agencies and public health organizations world-wide. Arsenic is classified by the International Agency for Research in Cancer as a human carcinogen (IARC 1987; IARC 2004). Epidemiological studies around the world show evidence of how arsenic exposures are associated with diseases such as skin, bladder and lung cancer, cardiovascular diseases, and skin disorders (Karagas et al. 2001; Chen 2003; Steinmaus et al. 2003; Stainmaus 2006; Bates et al. 2004; Tseng et al. 2005; Parvez et al. 2006). Arsenic has been detected at concentrations greater than the World Health Organization (WHO) limit of 10 μ g/L in countries such as Argentina, Bangladesh, Chile, China, Hungary, India, Mexico, Peru, Taiwan, Thailand, and the United States (WHO 2001). In the United States, the Environmental Protection Agency (EPA), estimates that over 13 million people are exposed to arsenic in drinking water over the 10 μ g/L established limit (EPA 2001).

Using this evidence the EPA made a cautious regulatory decision to lower the arsenic drinking water standard (maximum contaminant level, MCL) from 50 to 10 μ g/L in 2001. This rule became enforceable in 2006 and must be reexamined every 6 years. The research behind this rule comes mainly from Taiwan, Argentina, and Chile where arsenic levels in drinking water are higher than 150 μ g/L. Several concerns emerge from the use of this data to support risk assessment. Some studies had limited exposure assessment and the nutritional status of participants is different from the US population (NRC 1999; NRC 2001). In addition, there is not enough data from animal and human models to establish a dose-response curve. Furthermore, the exact arsenic carcinogenic and non-carcinogenic modes-of-action are still not known. Therefore, the estimated cancer risk from exposure to inorganic arsenic for the US population is made using linear extrapolations and default assumptions.

The level of uncertainty in risk assessment estimates increases in populations that are exposed to low levels of arsenic in drinking water (<100 μ g/L). Numerous questions have to be answered before the next rule's re-examination. For example, do the estimated effects still occur from exposure to inorganic arsenic below 10 μ g/L or should the standard go lower?; or are there not enough data to justify the actual standard? It is worth mentioning that the EPA drinking water standards only apply to public water systems while around 15% of the population uses well water (USGS 2005).

In order to answer these questions, research efforts have to be conducted where populations exposed to low levels of arsenic are examined. Inclusion of exposure

assessment tools is one of the areas that bring more uncertainties. In order to achieve high-quality risk assessment, adequate qualitative and quantitative information concerning exposure (e.g. sources), dose (e.g. metabolism), and response (e.g. adverse effects, biological variability and susceptibility) to the chemical should be drawn together (Hughes et al. 2007). The use of biomarkers of arsenic exposure to evaluate exposure through water and diet has become very valuable, although further development and refinement is needed. As a result, validating biomarkers to assess arsenic exposure is a main target for scientists investigating arsenic exposures, mechanisms of toxicity and related diseases.

II. Arsenic in the Environment

Arsenic is a natural element, ubiquitous in the environment, primarily in its organic forms. It is a metalloid, having metallic and non-metallic properties, and is the third element in Group VI of the periodic table. Table 1-1 shows other chemical properties of arsenic. More than 200 mineral species contain arsenic. Arsenic binds with iron and sulfur to form the most common arsenic mineral, arsenopyrite. In areas of volcanic activity, soil can contain up to 20mg/kg of arsenic.

The most common forms in nature are arsenite [+III] and arsenate [+V], arsenic (0) and arsine [-III]. In the aqueous environment, the arsenite oxy-anions H_2AsO_3^- , HAsO_3^{2-} and AsO_3^{3-} and arsenate species H_3AsO_4 , H_2AsO_4^- , HASO_4^{2-} , AsO_4^{3-} predominate (Welch et al. 1988).

Arsenic also has a rich history of uses ranging from poison to cures for multiple ailments. It was used in Persia since ancient times. Due to its use by the ruling class to murder one another because of its potency and discreetness, arsenic has been called the *Poison of Kings* and the *King of Poisons*. On the other hand, medicines such as Fowler's solution (1% potassium arsenite) and Donovan's solution (arsenic iodine) were used in the 19th and 20th century to treat rheumatism, malaria, arthritis, anemia, asthma, tuberculosis, and diabetes among others (Darmouth 2007). In addition, arsenic was used until recently (1960's) in insecticides/pesticides to control ticks, fleas, lice and to debark trees.

The residues of these insecticides and pesticides are considered anthropogenic contamination and represent only a portion of total arsenic in the earth. Large quantities of arsenic are released into the environment through industrial activities playing an important role in the contamination of soil and water sources (Nriagu 1989; Pacyna and Pacyna 2001). However, the primary route of exposure for the majority of the population in the world is the intake of naturally elevated arsenic concentrations in food and ground water (NRC 1999).

III. Arsenic in Michigan

According to the National Research Council (2001), the excess risks associated with lifetime exposure to 10µg/L of arsenic may be approximately 1-in-300. In areas where the population obtains drinking water from private wells, that risk may increase. Since arsenic is ubiquitous in the earth's crust, ground water supplies are most likely to

carry arsenic. In some regions of USA, well water contains high levels of arsenic (USGS 2008). In southeastern Michigan, drinking water is the largest source of inorganic arsenic intake (Meliker et al. 2006) (Fig 1-1). Persons obtaining drinking water from private wells or public supplies using groundwater are at risk of consuming arsenic-contaminated drinking water. Although the exact release mechanism of arsenic in the region is unknown, it is related to the presence of arsenic-rich pyrite in the Marshall Sandstone, the principal bedrock aquifer in the region (Kolker et al. 2003). Four mechanisms for arsenic release into the environment have been hypothesized. These include pyrite oxidation, carbonation of arsenic sulfide minerals, and reduction of arsenic-containing iron-oxyhydroxides (Apelo et al. 2002; Kim et al. 2000). An estimated 53-98% of the arsenic in the region occurs as As[III] (Kim et al. 2002).

IV. Michigan Bladder Cancer Case-Control Study

According to the Centers for Disease Control and Prevention (CDC), bladder cancer incidence in Michigan is higher compared with the whole of the USA and that difference is even higher in the male population (44.7 per 100,000 in Michigan vs. 37.1 per 100,000 in USA) (CDC 2005). There were over 68,810 expected new cases of bladder cancer in the United States for 2008 (NCI 2009), and the causes of this disease are not well-understood. Arsenic exposure has been associated with bladder cancer (Mink et al. 2008; Aposhian and Aposhian 2006; Chen et al. 2003; NRC 2001; Smith et al. 1998; Hopenhayn-Rich et al. 1996; Wu et al. 1989). Several risk factors may predispose an individual to bladder cancer such as tobacco use, certain infections (e.g. parasites), and

occupational exposures (e.g. rubber and leather industries). However, the relationship between arsenic at low levels and arsenic-related diseases is not well known.

Arsenic levels in Michigan are typically lower than 100µg/L. As part of the interest in investigating the potential health effects of arsenic at elevated levels, the National Cancer Institute (NCI) funded a research team at the University of Michigan to study the relationship between bladder cancer and arsenic in drinking water in southeastern Michigan.

The Michigan Bladder Cancer Case-Control Study is a multidisciplinary project which is the parent project of this dissertation. This dissertation only addresses urinary arsenic methylation profiles in the study population. Additional funding by the NCI was approved to complete this part of the study. The Michigan Bladder Cancer Case-Control Study consist of three elements: (a) assessment of current and lifetime exposure to arsenic in drinking water; (b) biomonitoring arsenic intakes by analyzing saliva, toenail and urine samples, and (c) modeling the relationships between arsenic exposure and bladder cancer in a cohort of 1,000 cases and controls.

Recruitment of cases and controls started in 2003 and cases include individuals diagnosed with bladder cancer between 2000 and 2004. Cases were recruited from the Michigan State Cancer Registry and eligibility requirements included no prior history of cancer, having lived in the study area at least five years prior to the cancer diagnosis, aged 21-80 when diagnosed, and being alive. Controls were recruited using a random-digit dialing-procedure from an age-weighted list and frequency matched to cases by

age (± 5 years), race, and gender. Controls had to comply with similar requirements as those for cases: no prior history of cancer and having lived in the study area at least five years prior to the telephone interview. For more details about cases and controls recruitment see Meliker (2007). The final number for recruitment of cases and controls was of 411 cases and 566 controls.

After recruitment, cases and controls completed a computer-aided telephone interview followed by a field visit to collect water, urine, toenails and saliva samples. Water and toenail samples were collected from all members of the study population, while urine and saliva samples were collected from only 40%. The phone interview collected information about medical history, current and past water consumption patterns, lifestyle risk factors, family history of cancer, and dietary habits (Meliker 2007). During the field visit, three questionnaires were submitted: occupational history, residential history, and a food frequency questionnaire (FFQ). The FFQ recorded foods, beverages, and certain vitamins and supplements consumed during the three days before urine samples were collected.

Water samples were collected from the kitchen tap, or primary source of water for drinking and cooking. Water collection containers were previously acid-washed for trace metals determination following the protocol by Nriagu et al. (1993). After collection, water samples were stored on ice, acidified with trace metal grade nitric acid and stored at 4°C until analysis. Toenails samples were collected in Teflon tubes, washed, dried, and digested (Slotnick et al. 2007). Collected water and toenails samples

were analyzed for total arsenic using ICP-MS. Saliva samples were collected using a mouthwash rinse. After collection, the DNA is extracted, using Puregene DNA Isolation Kits (Gentra Systems), and frozen. DNA pellets were analyzed for single nucleotide polymorphisms (SNPs) involved in arsenic metabolism, DNA repair and PAH/aromatic amine metabolism. This dissertation relies on the following information from the parent project: demography, water and toenail samples, lifestyles habits, medical history, and water consumption patterns.

V. Research Goals and Hypothesis

Epidemiological studies that evaluate urinary arsenic metabolites in the general population are necessary to better understand arsenic exposures and metabolism pathways. Urinary excretion of arsenic metabolites is the primary pathway for the excretion of arsenic from the human body (Vahter 1993; Le et al. 1994). This research is designed to investigate urinary arsenic metabolites as tools to determine arsenic exposure to arsenic poisoning. The goal of this study is to test the main hypothesis that urinary arsenic metabolites are biomarkers of arsenic exposure. To guide this hypothesis, I plan to advance the research of arsenic biotransformation and dose-response relationship for arsenic exposure at low levels (10-100 μ g/L) by:

1. Characterizing urinary arsenic profiles of inorganic and methylated arsenic species in a population exposed to low-to-moderate levels of arsenic.
2. Using individual characteristics and behaviors such as age, gender, diet, and smoking habits to explain variability in urinary arsenic profiles.

3. Assessing the relationships between arsenic in urine, toenail and water samples in an effort to validate these biomarkers of arsenic exposure.
4. Determining the association between arsenic metabolites excreted in urine with bladder cancer.

The urinary arsenic profiles were determined in a subsample of the study population from the Michigan Bladder Cancer Case-Control Study. Advances in analyzing and interpreting the biomarkers were highlighted using an accurate and advanced arsenic speciation analytical protocol. This research improved the knowledge about population- and individual-level susceptibilities to arsenic exposure.

This dissertation consists of seven chapters as described below. This chapter presents introductory material, describes the Michigan Bladder Cancer Case-Control Study, and establishes the goals and hypotheses of the research. More than a review, the second chapter is an explanation of how urinary arsenic metabolites are employed to explain arsenic metabolism. In Chapter 3, a protocol to analyze total arsenic in urine and water as well as speciated arsenic in urine is presented. This chapter is the analytical basis of the research and is supported by the literature review in Chapter 2. Chapter 4 evaluates the reliability of two different sampling strategies to better assess influences of metabolism in the biomarker. To test the hypothesis that the distribution of arsenic species excreted in urine is influenced by variables such as diet and gender, Chapter 5 includes an investigation of how diet and demographic characteristics influence arsenic methylation reflected in urine. In addition, this chapter assesses the relationships between urinary, toenail, and water arsenic as markers of low arsenic

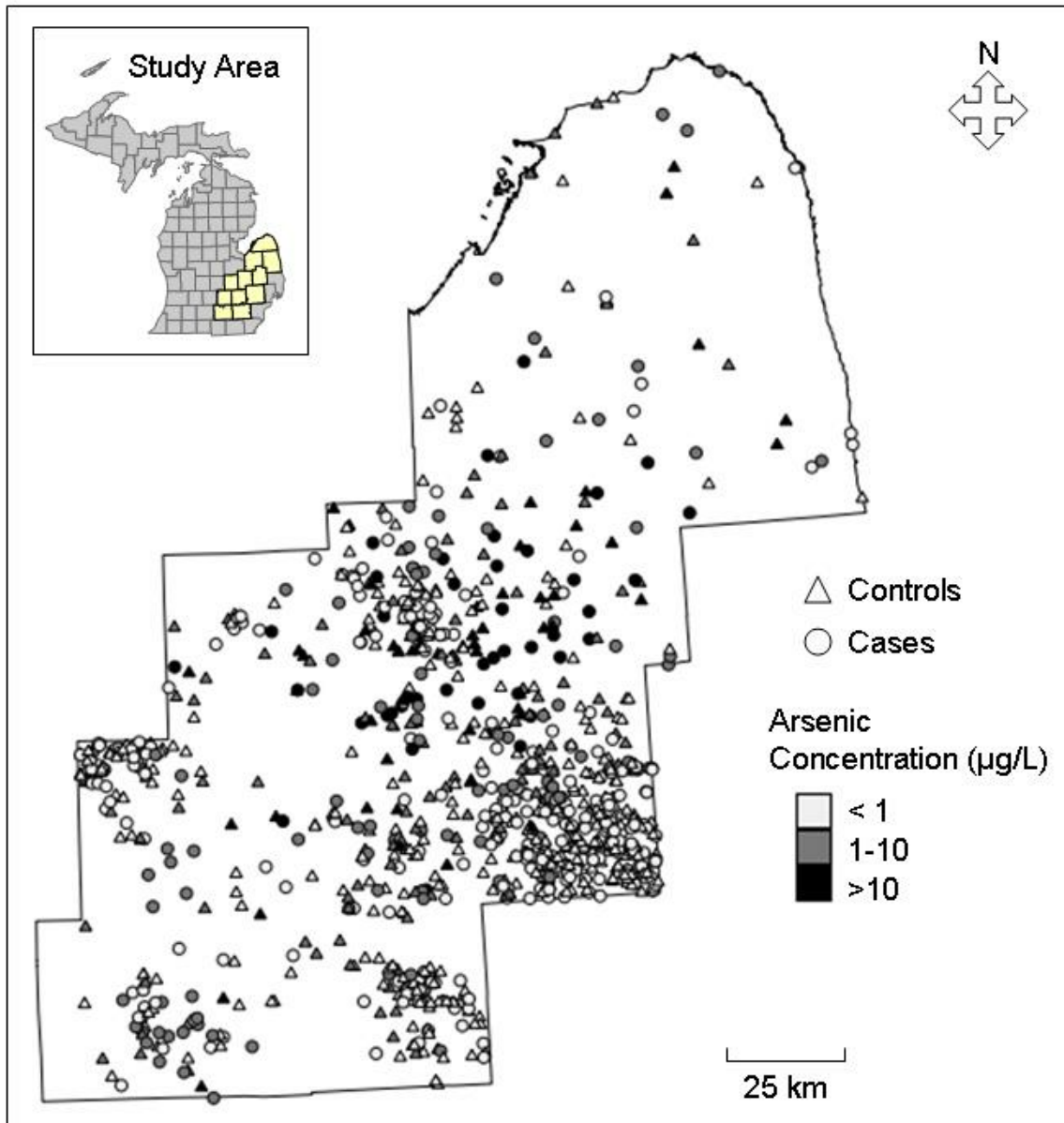
exposure. Chapter 6 evaluates urinary arsenic metabolites as biomarkers for bladder cancer. This chapters will test the hypothesis that exposure to inorganic arsenic is associated with bladder cancer. Finally, Chapter 7 provides conclusions and study limitations.

Table 1-1. Chemical Properties and Other Facts of Arsenic

Properties	Arsenic
Atomic number	33(5B)
Atomic weight	74.92
Oxidation state	V, III, I, 0, -I, -III
Van der Waals radius	1.85A
Ionization potential	9.81eV
Electronegativity	2.0
Major chemical form in water	Arsenite (As[III]), arsenate (As[V])
Safety limit for drinking water	10µg/L
Concentration in:	
Sea water	1-2µg/L
Marine algae	1>10mg/kg wet
Marine animals	0.5->10/kg wet
Soil	6mg/kg (average)
Terrestrial plants	≤0.1mg/kg dry

Sources: ATSDR 2005; WHO 2001

Figure 1-1. Arsenic concentrations and distribution of cases and controls in the study area in Southeastern Michigan (Meliker 2009).



References

- Aposhian HV, Aposhian MM. 2006. Arsenic toxicology: five questions. *Chemical Research in Toxicology* 19:1-15.
- Apelo CAJ, VanDerWeiden MJ, Torunassat C, Charlet L. 2002 Surface complexión of ferrous iron and carbonate on ferrihydride and the mobilization of arsenic. *Environmental Science Technology* 36:3096-3103.
- Bates M, Rey O, Biggs M, Hopenhayn C, Moore L, Kalma D, Steinmaus C, Smith A. 2004. Case-control of bladder cancer and exposure to arsenic in Argentina. *American Journal of Epidemiology* 159:381-389.
- Center for Disease Control and Prevention. 2005. State Cancer Facts. www.apps.nccd.cdc.gov. Department of Health and Human Services.
- Chen Y, Su H-J, Guo Y-L, Hsueh Y-M, Smith T, Ryan LM, Lee M-S, Christiani DC. 2003. Arsenic methylation and bladder cancer risk in Taiwan. *Cancer Causes and Control* 14:303-310.
- Dartmouth Toxic Metals Research Program. 2007. <http://www.dartmouth.edu/~toxmetal/TXSHas.htm>.
- Hopenhayn-Rich C, Biggs ML, Smith AH, Kalman DA, Moore LE. 1996. Methylation study of a population environmentally exposed to arsenic in drinking water. *Environmental Health Perspective*. 104:620-628.
- Hughes MF, Kenyon EM, Kitchin KT. 2007. Research approaches to address uncertainties in the risk assessment of arsenic in drinking water. *Toxicology and Applied Pharmacology* 222:399-404.
- International Agency for Research in Cancer. 2004. Some water drinking disinfectant and contaminants, including arsenic. IARC Monograph Evaluation.
- International Agency for Research in Cancer. Arsenic and arsenic compounds. 1987. IARC Monograph Evaluation.
- Karagas M, Stukel T, Morris J, Tosteson T, Weiss J, Spencer S, Greenberg E. 2001. Skin cancer risk in relation to toenails arsenic concentrations in a US population-based case-control study. *American Journal of Epidemiology* 153:559-565.
- Kim MJ, Nriagu JO, Haack S. 2000. Carbonate ion and dissolution of arsenic from ground water. *Environmental Science Technology* 34:3094-3100.

Kim MJ, Nriagu JO, Haack S. 2002. Arsenic species and chemistry in groundwater of southeast Michigan. *Environmental Pollution* 120:379-390.

Kolker A, Nordstrom DK. 2001. Occurrence and micro-distribution of arsenic pyrite [abstract] In: USGS Workshop on Arsenic in the Environment; 2001 Feb21-22; Denver.

Meliker JR. 2009. Arsenic concentrations and distribution of cases and controls in the study area in Southeastern Michigan. BMP Document.

Meliker JR, Franzblau A, Slotnick MJ, Nriagu JO. 2006. Major contributors to inorganic arsenic intake in southeastern Michigan. *International Journal of Environmental Health* 209:399-411.

Meliker J. 2007. Lifetime exposure to arsenic in drinking water: application to a bladder cancer case-control study. Saabruken, Germany: Mueller Press.

Mink PM, Alexander DD, Barraj LM, Kelsh MA, Tsuji JS. 2008. Low-level arsenic exposure in drinking water and bladder cancer: A review and meta-analysis. *Regulatory Toxicology and Pharmacology* 52:299-310.

National Cancer Institute. 2009. Bladder Cancer.

www.cancer.gov/cancertopics/types/bladder. US National Institute of Health.

National Research Council's Subcommittee on Arsenic in Drinking Water. *Arsenic in Drinking Water*. 1999. National Academy Press Washington DC pp. 177-192.

National Research Council's Subcommittee on Arsenic in Drinking Water. *Arsenic in Drinking Water 2001 Update*. 2001 National Academy Press Washington DC pp. 15-23.

Nriagu JO. 1989. Natural sources of trace metal emission to the atmosphere: global assessment. *Nature* 338:47-49.

Nriagu J, Lawson G, Wong H, Azcue J. 1993. A protocol for minimizing contamination in the analysis of trace metals in Great Lakes waters. *Journal of Great Lakes Research* 19:175-182.

Pacyna JM, Pacyna E. 2001. An assessment of global and regional emissions of trace metals to the atmosphere through anthropogenic sources worldwide. *Environmental Reviews* 9:269-298.

Parvez F, Chen Y, Argos M, Hussain A, Momotaj H, Dhar R, van Geen A, Graziano J, Ahsan H. 2006. Prevalence of arsenic exposure from drinking water and awareness of its risk in

- a Bangladeshi population: Results from a large population-based study. *Environmental Health perspectives* 114:355-359.
- Slotnick MJ, Meliker JR, AvRuskin GA, Ghosh D, Nriagu JO. 2007. Toenails as a biomarker of inorganic arsenic intake from drinking water and foods. *Journal of Toxicology and Environmental Health Part A* 70:148-158.
- Smith AH, Goycolea M, Haque R, Biggs ML. 1998. Marked increased in bladder and lung cancer mortality in a region of Northern Chile due to arsenic in drinking water. *American Journal of Epidemiology* 147:660-669.
- Steinmaus C, Yuan Y, Bates M, Smith A. 2003. Case-control study of bladder cancer and drinking water arsenic in the Western United States. *American Journal of Epidemiology* 158:1193-1201.
- Steinmaus C, Bates, Yuan Y, Kalman D, Atallah R, Rey O, Biggs M, Hopenhayn C, Moore L, Hoang B, Smith A. 2006. Arsenic methylation and bladder cancer risk in case-control studies in Argentina and the United States. *Journal of Occupational and Environmental Medicine* 48:478-488.
- Tseng CH, Huang Y.K., Huang Y.L., Chung C, Yang M, Chen C, Hsueh Y. 2005. Arsenic exposure, urinary arsenic speciation, and peripheral vascular disease in Blackfoot disease-hyperendemic villages in Taiwan. *Toxicology and Applied Pharmacology* 206:299-308
- US Environmental Protection Agency. Arsenic in Drinking Water. 2001.
<http://www.epa.gov/safewater/arsenic/regulations.html>
- US Geological Survey. 2008. Trace Elements National Synthesis Project.
www.water.usgs.gov/nawqa/trace/arsenic/
- Welch AH, Lico MS, Hughes JL. 1998. Arsenic in groundwater of the western United States. *Ground Water* 23:333-347.
- World Health Organization. 2001. Biomarkers in risk assessment: Validity and validation.
- Wu MM, Kuo TL, Hwang YH, Chen CJ. 1989. Dose-response relationship between arsenic concentration in well water and mortality from cancers and vascular diseases. *American Journal of Epidemiology* 130:1123-1132.

Chapter II

Urine as a Medium to Study Arsenic Metabolites

A. Urine Composition and Function

Urinary excretion of arsenic metabolites is the primary pathway for the elimination of arsenic from the human body (Vahter 1994a; Le et al. 1994). In addition, the collection of urine samples is a non-invasive procedure, and hence is an attractive methodology for large scale studies. Urine is the byproduct excreted by the kidneys, transported by the ureters to the urinary bladder where it is voided through the urethra (NCI 2001).

The composition of urine is mostly water (~95%) and contains urea, sodium, potassium, phosphate and sulfate ions, creatinine, and uric acid. The production of urine is a combination of three processes: filtration, reabsorption, and secretion (Solomon et al. 1993). Filtration is a non-selective process where 180 liters of fluid are filtered in the kidney every 24 hours; therefore 99% of that volume is reabsorbed into the blood (Solomon et al. 1993). This leaves only about 1.5 liters to be excreted as urine. The urine volume excreted varies by sex, age, and different health conditions (Perucca et al. 2006). Arsenic concentration in urine has a short half-life; thus urinary arsenic is often used as a measure of recent exposure to arsenic.

Several known factors can influence urine arsenic content and rates of excretion. Different health disorders change the filtration rate or vary the excretion of components or their amounts. For example, when a substance is in excess in the blood it will be excreted more in urine. If glucose is in excess in the blood it overflows into urine; this is a diagnostic tool for diabetic patients. Likewise, life-styles or medications vary the color, concentration, and odor of urine. To avoid biased results due to variation in urine dilution, an adjustment by urinary creatinine concentration is usually performed. Creatinine analysis is performed on the basis that its excretion is constant from the body. However, creatinine is formed by creatine and its excretion is related to muscle mass and meat intake (Worsfold et al. 1999; Hall Moran et al. 2001). Studies have shown that urinary creatinine varies by age, gender, body size, and diet (Boeniger et al. 1993; Suwazono et al. 2005). As a result, some studies propose the use of specific gravity instead of creatinine because of the strong correlation between specific gravity and creatinine (Moore et al. 1997; Parikh et al. 2002; Suwazono et al. 2005). Although specific gravity may also be influenced by age, gender, and body size, the influence is less than creatinine (Suwazono et al. 2005). The issue of creatinine versus specific gravity requires more consideration particularly when working in epidemiological studies where the individuals of studied populations may be different in age, gender, and body size and have different diets.

Diet is an important factor when using urine as a biomarker for arsenic exposure. Similar to toenails analysis, urine can reflect different exposure pathways including diet. Certain foods such as fish and seafood contain large amounts of arsenic. However,

arsenic contained in fish and seafood is mostly in non-toxic organic forms such as arsenobetaine (AsB) and arsenocholine (AsC) (Cullen and Reimer 1989; Ma and Le 1998; ATSDR 2005) and they are excreted from the human body without metabolism (Le et al. 1994; Le and Ma 1997; Vahter 1994b). In populations exposed to high levels of arsenic in their drinking water ($>100\mu\text{g/L}$), arsenic from food is probably negligible. In contrast, when analyzing urine samples in populations exposed to low levels of arsenic, diet may become an important factor.

B. Biomarkers of Exposure

Developing effective exposure assessment methods is one of the most difficult tasks to address in environmental health and epidemiological studies. It is, however, fundamental to determine the relationships between exposure to certain toxics and the dose needed to induce a particular health effect. Due to an increase in evidence of metal-related chronic diseases, exposure assessment tools in this area have become a key element in the research approaches of environmental epidemiology. In this context, biomarkers have emerged as essential tools to determine and interpret exposure to metals. In addition, they appear to reduce error measurement such as recall bias or aggregation of exposures (Decaprio 1997). Likewise, questionnaire data, environmental monitoring or modeling environmental pathways may provide no information about the actual absorbed dose of an individual or population (Silbergeld et al. 1994).

Biological markers of exposure have been defined by the National Research Council (1987) as exogenous substances or their metabolites, or the product of an

interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism. Dor et al. (1999) divided biomarkers of exposure into markers of internal dose and markers of effective dose. Markers of internal dose are indicators of occurrence and extent of exposure in a certain organism, and markers of effective dose represent the extent of exposure in a target organ, tissue or cell. Validity of these markers describes the extent to which a biomarker reflects a designated event in a biological system (WHO 2001).

Nevertheless, similar to other research tools, biomarkers of exposure also have some limitations including selection bias and validity. The selection and validity of a biomarker will depend on the intrinsic characteristics of the biomarker and those related to the analytical procedures (Dor et al. 1999). Intrinsic characteristics are those which refer to factors such as sensitivity, specificity in relation to the pollutant, significance in terms of exposure, existence of dose-response data, background in the general population, and knowledge of confounding factors (Dor et al. 1999). Characteristics related to the analytical procedures refer to the numbers of samples required to observe an effect, sample storage, detection limits, sample contamination, instrument sensitivity, among others (Dor et al. 1999). The development and validation of new biomarkers for metals exposure is currently a critical strategy to improve the knowledge of how metals interact with the human body.

Urinary arsenic metabolites have been used as biomarker of arsenic exposure worldwide. They have become useful tools to advance knowledge in arsenic metabolism

and toxicity. More investigations are needed to determine more specific analytical protocols, exposure assessment tools, variability among individuals and populations, how that variability affects the development of arsenic-related diseases and dose-response relationships.

C. Advantages of Urine over Other Biomarkers

Arsenic in urine has several advantages over other candidates as a biomarker. Arsenic is rapidly cleared from the blood in a few hours after the exposure. Therefore, blood arsenic is a good biomarker for acute exposures or poisonings. The largest amounts of arsenic in the human body can probably be found in hair and nails. Arsenic binds to keratine, which is a protein with disulfide bonds contained in hair and nails. Hair grows at different rates, may contain external contamination, and its pharmacokinetics are not fully understood (Harkin and Susten 2003). Therefore, it is not a reliable biomarker for arsenic exposure. Nails can reflect exposure that has occurred over the past 6-12 months (Goyer and Clarkson 2001) which is an advantage over blood, hair, and urine. However, nails are exposed to external contamination, and they grow at different rates in different individuals. In addition, different exposure routes may be reflected in human nails such as arsenic in air, soil, dust and food (Slotnick and Nriagu 2006). Urine composition and function is well known. The main attraction that urine offers as a biomarker of arsenic exposure is the characterization of arsenic species or metabolites. Even though arsenic speciation in human nails has been conducted (Lin et al. 1998), inorganic arsenic species have been reported to account for a smaller

percentage (~20%) of total arsenic in nails (Mandal et al. 2003). Arsenic speciation is an important tool linking arsenic exposure to bladder and skin cancer. Speciation of arsenic provides quantitative information on the amounts of arsenic metabolites. This data however, has to be validated in order to use arsenic species as indicators of intensity and duration of exposure to inorganic arsenic, and relate their distribution as specific biomarkers of any adverse health effect.

D. Arsenic in Human Urine

Metabolism of arsenic in mammalian species has been studied for a long time and there is significant variation among species. For instance, in the majority of mammalian species, most arsenic is methylated and excreted in urine as dimethylarsinic acid (DMA) and methylarsonic acid (MMA). Mice and dogs are very efficient in methylating arsenic to DMA, while chimpanzees lack the ability to methylate inorganic arsenic (Vahter 2002). Humans excrete more MMA in urine than other species (Vahter 1999).

The relative proportions of arsenic in human urine are usually 40-75% DMA (DMA[III]+DMA[V]), 20-25% inorganic arsenic (As[III]+As[V]) and 15-25% MMA (MMA[III]+MMA[V]) (Mandal et al. 2001; Hopenhayn et al. 2003; Loffredo et al. 2003). The main metabolite after long exposure is DMA with lower levels of inorganic arsenic and MMA (ASTDR 2005). However, evidence suggests particular exceptions that may involve genetic susceptibility. People living in certain arseniasis-hyperendemic villages in Taiwan excrete higher levels of MMA in urine (on average 25-30%) (Hsueh et al. 1998).

On the other hand, women living in northwestern Argentina in the Andes villages (Atacameños) excrete just about 2-3% of MMA in urine (median 2.2%) (Vahter et al. 1995; Hopenhayn-Rich et al. 1996). Atacameños living in Argentina and Chile have been exposed to arsenic for hundreds of years. This observation may provide evidence of a genetic-related path such as a genetic polymorphism in the enzyme pathway for the methylation of arsenic.

Marnell et al. (2003) studied populations exposed to $<100\mu\text{g/L}$ in Mexico. They found two subjects with very high amounts of inorganic arsenic (InAs) and very low amounts of the methylated species. When the genes of these two individuals were examined, the polymorphisms indicated that specific amino acids were deleted and in some areas replaced by a different one. They propose that differences in the MMA[V] reductase gene may be, in part, responsible for this variation. In addition, they show that the genotype E155del, Glu208Lys is associated with the inability to process inorganic As[V] normally. These variations in genotypes may explain the similarity in individual susceptibilities. Meza et al. (2005) also found that differences in the ratio of DMA[V] to MMA[V] were significantly associated with polymorphic sites in the CYT19 (As[III] methyltransferase) gene. These genetic associations are possible explanation for differences among individuals and population in arsenic metabolism. In order to better explain the existence of genetic determinants of inter-individual variability in human metabolism, additional research is needed. The determination of urinary arsenic profiles in different populations is conducted in order to elucidate the arsenic metabolism pathway in the human body.

E. Arsenic Metabolism

The classic arsenic biotransformation pathway in humans includes a series of reduction and oxidation reactions together with methylations (Fig 2-1). The conversion of arsenites, selenites, and tellurites to methylated species was first described by Frederic Challenger and colleagues in England during the 1930's and 1940's (Challenger 1945; Challenger and Higginbottom 1937). Decades later, Cullen and Reimer (1989) and Buchet and Lauwerys (1987) presented evidence for the mechanism of arsenic biotransformation. Two basic processes are involved: (1) oxidation/reduction reactions that interconvert arsenite (As[III]) and arsenate (As[V]), and (2) methylation reactions, which convert As[III] to dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA). The mechanism suggests that oxidation/reduction and methylation reactions follow five steps as shown in Fig 2-1: (1)As[V] is converted to As[III], if necessary, (2)methylation of As[III] to MMA[V], (3)reduction of MMA[V] to MMA[III], (4)methylation of MMA[III] to DMA[V], and (5)reduction of DMA[V] to DMA[III] .

In this mechanism, MMA[V] and DMA[V] are formed after MMA[III] and DMA[III]. In addition, this mechanism indicates that the methyl donor is S-adenosyl-L-methionine (SAM) and the reducing agent is glutathione (GSH). Two enzymes in the proposed pathway have been studied: MMA[V] reductase and arsenic methyltransferase (Zakharyan et al. 1995; Zakharyan et al. 1999; Zakharyan et al. 2001). In addition, a human methyltransferase (CYT19) produced by DNA recombinant technology has been studied (Li et al. 2005; Waters et al. 2004).

The arsenic species produced in the pathway in Fig 2-1 have different chemical characteristics. In sample preparation and storage studies, stability between each arsenic species varies. For example, As[III] and As[V] are more unstable than the methylated species DMA[V] and MMA[V] (Larsen et al. 1993). On the other hand, MMA[III] and DMA[III] are very unstable species (Gong et al. 2002; Mandal et al. 2001; Del Razo et al. 2001). In the cellular environment, MMA[III] and DMA[III] appear to be more unstable because they can be easily oxidized as well. There is, however, increasing interest in the determination of MMA[III] and DMA[III] since studies have shown that they are more toxic than As[III] and As[V] (Petrick et al. 2000; Styblo et al. 2000; Vega et al. 2001). Furthermore, some authors suggest that they can be used as indicators of increased susceptibility to toxic and cancer-promoting effects of arsenicals (Valenzuela et al. 2005).

The difference in toxicities between trivalent compounds and their pentavalent counterparts can be explained in part by the more efficient rate of uptake of trivalent compounds by cells (Dopp et al. 2004). Trivalent arsenic compounds that accumulate in cells have the potency to induce oxidative stress or reactive oxygen species such as hydroxyl radicals (ROS). Oxidative damage has been reported in epidemiological studies. In Inner Mongolia, increased lipid peroxide serum and decreased non-protein sulfhydryl serum levels were found in individuals living in a contaminated area (Pi et al. 2001). These two variations in human serum are indicators of oxidative stress. In addition, trivalent arsenicals are known to be highly toxic and reactive to thiol groups in proteins. Recently, studies have shown arsenic metabolites attached to proteins and forming

complexes with GSH during methylation (Naranmandura et al. 2006; Naranmandura et al. 2007; Hayakawa et al. 2005). This new picture of the arsenic methylation may better explain the differences in toxicities among arsenic species.

The methylation of arsenic was considered to be a detoxification pathway because DMA[V] and MMA[V] are less reactive and toxic species. After evidence of the high toxicity of MMA[III] and DMA[III] became known, the idea of detoxification has been questioned, proposing a possible bioactivation of arsenic. In 2005, Professor Toru Hayakawa and colleagues at Chiba University in Japan proposed a new arsenic metabolism pathway where arsenic methylation is indeed a detoxification pathway (Fig 2-2).

The main difference between the classical pathway and the new Hayakawa et al. (2005) proposal is that the trivalent species are formed before the pentavalent species. They described the formation of complexes of inorganic arsenic with glutathione such as arsenic triglutathione (ATG) and monomethylarsenic diglutathione (MAGD) instead of glutathione functioning as a simple reducing agent as described in Fig 2-1. These complexes are required before methylation occurred through CYT 19 because it is the lone pair from the thiol groups of the ATG or MAGD, the ones that attack SAM to obtain a methyl group. In other words, ATG and MAGD are substrates of the methyltransferase, not As[III].

In the Hayakawa et al. (2005) experiments a larger amount of DMA[V] was produced from As[III] than from MMA[V]. These results are contradictory with Fig 2-1

where the mechanism is a series of reduction, oxidation, and methylation reactions in which MMA[V] is a metabolite of As[III]. This implies that As[III] can be converted to DMA without outgoing MMA. Although metabolites from the older mechanisms are the same, the final products in the new proposed mechanism are the pentavalent DMA and MMA forms which are the major metabolites found in urine. DMA[V] and MMA[V] are less reactive than the trivalent species, and this is a reasonable explanation of why they may appear in human urine in large concentrations. Although MMA[III] and DMA[III] have been detected in human urine (Valenzuela et al. 2005; Le et al. 2000a; Le et al. 2000b Aposhian et al. 2000), most epidemiological studies just report As[III], As[V], MMA[V], and DMA[V] since the detection of the trivalent species is very difficult if they are not analyzed immediately. Moreover, in populations exposed to low levels of arsenic they may be undetectable since MMA[III] and DMA[III] have been detected in areas where residents have endemically been exposed to high levels of arsenic (>100µg/L) in drinking water (Mexico, West Bengal).

Another issue in detecting trivalent arsenic species is the recognition by some scientists of the mistaken identification of DMA[III] by sulfur-containing arsenicals (Hansen et al. 2004). The identification of arsenicals through laboratory analysis relies in the availability of genuine methylarsonous acid and dimethylarsinous acid for use as analytical standards. Since they are not commercially available, researchers produced their own standards in the laboratory. During this process sulfur containing reagents such as sodium thiosulfate or sodium-metabisulfite are used to reduce pentavalent arsenicals. These compounds may produce arsenic trivalents species or other

compounds that later are identified as MMA[III] or DMA[III]. Since identification of arsenicals in most laboratories only involved chromatographic retention time, more emphasis should be placed to address this issue. Identification by molecular weight and structure has to be performed to correctly identify the reaction products. Hansen et al. (2004) identified the thioarsenical dimethylarsinothioic acid as a natural metabolite in urine. This metabolite has not been described in any biotransformation pathway. Moreover, previous identification of DMA[III] in natural samples may be wrong. Naranmandura et al. (2007) also identified monomethylthio-arsonic acid (MMMTA[V]) and dimethylthio-arsinic acids (DMMTA[V] and DMDTA[V]) in urine of hamsters and rats. DMDTA[V] was much more excreted in urine which brings back the issue of accurate identification of species when using urinary arsenic metabolites to explain metabolic pathways. The issue of sulfur-containing arsenicals has been a critique (Lindberg et al. 2007) to an often-cited study where methylated trivalent metabolites were detected in 98% of the samples in an endemic region of central Mexico (Valenzuela et al. 2005)

In addition to the previously discussed urinary arsenic metabolites, ingested organic arsenicals such as arsenobetaine (AsB) and arsenocholine (AsC) are also excreted in urine. AsB is a tri-methylated pentavalent compound recognized as ubiquitous in the marine environment, while AsC is a precursor of AsB (Cullen and Reimer 1989; Edmonds and Francesconi 1988). Edmonds and Francesconi proposed that arsenobetaine is not synthesized from arsenate in marine organisms but transformed by microbial species from arsenosugars released by algae and then ingested by marine

animals. A study of arsenic species contained in cooked seafood, reveal that AsB was the only species detected in samples (Devesa et al. 2005). They purchased different seafood products on the basis of high domestic consumption in Spain. In an epidemiological study, Le et al. (1994) found that AsB was the "major" arsenical in urine after eating crabmeat or shrimps. These organic arsenicals appear to undergo little or no metabolic changes and therefore are excreted without any significant changes in structure (Borak et al. 2007). In Taiwan, dietary seafood intake was not correlated with excretion of inorganic arsenic, MMA or DMA (Hsueh et al. 2002). Although, AsB and other arsenicals found in seafood do not exert toxicity at low doses (Edmonds and Francesconi 1994), it is worthwhile to consider the potential role of dietary seafood because they may produce metabolites involved in the arsenic-induced carcinogenesis (Martin et al. 2005)

In addition to inorganic arsenic, DMA, MMA, arsenobetaine, and arsenocholine, several other arsenic species can be measured in urine. However, scientists often choose to measure As[III], As[V], DMA[V], MMA[V], DMA[III], and MMA[III] because they may reflect exposure to toxic arsenic and help to explain arsenic metabolism. A variety of analytical protocols to measure As[III], As[V], MMA[V], and DMA[V] are available while MMA[III] and DMA[III] are more difficult to assess.

F. Urinary Arsenic Metabolites as Biomarkers of Arsenic Exposure

Biomarkers studies have revealed positive and consistent relationships between arsenic in drinking water and arsenic in urine around the world. A greater number of studies have been conducted in areas with higher arsenic levels such as Taiwan and

Bangladesh. For example, in the Blackfoot disease-endemic area in Taiwan, arsenic concentrations in drinking water are up to 2,500µg/L (Lo 1977). Hsueh et al. (1997) found that people in this area have higher concentrations of arsenic and its metabolites in urine than persons exposed to lower levels of arsenic. In Bangladesh, a region where more than 100 million people drink water with more than 50µg/L of arsenic, a study showed that from 1,084 urine samples, 95.11% were above 50 µg/L (SOES & DCH 2000). A comparison between a group exposed to 150 to 600µg/L with a group exposed to <15µg/L results in higher significant correlations for those exposed to higher levels of arsenic in water in the northern desert region of Chile (Biggs et al. 1997). Also in Chile, Caceres et al. (2005) found positive relationships between arsenic excreted in urine and arsenic exposure through drinking water ($n=756$).

In West Bengal, a study reported an $R^2=0.410$ ($p<0.05$) for the relationship between arsenic in water and As[III] in urine; $R^2=0.115$ ($p<0.05$) between arsenic in water and As[V]; $R^2=0.379$ ($p<0.05$) between arsenic in water and MMA[V]; $R^2=0.474$ ($p<0.05$) between arsenic in water and DMA[V] ($n=51$) (Tokunaga et al. 2002). Certain regions of Europe are rich in arsenic as well. A recent study of cancer risk in Hungary, Romania and Slovakia ($n=537$) shows a significant correlation between arsenic in drinking water and arsenic in urine ($R^2=0.45$ $p<0.0001$) (Lindberg et al. 2006) (Table 2-2).

An increasing awareness of the potential chronic health effects of arsenic at low levels has emerged in new studies with populations exposed to low arsenic concentrations for long periods of time. Karagas et al. (2001) conducted an assessment

of cancer risk in New Hampshire where 99% of the studied population has arsenic concentrations less than 50 μ g/L in their drinking water. They found a correlation of 0.35 ($p=0.0024$) between arsenic in drinking water and urinary arsenic concentrations, and the association increased to 0.46 ($p=0.029$) when they limited the study to those who drank water with less than 1 μ g/L of arsenic. In Mexico, four towns were selected for an epidemiological study with arsenic in well water ranging from 5.5 to 43.3 μ g/L (Meza et al. 2004). This study ($n=53$) shows a correlation of 0.35 ($p=0.02$) between arsenic in water and arsenic in urine. This association increased, ($r=0.50$, $p<0.0001$), when the total arsenic intake by drinking water per day was correlated with the arsenic concentration in urine. In Bangladesh, the urinary arsenic level of individuals who switched from contaminated wells to 'safe wells' (<50 μ g/L) dropped from 375 μ g/g creatinine to 200 μ g/g creatinine (Chen et al. 2007). Calderon et al. (1999), found a quantitative relationship between the log of arsenic in drinking water and the log of arsenic in urine in a population where 86% of the individuals were exposed to concentrations less than 130 μ g/L. A mixed regression model showed that arsenic in drinking water is a better predictor than estimated intakes of arsenic (Calderon et al. 1999).

Even though there are a substantial number of studies showing significant associations between urine and water, just a few studies have evaluated the relationship between urine and toenails. Karagas et al. (2001b) found a correlation of 0.36 ($p=0.0012$) between urinary arsenic and toenails, however, this analysis just included 77 individuals. They attempted the same analysis later (Karagas et al. 2002) but

75% of the subjects had undetectable inorganic arsenic or MMA in urine. Using data from the Michigan Bladder Cancer Case-Control Study, Slotnick et al. (2007) found a significant correlation of 0.32 between drinking water and arsenic in toenails ($n=430$). Moreover, they extended the analysis to evaluate additional variables such as food and estimates of water consumption at home and work. They found that total water intake should be considered in order to establish better prediction of arsenic in toenails.

The degree to which risk estimates from epidemiological studies reflects true risks depends on the accuracy of the exposure estimates (Cantor and Lubin 2007). The accuracy of exposure estimates directly relies on laboratory precision, selection of tools to examine exposure, exposure misclassification, and identification of modifiers of the exposure-biomarker relationship. These are critical pieces in the biomarker validation process. Uncertainties rising from the biomarker validation process have to be elucidated before extending and applying the existent exposure estimates emerging from epidemiological studies.

G. Urine Analysis

1. *Sampling Strategies for Arsenic Speciation*

Sample collection strategies will vary depending on the purpose of the study, the logistic groundwork, and the budget constrain. Three possibilities are common: 24 hours sample, first morning void sample (FMV), and spot sample. In addition, researchers can consider 72-, 96- or 120-hour sample. Generally, 24- to 120-hour samples are used to assess within-day and between-day variation and intra-individual variability.

Nonetheless, 24-hour samples and first morning void samples are more expensive and

difficult to collect compared with spot samples. Samples collected during a period of 24 to 120 hours increase precision when assessing individuals but a group average of many individual spot urine samples may also provide good precision (Smith and Steinmaus 2000). Valenzuela et al. (2005) used spot samples when they claimed identification of trivalent arsenic species in 98% of the samples ($n=104$). On the other hand, a study by Calderon et al. (1999) shows that one first FMV sample is a reasonably good estimate of exposure. They argue that a comparison between 24-hour and FMV indicated that there was little day-to-day variation in the concentration of arsenic in urine. In addition, FMV samples are usually selected because they are more concentrated, and the metabolites obtained from the analysis are known to be in contact with the bladder for a longer period. Therefore, more than a logistic or budgetary issue, the selection of a sample strategy may help to understand arsenic exposures and to elucidate arsenic metabolism pathways. In theory it is possible that different methods will yield different concentration of urinary arsenic species, but no study has yet compared the effects of different methods of urinary collection (Tseng 2009).

2. Storage

After collection, storage in a cold environment is required to preserve the sample because in epidemiological studies, usually, samples are not analyzed immediately after collection. This does not follow the usual rule of analyzing clinical samples since fresh cells and residues begin to disintegrate within 1-3hrs. Urine refrigeration at 2-8°C usually prevents decomposition for up to 48hrs (Palacios et al. 1997). The importance of the storage is to maintain the original arsenic chemical species

in the sample before analysis (Feldelmann et al. 1999; Gong et al. 2002). For total arsenic the requirements are not as critical as speciation analysis because metabolites change over time through oxidation-reduction reactions (Gong 2002; Palacios et al. 1997; Larsen et al. 1993). Feldelmann et al. (1999) reported that the appropriate storage conditions for urine samples are either 4°C or -20°C for up to two months, while Chen et al. (2002) and Palacios et al. (1997) reported -20°C or lower for up to six months to measure As[III], As[V], MMA[V], and DMA[V].

Storage becomes a major limitation when developing a method for the analysis of trivalent species. Since trivalent species are not stable, the probability of finding MMA[III] or DMA[III] after 72 hours is very low. In an experiment performed by Gong et al. (2001), MMA[III] was quickly converted to MMA[V] after 3 days of storage at 25°C or 4°C. Moreover, after a longer storage (4 months), 96% of the sample was found to contain MMA[V], DMA[V], and As[III]. Similar to MMA[III], DMA[III] has an even shorter storage life. In the experiment mentioned before, Gong et al. (2001) observed that DMA[III] is completely converted to DMA[V] within 3 hours. When the samples were frozen at -20°C, DMA[III] was completely converted to DMA[V] within 17 hours. In conclusion, DMA[III] is extremely unstable, even at -20°C. On the other hand, when samples are analyzed immediately (less than 6 hours) trivalent species may appear (Valenzuela et al. 2005). In that study, subjects were scheduled for a urine sample collection in the morning at the local health center (Hidalgo, Mexico). The samples were immediately frozen in dry ice and analyzed within 6 hours after collection. As[III],

MMA[III], and DMA[III] were the predominant species, being DMA[III] the major metabolite (49% of samples).

In epidemiological studies, monetary constraints and logistic planning may be over-riding factors in deciding when, how, and where samples are being collected and analyzed. If the study has 100 individuals the probabilities of immediate analysis are higher than a 600 person study. The question of how trivalent species can be detected in a protocol designed to work along with a large epidemiological study is still under debate.

3. Separation and Detection

i. Total Arsenic

In order to achieve selectivity and sensitivity during analysis, different analytical techniques are applied. The separation and detection techniques are as reliable as the sample procedure used (Gong et al. 2002). For total arsenic analysis no separation technique is needed and detection can be accomplished with several instruments. The selection of the appropriate instrumentation will be determined by the sensitivity needed to detect arsenic. For example, fluorescence atomic absorption spectrometry (FAAS) use has declined because it suffers from low sensitivity and high background noise and inductively coupled plasma atomic emission spectrometry (ICP-AES) does not provide sufficient sensitivity for samples containing lower levels of arsenic (Gong et al. 2002; Francesconi and Kuehnelt 2004). The most widely used instrumentation for the analysis of total arsenic in urine is atomic fluorescence spectrometer (AFS) (ASTDR 2005). When the AFS is coupled with a hydride generation unit (HGAFS), it can be use

for speciation of inorganic arsenic. In the HGAFS system, arsenic compounds are reduced to gaseous arsines which are transported to the detector (Stockwell and Corns 1994).

ii. Arsenic Speciation

Various techniques are available for speciation of arsenic compounds in urine. These techniques have been a subject of extensive research and successful improvements. The most common technique for the separation of arsenic species is high performance liquid chromatography (HPLC) because of the ease of coupling it with most detectors (McSheehy 2003). Ion-pair and ion-exchange chromatography have been used in the analysis of arsenic species in urine. The ion pair columns allow the separation of complex mixture of polar and ionic molecules through a determined mobile phase. Ion-exchange columns depend more on different charges in solutes but both columns work on the basis of reversible absorption of charge molecules. Table 2-1 shows several columns and conditions used to identify arsenic species in urine. Most methods used today in the speciation of arsenic species using chromatography are adaptations of these well recognized protocols.

In ion-pair columns, the elution order is As[III], MMA[V], DMA[V], and As[V] independent of the different reverse-phase columns used (Gong et al. 2002). The resolution of the arsenic species depends on the concentration of the ion-pair reagent, flow rate, ionic strength, and pH of the mobile phase (Wangkarn and Pergantis 2000; Kiseleva and Nesterenko 2001). When analyzing DMA[III] and MMA[III], the elution order is As[III], MMA[III], DMA[V] MMA[V], DMA[III], and As[V] (Le et al. 2000a) (Table 2-

1). In addition to ion-pair and ion-exchange columns, anion and cation-exchange columns have been used for arsenic speciation as well. Cation-exchange columns are useful to separate AsB and AsC (Zheng et al. 1998; Mandal et al. 2001).

As an alternative to chromatography, the cryogenic trapped system is also used. The hydride generation-cryogenic trapping was the first speciation method (Braman and Foreback 1973). Actually, the cryogenic trap is still implemented for inorganic arsenic speciation in urine samples with a few changes (Steinmaus et al. 2006; Hopenhayn et al. 2003; Hopenhayn-Rich et al. 1996; Ranft et al. 2003; Biggs et al. 1997). During the separation through a cryogenic trap, arsenic species are trapped in a U-shape tube immersed in liquid nitrogen. Once they are all trapped, the nitrogen is removed and the U-tube is warmed up. Since boiling points are different for each species, they evaporate and are transported to the detector sequentially.

The separation system is coupled to a detection system in order to identify and quantify arsenic species. The most common detectors for urine analysis are atomic fluorescence spectrometry (AFS), atomic absorption spectrometry (AAS) and inductively coupled plasma mass spectrometry (ICP-MS). Actually, HPLC coupled with ICPMS is the most effective tool in many arsenic research laboratories (Gong et al. 2002; Hymer and Caruso 2004). ICP-MS offers extremely high sensitivity, large dynamic range, and isotope ratio measurement. However, ICPMS is expensive and requires high maintenance, and for that reason AFS and AAS have been the preferred tools for arsenic speciation. HGAFS and HGAAS have been used widely in urine analysis for arsenic speciation (Le et al.

2000a; Le and Ma 1998; Chen et al. 2003; Calderon et al. 1999; Valenzuela et al. 2005; Steinmaus et al. 2006; Biggs et al. 1997; Karagas et al. 2001b; Hopenhayn et al. 2003). Hydride generation (HG) is an interface between HPLC and AAS or AFS and enhances sensitivity because only gaseous hydrides are introduced into the detector while the sample matrix is left in the liquid waste. Therefore, spectral and chemical interferences are basically eliminated when using HG. HGAFS lowers the detection limits obtained from HG-AAS (Gong et al. 2002), hence providing a better choice for exposure levels lower than 100µg/L.

Figure 2-1. Classical Inorganic Arsenic Biotransformation Pathway (Cullen and Reimer 1989).

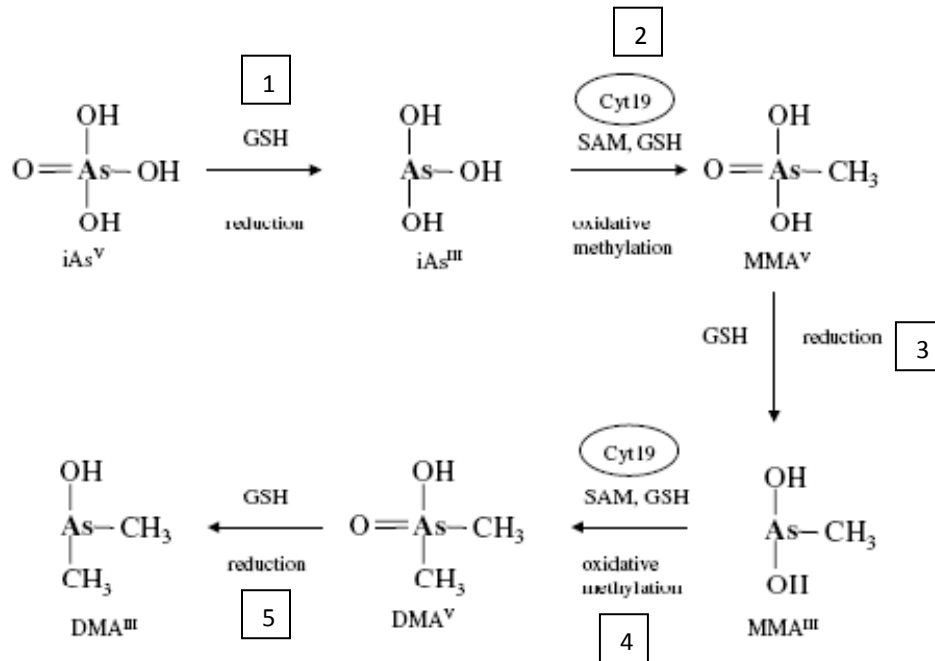


Figure 2-2 New Inorganic Arsenic Biotransformation Pathway (Hayakawa et al. 2005).

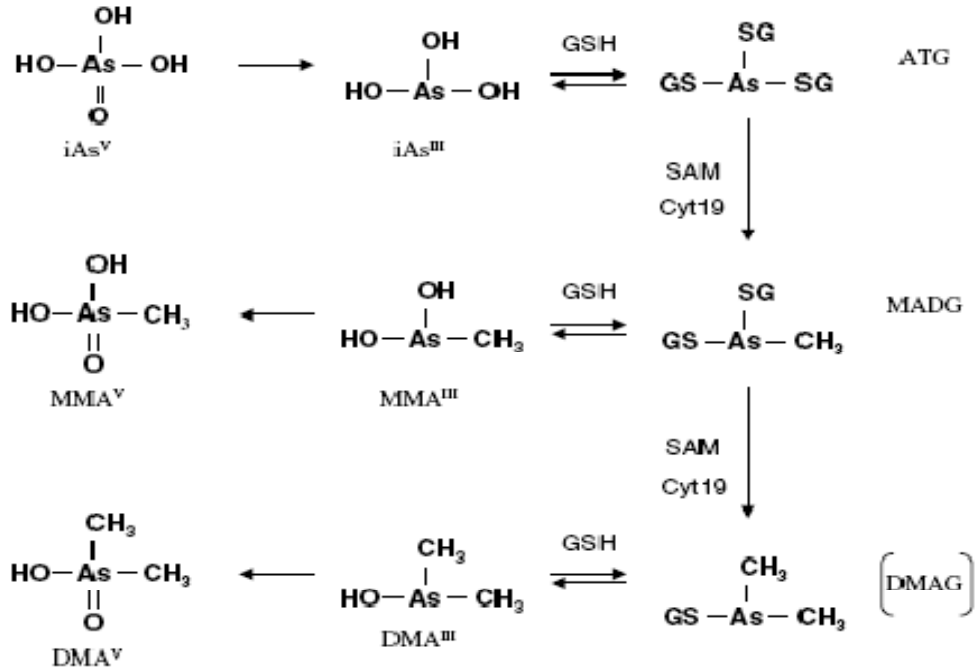


Table 2-1 Most common chromatography columns and conditions used in arsenic speciation.

Column	Mobile Phase	Flow Rate (mL/min)	Detection Method	Detectable Arsenic Species	Reference
Ion-pair Chromatography					
Phenomenex ODS 3 (150x4.6mm, 3µm)	5mM ^a TBHA, 4mM malonic acid, 5% methanol, pH 5.9	1.50	^b HGAFS	As[III], As[V], MMA[V], DMA[V]	Le et al. 1998
Phenomenex ODS 3 (150x4.6mm, 3µm)	5mM TBHA, 4mM malonic acid, 5% methanol, pH 5.85	1.20	HGAFS	As[III], As[V], MMA[V], DMA[V], MMA[III]	Le et al. 2000b
Phenomenex ODS 3 (150x4.6mm, 3µm)	5mM TBHA, 3mM malonic acid, 5% methanol, pH 5.85	1.20	HGAFS	As[III], As[V], MMA[V], DMA[V], MMA[III], DMA[III]	Le et al. 2000a
Ion-exchange Chromatography					
Hamilton PRPX100 anion exchange (250x4.1mm) 10µm	Gradient elution	1.0 or 1.5	^c ICPMS	AsB, AsC, As[III], AsV, MMA[V], DMA[V]	Zheng et al. 1998
Shodex Asahipak ES-502N 7C Anion exchange (100x7.6mm)	15mM citric acid adjusted pH 1.0 with 10%HNO3	1.0	ICPMS	AsB, AsC, As[III], As[V], MMA[V], DMA[V], MMA[III], DMA[III]	Mandal et al. 2001
Shodex Asahipak NN-614 cation exchange (150x6.0mm)	36mM formic acid 2mM ammonium formate pH 2.8	0.8	ICPMS	AsB, As[III], As[V], MMA[V], DMA[V], MMA[III], DMA[III]	Suzuki et al. 2002

^aTetrabutylammonium hydroxide ^bHydride Generation Atomic Fluorescence Spectrometry ^cIon Coupled Plasma Mass Spectrometer

Table 2-2. Studies reporting individual level exposure-biomarker association for arsenic

Urine sample	Exposure measure	Exposure concentration	Association	Study
^a FMV	drinking water	<95µg/L	0.45(<i>p</i> <0.0001)	Lindberg et al. 2006
FMV	drinking water	^b 3.5µg/L	0.35 (<i>p</i> =0.0024)	Karagas et al. 2001a
FMV	drinking water	9-50µg/L	0.496(<i>p</i> =0.007)	Meza et al. 2004
FMV	drinking water	^b 9.89µg/L	0.40(<i>p</i> =0.0004)	Karagas et al. 2002b
FMV	drinking water	^b 43.8µg/L	0.50(<i>p</i> <0.0001)	Hinwood et al. 2003
spot	drinking water	<991µg/L	0.50(<i>p</i> <0.0001)	Ahsan et al. 2000
spot	drinking water	-	For every 1 µg/L increment in As in well water, there was an increase of 0.18 µg/L InAs metabolite in urine	Chen et al. 2005
spot	air	2-850µg/m ³	0.723 (<0.0001)	Jakubowski et al. 1998

^aFirst Morning Void Sample ^bArithmetic Mean

References

Agency for Toxic Substances and Disease Registry. Toxicological Profile for Arsenic Draft for Public Comments. 2005 Division of Toxicology and Environmental Medicine, Atlanta, GA.

Ahsan H, Perrin M, Rahman A, Parvez F, Stute M, Zheng Y, Milton Abul, Brandt-Rauf P, van Green A, Graziano J. 2000. Associations between drinking water and urinary arsenic levels and skin lesions in Bangladesh. *Journal of Occupational and Environmental Medicine* 42:1195-1201.

Aposhian HV, Gurzau ES, Le XC, Gurzau A, Healy SM, Lu X, Ma M, Yip L, Zakharyan RA, Maiorino RM, Dart R, Tircus MG, Gonzalez-Ramirez D, Morgan D, Avram D, Aposhian M. 2000. Occurrence of monomethylarsonous acid in urine of humans exposed to inorganic arsenic. *Chemical Research in Toxicology* 12:693-697.

Biggs ML, Kalman D, Moore LE, Hopenhayn-Rich C, Smith MT, Smith A. 1997. Relationship of urinary arsenic to intake estimates and a biomarker of effect, bladder cell micronuclei. *Reviews in Mutation Research* 386:185-195.

Borak J, Hosgood HD. 2007. Seafood arsenic: implications for human risk assessment. *Regulatory Toxicology and Pharmacology* 47:204-212.

Braman RS, Foreback CC. 1973. Methylated forms of arsenic in the environment. *Science* 182:1247-1249.

Buchet J, Lauwerys R. 1987. Study of factors influencing the in vivo methylation of inorganic arsenic in rats. *Toxicology Applied Pharmacology* 91:65-74.

Caceres D, Pino P, Montesinos N, Atalah E, Amigo H, Loomis D. 2005. Exposure to inorganic arsenic in drinking water and total urinary arsenic concentration in a Chilean population. *Environmental Research* 98:151-159.

Calderon R, Hudgens E, Le X.C., Schreinemachers D, Thomas D. 1999. Excretion of arsenic in urine as a function of exposure to arsenic in drinking water. *Environmental Health Perspectives* 107:663-667.

Caceres SD, Pino P, Montesinos N, Atalah E, Amigo H, Loomis D. 2005. Exposure to inorganic arsenic in drinking water and total urinary arsenic concentration in a Chilean population. *Environmental Research* 98:151-159.

Cantor K, Lubin J. 2007. Arsenic, internal cancers, and issues in inference from studies of low-level exposure in human populations. *Toxicology and Applied Pharmacology* 222:252-257.

Challenger F and Higginbottom C. 1937. Arsenic methylation. *Biochemistry Journal*. 29:1757-1761.

Challenger F. 1945. Biological methylation. *Chemical Reviews* 36:315-361 .

Chen Y, Amarasiriwardena C, Hsueh Y, Christiani D. 2002. Stability of arsenic species and insoluble arsenic in human urine. *Cancer Epidemiology, Biomarkers & Prevention* 11:1427-1433.

Chen Y, Guo L Su J, Hsueh Y, Smith T, Ryan L, Lee M, Chao S, Lee J, Christiani D. 2003. Arsenic methylation and skin cancer risk in southwestern Taiwan. *Journal of Occupational and Environmental Medicine* 45:241-248.

Chen C-J, Hsu L-I, Wang C-H, Shih W-L, Hsu Y-H, Tseng M-P, Lin Y-C, Chou W-L, Chen C-Y, Lee C-Y, Wnag L-H, Cheng Y-C, Chen C-L, Chen S-Y, Wang Y-H, Hsueh Y-M, Chiou H-Y, Wu M-M. 2005. Biomarkers of exposure, effect and susceptibility of arsenic-induced health hazards in Taiwan. *Toxicology and Applied Pharmacology* 206:198-206.

Chen Y, van Geen A, Graziano JH, Pfaff A, Madajewicz M, Parvez F, Iftexhar Hussain AZM, Slavkovich V, Islam T, Ahsan H. 2007. Reduction in urinary arsenic levels in response to arsenic mitigation efforts in Araihasar, Bangladesh. *Environmental Health Perspectives* 115:917-923.

Cullen W, McBride B, Reglinski J. 1984. The reduction of trimethylarsine oxide to trimethylarsine by thiols: a mechanistic model for biological reduction of arsenicals. *Journal of Inorganic Biochemistry* 21:45-60.

Cullen W, Reimer K. 1989. Arsenic speciation in the environment. *Chemical Reviews* 89:713-764.

Decaprio A. 1997. Biomarkers: coming of age for environmental health and risk assessment. *Environmental Science Technology* 31:1837-1848.

Del Razo LM, Styblo M, Cullen WR, Thomas DJ. 2001. Determination of trivalent arsenicals in biological matrices. *Toxicology and Applied Pharmacology* 174:282-293.

- Devesa V, Suner MA, Algora S, Velez D, Mоторo R, Jalon M, Urieta I, Macho ML. 2005. Organoarsenicals species contents in cooked seafood. *Journal of Agriculture and Food Chemistry* 53:8813-8819.
- Dopp E, Hartmann L, Florea LM, Von Recklinghausen U, Piper R, Shokouhi B, Rettenmeir A, Heirner AV, van Obe G. 2004. Uptake of inorganic and organic derivatives of arsenic associated with induced cytotoxic and genotoxic effects in Chinese hamster ovary (CHO) cells. *Toxicology and Applied Pharmacology* 201:156-165.
- Dor F, Dab W, Empereur-Bissonnet P, Zmirou D. 1999. Validity of biomarkers in environmental health studies: the case of PAH's and benzene. *Critical Reviews in Toxicology* 29:129-168.
- Edmonds JS, Francesconi KA. 1988. The origin of arsenobetaine in marine animals. *Applied Organometallic Chemistry* 2:297-302.
- Edmonds JS, Francesconi KA. 1994. Arsenic in seafood: human health aspects and regulations. *Marine Pollutants Bulletin* 26:665-674.
- Feldmann J, Lai VW, Cullen WR, Ma M, Lu X, Le XC. 1999. Sample preparation and storage can change arsenic speciation in human urine. *Clinical Chemistry* 45:1988-1997.
- Francesconi KA, Kuehnelt D. 2004. Determination of arsenic species: A critical review of methods and applications, 2000-2003. *The Analyst* 129:373-395.
- Gong Z, Lu A, Cullen WR, Le XC. 2001. Unstable trivalent arsenic metabolites, monomethylarsonous acid and dimethylarsinous acid. *Journal of Analytical Atomic Spectrometry* 16:1409-1413
- Gong Z, Lu X, Ma M, Watt C, Le X.C. 2002. Arsenic speciation analysis. *Talanta* 58:77-96
- Goyer R, Clarkson T. Toxic effects of metals. In Klaassen, CD (Ed.) Casarett and Doull's *Toxicology: The basic science of poisons* sixth edition. McGraw-Hill, New York pp.811-867.
- Hall Moran V, Leathard H, Coley J. 2001. Urinary hormones levels during the natural menstrual cycle: the effect of age. *Journal of Endocrinology* 170:157-164.
- Hansen HR, Raab A, Jaspard M, Milne, Feldmann J. 2004. Sulfur-containing arsenicals mistaken for dimethylarsinous acid [DMA(III)] and identified as a natural metabolite in urine: major implications for studies on arsenic metabolism toxicity. *Chemical Research in Toxicology* 17:1086-1091.

- Harkins D, Susten A. 2003. Hair analysis: Exploring the state of the science. *Environmental Health Perspectives* 111:576-578.
- Hopenhayn C, Huan B, Christian J, Peralta C, Ferreccio C, Atallah R, Kalman D. 2003. Profile of urinary arsenic metabolites during pregnancy. *Environmental Health Perspectives* 111:1888-1891.
- Hopenhayn-Rich C, Biggs M.L., Smith A, Kalman D, Moore L. 1996. Methylation study of a population environmentally exposed to arsenic in drinking water. *Environmental Health Perspectives* 104:620-628.
- Hayakawa T, Kobayashi Y, Cui X, Hirano S. 2005. A new metabolic pathway of arsenite: arsenic-glutathione complexes are substrates for human arsenic methyltransferase CYT19. *Inorganic Compounds* 79:183-191.
- Hinwood AL, Sim M, Jolley D, De Klerk N, Bastone E, Gerostamoulos J, Drummer O. 2003. Risk factors for increased urinary inorganic arsenic concentrations from low arsenic concentrations in drinking water. *International Journal of Environmental Health Research* 13:271-284.
- Hopenhayn-Rich C, Biggs ML, Fuchs A, Bergoglio R, Tello EE, Nicolli H. 1996. Bladder cancer mortality associated with arsenic in drinking water in Argentina. *Epidemiology* 7:117-124.
- Hopenhayn C, Huang B, Christian J, Peralta C, Ferreccio C, Atallah R, Kalman D. 2003. Profile of urinary arsenic metabolites during pregnancy. *Environmental Health Perspectives* 111:1888-1891.
- Hsueh YM, Chiou HY, Huang YL, Wu WL, Huang CC, Yang MH, Lue LC, Chen GS, Chen CJ. 1997. Serum beta-carotene level, arsenic methylation capability, and incidence of skin cancer. *Cancer Epidemiology, Biomarkers and Prevention* 6:589-596.
- Hsueh Y-M, Huang Y-L, Huang C-C, Wu W-L, Chen H-M, Yang M-H, Lue L-C, Chen C-J. 1998. Urinary levels of inorganic and organic arsenic metabolites among residents in an arseniasis-hyperendemic area in Taiwan. *Journal of Toxicology and Environmental Health A* 54:431-444.
- Hsueh YM, Hsu MK, Chiou HY, Yang MH, Huang CC, Chen CJ. 2002. Urinary arsenic speciation in subjects with or without restriction from seafood dietary intake. *Toxicology Letters* 133:83-91.

Hymer CB, Caruso JA. 2004. Arsenic its speciation analysis using high-performance liquid chromatography and inductively coupled plasma mass spectrometry. *Journal of Chromatography A* 1045:1-13.

Jakubowski M, Trzcinka-Ochocka M, Razniewska G, Matczak W. 1998. Biological monitoring of occupational exposure to arsenic by determining urinary content of inorganic arsenic and its methylated metabolites. *International Archives in Occupational and Environmental Health* 71:S29-32.

Karagas M, Stukel T, Tosteson T. 2001a. Assessment of cancer risk and environmental levels of arsenic in New Hampshire. *International Journal of Hygiene and Environmental Health* 205:85-94.

Karagas M, Le C.X, Morris S, Blum J, Lu X, Spate V, Carey M, Stannard V, Klaue B, Tosteson T. 2001b. Markers of low level arsenic exposure for evaluating human cancer risk in a US population. *International Journal of Occupational Medicine and Environmental Health* 14:171-175.

Karagas M, Nelson H, Kelsey K, Morris S, Blum J, Tosteson T, Carey M, Lu X, Le X.C. 2002. Urinary arsenic species in relation to drinking water, toenail arsenic concentrations and genetic polymorphism in GSTMI in New Hampshire In: *Biomarkers of Environmentally Associated Disease* Wilson S. and Suk W. Lewis Publisher NY.

Kiseleva M.G., Nesterenko P.N. 2001. Novel stationary phase with regulated anion-exchange capacity. *Journal of Chromatography* 920:87-93.

Larsen EH, Pritzl G, Hansen SH. 1993. Speciation of eight arsenic compounds in human urine by high-performance liquid chromatography with inductively coupled plasma mass spectrometric detection using antimonite for internal chromatographic standardization. *Journal of Analytical Atomic Spectrometry* 8:557.

Le XC, Cullen W, Reimer K. 1994. Human urinary arsenic excretion following one-time ingestion of arsenosugars present in seaweed and arsenobetaine present in crab and shrimp. *Clinical Chemistry* 40:617-625.

Le XC Ma M. 1997. Speciation of arsenic compounds by using ion-pair chromatography with atomic spectrometry and mass spectrometry detection. *Journal of Chromatography* 764:55-64.

Le XC Ma M. 1998. Short-column liquid chromatography with hydride generation atomic fluorescence detection for the speciation of arsenic *Analytical Chemistry* 70: 1926-1933.

- Le XC, Lu X, Ma M, Cullen W, Aposhian HV, Zheng B. 2000a. Speciation of key arsenic metabolic intermediates in human urine. *Analytical Chemistry* 72:5172-5177.
- Le XC, MA M, Lu X, Cullen WR, Aposhian V, Zheng B. 2000b. Determination of monomethylarsonous acid, a key arsenic methylation intermediate, in human urine. *Environmental Health Perspectives* 108:1015-1018.
- Li J, Waters SB, Drobná Z, Devesa V, Styblo M, Thomas DJ. 2005. Arsenic (+3 oxidation state) methyltransferase and the inorganic arsenic methylation phenotype. *Toxicology and Applied Pharmacology* 204:164-169.
- Lin T, Huang Y, Wang M. 1998. Arsenic species in drinking water, hair, fingernails, and urine of patients with Blackfoot disease. *Journal of Toxicology and Environmental Health Part A* 53:85-93.
- Lindberg A-L, Goessler W, Gurzau E, Koppova K, Rudnai P, Kumar R, Fletcher T, Leonardi G, Slotova K, Gheorghiu E, Vahter M. 2006. Arsenic in Hungary, Romania and Slovakia. *Journal of Environmental Monitoring* 8:203-208.
- Lindberg AL, Goessler W, Grand'ér M, Nermell B, Vahter M. 2007. Evaluation of the three most commonly used analytical methods for determination of inorganic arsenic and its metabolites in urine *Toxicology Letters* 168:310–318.
- Lo MC, Hsen YC, Lin BK. 1977. Arsenic content of underground water in Taiwan: Second Report p1-23 Taichung, Taiwan: Taiwan Provincial Institute of Environmental Sanitation.
- Loffredo C, Aposhian HV, Cebrian M, Yamauchi H, Silbergeld E. 2003. Variability in human metabolism of arsenic. *Environmental Research* 92:85-91.
- Ma M, Le XC. 1998. Effect of arsenosugars ingestion on urinary arsenic speciation. *Clinical Chemistry* 44:539-550.
- Mandal BK, Ogra Y, Suzuki KT. 2001 Identification of dimethylarsinous and monomethylarsonous acids in human urine of the arsenic-affected areas in West Bengal, India. *Chemical Research in Toxicology* 14:371-378.
- Mandal BK, Ogra Y, Suzuki KT. 2003. Speciation of arsenic in human nail and hair from arsenic-affected area by HPLC-inductively couple argon plasma mass spectrometry. *Toxicology and Applied Pharmacology* 189:73-83.
- Marnell LL, Garcia-Vargas GG, Chowdhury UK, Zakharyan RA, Walsh B, Avram MD, Kopplin MJ, Cebrian ME, Silbergeld EK, Aposhian H. 2003. Polymorphism in the human

monomethylarsonic acid (MMAV) reductase/hGSTO1 gene and changes in urinary arsenic profiles. *Chemical Research in Toxicology* 16:1507-1513.

Martin SJ, Newcombe C, Raab A, Feldmann A. 2005. Arsenosugar metabolism not unique to the sheep of north Ronaldsay *Environmental Chemistry* 2:190-197.

McSheehy S, Szpunar J, Morabito R, Quevauviller P. 2003. The speciation of arsenic in biological tissues and the certification of reference materials for quality control. *Trends in Analytical Chemistry* 22:191-209.

Meza MM, Kopplin M, Burgess JL, Gandolfi AJ. 2004. Arsenic in drinking water exposure and urinary excretion among adults in the Yaqui Valley, Sonora, Mexico. *Environmental Research* 96:119-126.

Meza MM, Yu L, Rodriguez Y, Guild M, Thompson D, Gandolfi AJ, Klimecki WT. 2005. Developmentally restricted genetic determinants of human arsenic metabolism: association between urinary methylated arsenic and CYT19 polymorphisms in children. *Children's Health* 113:775-781.

Moore R, Hirata-Dulas C, Kasiske B. 1997. Use of urine specific gravity to improve screening for albuminuria. *Kidney International* 52:240-243.

Naranmandura H, Suzuki N, Suzuki KT. 2006. Trivalent arsenicals are bound to proteins during reductive methylation. *Chemical Research in Toxicology* 19:1010-1018

Naranmandura H, Suzuki N, Iwata K, Hirano S, Suzuki KT. 2007. Arsenic metabolism and thioarsenicals in hamsters and rats. *Chemical Research in Toxicology* 20:616-624.

National Cancer Institute. 2001. What do you need to know about bladder cancer. www.cancer.gov/cancertopics/wyntk/bladder. US National Institute of Health.

National Research Council. 1987. Biological markers in environmental health research. *Environmental Health Perspectives* 74:3-9.

Palacios MA, Gomez M, Camara C, Lopez M.A. 1997. Stability of arsenate, monomethylarsonate, dimethylarsinate, arsenobetaine and arsenocholine in deionized water, urine and clean-up dry residue from urine samples and determination by liquid chromatography with microwave-assisted oxidation-hydride generation absorption spectrometric detection. *Analytica Chimica Acta* 340:209-220.

Parikh C, Gyamlani G, Carvounis C. 2002. Screening for microalbuminuria simplified by urine specific gravity. *American Journal of Nephrology* 22:315-319.

Perucca J, Bouby N, Valeix P, Bankir L. 2006. Sex differences in urine concentration across differing ages, sodium intake, and level of kidney disease. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* 292:700-705.

Petrick JS, Ayala-Fierro F, Cullen WR, Carter DE, Aposhian HV. 2000. Monomethylarsonous acid (MMAIII) is more toxic than arsenite in Chang human hepatocytes. *Toxicology and Applied Pharmacology* 63:203-207.

Pi J, Yamauchi H, Kumagai Y, Sun G, Yoshida T, Aikawa H, Hopenhayn-Rich C, Shimojo N. 2002. Evidence for induction of oxidative stress caused by chronic exposure of Chinese residents to arsenic contained in drinking water. *Environmental Health Perspectives* 110:331-336.

Ranft U, Miskovic B, Jakubis P, Fabianova E, Keegan T, Hergemoller A, Jakubis M, Nieuwenhuijsen M.J., EXPASCAN Study Group. 2003. Association between arsenic exposure from a coal-burning power plant and urinary arsenic concentrations in Prievidza District, Slovakia. *Environmental Health Perspectives* 111:889-894.

School of Environmental Studies and Dhaka Community Hospital (SOES&DCH). 2000. Summary of 239 days field survey from August 1995 to February 2000. Ground Water Contamination in Bangladesh, A Survey Report Conducted by the School of Environmental Studies, Jadavpur University, Calcutta, India and Dhaka Community Hospital, Dhaka, Bangladesh.

Slotnick M, Nriagu J. 2006. Validity of human nails as a biomarker of arsenic and selenium exposures: A review. *Environmental Research* 102:125-139.

Slotnick MJ, Meliker JR, AvRuskin GA, Ghosh D, Nriagu JO. 2007. Toenails as a biomarker of inorganic arsenic intake from drinking water and foods. *Journal of Toxicology and Environmental Health Part A* 70:148-158.

Smith A, Steinmaus C. 2000. Arsenic in urine and drinking water. *Environmental Health Perspectives*. pA494.

Solomon E, Berg L, Martin D, Ville C. In *Biology*, 3rd Edition. 1993. Saunders College Publishing pp 993-1000.

Steinmaus C, Bates, Yuan Y, Kalman D, Atallah R, Rey O, Biggs M, Hopenhayn C, Moore L, Hoang B, Smith A. 2006. Arsenic methylation and bladder cancer risk in case-control studies in Argentina and the United States. *Journal of Occupational and Environmental Medicine* 48:478-488.

- Stockwell PB, Corns WT. 1994. Environmental sensors based on atomic fluorescence. *Analyst* 119:1641-1645.
- Stybło M, Del Razo LM, Vega L, Germolec DR, LeCluyse EL, Hamilton GA, Reed W, Wang C, Cullen WR, Thomas DJ. 2000. Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. *Archives of Toxicology* 74:289-299.
- Suwazono Y, Akesson A, Alfvén T, Jarup L, Vahter M. 2005. Creatinine versus specific gravity-adjusted urinary cadmium concentrations. *Biomarkers* 10:117-126.
- Suzuki KT, Mandal BK, Ogra Y. 2002. Speciation of arsenic in body fluids. *Talanta* 58: 111
- Tokunaga H, Roychowdhury T, Chandraskaran N, Uchino T, Ando M. 2002. Urinary arsenic species in an arsenic-affected area of West Bengal, India. *Applied Organometallic Chemistry* 16:406-414.
- Tseng, C-H. 2009 . A review on environmental factors regulating arsenic methylation in humans. *Toxicology and Applied Pharmacology* doi:10.1016/j.taap.2008.12.016
- Vahter M. 1994a. What are the chemical forms of arsenic in urine, and what can they tell us about exposure. *Clinical Chemistry* 40:617-624.
- Vahter M. 1994b. Species differences in the metabolism of arsenic compounds. *Applied Organometallic Chemistry* 8:175-182.
- Vahter M, Concha G, Nermell B, Nilsson R, Dulout F, Natarajan N. 1995. A unique metabolism of inorganic arsenic in Andean women. *European Journal of Pharmacology and Environmental Toxicology* 293:455-462.
- Vahter M. 1999. Methylation of inorganic arsenic in different mammalian species and population groups. *Science in Progress* 82:69-88.
- Vahter M. 2002. Mechanism of arsenic biotransformation. *Toxicology* 181-182:211-217.
- Valenzuela O, Borja-Aburto H, Garcia-Vargas G, Cruz-Gonzalez M, Garcia-Montalvo E, Calderon-Aranda E, Del Razo L.M. 2005. Urinary trivalent methylated arsenic species in a population exposed to inorganic arsenic. *Environmental Health Perspectives* 113:250-254.
- Vega L, Stybło M, Patterson R, Cullen W, Wang C, Germolec D. 2001. Differential effects on trivalent and pentavalent arsenicals on cell proliferation and cytokine secretion in

normal human epidermal keratinocytes. *Toxicology and Applied Pharmacology* 172:225-232.

Wangkarn S, Pergantis S.A. 2000. High-speed separation of arsenic compounds using narrow-bore high-performance liquid chromatography on-line with inductively coupled plasma mass spectrometry. *Journals of Analytical Atomic Spectrometry* 15:627-633.

Waters SB, Devesa V, Del Razo LM, Styblo M, Thomas DJ. 2004. Endogenous reductants support the catalytic function of recombinant rat CYT19, an arsenic methyltransferase. *Chemical Research in Toxicology* 17:404-409.

World Health Organization. 2001. Biomarkers in risk assessment: Validity and validation. 2001.

Worsfold M, Davie M, Haddaway M. 1999. Age-related changes in body composition, hydroxyproline, and creatinine excretion in normal women. *Calcified Tissue International* 64:40-44.

Zakharyan RA, Wu Y, Bogdan GM, Aposhian AV. 1995. Enzymatic methylation of arsenic compounds: Assay, partial purification, and properties of arsenite methyltransferase and monomethyl-arsenic acid methyltransferase of rabbit liver. *Chemical Research in Toxicology* 8:1029-1038.

Zakharyan RA, Ayala-Fierro F, Cullen W, Carter DM, Aposhian AV. 1999. Enzymatic methylation of arsenic compounds VII. Monomethylarsonous acid (MMAIII) is the substrate for MMA methyltransferase of rabbit liver and human hepatocytes. *Toxicology and Applied Pharmacology* 158:9-15.

Zakharyan RA, Sampayo-Reyes A, Healy SM, Tsaprailis G, Board PG, Liebler DC, Aposhian AV. 2001. Human monomethylarsonic acid (MMAV) reductase is a member of the glutathione-S-transferase superfamily. *Chemical Research In Toxicology* 14:1051-1057.

Zheng J, Goessler W, Kosmus W. 1998. Speciation of arsenic compounds by coupling high-performance liquid chromatography with inductively coupled plasma mass spectrometry. *Microchimica Acta* 130:71-79.

Chapter III

Low-Level Determination of Six Arsenic Species in Urine by High Performance Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometry- (HPLC-ICP-MS)

I. Introduction

Arsenic occurs in human urine in several chemical forms which have differing toxicities. The principal species that have been reported include the oxy-anions of As[III] (mainly arsenite) and As[V] (primarily arsenate) which are known carcinogens (IARC, 2004). Other important components are the various methylated arsenic species including monomethylarsonous acid (MMA[III]), dimethylarsinous acid (DMA[III]), monomethylarsonic acid (MMA[V]) and dimethylarsinic acid (DMA[V]) whose toxicities and carcinogenicities remain highly contentious (Suzuki 2005; Petrick et al. 2000; Styblo et al. 2000). Urine may also contain other organic arsenic species such as arsenobetaine, arsenocholine, arsenosugars and arsenolipids which are derived primarily from seafood and are much less toxic than inorganic arsenic (Francesconi and Kuehnelt 2002; Schmeisser et al. 2006). A single meal of seafood may increase total urinary arsenic concentrations several orders of magnitude (Vahter 1994).

In epidemiological studies, urinary arsenic has been used as a biomarker of exposure (Mushak and Crocetti 1995; Loffredo et al. 2003; Valenzuela et al. 2005). The importance of DNA methylation on the etiology and early detection (screening) of arsenic-induced cancers is currently an area of active research (Jensen et al. 2009; Vahter 2008; Salnikow and Zhitkovich 2008). A number of studies have also reported ethnic and hereditary differences in arsenic metabolism and methylation patterns of urinary arsenic (Loffredo et al. 2003; Lai et al. 2004; Brima et al. 2006). The validity of urinary arsenic as a biomarker is very much dependent on our ability to speciate the arsenic accurately. The determination of individual forms of arsenic at very low concentrations in urine still poses a challenge in terms of accuracy and precision for most analytical techniques. In the past, the most common method for determining arsenic species in urine involved high performance liquid chromatography (HPLC), with or without hydride generation (HG), coupled to an atomic absorption spectrometer (AAS). Hydride generation was used to discriminate between the so-called toxicologically relevant As forms (namely, As[III], As[V], MMA and DMA) from arsenobetaine and other organoarsenicals that do not form volatile arsines (Lai et al. 2004). Today, a combination of an HPLC with inductively coupled plasma-mass spectrometry (ICP-MS) has become the instrument of choice for ultratrace analysis of urinary arsenic, due mainly to the superior sensitivity, high samples throughput and wide linearity range of concentration of the system (Jarvis et al. 1992). The HPLC-ICP-MS technique, however, is limited by unwanted spectral and non-spectral interferences

which can change with the matrix of the sample being analyzed (Jakubowski et al. 1998). Spectral interference is particularly serious with quadrupole ICP-MS system because of its low resolution (D'Ilio et al. 2006).

A goal of this study is to develop a method for measuring the six arsenic species (As[III], As[V], MMA[III], MMA[V], DMA[V], and arsenobetaine (AsB) in urine samples at ultratrace levels found in an un-exposed population of southeast Michigan using the HPLC-ICP-MS technique. The study was prompted by the finding that the techniques used in many previous studies involved considerable overlapping of AsB and As[III] peaks and could have resulted in over-reporting of As[III] concentrations. Another goal of this report is to develop a simple method for overcoming the ArCl⁺ (caused by the chloride in the sample) spectral interference without resorting to the standard interference models. The method was applied to hundreds of samples with a view to identifying other arsenic species that may be present in human urine.

II. Methodology

Standards

Stock arsenic[III] and arsenic[V] standards were prepared by dissolving the correct amount of arsenic[III] oxide (Aldrich, 99.99%) and sodium arsenate dibasic heptahydrate (Aldrich, ACS Reagent), respectively, in water to obtain 1000 mg L⁻¹ As. Stock MMA[V] and DMA[V] solutions were prepared by dissolving the correct amount of monosodium acid methane arsonate sesquihydrate (Chem Service, 96.5%) and cacodylic acid (Sigma, 98%), respectively, in water to obtain 1000 mg L⁻¹ As. Stock arsenobetaine standard was

prepared by diluting arsenobetaine calibrant solution (European Commission, Community Bureau of Reference, 434 mg L⁻¹ As) to 10 mg L⁻¹ in water. The source of MMA[III] was the diiodomethylarsine (CH₃AsI), which was prepared following the procedure of Cullen et al. (1989), and was obtained from Dr. William Cullen at the University of British Columbia (Vancouver, CA). MMA[III] was prepared by dissolving the appropriate amount of CH₃AsI₂ in water to obtain 1000 mg L⁻¹ As. Purity was 98% with small traces of As[III] and MMA[V]. MMA [III] is formed after hydrolysis of CH₃AsI₂ in water. Ten mg L⁻¹ stock standards were prepared from the 1000 mg L⁻¹ standards and diluted daily to obtain the working standards. All standards were stored at 4°C.

Urine certified reference material (National Institute for Environmental Studies, NIES NO. 18, Tsukuba, Ibaraki, Japan) was prepared by dissolving the appropriate amount of urine powder in water to obtain 69 ± 12 µg L⁻¹ AsB, 36 ± 9 µg L⁻¹ DMA and 137 ± 11 µg L⁻¹ total As. A 2 mg L⁻¹ germanium internal standard solution was prepared from the stock Ge solution (Inorganic Ventures, 1000 mg L⁻¹ Ge) by dilution with the same mobile phase being used in the analytical column that day.

Reagents

Methanol (Fisher, certified electronic grade, 99.8%), tetrabutylammonium hydroxide solution (Fluka, puriss p.a., ~40% in water), ammonium phosphate monobasic (Fisher, 98.9%) and malonic acid (Fisher, reagent grade) were used to make the mobile phase. Mobile phase pH was adjusted using dilute solutions of sodium hydroxide (Fisher, certified ACS, 97.5%) or phosphoric acid (Mallinckrodt, AR, 85%) The mobile

phase was then filtered through a 0.2 µm pore diameter nylon membrane filter (Millipore, GNWP 047 00) and sonicated for 30 minutes to remove dissolved gases. Water was obtained from a Milli-Q Water Purification System using deionized water as the input.

Instrumentation

Details of the instrumentation are given in Table 3-1. The HPLC system included an Alltech model 426 isocratic HPLC pump, Alltech model 530 column heater set at 50°C, column with pre-column cartridge, and six-port manual injection valve. The columns used included a Phenomenex Gemini C18 analytical column (4.6 X 150 mm) with guard cartridge and a Phenomenex Gemini C18 analytical column (4.6 X 250 mm) with guard cartridge. The effluent from the column was injected directly into the ICP-MS nebulizer through PFA tubing.

The ICP-MS system used was an Agilent 7500c. The instrument was set to monitor signals at 75 amu (As), 72 amu (Ge) and 35 amu (Cl). Column eluent was introduced into the ICP-MS through a PFA sample line. The line was connected to a tee connector that also accepted the 2 mg L⁻¹ germanium ISTD diluted in mobile phase. A peristaltic pump supplied the ISTD to the tee. The ISTD solution was reduced to approximately 50 µg L⁻¹ through dilution by the eluent stream. The tee connector was grounded to the ICP-MS chassis through a Pt wire in contact with the sample solution to reduce signal fluctuations from any electrostatic charging caused by the ISTD peristaltic pump. After the tee, the sample was directed into a MicroMist micro-uptake glass

concentric nebulizer (Glass Expansion, Australia, AR35-1-FSS04EX). The peltier-cooled Scott-style spray chamber was jacketed and maintained at 2°C to increase signal stability and reduce water vapor in the sample mist. A quartz torch with a 2.5 mm id sample tube was used to prevent clogging caused by salt formation. Nickel-plated sample and skimmer cones (1.0 mm and 0.7 orifices, respectively) were used.

ICP-MS operating conditions and typical lens voltages are listed in Table 3-2. Conditions were optimized daily for high sensitivity of ^{59}Co and ^{89}Y while maintaining low oxide formation ($^{140}\text{Ce}^{16}\text{O}^+ / ^{140}\text{Ce}^+ > 1.0\%$) and low doubly-charged species ($^{140}\text{Ce}^{2+} / ^{140}\text{Ce}^+ < 2.0\%$) for a solution with $10 \mu\text{g L}^{-1}$ of Ce. To obtain the maximum counts without the ArCl⁺ interference, ^{59}Co and ^{89}Y were used to avoid increasing of the 75 peak trough tuning. The extraction lens voltage was set near 0.0 volts throughout the study to maintain stability in the instrument response despite the high dissolved solids in the eluent. The electron multiplier and detector were optimized daily using the Agilent Chemstation software and the pulse and analog detection modes of the detector were tuned to accommodate a large response range. Chromatograms for As, Ge and Cl were collected by the Agilent Chemstation software using time-resolved analysis. Arsenic chromatograms were adjusted by the software to account for signal drift relative to the standard set using the Ge ISTD counts. Chlorine was monitored to determine the position of chloride ion in order to ensure resolution with peaks of interest.

Sample Collection and Sample Preparation

Urine samples were collected from adults enrolled in a case-control study conducted in 11 counties of Southeastern Michigan. Drinking water in Southeastern Michigan contains elevated levels of arsenic (Haack and Trecanni 2000; Kim et al. 2002). Details on the Michigan Bladder Cancer Case-Control Study have been published elsewhere (Slotnick et al. 2007; Meliker et al. 2007). Briefly, cases were bladder cancer cases aged 21-80 (at the time of diagnosis) enrolled through the Michigan State Cancer Registry. Controls were selected from an age-weighted list using a random-digit dialing procedure and were matched to cases in terms of age, gender and race. Demographic, occupational and residential information were obtained through phone and personal interviews. The home of each participant was visited to collect water, toenails, urine, and saliva samples.

A sub-sample of 387 participants provided spot urine samples. The research team provided participants with a sample collection kit which included an acid washed 120 mL polyethylene cup in a plastic biohazard bag, disinfection wipes, gloves and instructions. Participants were asked to collect their urine samples, which were then picked up by the research staff. Samples were immediately frozen in dry ice and transported to the Trace Metal Laboratory at the University of Michigan, School of Public Health where they were stored at -20 °C until analysis. Before analysis samples were quickly thawed to room temperature and filtered through a 0.2 µm pore diameter

PTFE syringe filter (Whatman Puradisc, 6785-2502). No preservatives were added to samples, and were injected into the HPLC undiluted.

Quality Control/Quality Assurance

Calibration standards were prepared in water. Deionized water was sonicated and later used to prepare reference materials and mobile phase. The ICP-MS was equilibrated daily with the mobile phase to be used in each experiment. Standard and reference materials were injected 3 times into the 1% (vol/vol) carrier stream. Duplicate injections and duplicate preparations were analyzed daily along with the calibration check standards. Urine samples were frozen in dry ice immediately after collection and stored at -20°C until analysis. Samples were analyzed in batches ranging from 15-30 samples per day. At the end of the day calibration standards were checked to verify calibration and instrument performance.

III. Results and Discussion

This study grew out of our initial attempt to use the method for arsenic speciation developed by Le et al. (2000) with the exception that the column was switched to the Phenomenex Gemini, 5 μm , 150 X 4.6 mm column at a flow rate of 700 $\mu\text{L min}^{-1}$. The mobile phase was 5 mM tetrabutylammonium hydroxide, 3 mM malonic acid and 5% (v/v) methanol at pH 6.2. The column heater was set at 50°C and the output was connected to the ICP-MS nebulizer. The separation between most of the species, As[III], DMA[V], MMA[V] and As[V] was acceptable, with retention times of 2.5, 3.8, 5.1 and 7.6 minutes, respectively. However, we were unable to separate

arsenobetaine from As[III] by this method. This was not a problem for Le et al. (2000) as the HG-AFS system they used was not capable of detecting AsB.

Effect of pH. Changing the pH of the mobile phase was not effective in resolving the close similarity in the retention times of As[III] and AsB (Figure 3-1). Furthermore, DMA[V] and MMA[V] were co-eluted at higher pH values. The retention time for As[V] was found to increase with the increase in pH (Figure 3-1).

Effect of the mobile phase. Due to the necessity to resolve AsB from As[III], we decided to switch to another mobile phase system. For these experiments, we initially used the adapted ion-pairing chromatography method of Le et al. (2000) in conjunction with the phenomenex Gemini silica-organic column. This column can be used over a broad pH range, convenient for the deprotonation of arsenous acid (H_3AsO_2 , $\text{pK}_a = 9.3$) to H_2AsO_2^- and for enhanced separation of the various arsenic species. The mobile phase was 10 mM ammonium phosphate at pH 8.7 with 5 mM Tetrabutylammonium hydroxide (TBAH) added as the ion-pairing reagent and 4% (v/v) methanol added in order to enhance the sensitivity of the ICP-MS.

Four percent MeOH was chosen from past experience with the ICP-MS indicating that addition of alcohols to samples can cause a dramatic increase in ionization of some elements. Arsenic is very sensitive to this effect. The methanol concentration, combined with higher-than-usual ICP-MS sample flow rate ($700 \mu\text{L min}^{-1}$) and mobile phase matrix deposition can lead to faster-than-usual deterioration of sensitivity. However, because sensitivity was excellent, the increased maintenance time was

weighed against the need for achieving very low detection limits. For samples with high levels of arsenic, either the sample flow rate or MeOH concentration may be reduced to decrease routine maintenance of the ICP-MS sample introduction system.

Effect of retention times. It was crucial to ensure that chloride in the urine samples would not interfere with the detection and quantification of arsenic species, since ArCl^+ (75 amu) is not resolved with As (75 amu) with a quadrupole mass analyzer. Adequate resolution between the arsenic species is necessary for accurate quantification of those species. Initially, we used pH 8.7, which resulted in the elution of all species within 11 minutes with reasonable resolution. However, the chloride peak overlapped considerably with that of MMA[V]. By increasing the pH to 9.2, the retention time became longer resulting in adequate separation of MMA[V] and chloride peaks.

Furthermore, these conditions with the new pH provided for near-baseline separation of the AsB, As[III] and DMA[V] peaks. Using the mobile phase and pH, we were able to successfully resolve AsB, As[III], DMA[V], MMA[V] and As[V] peaks with eluting times of 2.4, 3.1, 3.8, 6.9 and 10.3 minutes, respectively, as shown in Figure 3-2. The total runtime was approximately 13 minutes.

Figure 3-3 shows a chromatogram of a urine sample spiked with five arsenic species. The chloride peak, monitored at 35 amu, is shown as a dotted line. As can be seen, the chloride peak was perfectly resolved from those of arsenic species of interest at a retention time of 9.8 minutes, permitting the use of normal mode operation (i.e. no reaction gases, such as helium) and the omission of interference equations. The

separation between DMA[V], Cl⁻ and MMA[V] was good enough that even large changes in Cl⁻ concentration in different urine samples (resulting in a broader Cl⁻ elution and potential for interference) were non-interfering. This method, which is relatively rapid, can be used in studies where the presence of MMA[III] and DMA[III] is not expected in the sample.

Separation of MMA[III]. When a standard solution containing As[III] and MMA[III] was injected into the 150 mm Gemini column which had provided adequate resolution of the peaks of the five arsenic species and allowed for complete separation of the ArCl⁺ interference, one large, broad peak at approximately 3.2 minutes was obtained. Since it was not possible to change the pH of the mobile phase using the 150 mm Gemini without affecting the separation of the other peaks, it became necessary to switch to a longer column while maintaining the same conditions (10 mM ammonium phosphate, 5 mM tetrabutylammonium hydroxide and 4% (v/v) methanol at pH 9.2). The switch to a Phenomenex Gemini C18, 5 μm, 250 x 4.6 mm column increased the runtime significantly. At pH 9.2, however, chloride interfered with MMA[V] peak and the MMA[III] and As(III) peaks were not adequately resolved. When the pH adjusted up to 9.5, all peaks for the seven species were resolved and there was no overlap with the chloride peak (Figure 3-4). The eluting times for AsB, MMA[III], As[III], DMA[V], MMA[V] and As[V] were 3.87, 5.02, 5.48, 6.66, 12.35 and 19.38 minutes, respectively. Total runtime was approximately 23 minutes.

Recoveries and detection limits. The five arsenic species exhibit a small difference in sensitivity, 2.5, 1.8, 2.2, 2.0 and 2.3 X 10⁵ counts ppb⁻¹ for AsB, As[III], DMA[V], MMA[V] and As[V], respectively. This might be due to a difference in the ionization potentials of the different species using the current mobile phase and plasma conditions. As can be seen, the ordinary linear regression of the data for the species gave correlation coefficients of at least 0.99 using equally-spaced standards. The peak area relative standard deviation for three injections of a 20 µg L⁻¹ aqueous standard was less than 2% for all but As[III], which had an acceptable relative standard deviation (RSD) of 5.7%.

Table 3-3 shows the results of a urine sample spiked with 10 µg L⁻¹ of each of the five main arsenic species and recoveries for certified reference material injections. Spike sample peak area precision was very good, less than 2.5% for all five of the main arsenic species. Spike sample peak recoveries were all within 10%. The certified reference material (NIES no. 18) showed good recovery for AsB and DMA[V] at 89% and 94%, respectively, the only two species certified in that standard. While the CRM contained only two arsenic species, the spiked urine samples showed excellent recovery for all five of the main arsenic species found in urine. Thus, we are confident that the matrix has a minimal effect on sample recovery relative to the aqueous standards.

The detection limits of speciation were taken to be three times the standard deviation of the background fluctuation of a 10 µg L⁻¹ standard solution, divided by the sensitivity of the instrument for each species on any given day based upon the peak height of each species in the standard solution. The detection limit for all species was

less than $0.15 \mu\text{g L}^{-1}$ using a $20 \mu\text{L}$ injection volume. The high sensitivity of this method is due to three main factors: (i) the complete separation between all of the peaks and chloride permits the omission of any interference equations or reaction gas (helium) both of which increase detection limits relative to normal mode operation if it can be done interference-free; (ii) the combination of a highly sensitive ICP-MS instrument with the addition of methanol to the mobile phase allows for very low detection limits for arsenic compounds; and (iii) the low background fluctuation of the chromatographic trace reduces instrumental noise, primarily due to the steady input flow from the HPLC system and the very good precision afforded by the MicroMist nebulizer in conjunction with the Peltier-cooled spray chamber of the ICP-MS.

Urine Sample Results

Most of the urine samples analyzed showed excellent peak separation. During the course of analysis, were able to detect two hitherto unknown arsenic peaks. One of the peaks occasionally co-eluted with DMA[V], between 6 and 8 minutes. Few of the previous studies had the resolution capacity of our method, hence this particular compound was probably misclassified and reported as DMA[V]. The other unknown occurred after MMA[V], near 12-14 minutes and was completely separated from the analyte peaks. A chromatogram of a urine sample showing the co-eluting peak near DMA[V] as well as the resolved unknown near 12.5 minutes is shown in Figure 3-5.

Results of arsenic species in spot urine samples from 387 adults who participated in the study are shown in Table 3-4. The concentrations presented were adjusted by specific gravity to account for the dilution factor. Arsenic values were transformed to a

log scale in order to perform statistical analysis. DMA[V] was detected in 99.2% of samples, AsB in 98.2%, MMA[V] in 73.4%, As[III] in 45.0%, and As[V] in 27.1%. This sensitive method enabled us to achieve a higher percent of samples with detectable inorganic arsenic and MMA[V] than what has previously been reported in similar epidemiological studies (Karagas et al. 2002) Neither MMA[III] nor DMA[III] was detected in any urine sample even those that were processed in less than 24 hours after collection (n=123).

Although MMA[III] and DMA[III] have been reported in urine of people exposed to high levels of arsenic in their drinking water (Mandal et al. 2001; Del Razo et al. 2001; Valenzuela et al. 2005), many other studies have not been able to detect these urinary arsenic species especially in un-exposed populations (Lindberg et al. 2006; Meza et al. 2004; Hopenhayn et al. 2003). One reason may be the fact that these species are very unstable and can be completely oxidized to DMA within a day even when stored at -20°C (Gong et al. 2001). It has also been suggested that use of uncharacterized standard prepared by treating DMA with sulfur-containing reductants has led many previous studies to misclassify thio-DMA as DMA[III] in human urine (Hansen et al. 2004). Besides the misidentification of DMA[III] because of its chromatographic properties, a recent paper by Lindberg et al. (2007) suggests that thio-DMA was at least contributing to the analytical signal assigned to DMA[III] in the often-cited study of Valenzuela et al. (2005) which found high levels of DMA[III] in fresh urine samples that were analyzed within 6 hours after collection. Considerable uncertainty thus still

surrounds the measurements of MMA[III] and DMA[III] that have been reported in the literature.

Even the metabolic role of MMA[III] and DMA[III] in the biotransformation of arsenic and the accumulation of these species in urine are being challenged. The Challenger (1945) pathway produces “free” DMA[III] and MMA[III] in a series of oxidation/reduction steps that involve As[III], MMA[V], and DMA[V] en route to trimethylarsine oxide (TMAO). Since TMAO is not common in urine of people exposed to inorganic arsenic, the Challenger pathway basically leads to the highly reactive DMA[III] as the end product, which would seem rather odd. The recently proposed Hayakawa pathway involves non-oxidative methylation of arsenic and places MMA[III] as precursor of MMA[V] and DMA[III] as precursor of DMA[V], hence provides a better explanation for the high levels of DMA[V] and MMA[V] and absence of TMAO in human urine which have been reported in many studies (Hayakawa et al. 2005; Lindberg et al. 2007). Our results are consistent with the Hayakawa model in the sense that we were unable to detect any MMA[III] or DMA[III] even in fresh urine samples analyzed within six hours after collection.

Lindberg et al. (2007) have recently reported the presence of thio-DMA[V] in 44% of urine samples from women in Bangladesh exposed to high levels of arsenic in their drinking water. We believe that one of the unidentified peaks in our chromatograms is probably due to this compound. Because Lindberg et al. (2007) used different column and mobile phase, the chromatographic characteristics of their

peak are not readily comparable with those of the present study and hence cannot be used to confirm the identity of our mysterious peaks.

Table 3-1. Final HPLC operating conditions.

HPLC	
Column	Phenomenex Gemini C18, 5 μm , 250 x 4.6 mm (00G-4435-E0)
Pre-column	Phenomenex SecurityGuard Cartridges (AJ0-7596)
Column heater	50°C with mobile phase pre-heating loop
Mobile phase	4% (v/v) MeOH, 5 mM TBAH, 10 mM ammonium phosphate, pH 9.5
Flow	700 $\mu\text{L min}^{-1}$
Injection volume	20 μL

Table 3-2. Final ICP-MS operating conditions.

ICP-MS	
Mode	Normal mode, no reaction gas
Forward power	1550 W
Plasma gas	15 L min ⁻¹ Ar
Intermediate gas	1 L min ⁻¹ Ar
Carrier gas	0.85 L min ⁻¹ Ar
Auxiliary gas	0.2 L min ⁻¹ Ar
Sample depth	8.0 mm
Extraction lens	0.0 V
Einzel 1,3	-80 V
Einzel 2	12 V
Cell entrance	-15 V
Cell exit	-10 V
Quadrupole bias	-3 V
Octopole bias	-6 V
Plate bias	-45 V
Nebulizer	MicroMist (Glass Expansion)
Cones	nickel-plated, sampler (1.0 mm orifice) and skimmer (0.7 mm orifice)
Oxides	$(^{140}\text{Ce}^{16}\text{O}^+ / ^{140}\text{Ce}^+) > 1.0\%$
Doubly-Charged	$(^{140}\text{Ce}^{2+} / ^{140}\text{Ce}^+) < 2.0\%$
Acquisition	Time-Resolved Analysis mode with 0.1,0.1 and 0.5 seconds/point for Cl, Ge and As, respectively

Table 3-3. Quantitative results for arsenic speciation method in spiked sample and CRM

	AsB	As[III]	DMA[V]	MMA[V]	As[V]
Spiked urine sample (10µg/L):					
Original Result (µg/L)	3.7	0.9	11.4	6.2	2.1
Average Spike Result	13.2	11	22.4	16.6	12.6
Peak Area RSD	1.6	1.9	0.9	2.5	2.4
Average Recovery (%)	96	101	110	104	105
Reference Material (NIES No. 18):					
Certified (µg /L)	69 ± 12		36 ± 9		
Measured (µg/L) ^a	61 ± 0.6		34 ± 0.4		
Recovery (%)	89		94		

^a 99% confidence interval

Table 3-4. Summary of arsenic species in urine samples.

Arsenic Species	A Mean (µg /L)	G Mean (µg /L)	Range (µg /L)	% < DL	Percentiles		
					5%	75%	95%
As[III]	0.34	0.266	BDL-3.40	55	BDL	0.29	1.0
As[V]	0.31	0.244	BDL-7.0	73	BDL	0.17	0.75
DMA[V]	5.57	3.73	0.15-74.0	0.8	0.88	7.2	16.0
MMA[V]	1.0	0.590	BDL-18.0	26	BDL	1.0	3.4
AsB	13.6	4.44	0.17-257.0	1.8	0.54	9.71	72.12
TotAs	7.18	5.17	0.75-74.6	-	-	-	-

AsB=Arsenobetaine

TotAs=As[III]+As[V]+DMA[V]+MMA[V]

A Mean=Arithmetic Mean;

G Mean= Geometric Mean

DL=Detection limit

BDL=Below the detection limit

Figure 3-1. Change in retention time of five arsenic species with increasing mobile phase pH using the Gemini, 5 μ m, 150 X 4.6 mm column with 5 mM tetrabutylammonium hydroxide, 3mM malonic acid and 5% (v/v) methanol.

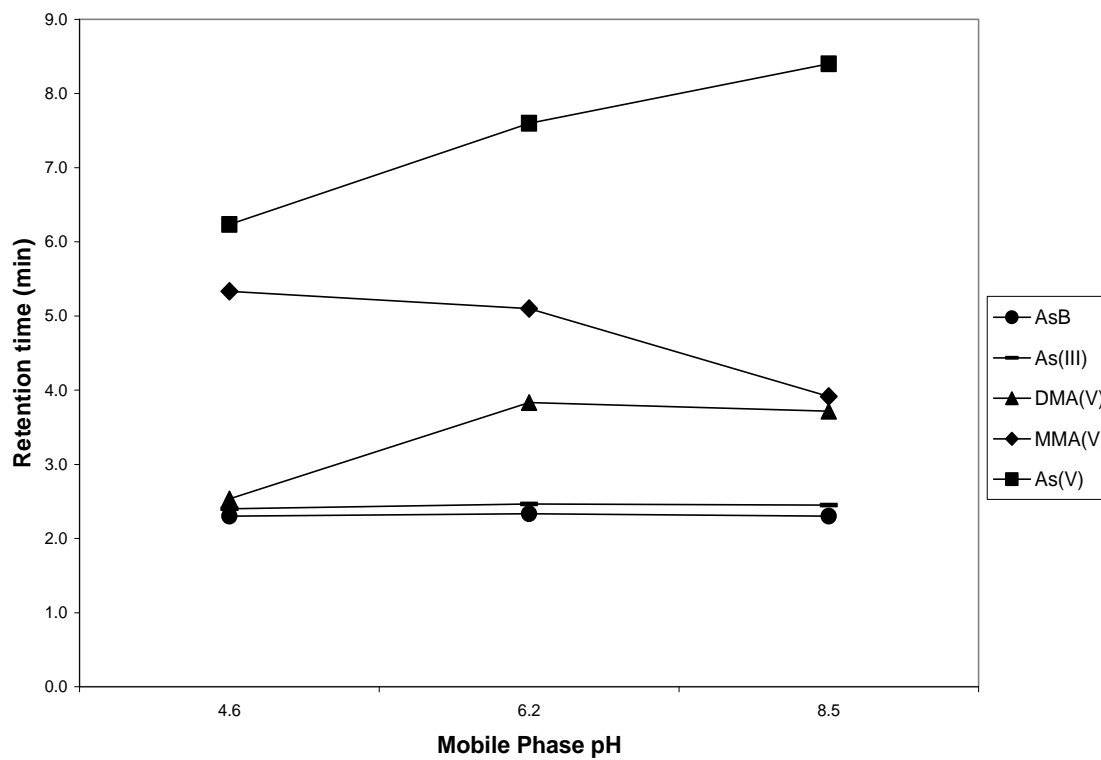


Figure 3-2. Chromatogram of a urine sample using the Gemini, 5 μ m, 150 X 4.6 mm column with 10 mM ammonium phosphate, 5 mM tetrabutylammonium hydroxide and 4% (v/v) methanol at pH 9.2.

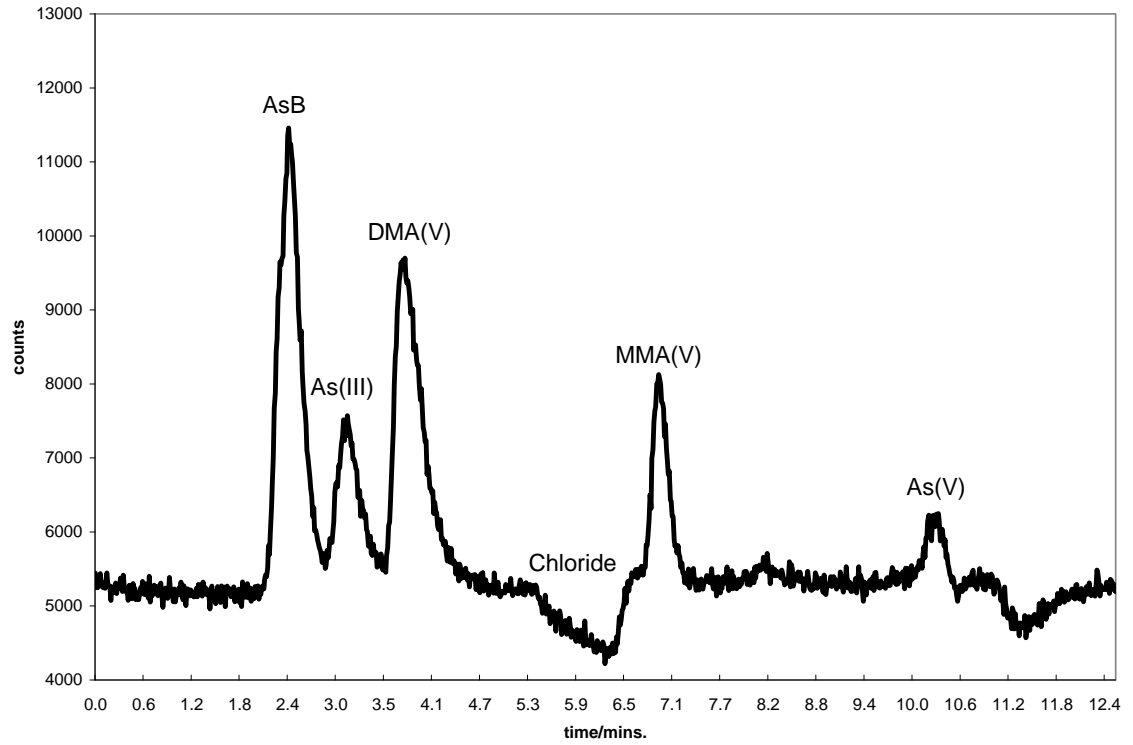


Figure 3-3. Chromatogram of a urine sample, spiked with $10 \mu\text{g L}^{-1}$ of AsB, As(III), DMA(V), MMA(V), As(V) using the Gemini, $5 \mu\text{m}$, $250 \times 4.6 \text{ mm}$ column with 10 mM ammonium phosphate, 5 mM tetrabutylammonium hydroxide and $4\% \text{ (v/v)}$ methanol at pH 9.5.

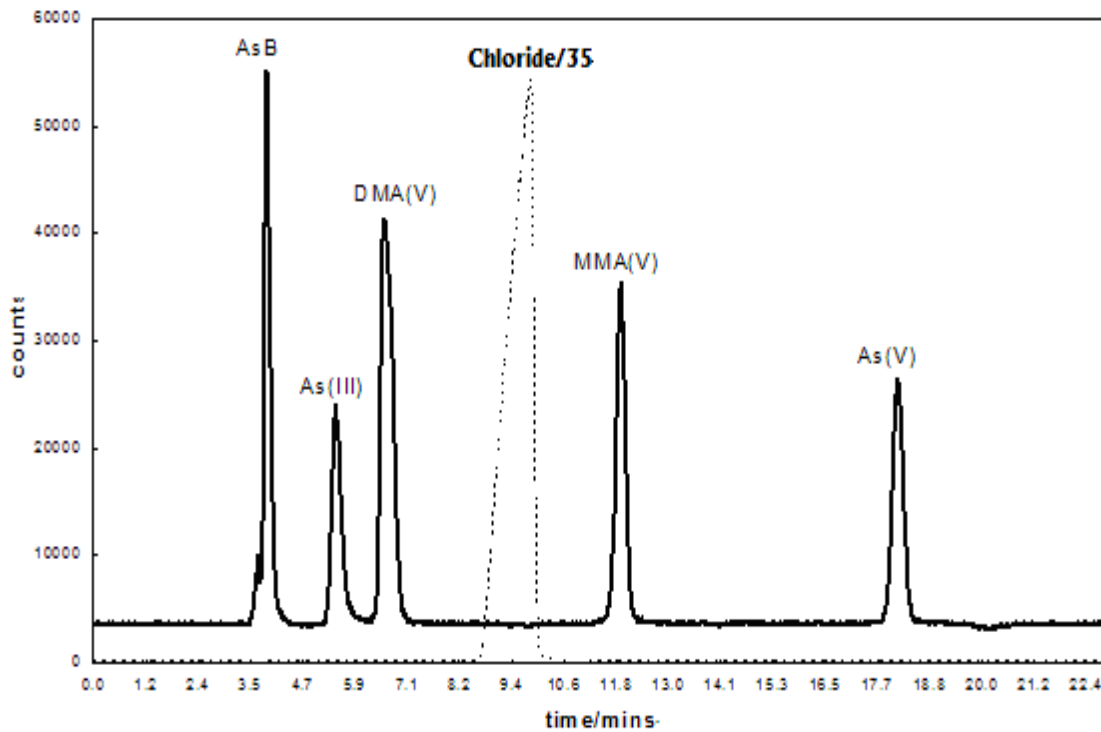


Figure 3-4. Chromatogram of aqueous solution of AsB, As(III), DMA(V), MMA(V), As(V), $10 \mu\text{g L}^{-1}$, and MMA(III), unknown concentration using the Gemini, $5 \mu\text{m}$, $250 \times 4.6 \text{ mm}$ column with 10 mM ammonium phosphate, 5 mM tetrabutylammonium hydroxide and $4\% \text{ (v/v)}$ methanol at pH 9.5.

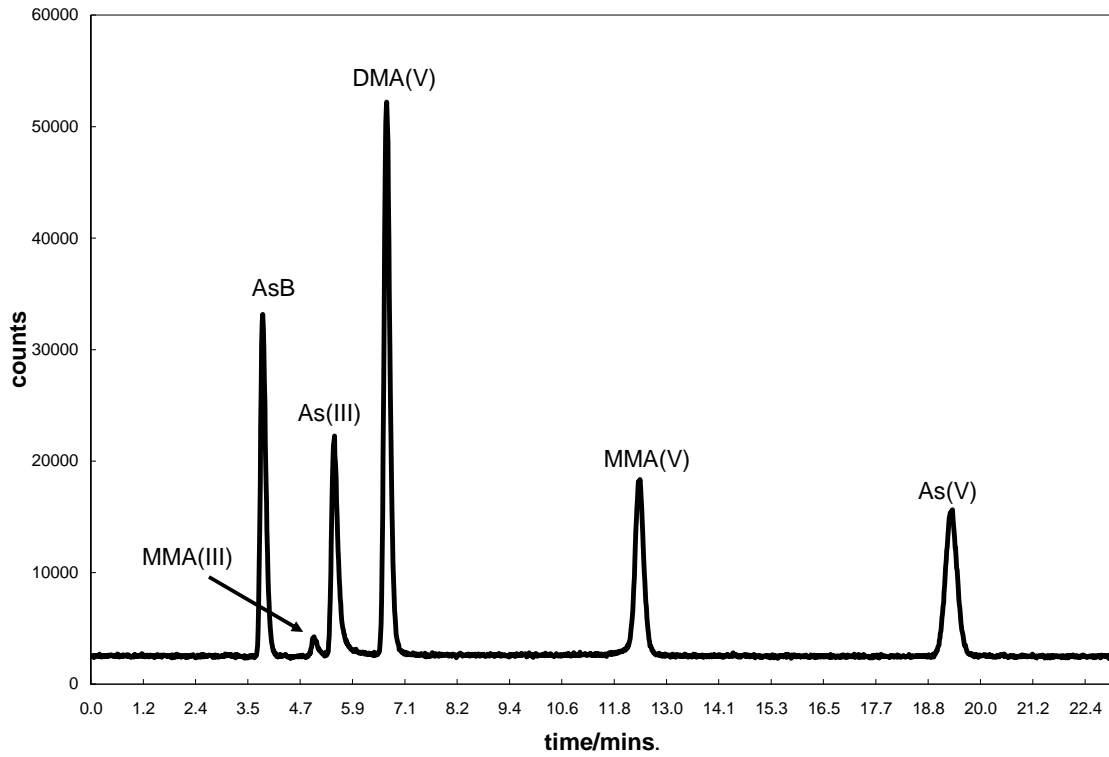
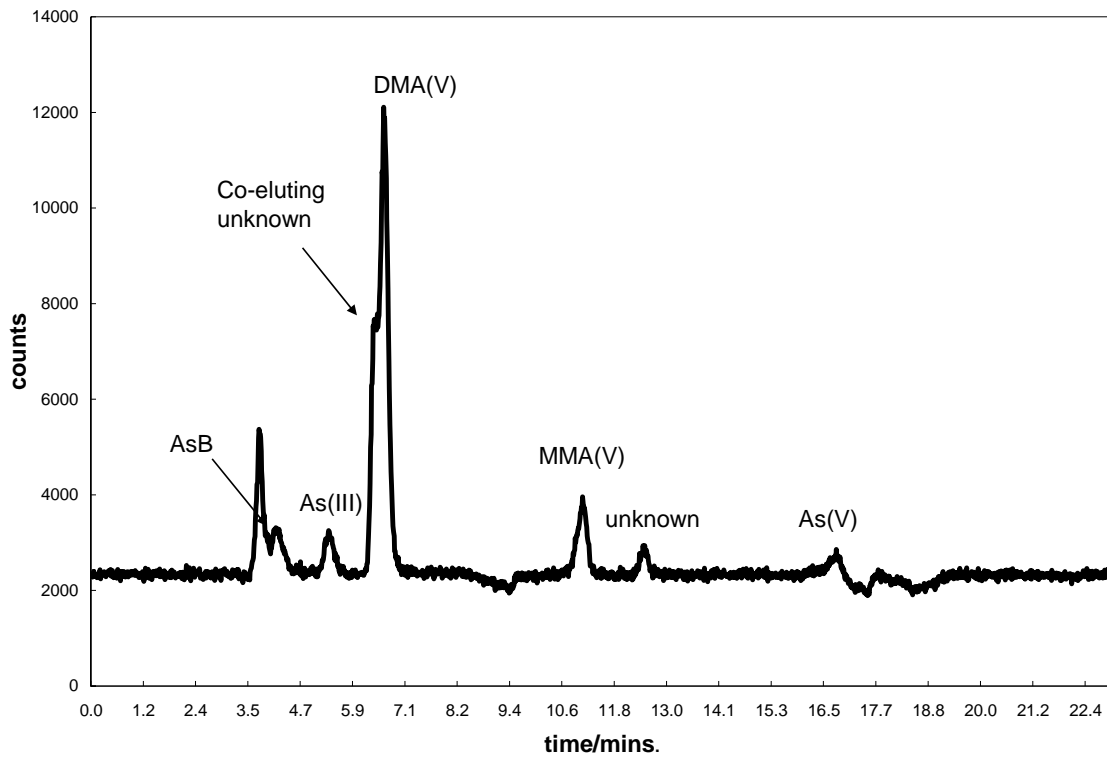


Figure 3-5. Urine sample showing two unknown arsenic species, one co-eluting with DMA[V], the other near 12.7 minutes.



References

- Brima EI, Haris PI, Jenkins RO, Polea DA, Gault AG, Harrington CF. 2006. Understanding arsenic metabolism through a comparative study of arsenic levels in the urine, hair and fingernails of healthy volunteers from three unexposed ethnic groups in the United Kingdom. *Toxicology and Applied Pharmacology* 216:122-130.
- Chen Y, Su H-J, Guo Y-L, Hsueh Y-M, Smith T, Ryan LM, Lee M-S, Christiani DC. 2003. Arsenic methylation and bladder cancer risk in Taiwan. *Cancer Causes and Control* 14:303-310.
- Del Razo LM, Garcia-Vargas G.G, Vargas H, Albores A, Gonesbatt ME, Montero R, Ostrowsky-Wegman P, Kelsh M, Cebrian ME. 1997. Altered profile of urinary arsenic metabolites in adults with chronic arsenism. A pilot study. *Archives of Toxicology* 71:211-217.
- Del Razo LM, Styblo M, Cullen WR, Thomas DJ. 2001. Determination of trivalent arsenicals in biological matrices. *Toxicology and Applied Pharmacology* 174:282-293
- D'Illio S, Violante N, Caimi S, Di Gregorio M, Petrucci F, Senofonte O 2006. *Analitical Chimica Acta* 573:432-438.
- Feldmann J, Lay V, Cullen W, Ma, M, Lu X, Le X.C. 1999. Sample preparation and storage can change arsenic speciation in human urine. *Clinical Chemistry* 45:1988-1997.
- Francesconi KA and Kuehnelt D. 2004. Determination of arsenic species: a critical review of methods and applications, 2000-2003. *Analyst* 129: 373-395.
- Gong Z, Lu X, Cullen W, Le X.C. 2001. Unstable trivalent arsenic metabolites, monomethylarsonous acid and dimethylarsinous acid. *Journal of Analytical Atomic Spectroscopy* 16:1409-1413.
- Haack, SK, Trecanni, SL, 2000. Arsenic Concentration and Selected Geochemical Characteristics for Ground Water and Aquifer Materials in Southeastern Michigan. US Geological Survey Water Resources Investigations Report 00-4171.
- Hansen HR, Raab A, Jaspas M, Milne, Feldmann J. 2004. Sulfur-containing arsenicals mistaken for dimethylarsinous acid [DMA(III)] and identified as a natural metabolite in urine: major implications for studies on arsenic metabolism toxicity. *Chemical Research in Toxicology* 17:1086-1091.

Hopenhayn C, Huang B, Christian J, Peralta C, Ferreccio C, Atallah R, Kalman D. 2003. Profile of urinary arsenic metabolites during pregnancy. *Environmental Health Perspectives* 111:1888-1891.

International Agency for Research in Cancer. 2004. Some water drinking disinfectant and contaminants, including arsenic. IARC Monograph Evaluation

Jakubowski M, Trzcinka-Ochocka M, Razniewska G, Matczak W. 1998. Biological monitoring of occupational exposure to arsenic by determining urinary content of inorganic arsenic and its methylated metabolites. *International Archives in Occupational and Environmental Health* 71:S29-32.

Jarvis KE, Gray AL, Houk RS. 1992. *Handbook of Inductively Coupled Plasma Mass Spectrometry*, Blackie, London.

Jensen TJ, Novak P, Eblin KE, Gandolfi AJ, Futscher BW. 2009. Epigenetic remodeling during arsenical-induced malignant transformation. *Carcinogenesis* 235:39-46.

Karagas MR, Stukel TA, Tosteson TD. 2002. Assessment of cancer risk and environmental levels of arsenic in New Hampshire. *International Journal of Hygiene and Environmental Health* 205:85-94.

Kim MJ, Nriagu JO, Haacks S. 2002. Arsenic species and chemistry in groundwater of southeast Michigan. *Environmental Pollution* 120:379-390.

Larsen EH, Pritzi G, Hansen SH. 1993. Speciation of eight arsenic compounds in human urine by high-performance liquid chromatography with inductively coupled plasma mass spectrometric detection using antimonite for internal chromatographic standardization. *Journal of Analytical Atomic Spectrometry* 8:557-563.

Lai VW, Sun Y, Ting E, Cullen WR, Reimer KJ. 2004. Arsenic speciation in human urine: are we all the same? *Toxicology and Applied Pharmacology* 298:297-306.

Le XC, Lu X, Ma M, Cullen WR, Aposhian HV, Zheng B. 2000. Speciation of key arsenic metabolic intermediates in human urine. *Analytical Chemistry* 72:5172-5177.

Lindberg AL, Goessler W, Grand´er M, Nermell B, Vahter M. 2007. Evaluation of the three most commonly used analytical methods for determination of inorganic arsenic and its metabolites in urine *Toxicology Letters* 168:310–318.

Lindberg AL, Goessler W, Gurzau E, Koppova K, Rudnai P, Kumar R, Fletcher T, Leonardi G, Slotova K, Bheorghiu E, Vahter M. 2006. Arsenic exposure in Hungary, Romania and Slovakia. *Journal of Environmental Monitoring* 8:203-208.

- Loffredo C, Aposhian HV, Cebrian M, Yamauchi H, Silbergeld E. 2003. Variability in human metabolism of arsenic. *Environmental Research* 92:85-91.
- Mandal BK, Ogra Y, Suzuki KT . 2001. Identification of dimethylarsinous and monomethylarsonous acids in human urine of the arsenic-affected areas in West Bengal, India. *Chemical Research in Toxicology* 14:371-378.
- Meliker JR, Slotnick MJ, Avruskin GA, Haufmann A, Fedewa SA, Goovaerts P, Jacquesz GJ, Nriagu JO. 2007. Individual lifetime exposure to inorganic arsenic using a space-time information system. *International Archives of Occupational and Environmental Health* 80:184-197.
- Meza MM, Kopplin MJ, Burgess JL, Gandolfi AJ. 2004. Arsenic drinking water exposure and urinary excretion among adults in the Yaqui Valley, Sonora, Mexico. *Environmental Research* 96:119-126.
- Mushak P and Crocetti AF. 1995. Risk and revisionism in arsenic cancer risk assessment. *Environmental Health Perspectives* 103:684-689.
- Petrick JS, Ayala-Fierro F, Cullen WR, Carter DE, Aposhian HV. 2000. Monomethylarsonous acid (MMAIII) is more toxic than arsenite in Chang human hepatocytes. *Toxicology and Applied Pharmacology* 163:203-207.
- Salnikow K. and Zhitkovich A. 2008. Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. *Chemical Research in Toxicology* 21:28-44.
- Schmeisser E, Goessler W, Francesconi KA. 2006. Human metabolism of arsenolipids present in cod liver. *Analytical and Bioanalytical Chemistry* 385:367-76.
- Slotnick MJ, Meliker JR, Nriagu JO. 2008. Intra-individual variability in toenail arsenic concentrations in a Michigan population, USA. *Journal of Exposure Science and Environmental Epidemiology* 18:149-157.
- Steinmaus C, Bates, Yuan Y, Kalman D, Atallah R, Rey O, Biggs M, Hopenhayn C, Moore L, Hoang B, Smith A. 2006. Arsenic methylation and bladder cancer risk in case-control studies in Argentina and the United States. *Journal of Occupational and Environmental Medicine* 48:478-488.
- Suzuki KT. 2005, Metabolomics of arsenic based on speciation studies. *Analytica Chimica Acta* 540:71-76.

Styblo M, Del Razo LM, Vega L, Germolec DR, LeCluyse EL, Hamilton GA, Reed W, Wang C, Cullen WR, Thomas DJ. 2000. Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. *Archives of Toxicology* 74:289-299

Vahter M. 1994. What are the chemical forms of arsenic in urine, and what can they tell us about exposure? *Clinical Chemistry* 40:679-680.

Vahter M. 2008. Health effects of early life exposure to arsenic. *Basic and Clinical Pharmacology and Toxicology* 102:204-211.

Valenzuela O, Borja-Aburto H, Garcia-Vargas G, Cruz-Gonzalez M, Garcia-Montalvo E, Calderon-Aranda E, Del Razo L.M. 2005. Urinary trivalent methylated arsenic species in a population exposed to inorganic arsenic. *Environmental Health Perspectives* 113:250-254.

Vega L, Styblo M, Patterson R, Cullen W, Wang C, Germolec D. 2001. Differential effects on trivalent and pentavalent arsenicals on cell proliferation and cytokine secretion in normal human epidermal keratinocytes. *Toxicology and Applied Pharmacology* 172:225-232.

Chapter IV

Reliability of Spot Urine Samples in Assessing Arsenic Exposure

I. Introduction

The concentrations and forms of arsenic in urine are often used as biomarkers of arsenic exposure (ATSDR 2005). Although differences in urinary arsenic metabolites may stem from sampling and storage procedures (Michalke 2003), the validation of common and consistent methodologies which can lead to reduced variance in measured levels of urinary arsenic metabolites among different individuals has not been attempted in most previous studies. Sample collection strategies vary depending on the purpose of the study, the logistics, and budget constraints. Three urine collection methods are common: a 24-hour sample, a first morning void (FMV) sample, and a spot urine sample (Calderon et al. 1999; Smith and Steinmaus 2000). The 24-hour urine is often considered to be the most reliable sample type (Cornelius et al. 1996). However, they are infrequently used in epidemiological studies involving a large number of participants due to complex logistics. Participants have to commit to a greater extent with the project in order to provide the 24-hour sample. FMV samples are more frequently used in epidemiological studies to evaluate arsenic exposure (Caceres et al. 2005; Karagas et

al. 2001; Meza et al. 2003), because they are more feasible to collect than 24-hour samples.

First morning voids are attractive because they are more concentrated, and the metabolites obtained from the analysis are known to be in contact with the bladder for a longer period. Calderon et al. (1999) showed that one FMV sample is a reasonably good estimate of exposure but Smith and Steinmaus (2000) reported that a group average of many individual spot urine samples also provides a good estimate of exposure. Researchers have also used 72-, 96- or 120-hour samples (Calderon et al. 1999). Samples collected during a period of 24 to 120 hours show increased reliability when assessing within-day and between-day variation and intra-individual differences. These multi-day samples are more expensive and difficult to collect, however. This chapter examines the differences in arsenic metabolites in FMV and spot urine samples from an adult population in southeastern Michigan. The urine samples were analyzed for five different arsenic species: arsenobetaine (AsB), arsenite (As[III]), arsenate (As[V]), methylarsenic acid (MM[V]), and dimethylarsenic (DMA[V]). Arsenic speciation is useful to distinguish exposure to organic and inorganic arsenic and to assess arsenic metabolism in the human body. The goal of the study is to evaluate arsenic species in FMV and spot urine samples as adequate biomarkers using reliability and agreement methods to assess variability. In addition, total arsenic concentration in toenails will be compared to urinary arsenic to evaluate the correlation between the two biomarkers.

II. Methodology

Study Population and Sample Collection

First morning void and spot urine samples were collected from a sub-sample of participants enrolled in a large case-control study of arsenic exposure and bladder cancer in 11 counties of southeastern Michigan. Project details including recruitment strategies have been published elsewhere (Meliker 2007). Briefly, cases were obtained from the Michigan State Cancer Registry and were frequency-matched by age, race and gender with controls selected through a random-digit dialing procedure. The sub-sample used in this study included those individuals from the main study that had both type of samples, FMV and spot. Participants were recruited from June 2005 to May 2007. The total number of individuals was 131 of which 50 were cases and 81 were controls. Initially, individuals completed a phone interview to obtain information on medical history, life-styles habits, and water consumption patterns. Subsequently, water, toenails and urine samples were collected during visits to participants' homes.

The research team provided two different 'urine collection kits' to participants along with materials and instructions for urine collection. Kit A (the FMV sample) included a plastic bag packed with an acid-washed polyethylene urine cup, antibacterial wipes, gloves, and urine collection instructions. Kit A also included a small foam cooler with dry ice into which the urine sample would be placed. Kit B (the spot sample) included only the plastic bag with collection materials with no dry ice and foam cooler. Each participant was asked to use Kit A to collect FMV sample first thing in the morning

and immediately fast-freeze it by immersing the container in dry ice. FMV samples were picked up (frozen) the morning they were collected and transported to the laboratory. Participants were asked to collect spot urine samples using Kit B during field visits. Spot samples were immediately frozen in dry ice brought along by the research team and then transported to the laboratory. The FMV and spot samples were stored in the laboratory at -20°C until analysis. Each of the participants provided both spot and FMV urine samples.

In addition to urine samples, toenail samples were also collected by researchers during field visits. Participants were asked to clip toenails with materials provided by the research team using the methodology described by Slotnick et al. (2007).

Sample Preparation and Analysis

Urine samples were quickly thawed using a water bath. After they achieved room temperature, samples were filtered using a 0.22µm syringe filter and no preservatives were added. Specific gravity measures were taken immediately after samples were thawed and reached room temperature; freezing urine samples apparently does not change the specific gravity (Nermell et al. 2008). Specific gravity measures provides a measure of dissolved material and particles concentration in urine (Flasar 2008). Specific gravity measurements were conducted with a pycnometer (Fisher Scientific) using the density of water as a reference. Three weight measurements were taken using a pycnometer: the empty flask, flask with water, and flask with urine sample. The weight of the urine was obtained by subtracting the weight of the empty

flask and its specific gravity was calculated by dividing the urine by the weight of the water previously obtained. Samples with specific gravity above 1.03 or below 1.01 were considered to concentrated or to diluted and were excluded from the analysis (n=2) (Teass et al. 2003).

Immediately after filtration, samples were analyzed using a High Performance Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometry (HPLC-ICP-MS) system following an adaptation protocol from Le et al. (2000) and described on Chapter 3. Briefly, urine samples were filtered through a 5 μ m 250x4.6mm column (Phenomenex, Torrance, CA, USA). The mobile phase contained 4% (v/v) methanol, 5mM tetrabutylammonium hydroxide (TBAH), 10 mM ammonium phosphate at pH 9.5. The HPLC system (Alltech) was coupled to an ICP-MS (Agilent Technologies Model 7500c) unit. Detection limits following the method were: AsB, 0.06 μ g/L; As[III], 0.112 μ g/L; As[V], 0.147 μ g/L; MMA[V], 0.117 μ g/L; and DMA[V], 0.076 μ g/L, with recovery rates ranged between 96% and 105% respectively. Urine certified reference material (National Institute for Environmental Studies, NIES NO. 18, Tsukuba, Ibaraki, Japan) was prepared by dissolving the appropriate amount of urine powder in water to obtain 69 \pm 12 μ g/L AsB, 36 \pm 9 μ g/L DMA and 137 \pm 11 μ g/L total As. A 2mg/L germanium internal standard solution was prepared from the stock Ge solution (Inorganic Ventures, 1000 mg L⁻¹ Ge) by dilution with the same mobile phase being used in the analytical column.

Toenail samples, after being collected, were washed and dried overnight in a 60°C oven. Following the drying procedure, toenails were digested using Optima HNO₃

(Fisher Chemical) and Suprapur H₂O₂ (Merk). Toenail samples were analyzed for total arsenic using ICP-MS and calibration standards were prepared prior to analysis. A complete description of toenail sample preparation and analysis has been described in Slotnick et al. (2007).

Statistical Analysis

Descriptive statistics were calculated for urinary arsenic metabolites as well as for the participants' attribute data. Histograms and normal probability plots revealed deviations from normal distribution for all urinary arsenic metabolites and toenail arsenic concentrations. Log₁₀-transformations were therefore applied to the data before performing statistical analysis. No difference was found between spot samples collected in the morning and those collected in the afternoon. For this paper, the sum of As[III], As[V], MMA[V] and DMA[V] was used to designate the total toxic arsenic species (TotAs). The sum of As[III] and As[V] was used as the total of inorganic arsenic (InAs) because it provides a more stable measure of inorganic arsenic in urine, since these two species may interconvert while in urine. MMA[III] and DMA[III] were not detected in any of the samples. MMA and DMA will refer to the pentavalent species of MMA and DMA, unless otherwise stated. Concentrations below the detection limit were set at one half the detection limit (Table 4-1). The relative proportion of arsenic in each species (%InAs, %MMA, and %DMA) was calculated by dividing the concentration of arsenic in each species by the concentration of InAs, MMA, and DMA combined.

The association of each arsenic species with variables such as disease status, gender, age, and smoking were assessed using univariate analyses. Student's *t* test was used to compare category means. Paired *t*-tests were conducted using log-transformed values for both FMV and spot urine samples. Since a significant difference was detected between FMV and spot sample for TotAs, the Cohen's *d* coefficient was calculated to evaluate the magnitude of difference, independent of sample size, between samples. In this analysis, effect size calculations were used to determine whether the statistically significant difference between FMV and spot samples is a difference of concern. Cohen's *d* is defined as the difference between two means divided by the pooled standard deviation for those means (Cohen 1992). A $d=0.20$ indicates a small effect size, $d=0.50$ indicates a medium effect size, and $d=0.80$ a large effect size (Cohen 1992).

In order to evaluate reliability for each species in FMV samples and spot samples, the intraclass correlation coefficient (ICC) was calculated. The ICC was used to estimate the correlation of each species among individuals within the same group of samples (FMV or spot) and it is different from the Pearson correlation coefficient where the variables of interest are modeled as two distinct traits. The ICC shows how much of the variance comes from between subjects and not within subjects. To evaluate the degree of agreement, a Bland-Altman plot of the difference between the urinary arsenic concentration of both sample types against the mean of both sample types (FMV-spot $\pm 2SD$) was constructed (Bland and Altman 1986). Pearson correlation coefficients (*r*) were generated to compare arsenic concentration and species in each sampling

method. Pearson correlation coefficients were also generated to compare arsenic in toenails with total urinary arsenic metabolites for each sample type.

The ICC and Pearson correlation coefficient were performed for all individual species and TotAs. The Bland-Altman plot was performed for TotAs. Since no significant differences among disease status, gender, age, and smoking status were seen these groups were combined for the correlation coefficients presented in this chapter. All statistical analyses were run using SAS statistical software, version 9.1 (SAS Institute, Inc., Cary, NC).

III. Results

Demographic information and arsenic average concentrations for participants who provided urine samples are shown in Table 4-2. A total of 131 FMV and corresponding spot samples were used in this study. The majority of participants included in this research were men (86%) and the group's average age was 65.7; the age distribution reflects the fact that bladder cancer is predominantly a disease of elderly white men. Table 4-2 also shows TotAs for all groups. Cases and ever smokers have a higher arsenic concentration in both samples, FMV and spot, than controls and never smokers.

Table 4-3 shows the average concentration for each species in FMV and spot samples. The average storage time for samples was 41 days (0-189days). Neither MMA[III] nor DMA[III] was detected in any of the samples. Overall, spot urine samples had more detectable arsenic concentrations of all species than FMV samples except for

arsenobetaine. Geometric means for first morning voids of As[III], As[V], MMA, DMA and AsB were 0.13 µg/L, 0.12 µg/L, 0.69 µg/L, 3.6µg/L, and 2. 7µg/L respectively. Geometric means for spot samples of As[III], As[V], MMA, DMA and AsB were 0.21 µg/L, 0.16 µg/L, 0.94 µg/L, 4.1 µg/L, and 3.0 µg/l respectively.

TotAs, As[III], As[V], MMA[V], DMA[V], and arsenobetaine were significantly different between FMV and spot samples according to pair-t-tests analysis. To further investigate these differences, the Cohen's *d* was calculated to evaluate the effect size that may influence the finding of a significant difference between samples. The calculated *d* for TotAs was 0.23 which indicates that the difference between the samples is small. Calculated *d*'s for individual samples were 0.25, 0.29, 0.21, 0.17, 0.18 for As[III], As[V], MMA, DMA, and arsenobetaine respectively. Therefore, the difference between FMV and spot urine samples (according to the paired t-test) might be attributed to the large sample size, not an effect difference between the geometric means of the two types of samples being compared.

To further evaluate the degree of agreement, a Bland-Altman plot was constructed (Fig 4-1). The mean difference in TotAs between samples was -1.11 µg/L (95% CI -1.89,-0.33). The Bland-Altman plot showed a high degree of agreement with 92% of samples within the limits of agreements determined by two standard deviations. This indicates that even though TotAs was significantly different between the geometric means of the two sample types, there is little difference between FMV and spot urine levels for 92% of the samples.

Table 4-4 shows the Pearson correlation coefficients and ICC for associations of each arsenic species between FMV and spot samples. Arsenic concentrations (TotAs) between spot and FMV samples were strongly correlated ($r=0.80$) (Fig 4-2). Individual arsenic species were also correlated. Methylated metabolites, MMA and DMA showed a higher correlation between FMV and spot samples than inorganic arsenic (As[III] and As[V]). Spot and FMV samples were the most correlated for MMA ($r=0.83$), and DMA was also strongly correlated ($r=0.77$). The ICC between TotAs in FMV and TotAs in spot samples was 0.90. The ICC shows that 90% of variation comes from between individuals and not within individuals. ICC's were 0.88, 0.91, 0.91 for InAs, MMA and DMA respectively.

Given the interest in arsenic methylation, the urinary arsenic profile for the 131 individuals was determined using FMV and Spot samples separately and is presented in Table 4-5 . The relative proportion of FMV samples for % InAs, %MMA, and %DMA were 7.9%, 16.0%, and 76.0%, respectively. The relative proportion of spot samples for %InAs, %MMA, and %DMA were 9.6%, 18.1%, and 72.3%, respectively. To better evaluate the methylation profiles, Pearson correlation coefficients between FMV and spot samples are presented in Table 4-6 for all individual groups. All correlations were significant ($\alpha=0.05$). Overall, correlations for the relative proportion values were higher than correlations for the absolute values. The smallest correlation was shown by the 'Female' group with respect to %InAs. The group 'Age ≥ 75 ' has the lower correlation across all absolute values and relative proportions.

In order to compare biomarkers of arsenic exposure, data on toenail arsenic levels were compared with spot and FMV urine samples (Table 4-7). Spot samples and FMV samples were significantly correlated to arsenic in toenails. However, the correlation for FMV samples was higher than for spot samples. In addition, arsenic concentrations in toenails were compared with arsenic concentrations in FMV samples and spot samples. All correlations were positively significant and similar between the two groups.

IV. Discussion

Our analysis of 131 paired urine samples shows that arsenic species in spot urine samples are adequate biomarkers to measure arsenic exposure. To our knowledge, this is the first reported study evaluating FMV vs. spot urine samples with a large sample size where 99% of participants had detectable total arsenic concentrations. Furthermore, this research measured several urinary arsenic metabolites in order to better compare sampling methods. Although 24-hours samples are considered the most reliable sample type (Cornelius et al. 1996), the search for less-expensive and more logistically efficient methods is always a goal. Spot sample collection, instead of FMV, will decrease budgetary and logistical issues.

Dilution variation adjustments and arsenic metabolism are often discussed as confounders in biomarker validation. Data interpretation of 24-hour, FMV, and spot urine samples are influenced by factors such as urine concentration and volume. To avoid biased results due to variation in urine dilution, an adjustment by urinary

creatinine concentration is usually performed (Hinwood et al. 2002). Creatinine analysis is performed on the basis that its excretion is constant from the body. However, creatinine is formed by creatine and its excretion is related to muscle mass and meat intake (Hall Moran et al. 2001; Worsfold et al. 1999). Studies have shown that urinary creatinine excretion varies by age, gender, body size, and diet (Boeniger et al. 1993; Suwazono et al. 2005). As a result, some studies have proposed the use of specific gravity instead of creatinine for normalization purposes, because of the strong correlation between specific gravity and creatinine (Moore et al. 1997; Parikh et al. 2002; Suwazono et al. 2005). Although specific gravity may also be influenced by age, gender, and body size, the influence of these is less than for creatinine (Nermell et al. 2008).

After adjustment by specific gravity, geometric mean values of FMV and spot urine samples were found to be significantly different for measurements of TotAs ($As[III]+As[V]+MMA[V]+DMA[V]$). However, given the strong correlation between the two samples, we sought a greater understanding of this significant value. Two factors influence p -values: sample size and effect size. Since 131 samples have enough power to perform correlation analyses (62 samples for 80% when $\alpha = 0.05$), the magnitude of the difference independent of sample size was evaluated. The effect size needs only be small to produce a significant result, because when using as many samples as were included in this study, significant differences may arise due to a large sample size (Baghi et al. 2007). The Bland-Altman plot showed a high degree of agreement between sample types, in spite of the significant difference between geometric means of the

samples, indicating that spot samples are adequate to evaluate arsenic exposure. In addition to the strong correlations presented here, the ICC's for TotAs, InAs, MMA, DMA, and AsB indicates that most of the variance between FMV and spot urine samples is due to subject-to-subject variability. The same results are true for methylation patterns where just ~10% of the variation is due to other sources. A previous study where two or three samples were collected over time from the same individuals revealed that beside within-person variation, laboratory imprecision makes a substantial contribution to the total variance not due to between-subject variability (Steinmaus et al. 2005). They estimated that ~45% of variance in their analysis was due to laboratory imprecision. Another study that evaluates intraindividual variability, (Concha et al. 2002), found considerable between-person variability in the urinary excretion while little day-to-day variation was seen among arsenic metabolites.

I also observed a moderate correlation between urinary arsenic (TotAs) and arsenic in toenails (Table 4-7). Even though urinary arsenic is a short term biomarker and arsenic in toenails is a longer term biomarker, the fact that arsenic concentrations of public and private water sources are stable over time (Francesconi and Kuehnelt 2004; Steinmaus et al. 2005) may influence the correlation between the two biomarkers. Total arsenic concentrations in toenails were found significantly correlated with those of both FMV and spot samples. FMV vs. total toenail concentrations were more strongly correlated than spot vs. toenails concentrations. However, when outlying FMV samples were removed from the analysis, the correlation was lower ($r=0.39$), very similar to the correlation for arsenic toenails vs. spot samples ($r=0.38$). The correlations

of each urinary arsenic species, including arsenobetaine, with total arsenic concentration in toenails also confirm that toenail arsenic reflects mostly inorganic arsenic. Urine reflects exposures occurring in the past days or weeks while toenails may reflect a longer period of exposure (NRC 2001). This may be the reason why AsB shows, even low still significant, a negative correlation between the urine and toenail biomarkers. Organic arsenicals, including AsB appear to undergo little or no metabolism and therefore are excreted without any significant changes in structure (Borak et al. 2007). AsB is excreted after two or three days following ingestion, therefore it will be reflected in urine not in the toenail biomarker since the later reflect a longer period of exposure reflecting mostly inorganic arsenic. Inorganic arsenic is thought to bind to keratin proteins in nails (Mandal et al. 2003; NRC 1999). The correlation of arsenic in urine and toenails is notable considering the lifetimes of these biomarkers and the many variables that can moderate the arsenic burden in each biomarker (Slotnick et al. 2007).

Although MMA[III] and DMA[III] have been reported in human urine (Aposhian et al. 2000; Valenzuela et al. 2005) most epidemiological studies have recorded only As[III], As[V], MMA[V], and DMA[V] (Lofredo et al. 2003; Steinmaus et al. 2006) since the detection of the trivalent species is very difficult if samples are not analyzed immediately (Gong et al. 2001; Mandal et al. 2001). Moreover, in populations exposed to low levels of arsenic, they may be undetectable since MMA[III] and DMA[III] have been observed only in areas where residents have endemically been exposed to high levels of arsenic (>100µg/L) in drinking water (e.g. Mexico, India). Recently, a model has been proposed which questions the formation of MMA[III] and DMA [III] as significant

intermediates during arsenic metabolism in humans (Slejkovec et al. 2008). The levels of pentavalent methylated species were highly correlated between FMV and spot samples, especially MMA. MMA[V] excretion has been related to an increased risk of arsenic-related diseases such as bladder and skin cancer (ATSDR 2005; Chen et al. 2003; Hopenhayn-Rich et al. 1996; Steinmaus et al. 2006).

Two different pathways have been proposed to explain arsenic metabolism. The first one, described by Cullen and Reimer (1986) explained arsenic biotransformation in two stages: oxidation/reduction followed by methylation reactions. The second pathway was described more recently by Hayakawa et al. (2005). The main difference between the two is that Hayakawa et al.'s model involves preferential formation of trivalent methylated species before the pentavalent methylated species. At the present time, the precise mechanisms of arsenic biotransformation and the enzymes involved are still unclear. The urinary arsenic profiles observed in this study do not provide confirmatory evidence that arsenic metabolism follows either the Cullen and Reimer (1986) or Hayakawa et al. (2005) biotransformation pathways. However, collection of urine samples using the methods described here, combined with genetic information may provide insight on the metabolic processes.

In conclusion, the results presented here suggest that FMV and spot samples can be used without preference when evaluating arsenic exposure in epidemiological studies. Spot urine samples, as opposed to 24-hour or FMV urine samples, are simpler to obtain and less expensive to collect, making them a good biomarker candidate for

epidemiological studies. Nonetheless, factors such as laboratory imprecision and stability of methylation patterns may result in some within-individual variability.

Table 4-1 Arsenic concentrations below detection limit for each arsenic species

Arsenic Species	Number of Samples below the Detection Limit	%	Detection Limit (µg/L)
InAs ^{FMV}	73	55.6	0.112
InAs ^{SPOT}	44	33.6	0.112
MMA ^{FMV}	8	6.1	0.117
MMA ^{SPOT}	5	3.8	0.117
DMA ^{FMV}	1	0.76	0.076
DMA ^{SPOT}	0	0.0	0.076
AsB ^{FMV}	0	0.0	0.060
AsB ^{SPOT}	1	0.76	0.060

Table 4-2. Univariate analysis for total arsenic concentration (TotAs) of FMV, spot urine samples and total arsenic concentration toenails

Variable	n	%	TotAs ^a FMV Urine (µg/L)		TotAs ^a Spot Urine (µg/L)		[As] Toenails (µg/g)	
			A ^b Mean	G ^c Mean	A ^b Mean	G ^c Mean	A ^b Mean	G ^c Mean
All Individuals	131	100	6.9	4.7	8.0	5.7	0.20	0.13
Disease Status								
Case	50	38.2	7.6	5.1	8.3	6.3	0.25	0.16
Control	81	61.8	6.4	4.5	7.8	5.4	0.16	0.12
Gender								
Female	18	13.7	6.9	3.8	8.0	5.0	0.26	0.14
Male	113	86.3	6.7	4.9	8.2	5.8	0.19	0.13
Age								
<65	50	38.2	7.6	5.1	9.0	6.3	0.24	0.17
65-75	55	42.0	6.5	4.4	7.3	5.4	0.19	0.12
>75	26	19.8	6.1	4.8	7.2	5.4	0.13	0.10
Smoking								
Never	43	32.8	6.3	4.7	7.8	5.6	0.18	0.13
Ever	87	66.4	7.2	4.8	8.1	6.0	0.20	0.13
Race								
White	123	93.9	6.8	4.7	7.8	5.6	0.20	0.13
Other	8	6.1	8.4	5.5	11.3	7.0	0.18	0.12

^aTotAs=As[III]+As[V]+MMA[V]+DMA[V]

^bArithmetic Mean

^cGeometric Mean

Table 4-3. Table Arsenic species concentration in FMV and spot urine samples

Variable	TotAs FMV Urine (µg/L)		TotAs Spot Urine (µg/L)	
	A ^a Mean	G ^b Mean	A ^a Mean	G ^b Mean
InAs	0.50	0.25	0.74	0.37
MMA	1.1	0.69	1.5	0.94
DMA	5.2	3.6	5.8	4.1
Asb	8.4	2.7	7.8	3.0

^aTotAs=As[III]+As[V]+MMA[V]+DMA[V]

^bArithmetic Mean

^cGeometric Mean

Table 4-4. Pearson correlation coefficients and ICC between FMV and Spot samples for each arsenic species

FMV correlated with Spot sample	Pearson's correlation coefficient	<i>p</i>-value	<i>ICC</i>
TotAs	0.82	<0.0001	0.90
InAs	0.72	<0.0001	0.88
DMA	0.78	<0.0001	0.91
MMA	0.83	<0.0001	0.89
AsB	0.84	<0.0001	0.93

Table 4-5. Proportion of arsenic species in FMV and spot urine samples.

Variable	N	%	%InAs FMV	%InAs SPOT	%DMA FMV	%DMA SPOT	%MMA FMV	%MMA SPOT	SMI ^a FMV	SMI ^a SPOT
All	131	100	7.9	9.6	76.0	72.3	16.0	18.1	6.7	5.6
Bladder Cancer										
Cases	50	38.2	7.0	9.4	77.7	73.0	15.2	17.5	6.4	4.7
Controls	81	61.8	8.6	9.9	74.9	71.7	16.6	18.4	6.8	6.2
Gender										
Women	18	13.7	9.7	10.2	76.0	72.9	14.3	16.9	6.8	5.2
Men	113	86.3	7.7	9.6	75.9	72.1	16.3	18.3	6.6	5.7
Age										
<65	50	38.2	8.2	9.8	75.9	71.8	16.0	18.4	6.7	5.5
65-75	56	42.0	8.7	10.1	74.7	70.9	16.2	19.0	5.8	4.8
>75	26	19.8	6.0	8.5	79.0	76.1	15.0	15.4	8.4	7.6
Smoking										
Never	43	32.8	8.2	8.9	75.5	72.2	16.2	18.8	5.2	4.7
Ever	88	66.4	7.9	9.9	76.2	72.5	15.9	17.6	7.3	6.1
Race										
White	123	93.9	7.9	9.6	76.2	72.3	16.1	18.0	6.7	5.7
Other	8	6.1	8.5	9.6	75.7	71.6	15.6	18.3	4.0	4.7

^aSecondary Methylation Index (DMA/MMA)

Table 4-6. Pearson correlations coefficients (*r*) between FMV and spot urine samples for each arsenic species between FMV and Spot samples by groups

Group	TotAs	Absolute Values			Relative Proportions		
	(InAs+MMA+DMA)	InAs	MMA	DMA	%InAs	%MMA	%DMA
All Individuals	0.82	0.72	0.83	0.78	0.71	0.69	0.75
Cases	0.80	0.65	0.86	0.78	0.71	0.55	0.73
Controls	0.79	0.76	0.83	0.79	0.73	0.74	0.76
Males	0.79	0.70	0.84	0.77	0.77	0.70	0.76
Females	0.80	0.80	0.81	0.78	0.33	0.64	0.71
Age Group <65	0.88	0.81	0.91	0.86	0.82	0.54	0.66
Age Group 65-75	0.84	0.70	0.90	0.82	0.68	0.87	0.83
Age Group >75	0.46	0.50	0.61	0.43	0.63	0.53	0.58
Ever Smokers	0.82	0.77	0.88	0.80	0.72	0.69	0.76
Never Smokers	0.73	0.69	0.67	0.72	0.73	0.71	0.77
Race Group Whites	0.78	0.70	0.82	0.76	0.70	0.68	0.74
Race Group Other	0.96	0.85	0.98	0.96	0.98	0.99	0.99

Table 4-7 Association between TotAs in urine and total arsenic concentration in toenails

FMV and Spot sample correlated with total arsenic in toenails samples	Pearson's correlation coefficient	P-value
TotAs ^{FMV}	0.45	<0.0001
TotAs ^{SPOT}	0.38	<0.0001
InAs ^{FMV}	0.40	<0.0001
InAs ^{SPOT}	0.31	<0.0001
MMA ^{FMV}	0.42	<0.0001
MMA ^{SPOT}	0.40	<0.0001
DMA ^{FMV}	0.43	<0.0001
DMA ^{SPOT}	0.35	<0.0001
AsB ^{FMV}	-0.10	<0.0001
Asb ^{SPOT}	-0.15	<0.0001

Figure 4-1 Bland-Altman plot of difference between FMV and spot TotAs($\mu\text{g/L}$) urine samples (n=131).

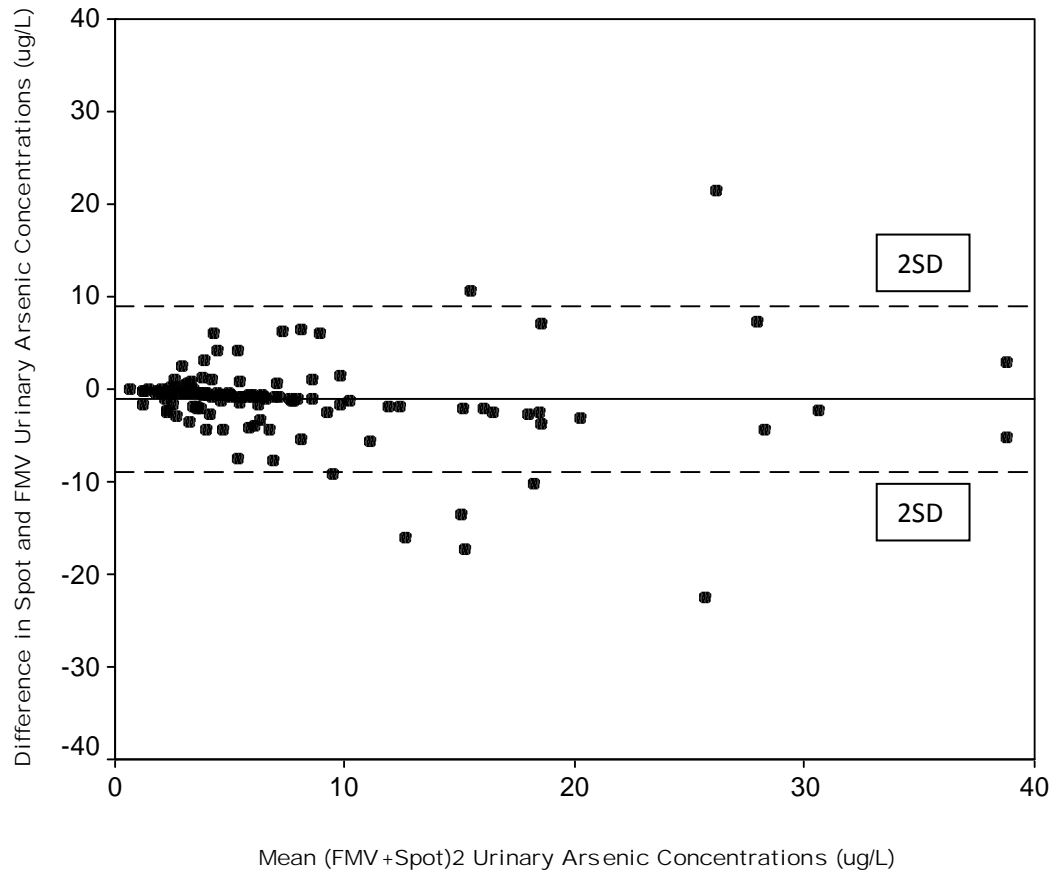
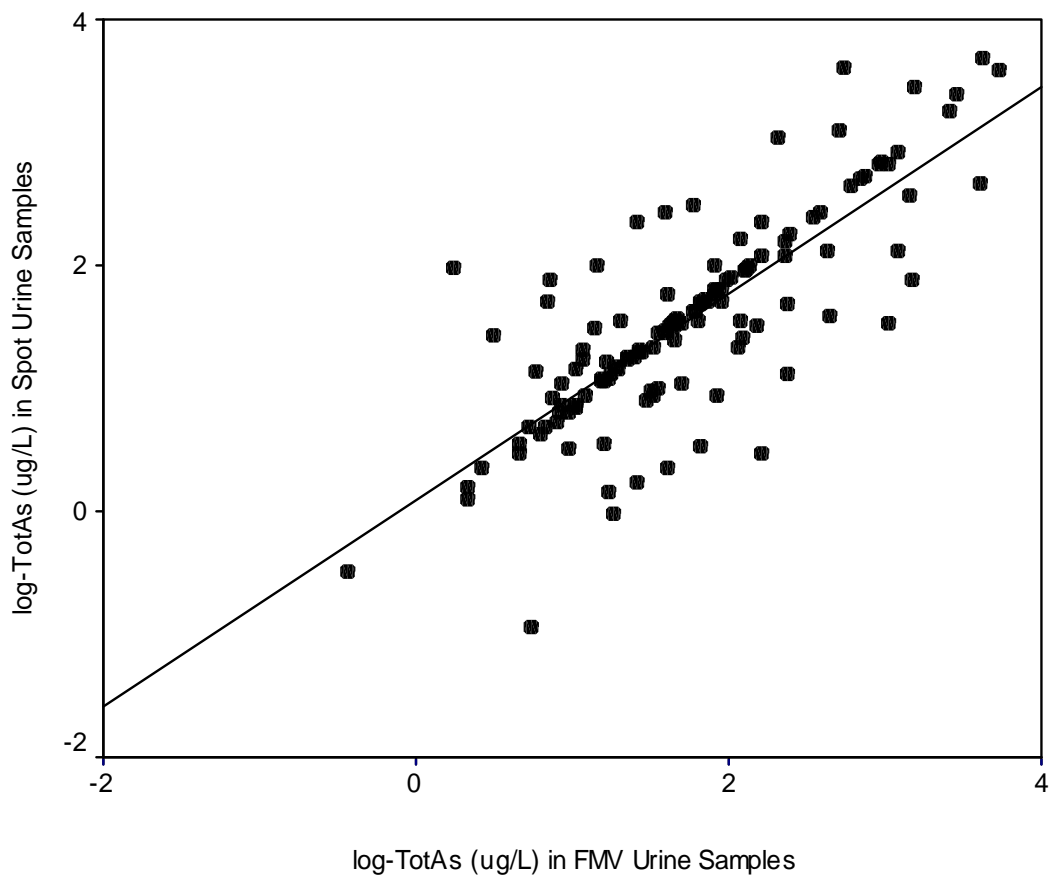


Figure 4-2 Correlation between FMV and Spot samples for Log₁₀-TotAs



References

- Agency for Toxic Substances and Disease Registry A. 2005. Toxicological Profile for Arsenic.
- Aposhian H, Gurzau E, Le XC, Gurzau A, Healy S, Lu X, Ma M, Yip L, Zakharyan R, Maiorino R and others. 2000. Occurrence of monomethylarsonous acid in urine of humans exposed to inorganic arsenic *Chemical Research in Toxicology* 12:693-697.
- Baghi H, Noorbaloochi S, Moore J. 2007. Statistical and non-statistical significance: implication for health care researchers. *Quality Management in Health Care* 16:104-112.
- Bland J, Altman D. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1:307-310.
- Boeniger M, Lowry L, Rosenberg J. 1993. Interpretation of urine results used to assess chemical exposure with emphasis on creatinine adjustments: a review. *American Industrial Hygiene Association Journal* 54:201-212.
- Borak J, Hosgood H. 2007. Seafood arsenic: implications for human risk assessment. *Regulatory Toxicology and Pharmacology* 47:204-212.
- Caceres D, Pino P, Montesinos M, Atalah E, Amigo H, Loomis D. 2005. Exposure to inorganic arsenic in drinking water and total urinary arsenic concentration in a Chilean population. *Environmental Research* 98:151-159.
- Calderon R, Huggens E, Le XC, Schreinemachers D, Thomas DJ. 1999. Excretion of arsenic in urine as a function of exposure to arsenic in drinking water. *Environmental Health Perspectives* 107:663-667.
- Chen Y, Su H-J, Guo Y-L, Hsueh Y-M, Smith T, Ryan L, Lee M-S, Christiani D. 2003. Arsenic methylation and bladder cancer risk in Taiwan. *Cancer, causes and controls* 14:303-310.
- Cohen J. 1992. A power primer. *Psychological Bulletin* 112:155-159.
- Cornelius R, Heinzow B, Herber R, Christensen JM, Poulson O, Sabbioni E, Templeton D, Vahter M, Vesterberg O. 1996. Sample collection guidelines for trace elements in blood and urine. *Journal of Trace Elements in Medicine and Biology* 10:103-127.
- Cullen W, Reimer K. 1986. Arsenic speciation in the environment. *Chemical Reviews* 89:713-764.

- Flasar C. 2008. What is specific gravity? *Nursing* 38:14.
- Gong Z, Lu X, Cullen W, Le XC. 2001. Unstable trivalent arsenic metabolites, monomethylarsonous acid and dimethylarsinous acid. *Journal of Analytical Atomic Spectroscopy* 16:1409-1413.
- Hall Moran V, Leathard H, Coley J. 2001. Urinary hormones levels during the natural menstrual cycle: the effect of age. *Journal of Endocrinology* 170:157-164.
- Hayakawa T, Kobayashi Y, Cui X, Hirano S. 2005. A new metabolic pathway of arsenite: arsenic-glutathione complexes are substrates for human arsenic methyltransferase CYT19. *Inorganic Compounds* 79:183-191.
- Hinwood AL, Sim MR, de Klerk N, Drummer O, Gerostamoulos J, Bastone EE. 2002. Are 24-hour urine samples and creatinine adjustment required for analysis of inorganic arsenic in urine in population studies? *Environmental Research* 88:219-224.
- Hopenhayn-Rich C, Biggs M, Smith A, Kalman D, Moore L. 1996. Methylation study of a population environmentally exposed to arsenic in drinking water. *Environmental Health Perspectives* 104:620-628.
- Karagas MR, Le XC, Morris S, Blum J, Lu X, Spate V, Carey M, Stannard V, Klaue B, Tosteston T. 2001. Markers of low level arsenic exposure for evaluating human cancer risks in a US population. *International Journal of Occupational Medicine and Environmental Health* 14:171-175.
- Mandal B, Ogra Y, Suzuki K. 2001. Identification of dimethylarsinous and monomethylarsonous acids in human urine of the arsenic-affected areas in West Bengal, India. *Chemical Research in Toxicology* 14:371-378.
- Mandal B, Ogra Y, Suzuki K. 2003. Speciation of arsenic in human nail and hair from arsenic-affected area by HPLC-inductively coupled plasma mass spectrometry. *Toxicology and Applied Pharmacology* 189:73-83.
- Meliker J. 2007. Lifetime exposure to arsenic in drinking water: Application to a bladder cancer case control study. Saarbruecken, Germany: VDM Verlag Dr. Mueller Press.
- Meza M, Kopplin M, Burgess J, Gandolfi A. 2003. Arsenic drinking water exposure and urinary excretion among adults in the Yaqui Valley, Sonora, Mexico. *Environmental Research* 96(2):119-126.
- Michalke B. 2003. Element speciation definitions, analytical methodology, and some examples. *Ecotoxicology and Environmental Safety* 56:122-139.

- Moore R, Hirata-Dulas C, Kasiske B. 1997. Use of urine specific gravity to improve screening for albuminuria. *Kidney International* 52:240-243.
- Nermell B, Lindberg A-L, Rahman M, Berglund M, Persson LA, Arifeen SE, Vahter M. 2008. Urinary arsenic concentration adjustment factors and malnutrition. *Environmental Research* 106:212-218
- NRC. 1999. Arsenic in Drinking Water. In: Water SoAiD, editor. Washington, DC: National Academy Press.
- NRC. 2001. Arsenic in Drinking Water 2001 Update. In: Water SoAiD, editor: National Academy Press. p 15-23.
- Parikh C, Gyamlani G, Carvounis C. 2002. Screening for microalbuminuria simplified by urine specific gravity *American Journal of Nephrology* 22:315-319.
- Slejkovec Z, Falnoga I, Goessler W, van Elteren J, Raml R, Podgornik H, Cernelc P. 2008. Analytical artifacts in the speciation of arsenic in clinical samples. *Analytica Chimica Acta* 607(1):83-91.
- Slotnick M, Meliker J, Nriagu J. 2007. Intra-individual variability in toenails arsenic concentrations in a Michigan population, USA. *Journal of Exposure Science and Environmental Epidemiology* 1-9.
- Smith AH, Steinmaus C. 2000. Arsenic in urine and drinking water. *Environmental Health Perspectives*:A494.
- Steinmaus C, Yuan Y, Kalman D, Attallah R, Smith AH. 2005. Intraindividual variability in arsenic methylation in a US population. *Cancer Epidemiology, Biomarkers & Prevention* 14:919-924.
- Steinmaus C, Bates M, Yuan Y, Kalman D, Attallah R, Rey O, Biggs M, Hopenhayn C, Moore L, Hoang B and others. 2006. Arsenic methylation and bladder cancer risk in case-control studies in Argentina and the United States. *Journal of Occupational and Environmental Medicine* 48:478-488.
- Suwazono Y, Akesson A, Alfvén T, Jarup L, Vahter M. 2005. Creatinine concentrations versus specific gravity-adjusted urinary cadmium concentrations. *Biomarkers* 10:117-126.
- Teass A, Biagini R, DeBord G, Hull R. 2003. Application of biological monitoring methods. In: US Department of Health and Human Services PS, Centers for Disease Control and

Prevention, National Institute of Occupational Safety and Health, editor. NIOSH Manual of Analytical Methods. Fourth Edition ed. Cincinnati, OH: Eller PM.

Valenzuela O, Borja-Aburto H, Garcia-Vargas G, Cruz-Gonzalez M, Garcia-Montalvo E, Calderon-Aranda E, Del Razo L. 2005. Urinary trivalent methylated species in a population exposed to inorganic arsenic. *Environmental Health Perspectives* 113:250-254.

Worsfold M, Davie M, M H. 1999. Age-related changes in body composition, hydroxyproline, and creatinine excretion in normal women. *Calcified Tissue International* 64:40-44.

Chapter V

Urinary Arsenic Species, Toenail Arsenic, and Estimates of Arsenic Intake in a Southeastern Michigan Population with Low-To-Moderate Exposure to Arsenic in Drinking Water

I. Introduction

Urinary arsenic is a well-documented biomarker of exposure. Urinary excretion of arsenic is the primary pathway for the elimination of arsenic from the human body (Le et al. 1994; Vahter 1994b). In addition, the collection of urine samples is a non-invasive procedure, and hence is an attractive methodology for large-scale studies. Epidemiological studies have shown positive and consistent relationships between arsenic in drinking water and arsenic in urine around the world. A greater number of studies have been conducted in areas with higher arsenic levels such as Taiwan and Bangladesh. In Taiwan, Hsueh et al. (1997) found that people have higher concentrations of arsenic and its metabolites in urine than persons exposed to lower levels of arsenic. After termination of arsenic exposure through drinking water, populations show a decrease in urinary arsenic metabolites concentration (Tseng et al., 2005). A study of cancer risk in Hungary, Romania and Slovakia ($n=537$) showed a significant correlation between arsenic in drinking water and arsenic in urine ($R^2=0.45$

$p < 0.0001$) (Lindberg et al. 2006). An increasing awareness of the potential chronic health effects of arsenic at low levels has emerged in new studies with populations exposed to low arsenic concentrations for long periods of time. Karagas et al. (2001) conducted an assessment with New Hampshire population where 99% of the studied population has arsenic concentrations of less than $50\mu\text{g/L}$ in their drinking water. They found a correlation of 0.35 ($p=0.0024$), and the association increased to 0.46 ($p=0.029$) when they excluded drinking water with less than $1\mu\text{g/L}$ of arsenic (Karagas et al. 2002). In Mexico, a study showed that including total arsenic intake per day influence the correlation between arsenic concentrations in water and urinary arsenic (Meza et al. 2004). This finding may suggest that incorporating water consumption may improve prediction of arsenic in urine. Intake of water and beverages made with tap water and consumption of certain foods may influence the amount of arsenic excreted in urine. Evidence suggests that volume of water intake may be an influential modifier of ingestion exposures (Wright et al. 2006). To better assess exposure in epidemiological studies it is critical to correctly measure individual exposure to water contaminants to reduce exposure misclassification (Maskiell et al. 2006). The use of findings from human studies conducted at the low levels of arsenic to guide risk assessment is limited by exposure misclassification (Cantor and Lubin 2007). The main attraction that urine offers as a biomarker for arsenic exposure is the characterization of arsenic species or metabolites. Organic species derived primarily from seafood such arsenobetaine or arsenosugars can be identified while the concentrations of toxic species can be determined.

A few studies have also evaluated the relationship between urine and toenails. Karagas et al. (2001) found a correlation of 0.36 ($p=0.0012$) between urinary arsenic and toenails, however, this analysis included only 77 individuals. In some areas of southeastern Michigan arsenic concentrations in ground water frequently exceed the Environmental Protection Agency (EPA) maximum contaminant limit of $10\mu\text{g/L}$ (Haack and Treccani 2000). Using data from the Michigan Bladder Case-Control Study, Slotnick et al. (2007) found a significant correlation of 0.32 between drinking water and arsenic in toenails ($n=430$). Moreover, they extended the analysis to evaluate additional variables such as food and estimates of water consumption at home and work. They found that total water intake was a better predictor of arsenic in toenails. Now that data have been obtained for urinary arsenic metabolites, it is important to evaluate urinary arsenic as a biomarker of arsenic exposure for this population as well. The primary goal of this study is to evaluate the impact of possible predictors including water intake and food, in addition to arsenic in drinking water, on the ability of urinary arsenic to reflect exposure. A secondary goal is the comparison of the differences and similarities of urinary arsenic and arsenic in toenails as biomarkers of exposure in the same population. This study explores the ability of two well-recognized biomarkers to reflect arsenic ingestion, consequently adding to biomarker validation and improving exposure assessment efforts.

II. Methods

Study Subjects

Spot urine samples were collected from a sub-sample of participants in southeastern Michigan enrolled in a case-control study of arsenic exposure and bladder cancer (Meliker 2007). Cases were obtained from the Michigan State Cancer Registry and were frequency-matched by age, race and gender with controls selected through a random-digit dialing procedure. Cases, as well as controls, were recruited on the basis that they had been living in the study area for at least five consecutive years prior to recruitment. The study area included the southeastern counties of Genesee, Huron, Ingham, Jackson, Lapeer, Livingston, Oakland, Salinac, Shiawassee, Tuscola, and Washtenaw. The sub-sample used in this study included 151 cases and 192 controls recruited from June 2005 through May 2007. Each participant signed an informed consent form and random identification numbers were assigned to each participant to maintain confidentiality. Further details on the selection of subjects are described elsewhere (Meliker 2007).

Interview Data

Initially, phone interviews to obtain demographic information, medical history, life-styles habits, and water consumption patterns of individuals were conducted (Appendix A) (Slotnick et al. 2007). For the purpose of this study, exposure data was obtained from a Food Frequency Questionnaire (FFQ) derived from instruments used in previous studies of arsenic exposure in the United States (Appendix B) (Karagas et al. 2004; Steinmaus et al. 2005). The questionnaire was administered during personal

home interviews. During these home interviews, we obtained information on consumption of specific food items, home tap water, vitamins, smoking and drinking habits. The questionnaire was limited to three days prior to the urine sample collection. Food items in the survey included rice, chicken, mushrooms, fish (e.g. cod, salmon, haddock, trout), shellfish (e.g. shrimp, lobster, clams), and sushi among others. Specifically, participants were asked how many servings of each food item they had consumed in the past three days prior to the study. Likewise, they were asked about home tap water consumption per day: plain tap water at home and beverages made with tap water at home (e.g. coffee, hot or iced tea). Data on cigarettes and alcohol consumption per day during the three days prior to the urine sample collection were also collected.

Collection and Analysis of Water Samples

During home visits, research team members collected tap water samples. Participants identified their main drinking water sources and additional sources that they use for cooking and coffee, if different. Water samples were collected in low-density polyethylene (LDPE) bottles and acid-washed for determination of trace metals (Nriagu et al. 1993). Samples were immediately stored on ice until delivered to the laboratory where they were acidified with 100µg/L trace-metal grade HNO₃ (Fisher Chemical) and stored until analysis. Throughout water collection procedures, non-powdered vinyl gloves were worn and water samples were stored in plastic zippered bags to minimize contamination. In addition, field blanks and replicates were collected

every day. For each batch, an average blank value was calculated and subtracted from the concentration of each sample.

All samples were analyzed at the University of Michigan, School of Public Health using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent Technologies Model 7500c). To minimize interferences, samples higher than 50 $\mu\text{g/L}$ were diluted (1:10) and rerun. The ICP-MS was also equipped with a collision cell for the purpose of minimizing interference. Dilutions of the Agilent multi-element calibration standard were prepared in 5% trace metal HNO_3 before analysis. In addition, NIST SRM 1640, Trace Elements in Natural Water, was used to validate the calibration. The SRM 1640 was always within 10% of the certified value for arsenic. The arsenic detection limit for water was three times the standard deviation of the calibration standard diluted to 0.050 $\mu\text{g/L}$. The minimum detection level (MDL) for this set of samples was calculated as 0.046 $\mu\text{g/L}$. Samples below detection limit were assigned as half the detection limit for modeling purposes.

Collection and Analysis of Toenail Samples

Specific methods on collection, preparation and analysis of toenails samples in this study have been published previously (Slotnick et al. 2007). Briefly, clipping materials and instructions were mailed to participants after enrollment into the study. Participants were asked to clip all their 10 toenails after bathing or showering, and record the date of clipping and time since their last clipping. During home visits, the research team collected the toenail clippings. After collection, samples were washed

and digested following modified protocols (Das et al. 1995; Karagas et al. 2000). Toenails were analyzed for arsenic using ICP-MS, toenail MDL for this set of samples was 0.124 $\mu\text{g/g}$.

Collection and Analysis of Urine Samples

The research team provided participants with materials and instructions for urine collection. Materials included a plastic bag packed with an acid-washed polyethylene urine cup, antibacterial wipes, gloves, and urine collection instructions. Participants were asked to collect a spot urine sample during field visits. Spot samples were immediately frozen in dry ice brought along by the research team and then transported to the laboratory. Spot samples were stored in the laboratory at -20°C until analysis. Before analysis, samples were filtered using a 0.22 μm syringe filter and no preservatives were added.

Urine samples were passed through a 5 μm 250x4.6mm column (Phenomenex, Torrance, CA, USA). The mobile phase contained 4% (v/v) methanol, 5mM tetrabutylammonium hydroxide (TBAH), and 10 mM ammonium phosphate at pH 9.5. The HPLC system (Alltech) was coupled to the ICP-MS unit. Detection limits for arsenic species by the method used were as follows: AsB, 0.06 $\mu\text{g/L}$; As[III], 0.112 $\mu\text{g/L}$; As[V], 0.147 $\mu\text{g/L}$; MMA[V], 0.117 $\mu\text{g/L}$; and DMA[V], 0.076 $\mu\text{g/L}$. Urine certified reference material from the Japanese National Institute for Environmental Studies (NIES NO. 18, Tsukuba, Ibaraki, Japan) were used as reference standards. Details on this method are explained in Chapter 2. Specific gravity measures were taken immediately after samples

were thawed and reached room temperature; freezing a urine sample does not change its specific gravity (Nermell et al. 2008). Details on specific gravity measurements are discussed on Chapter 4.

Calculation of Exposure Metrics

Inorganic arsenic in different foods was estimated from the literature (Table 5-2). Comparable methods have been employed (Meacher et al. 2002) and used in previous published papers by our team (Slotnick et al. 2007). Inorganic arsenic in each food item was averaged across cited articles. Estimated intake ($\mu\text{g}/\text{L}$) in Metric 4 was calculated by multiplying food consumption (g/d) by inorganic arsenic concentrations ($\mu\text{g}/\text{g}$). From all the different food items included in the FFQ, only those with more than 10% response were included in the final analysis. These foods included rice, mushrooms, chicken, and canned fish. Other foods such as individual kind of fish (haddock, salmon, cod), shellfish (shrimp, clams, lobsters), sushi or seaweed were not reportedly consumed by more than 4% of the respondents (Table 5-3). These items were included in the food metric as “any seafood” which contained consumption of any fish or shellfish including canned fish. Rice, mushrooms, chicken and seafood were evaluated as single variables and as part of the exposure metrics. The food data was collected using the amount of serving sizes to obtain how much of a food item the participant ate during any of the three days requested in the questionnaire. The serving amounts were provided in ounces. The amount of servings were converted to the metric system and multiplied by the amount of arsenic reported in the literature. Individual analyses were done for each of the three

days for water and food intakes. There were no differences in the results of individual days or in combining all three days together. In addition, there were no differences in the analysis when including individual food items as different metrics or all four food items together in one metric. To incorporate this data to the exposure metric analysis, results from the three-day period were added and incorporated as one total amount of arsenic in food. Total arsenic in food was included as a separate metric in the exposure metric. In addition, individual food items were also assessed separately using single and multiple regression models.

Exposure metrics for each participant were calculated using arsenic concentration in drinking water, drinking water intake, and FFQ data. Four different metrics were developed to explain arsenic exposure: 1) arsenic concentration in drinking water at home ($\mu\text{g/L}$), 2) arsenic intake ($\mu\text{g/d}$) from plain drinking tap water at home, 3) arsenic intake ($\mu\text{g/d}$) from tap water and beverages made with tap water at home, and 4) arsenic intake ($\mu\text{g/d}$) from food and all tap water sources at home (Table 5-1). Water intake in Metrics 2 and 3 was calculated by multiplying water consumption (L/d) by arsenic in respective water concentrations ($\mu\text{g/L}$).

Statistical Analysis

Descriptive statistics were calculated for arsenic concentrations in drinking water, toenails, and urine. Histograms and normal probability plots revealed deviations from a normal distribution for water arsenic concentrations, toenails arsenic concentrations, and all urinary arsenic metabolites. For this paper, the sum of As[III],

As[V], MMA[V] and DMA[V] was \log_{10} -transformed and used to designate total toxic arsenic species (*TotAs*). AsB was not included in *TotAs* in order to distinguish between the toxic inorganic arsenic compounds and the less toxic AsB. When AsB was included in the \log_{10} -transformed sum it was designated *As*. The sum of As[III] and As[V] was used as the total of inorganic arsenic (*InAs*) because it provides a more stable measure of inorganic arsenic in urine, since these two species may interconvert while in urine. Since MMA[III] and DMA[III] were not detected in any of the samples, MMA and DMA refer only to the respective pentavalent species, unless otherwise stated. Concentrations below the detection limit were set at one half the detection limit (Table 5-4).

The association of urinary arsenic with demographic variables such as disease status, gender, age, and smoking were assessed using univariate analyses. Student's t test was used to compare category means. ANOVA was used when three or more means were compared. A smoker was defined as a person that reported at least one cigarette-a-day in the FFQ. Age, gender, smoking and BMI were evaluated as single variables as well as in multiple regression models in order to assess confounding.

Linear regression models were used to evaluate exposure metrics and single variables in order to predict arsenic in urine. Log-transformed urinary arsenic concentration (*TotAs*) was modeled as the dependent variable in the regression analyses. Pearson correlation procedures were applied to investigate the relationship between urinary arsenic concentration and water arsenic concentrations as well as urinary arsenic concentration and toenail arsenic concentration.

Separate models were run in the exposure metric analyses to assess different groups. To assess different exposure levels we dichotomized individuals according to arsenic in water: individuals with arsenic $<1\mu\text{g/L}$ ($n=206$) and individuals with arsenic in water $\geq 1\mu\text{g/L}$ ($n=137$). This stratification was also performed for comparison with previous studies (Karagas et al. 2002; Slotnick et al. 2007). Individuals were also stratified in terms of plain water intake, greater than and less than median plain water intake ($n=118$). All statistical analyses were run using SAS statistical software, version 9.1 (SAS Institute, Inc., Cary, NC).

III. Results

A total of 343 individuals were interviewed and provided the research group with urine, water and toenail samples. Descriptive characteristics for the 343 individuals are presented in Table 5-5. More than half of the participants included in this research were men (67.6%) and the group's average age was 65.7 years; the age and race distribution reflects the fact that bladder cancer is predominantly a disease of elderly white men.

The average storage time for samples was 73 days (0-278days). Neither MMA[III] nor DMA[III] was detected in any of the samples. Table 5-5 also shows urinary arsenic (TotAs), toenails, and water arsenic concentrations. There were no significant differences in urinary arsenic, drinking water arsenic concentration, and arsenic concentration in toenails between cases and controls, males and females, or race groups.

Table 5-6 shows the distribution of urinary arsenic metabolite for the study population. TotAs ranged from 0.22µg/L to 74.2µg/L for urine samples. Total arsenic in toenails ranged from below detection limit to 2.1µg/g and total arsenic in water ranged from below detection limit to 70.7µg/L. In addition, Table 5-6 shows averages arsenic concentrations in urine for the 2003-2004 National Health and Nutrition Examination Survey (NHANES) (Caldwell et al. 2009) for comparison purposes ($n=2557$). Overall, average arsenic concentrations from southeastern Michigan are similar from those reported in the NHANES. NHANES, however, does not report geometric mean concentrations or percentiles for As[III] and As[V] because arsenic was detected in a very small percentage of their samples, 7.6% and 4.6% respectively. We report means for these two metabolites with detectable levels of 44.3% for As[III] and 23.3% for As[V]. For MMA[V], NHANES reported a 35% arsenic detection for participants 6 years and older. The 75th percentile was 1.2µg/L and the 95th percentile was 2.4µg/L while we report 83.1% of detectable arsenic in samples and 1.15µg/L and 3.68µg/L for the 75th and 95th percentiles respectively. DMA concentrations are very comparable, while arsenobetaine (AsB) is the metabolite that presented the largest difference between the study sample and NHANES.

Population characteristics, age, gender, BMI, and smoking were explored as predictors of urinary arsenic. None of these were significant predictors of TotAs except for smoking status ($p<0.05$) (Table 5-7). Correlation procedures show a positive, significant association between TotAs and arsenic concentrations in water ($r=0.42$, $p<0.0001$) (Fig 5-1). The correlation increased ($r=0.45$, $p<0.0001$) when analyses were

run including only individuals with drinking water concentrations exceeding 1µg/L and when including only individuals with drinking water concentrations exceeding 1µg/L and water intake higher than median ($r=0.63$ $p<0.0001$) (Fig 5-2-5-3).

Simple and multiple linear regression models were applied to evaluate predictors of urinary arsenic. All exposure metrics were significant predictors of arsenic concentrations in urine (Table 5-8). The percentage of variation in TotAs explained by the metrics was similar across all exposure metrics when all participants were included. There was a substantial increase, however, in the value of Metric 1 (arsenic in drinking water) for participants with drinking water concentrations $\geq 1\mu\text{g/L}$ and above median water intake ($\geq 2.4\text{L/d}$); Metric 2 and 3, which included a continuous estimate of water intake did not correlate as well with TotAs. Tables 5-9-5-11 show the multiple regression analysis for each of the arsenic species (InAs, MMA, DMA). Individual metabolites present the same trend, where Metric 1 increased for participants with drinking water concentrations $\geq 1\mu\text{g/L}$ and above median water intake ($\geq 2.4\text{L/d}$). However, metric values for DMA were relatively lower than for MMA.

Since Metric 4 does not explain additional variability of urinary arsenic as well as arsenic in drinking water, a separate multiple regression analysis was performed to explore food consumption and arsenic in urine. Categorizing food consumption of people reporting eating a single item more than one time in the three days prior to the urine sample collection did not change the results. This maybe due to the fact that people who reported eating a specific food item during Day 1 also reported eating the

same item on Day 2 and vice versa. This happened 91% of the time. To explore individual food items as predictors of TotAs, single regression analyses were performed. Intake of rice, chicken and canned fish was positively associated with TotAs (Table 5-12). Exploratory analyses using fish or shellfish did not produce significant results. Since food has been associated with AsB excretion, individual food items were compared with urinary AsB concentrations. Correlation procedures performed using only individuals who consumed some type of shellfish any time during the three days ($n=48$) produced a low but significant correlation between shellfish consumption and AsB urine concentration ($r=0.15$, $p=0.0055$). These individuals also had a significantly higher AsB average concentration ($21.6\mu\text{g/L}$) compared with the rest of the population ($13.3\mu\text{g/L}$). Individuals who consumed any type of fish at some point during the three days had also a significant correlation between fish consumption and AsB excretion ($r=0.27$, $p<0.0001$). Similar to shellfish, AsB average concentration were significantly higher for individuals consuming any type of fish ($27.7\mu\text{g/L}$) compared to the rest of the population ($13.3\mu\text{g/L}$). In addition to shellfish and fish, rice was significantly correlated with urinary AsB concentrations ($r=0.11$, $p=0.0437$). None of the other single food items were significantly correlated with AsB.

Pearson correlation procedures were applied using arsenic in drinking water and individual urinary arsenic metabolites (Table 5-13). Water arsenic concentrations showed a positive and significant relationship with each arsenic metabolite except for arsenobetaine, with MMA having the highest correlation ($r=0.46$). Similarly, toenail arsenic concentrations showed significant relationships with each urinary arsenic

metabolite as well as TotAs (Table 5-13). In Table 5-14 the two biomarkers, TotAs and total toenail concentrations, are compared as biomarkers for arsenic in drinking water. After adjusting for age, gender, smoking status, toenail arsenic concentration has a higher R^2 value than TotAs. When this analysis was done using only those individuals with $\geq 1\mu\text{g/L}$ of arsenic in drinking water and water intake greater than median intake, the R^2 increased from 0.29 to 0.44 showing that arsenic in drinking water explained almost half of the variability in the urine biomarker in these individuals.

IV. Discussion

These results contribute to the existing literature on the use of urinary arsenic concentrations as a biomarker of arsenic exposure. Consistent with previous findings, arsenic concentration in drinking water was found to be an important factor in predicting urinary TotAs. In addition, we found incorporating home water consumption using categorical cut-offs at the median improved the prediction of urinary TotAs. We also identified arsenobetaine in urinary samples of non-fish eaters, likely pointing to additional sources of this organo-arsenical.

Comparing different exposure metrics indicate that arsenic in drinking water is an important predictor of urinary TotAs. Metric 1 increases when arsenic in drinking water and water intake increases. In a population with similar exposure (arsenic in water ranged from $3.3\mu\text{g/L}$ to $49.3\mu\text{g/L}$), an increase in water consumption was reflected in an increase in urinary arsenic (Meza et al. 2004). Inclusion of drinking water intake, together with intake from other beverages, did not improve the ability of the

exposure metric to predict urinary arsenic (Metric 2 and 3). However, when the exposure was stratified by water consumption the explained variability increased. This may suggest that categorical estimates of water consumption provide better characterization of intake, potentially attributed to misclassification in self-reported water consumption.

Several factors can impact ingestion exposure estimates including volume of water intake, bottled and filtered water consumption and effectiveness of point-of-use filtration at home (Maskiell et al. 2006). The Environmental Protection Agency (EPA) has conducted research using simulations to assess Disinfection Byproducts (DBP) exposure in pregnant women (Wright et al. 2006). They found that bottled and filtered water use resulted in considerably less DBP intake. They concluded that most of the misclassified subjects in their simulation had system average concentrations larger than their true exposures. In our study, intakes from fluids other than water sources at home (eg. water at work) may also influence the application of Metrics 2 and 3. In addition, accuracy in fluid intake or identification of water sources may also affect the metrics. Using categorical measures instead of continuous values may minimize misclassification as suggested by Wright et al. (2006) in water intake simulations of drinking water contaminants.

Independent of water volume, urinary arsenic metabolites showed positive and significant correlations with arsenic concentration in drinking water. Since they are all similarly correlated it is hard to determine which metabolite has the largest

contribution. It is worth mentioning, however, that MMA has the highest correlation coefficient among the metabolites. MMA excretion has been associated with an increased risk of arsenic-related diseases such as bladder and skin cancer (Chen et al. 2003; Hopenhayn-Rich et al. 1996; Steinmaus et al. 2006). This information supports the hypothesis that arsenic concentrations in drinking water are to some extent good surrogates of arsenic exposure, even in populations exposed to low levels of arsenic in drinking water.

The arsenic exposure metrics did not include arsenobetaine because it is a less toxic metabolite. Arsenobetaine does not seem to be metabolized by the human body, but is excreted in urine, and is rapidly eliminated unchanged from its consumed form (Le et al. 1993). In addition, AsB was not significantly correlated with arsenic concentrations in water ($r=-0.15$, $p=0.1107$) (Table 5-13). Furthermore, when AsB was included in the sum of total arsenic (As) the correlation between urinary arsenic and arsenic concentration in water dropped substantially from $r=0.42$, ($p<0.0001$) to $r=0.12$, ($p=0.0024$) because AsB mainly enters the body through diet, not drinking water. Nonetheless, the identification of organoarsenicals such as arsenobetaine assists in the study of dietary effects and arsenic metabolism. In addition, it is worthwhile to consider the potential role of AsB because it may influence other arsenic metabolites such as DMA (Francesconi et al. 2002). Given that the metric estimates for DMA were relatively lower than for MMA, we decided to explore the correlations between AsB and the two methylated species MMA and DMA. AsB and DMA showed a positive and significant correlation ($r=0.41$, $p<0.0001$) while AsB was not correlated with MMA ($r=-0.006$,

$p=0.9138$). This may suggest, in fact, that AsB influence the concentrations of DMA in urine. It has been suggested that urinary DMA[V] percentage is an indicator of methylation efficiency (Vahter, 1999). However, if organoarsenicals increase the excretion of DMA, the DMA role in methylation efficiency need further review. On the other hand, DMA is a known rat urinary bladder carcinogen (Ma and Le, 1998). If ingestion of organoarsenical forms produce DMA, their inclusion together with inorganic forms in toxicological and epidemiological studies is critical.

In populations exposed to elevated levels of arsenic in drinking water (10-50 $\mu\text{g/L}$), contribution from specific foods can increase the total urinary arsenic due to contribution of organic forms such as AsB. The mean for total arsenic in this study is relatively higher than the total arsenic mean in NHANES. That difference is mostly due to the higher levels of AsB in our study population. This population does not show, based on the FFQ, a significant consumption of fish or shellfish. However, when individuals who consumed any type of fish or shellfish were analyzed individually, significant correlations were shown between fish or shellfish consumption and AsB excretion. This may be the reason why the food metric does not explain additional variability in the urine biomarker, since TotAs instead of As was used as dependent variable in the multiple regression models. A single meal of seafood may increase total urinary arsenic concentrations by several orders of magnitude (Vahter 1994). Several studies have also reported high concentrations of urinary AsB in populations that consumed little or no fish or seafood (Brima et al. 2006). Lai et al (2004) suggest that slow excretion of AsB or the ability to metabolize arsenic compounds through different

pathways may be the reason for high amounts of AsB in volunteers who refrained from eating fish or seafood three days prior to the urine collection. Another explanation for high AsB levels is the consumption of hidden fish products (Ritsema et al. 1998).

Chicken consumption had a significant correlation with AsB excretion as well. Higher amounts of arsenic are found in chicken when compared with other poultry or meat products (Polatajko and Szpunar 2004). This is consistent with the use of chicken feed containing additives that contain arsenic compounds (Lasky et al. 2004). Urinary arsenic species attributed to chicken consumption are not known. Other studies have also reported high concentrations of urinary AsB in populations that consumed little or no fish or seafood (Brima et al. 2006). Lai et al (2004) explain that slow excretion of AsB or the ability to metabolize arsenic compounds through different pathways may be the reason for high amounts of AsB in volunteers who refrained from eating fish or seafood three days prior to the urine collection. In addition, this population has a relatively high consumption of mushrooms (>28% of respondents), one of the foods known to contain AsB (Byrne et al. 1995; Byrne et al. 1991). Several mushrooms varieties such as *Sarcosphaera*, *Entoloma*, and *Agaricus* have been identified as genera that accumulate arsenic compounds (Byrne et al. 1995; Smith et al. 2007). Finally, Goessler et al. (1997) suggested that AsB can be synthesized by humans from trimethylarsine (TMA). They propose that the human body can metabolize forms other than MMA and DMA but this needs further investigation.

Similar to urinary arsenic, toenail arsenic concentration has been used to determine arsenic exposure. In addition to the correlation of water and urinary arsenic, toenail arsenic concentration was correlated with TotAs. The comparatively lower values are more likely due to the fact that it is a correlation of outcomes not a source-and-effect correlation. In spite of these lower correlations, toenails, when regressed against water, were a better explanation of arsenic concentrations in water than the explanation provided by TotAs. (Table 5-14). The correlation between arsenic concentration in water and arsenic concentration in toenails was $r=0.49$ ($p<0.0001$), slightly higher than for arsenic concentrations in water and TotAs $r=0.42$ ($p<0.0001$). Toenail arsenic concentrations are less-affected by organic arsenic species that are more rapidly excreted and less-absorbed (NRC 1999). Nevertheless, when the exposure is categorized by arsenic concentrations in water and water intake, urinary arsenic explains more variability than toenail arsenic concentrations. Toenail arsenic concentrations may be affected by longer-term temporal variation in exposure, such as participant mobility or variation in drinking-water arsenic concentrations (Slotnick et al. 2007). Urinary arsenic is a short-term biomarker while toenails may reflect exposure in the past year. The main attraction of urinary arsenic is the characterization of its metabolites. Through arsenic speciation, organoarsenicals can be identified and therefore, contribution from diet can be determined. When these studies are done in different populations, results contribute to the understanding of the arsenic metabolism. Nonetheless, the correlation of arsenic in urine and toenails is notable

considering the lifetimes of these biomarkers and the many variables that can moderate the arsenic burden in each biomarker.

There were some limitations that can be identified in this study. Recall error may have been introduced through the FFQ when information on diet and water intakes was collected. Exposure misclassification may also be present due to arsenic in food data obtained from the literature. Data on arsenic in consumed foods in the USA is limited and varies by regions (Schoof et al. 1999). The lack of available data may affect the results related to inorganic arsenic and food consumption. In addition, other food-sources of arsenic may not be represented in the FFQ. In spite of these limitations, this study reveals positive and significant correlation between urinary arsenic and drinking water arsenic concentrations at home in a population exposed to low levels of arsenic in their drinking water. This finding adds to the validity of the urine biomarker ability to reflect drinking-water arsenic concentrations. The use of the FFQ, where exposure through other pathways was examined, adds information about exposure besides arsenic in drinking water alone. Results on exposure metrics help to better understand how exposure is reflected. In the same way, the fact that this population was not a high consumer of fish or seafood and still had relatively high levels of AsB, indicates that even small consumption of fish or shellfish may increased AsB excretion. In addition, there are other sources for organoarsenicals that have to be accounted for in future exposure assessment work such as chicken and mushroom consumption. This work also adds knowledge to better characterize toenail arsenic concentrations and urinary arsenic as biomarkers of arsenic exposure. This will lead to better selection of biomarkers in future

research, accounting for logistical issues, budgetary concerns and laboratory facilities knowing the potential that each biomarker offers.

Table 5-1. Arsenic exposure metrics.

Exposure Metric	Calculation	Units	Mean
1	AsDW $\mu\text{g/L}$	$\mu\text{g/L}$	5.01
2	AsDW($\mu\text{g/L}$) \times DWI(L/d)	$\mu\text{g/d}$	7.34
3	Metric 2 + BI(L/d)	$\mu\text{g/d}$	13.1
4	Metric 3 + FoodAs($\mu\text{g/d}$)	$\mu\text{g/d}$	13.2

AsDW=arsenic concentration in home drinking-water sample

DWI=Intake of plain drinking water at home

BI=Intake of beverages other than plain water made from tap water at home

FoodAs=estimated concentration of arsenic in food

Table 5-2. Inorganic arsenic concentration estimates.

Food	Inorganic As Estimate	Total As Estimate	Sources
Rice	0.086	0.238	(Heitkemper et al. 2001; Lamont 2003; Phuong et al. 1999; Schoof et al. 1999)
Mushrooms	0.027	0.091	(Dabeka et al. 1993; Wuilloud et al. 2004)
Chicken	0.072	0.081	(Lasky et al. 2004; Schoof et al. 1999)
Fish	0.010	1.59	(Schoof et al. 1999; Tao and Bolger 1998)
Shellfish	0.040	1.85	(Schoof et al. 1999; Tao and Bolger 1998)

Table 5-3. Percent of participants self-reported food consumptions during three days prior to the urine sample collection.

Food Item	# Participants Day 1	# Participants Day 2	# Participants Day 3	Overall %
Rice	38(11.1%)	29(8.6%)	19(5.5%)	8.4
Rice Cereal	18(5.2%)	12(3.5%)	10(2.9)	3.9
Mushrooms	101(29.4%)	96(28.0%)	11(3.2%)	20.2
Clam/Fish Chowder	2(.58%)	1(0.30%)	0(0%)	0.3
Chicken	96(28.0%)	90(26.2%)	20(5.8%)	20.0
Canned Fish	30(8.7%)	20(5.8%)	7(2.0%)	16.6
Sushi	0(0%)	0(0%)	0(0%)	0.0
Seaweed	2(.58%)	1(0.30%)	0(0%)	0.3
Cod	2(.58%)	5(1.4%)	1(0.30%)	0.78
Haddock	0(0%)	1(0.28%)	0(0%)	0.0
Salmon	14(4.1%)	6(1.7%)	2(0.58%)	2.1
Scup	1(0.23%)	0(0%)	0(0%)	0.0
Swordfish	0(0%)	0(0%)	0(0%)	0.0
Sole	0(0%)	0(0%)	0(0%)	0.0
Sea bass	1(0.23%)	0(0%)	1(0.30%)	0.0
Tuna	0(0%)	2(0.56%)	0(0%)	0.0
Other Fish	11(3.2%)	7(2.0%)	2(0.58%)	1.9

Table 5.3. Percent of participants self-reported food consumptions during three days prior to the urine sample collection (Continuation).

Food Item	# Participants Day 1	# Participants Day 2	# Participants Day 3	Overall %
Shrimp	12(3.5%)	14(4.1%)	2(0.58%)	2.7
Clams	0(0%)	1(0.28%)	0(0%)	0.0
Oysters	0(0%)	0(0%)	0(0%)	0.0
Scallops	0(0%)	2(0.58%)	0(0%)	0.0
Mussels	0(0%)	0(0%)	0(0%)	0.0
Crabs	0(0%)	2(0.58%)	0(0%)	0.0
Lobster	0(0%)	1(0.30%)	0(0%)	0.0
Other Shellfish	1(0.30%)	2(0.58%)	0(0%)	0.0
Any Seafood	74(21.6%)	63(18.4%)	15(4.4%)	14.8

Table 5-4. Amount of samples below detection limits by individual species.

Arsenic Specie	Number of Samples below the Detection Limit	%	Detection Limit (µg/L)
InAs	160	46.6	0.112
MMA	58	16.9	0.117
DMA	1	0.29	0.076
AsB	2	0.58	0.060

Table 5-5. Average concentrations of arsenic in Urine (TotAs), arsenic in water and arsenic in toenail for the study population.

Population Characteristics	n	%	TotAs µg/L	Arsenic in Water µg/L	Arsenic in Toenails µg/g
All	343	100	7.01	5.01	0.15
Disease Status					
Case	151	44.0	6.57	5.38	0.16
Control	192	66.0	7.52	4.72	0.14
Age					
<55	49	14.3	6.82	5.86	0.17
55-64	84	24.4	5.86	6.69	0.21
65-74	114	33.3	6.15	3.49	0.13
≥75	96	28.0	7.94	4.95	0.12
Gender					
Male	232	67.6	7.13	5.34	0.16
Female	111	32.3	7.05	4.30	0.13
Race					
White	324	95.3	7.02	4.84	0.15
Other	15	4.7	9.95	10.62	0.22
Current Smoker					
Yes	37	10.7	5.09	3.85	0.15
No	306	89.2	7.35	5.15	0.15
Drinking Water Source					
Public Supply-Surface	102	29.7	5.43	0.91	0.07
Public Supply-Well	5	1.4	4.55	1.59	0.10
Private Well	202	58.1	8.26	7.86	0.20
Bottle	33	9.6	5.72	0.63	0.07
Missing	1	0.3	2.30	0.49	0.07

Table 5-6. Average arsenic concentrations in urine ($\mu\text{g/L}$), toenails ($\mu\text{g/g}$) and water samples ($\mu\text{g/L}$) from Michigan samples and NHANES (Caldwell et al. 2009).

Arsenic	N	^a A Mean	Range	^b G Mean	^b G Mean	25th		75th		95th	
	MI	MI	MI	MI	*NHANES	MI	*NHANES	MI	*NHANES	MI	*NHANES
Urinary As[III]	343	0.29	<lod-3.40	0.14	-	< lod	-	0.35	-	1.09	-
Urinary As[V]	343	0.19	<lod-2.14	0.12	-	< lod	-	< lod	-	0.82	-
Urinary MMA[V]	343	1.09	<lod-18.0	0.56	-	0.345	-	1.2	1.2	3.68	2.86
Urinary DMA[V]	343	5.53	<lod-74.0	3.81	3.79	2.18	2.26	6.8	5.95	15.4	13.5
Urinary AsB	343	13.3	<lod-257.0	4.40	1.79	1.77	<lod	10.3	5.91	47.1	30.0
^c As	343	20.4	<lod-331.2	11.5	8.64	5.81	4.26	21.4	15.4	61.7	53.9
^d TotAs	343	7.1	0.224-74.2	5.01	-	2.95	-	8.5	-	20.4	18.9

^aA Mean=Arithmetic Mean

^bG Mean= Geometric Mean

^c(As[III]+As[V]+MMA[V] +DMA[V]+AsB)

^d(As[III]+As[V]+MMA[V] +DMA[V])

*In the age group \geq 20y

lod=limit of detection

Table 5-7. Study population characteristics as predictors of TotAs ($\mu\text{g/L}$).

Variable	Parameter Estimate	Model R²	<i>p</i>-value
Status	0.08342	0.0038	0.2519
Gender	-0.06459	0.0020	0.4003
Age	-0.00197	0.0010	0.5560
Smoking Status	-0.23700	0.0121	0.0415

Table 5-8. Relationship between urinary arsenic concentrations (TotAs $\mu\text{g/L}$) and estimated arsenic exposure, stratified by arsenic drinking-water concentration and water intake.

Drinking Water Arsenic Concentration and Intake	N	Metric	β	*Model results R^2
All samples	339	1	0.2523	0.1727
	339	2	0.0162	0.1875
	339	3	0.0097	0.1867
	339	4	0.0098	0.1860
$\geq 1\mu\text{g/L}$	137	1	0.3830	0.2363
	137	2	0.0144	0.2438
	137	3	0.0087	0.2361
	137	4	0.0087	0.2365
$\geq 1\mu\text{g/L}$ and Plain Water Intake \geq Median (2.4L)	80	1	0.4982	0.3916
	80	2	0.0141	0.3141
	80	3	0.0086	0.2984
	80	4	0.0087	0.2990

*All values significant at $\alpha=0.05$

Table 5-9. Relationship between InAs arsenic concentrations in urine ($\mu\text{g/L}$) and estimated arsenic exposure, stratified by arsenic drinking-water concentration and water intake.

Drinking Water Arsenic Concentration and Intake	N	Metric	β	*Model results R^2
All samples	339	1	0.0318	0.1288
	339	2	0.0163	0.1019
	339	3	0.0109	0.1236
	339	4	0.0109	0.1207
$\geq 1\mu\text{g/L}$	137	1	0.0286	0.1547
	137	2	0.0136	0.1167
	137	3	0.0095	0.1503
	137	4	0.0095	0.1503
$\geq 1\mu\text{g/L}$ and Plain Water Intake \geq Median (2.4L)	80	1	0.0424	0.2758
	80	2	0.0133	0.1319
	80	3	0.0099	0.1884
	80	4	0.0099	0.1884

*All values significant at $\alpha=0.05$

Table 5-10. Relationship between MMA arsenic concentrations ($\mu\text{g/L}$) in urine and estimated arsenic exposure, stratified by arsenic drinking-water concentration and water intake.

Drinking Water Arsenic Concentration and Intake	N	Metric	β	*Model results R^2
All samples	339	1	0.0196	0.1913
	339	2	0.0120	0.2156
	339	3	0.0076	0.2338
	339	4	0.0077	0.2377
$\geq 1\mu\text{g/L}$	137	1	0.0162	0.1990
	137	2	0.0103	0.2654
	137	3	0.0067	0.2937
	137	4	0.0067	0.2939
$\geq 1\mu\text{g/L}$ and Plain Water Intake \geq Median (2.4L)	80	1	0.0310	0.4403
	80	2	0.0111	0.3297
	80	3	0.0072	0.3739
	80	4	0.0072	0.3741

*All values significant at $\alpha=0.05$

Table 5-11. Relationship between DMA arsenic concentrations ($\mu\text{g/L}$) in urine and estimated arsenic exposure, stratified by arsenic drinking-water concentration and water intake.

Drinking Water Arsenic Concentration and Intake	N	Metric	β	*Model results R^2
All samples	339	1	0.0230	0.1312
	339	2	0.0152	0.1717
	339	3	0.0091	0.1678
	339	4	0.0091	0.1667
$\geq 1\mu\text{g/L}$	137	1	0.0198	0.1414
	137	2	0.0137	0.2273
	137	3	0.0082	0.2154
	137	4	0.0082	0.2158
$\geq 1\mu\text{g/L}$ and Plain Water Intake \geq Median (2.4L)	80	1	0.0340	0.2849
	80	2	0.0133	0.2637
	80	3	0.0078	0.2368
	80	4	0.0078	0.2373

*All values significant at $\alpha=0.05$

Table 5-12. Estimates of arsenic in food as predictors of TotAs ($\mu\text{g/L}$).

Variable	Parameter Estimate	Model R²	p-value
<i>All Individuals</i>			
<i>Total Food</i>	0.64524	0.0246	0.0148
<i>Subjects with As in drinking water $\geq 1\mu\text{g/L}$</i>			
<i>Total Food</i>	1.01712	0.0213	0.0488
<i>Individual Items</i>			
Rice	7.68351	0.0080	0.0965
Chicken	1.45278	0.0203	0.0223
Mushrooms	-8.38878	0.0381	0.0456
Canned Fish	3.49924	0.0293	0.1924
Shellfish	0.07434	0.0000	0.9975
Fish	-0.22018	0.0010	0.7123
Any Seafood	-0.22100	0.0010	0.7117
<i>Only Individuals who consumed any type of seafood (n=77)</i>			
<i>Predictor of TotAs</i>			
Any Seafood	0.71339	0.0152	0.0223
<i>Predictor of As</i>			
Any Seafood	1.97649	0.0529	<0.0001
<i>Predictor of AsB</i>			
Any Seafood	2.55831	0.0715	<0.0001

Table 5-13. Correlations of urinary arsenic metabolites with arsenic in water and toenail.

Marker	Arsenic Metabolites in Urine					
	As[III]+As[V]	MMA[V]	DMA[V]	AsB	As	TotAs
Water	0.370	0.463	0.387	-0.147	0.122	0.416
	p<0.0001	p<0.0001	p<0.0001	p=0.1107	p=0.024	p<0.0001
Toenails	0.351	0.369	0.312	-0.092	0.125	0.345
	p<0.0001	p<0.0001	p<0.0001	p=0.2905	p=0.0217	p<0.0001

Table 5-14. Arsenic in drinking water ($\mu\text{g/L}$) as a predictor of urinary arsenic and toenail arsenic concentration ($\mu\text{g/g}$).

Biomarker	β	Model R^2	p-value
<i>All Individuals (n=343)</i>			
TotAs	0.2504	0.1839	<0.0001
Total Arsenic in Toenails	0.4506	0.2518	<0.0001
<i>Individuals with $\geq 1\mu\text{g/l}$ arsenic in drinking water (n=80) and water intake > median</i>			
TotAs	0.48825	0.4404	<0.0001
Total Arsenic in Toenails	1.93630	0.2915	<0.0001

Adjusted by age, gender, and smoking status.

Figure 5-1. Urinary arsenic vs. arsenic in drinking water (n=343).

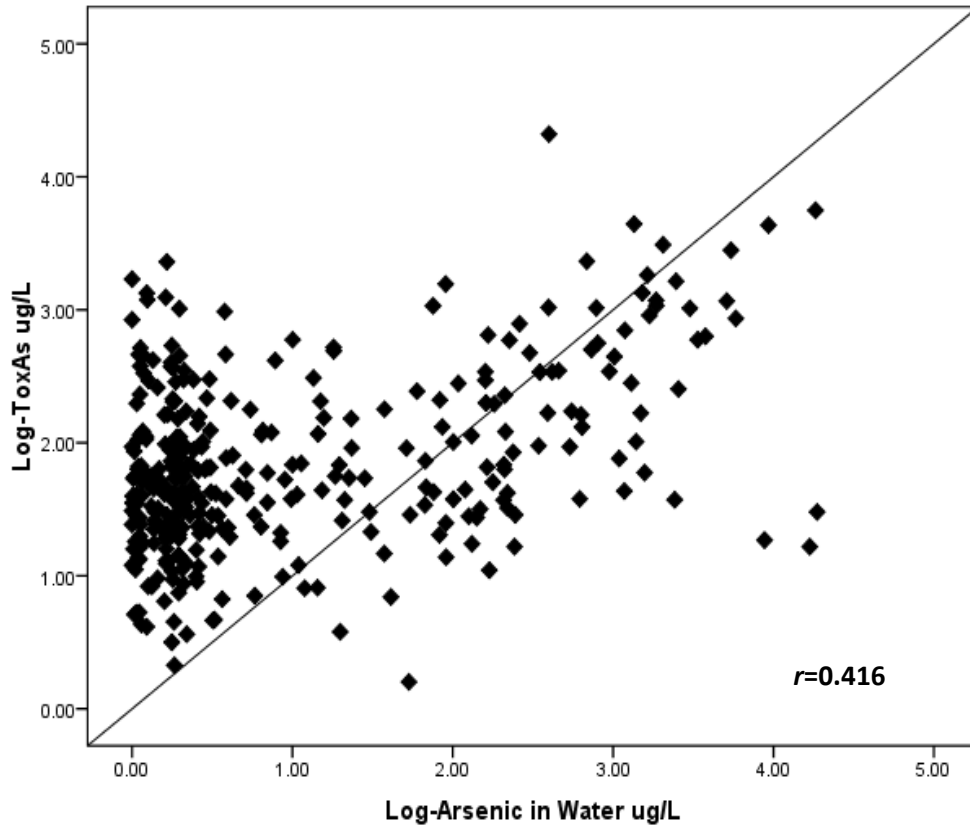


Figure 5-2. Urinary Arsenic vs. Arsenic in drinking water ($\geq 1\mu\text{g/L}$) (n=137).

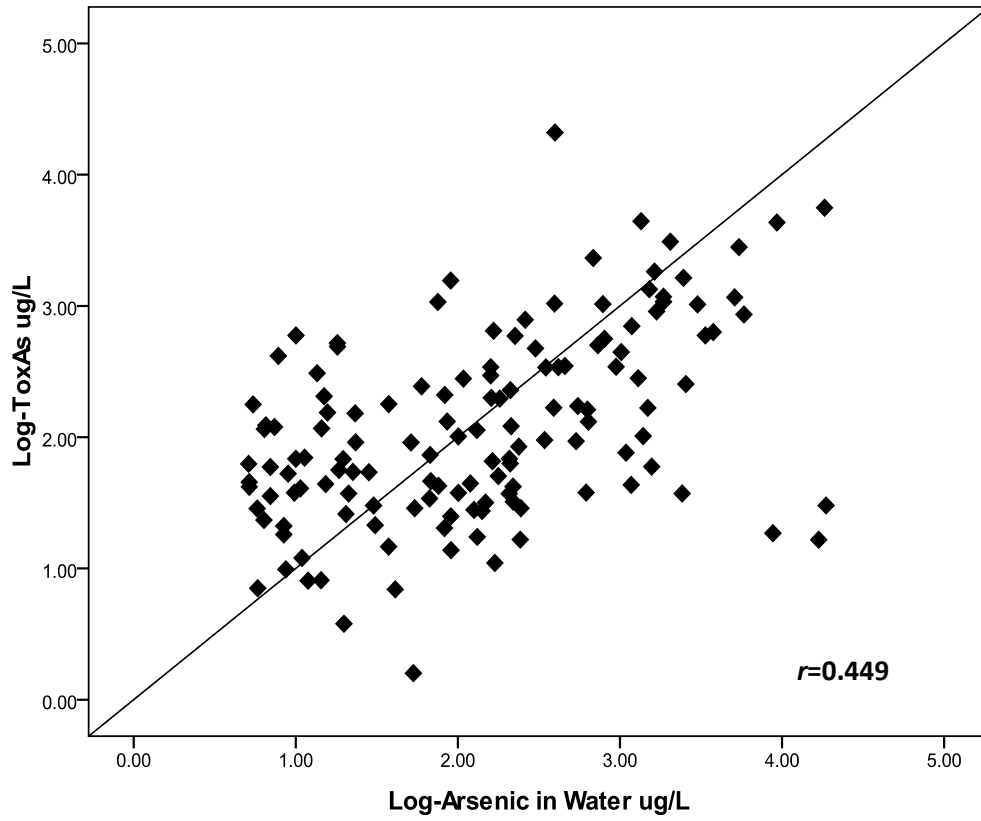
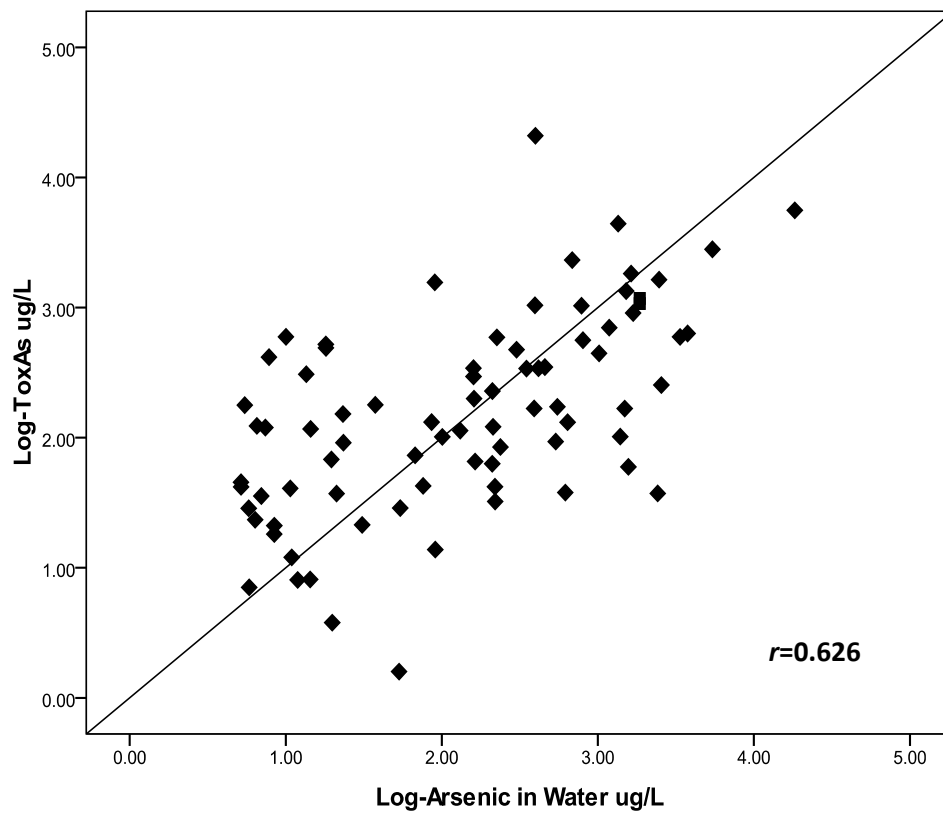


Figure 5-3. Urinary Arsenic vs. Arsenic in drinking water ($\geq 1\mu\text{g/L}$) and among those with water intake above median ($n=80$).



References

Adair B, Hudgens E, Shmitt M, Calderon R, Thomas D. 2006. Total arsenic concentrations in toenails quantified by two techniques provide a useful biomarker of chronic arsenic exposure in drinking water. *Environmental Research* 101:213-220.

Brima E, Parvez H, Jenkins R, Polya D, Gault A, Harrington C. 2006. Understanding arsenic metabolism through a comparative study of arsenic levels in the urine, hair and fingernails of healthy volunteers from three unexposed ethnic groups in the United Kingdom. *Toxicology and Applied Pharmacology* 216:122-130.

Byrne A, Slejkovec Z, Stijve T, Fay L, Gossler W, Gailer J, Irgolic K. 1995. Arsenobetaine and other arsenic species in mushrooms. *Applied Organometallic Chemistry* 9:305-313.

Byrne A, Tugek-hidaric M, Puri B, Irgolic K. 1991. Studies of the uptake and binding of trace metals in fungi Part II. Arsenic compounds in *Laccaria arnethystina*. *Applied Organometallic Chemistry* 5:25-32.

Caldwell K, Jones R, Verdon C, Jarrett J, Caudill S, Osterloh J. 2009. Levels of urinary total and speciated arsenic in the US population: National Health and Nutrition Examination Survey 2003-2004. *Journal of Exposure Science and Environmental Epidemiology* 19:59-68

Cantor K, Lubin J. 2007. Arsenic, internal cancers, and issues in inference from studies of low-level exposures in human populations. *Toxicology and Applied Pharmacology* 222:252-257.

Chen Y, Su H-J, Guo Y-L, Hsueh Y-M, Smith T, Ryan L, Lee M-S, Christiani D. 2003. Arsenic methylation and bladder cancer risk in Taiwan. *Cancer Causes and Control* 14:303-310.

Dabeka R, McKenzie A, Lacroix G, Cleouroux C, Bowe S, Graham R, Conacher H, Verdier P. 1993. Dietary intakes of lead, cadmium, arsenic, and fluoride by Canadian adults: A 24-hour duplicate diet study *Food Additives and Contamination* 4:89-102.

Das D, Chatterjee A, Mandal B, Samata G, Chakraborti D. 1995. Arsenic in ground water in six districts of West Bengal, India: The biggest arsenic calamity in the world. Part 2. Arsenic concentration in drinking water, hair, nails, urine, skin-scale and liver tissue (biopsy) of the affected people. *Analyst* 120:917-924.

Francesconi K, Tanggaar R, McKenzie CJ, Goessler W. 2002. Arsenic metabolites in human urine after ingestion of an arsenosugar. *Clinical Chemistry* 48:92-101.

- Haack S, Treccani S. 2000. Arsenic concentration and selected geochemical characteristics for ground water and aquifer materials in Southeastern Michigan. . In: US Geological Survey LM, editor.
- Heitkemper D, Vela N, Stewart K, Westphal C. 2001. Determination of total and speciated arsenic in rice by ion chromatography and inductively coupled plasma mass spectrometry. *Journal of Analytical Atomic Spectrometry* 16:299-306.
- Hopenhayn-Rich C, Biggs M, Smith A, Kalman D, Moore L. 1996. Methylation study of a population environmentally exposed to arsenic in drinking water. *Environmental Health Perspectives* 104:620-628.
- Karagas M, Tosteson T, Blum J. 2000. Measurement of low levels of arsenic exposure: A comparison of water and toenail concentrations. *American Journal of Epidemiology* 152:84-90.
- Karagas M, Stukel T, Tosteson T. 2001. Assessment of cancer risk and environmental levels of arsenic in New Hampshire. *International Journal of Hygiene and Environmental Health* 205:85-94.
- Karagas M, Nelson H, Kelsey K, Morris S, Blum J, Tosteson T, Carey M, Lu X, Le X. 2002. Urinary arsenic species in relation to drinking water, toenail arsenic concentrations and genetic polymorphism in GSTM1 in New Hampshire. In: Wilson S, Suk W, editors. *Biomarkers of Environmentally Associated Disease* NY: Lewis Publisher.
- Karagas M, Tosteson T, Morris JS, Demidenko E, Mott L, Heaney J, Schned A. 2004. Incidence of transitional cell carcinoma of the bladder cancer and arsenic exposure in New Hampshire. *Cancer, Causes and Control* 15:465-462.
- Lamont W. 2003. Concentration of inorganic arsenic in samples of white rice from the United States. *Journal of Food Composition and Analysis* 16:687-695.
- Lasky T, Sun W, Kadry A, Hoffman M. 2004. Mean total arsenic concentrations in chicken 1989-2000 and estimated exposure for consumer of chicken. *Environmental Health Perspectives* 112(1):18-21.
- Le X, Cullen W, Reimer K. 1993. Determination of urinary arsenic and impact of dietary arsenic intake. *Talanta* 40(2):185-193.
- Le X, Cullen W, Reimer K. 1994. Human urinary arsenic excretion following one-time ingestion of arsenosugars present in seaweed and arsenobetaine present in crab and shrimp. *Clinical Chemistry* 40:617-625.

Lindberg A, Goessler W, Gurzau E, Koppova K, Rudnai P, Kumar R, Fletcher T, Leonardi G, Slotova K, Gheorghiu E and others. 2006. Arsenic in Hungary, Romania and Slovakia. *Journal of Environmental Monitoring* 8:203-208.

Ma M, Le XC. 1998. Effect of arsenosugars ingestion on urinary arsenic speciation. *Clinical Chemistry* 44:539-550

Maskiell K, Heyworth J, McCaul K. 2006. Validation of a water consumption questionnaire for a study of the adverse health outcomes associated with disinfection by-products. *International Journal of Environmental Health Research* 16(2):145-153.

Meacher D, Dillencourt D, Bic L, Schoof R, Yost L, Eickhoff J, Farr C. 2002. Estimation of multimedia inorganic arsenic in take in the US population. *Human and Ecologic Risk Assessment* 8:1697-1721.

Meliker J. 2007. Lifetime exposure to arsenic in drinking water: application to a bladder cancer case-control study. Saabruken, Germany: Mueller Press.

Meza M, Kopplin M, Burgess J, Gandolfi A. 2004. Arsenic drinking water exposure and urinary excretion among adults in the Yaqui Valley, Sonora, Mexico. *Environmental Research* 96:119-126.

Nermell B, Lindberg A, Rahman M, Berglund M, Persson L, Arifeen S, Vahter M. 2008. Urinary arsenic concentration adjustment factors and malnutrition. *Environmental Research* 106(2):212-218.

Nriagu JO, Lawson G, Wong H, Aczue J. 1993. A protocol for minimizing contamination in the analysis of trace metals in Great Lakes waters. *Journal of Great Lakes Research* 19:175-182.

Phuong T, Chuong P, Khiem D, Kokot S. 1999. Elemental content of Vietnamese rice Part 1. Sampling, analysis and comparison with previous studies. *The Analyst* 124:553-560.

Polatajko A, Szpunar J. 2004. Speciation of arsenic in chicken meat by anion-exchange liquid chromatography with inductively coupled plasma-mass spectrometry. *Journal of AOAC International* 87(1):233-237.

Ritsema R, Dukan L, Roig-i Navarro T, van Leeuwen W, Oliviera N, Wolfs P, Lebet E. 1998. Speciation of arsenic compounds in urine by LC-ICP MS. *Applied Organometallic Chemistry* 12:591-599.

Schoof R, Yost L, Eickhoff J, Crecelius E, Cragin D, Meacher D, Menzel D. 1999. A market basket survey of inorganic arsenic in food. *Food Chemistry and Toxicology* 37:839-846.

- Slotnick MJ, Meliker J, AvRuskin GA, Ghosh D, Nriagu JO. 2007. Toenails as a biomarker of inorganic arsenic intake from drinking water and foods. *Journal of Toxicology and Environmental Health, Part A* 70:148-158.
- Smith P, Koch I, Reimer K. 2007. Arsenic speciation analysis of cultivated white button mushrooms (*Agaricus bisporus*) using high performance-liquid chromatography-inductively coupled plasma mass spectrometry and x-ray absorption spectroscopy. *Environmental Science Technology* 41:6947-6954.
- Steinmaus C, Bates M, Yuan Y, Kalman D, Atallah R, Rey O, Biggs M, Hopenhayn C, Moore L, Hoang B and others. 2006. Arsenic methylation and bladder cancer risk in case-control studies in Argentina and the United States. *Journal of Occupational and Environmental Medicine* 48:478-488.
- Steinmaus C, Carrigan K, Kalman D, Atallah R, Yuan Y, Smith A. 2005. Dietary intake and arsenic methylation in a U.S. population. *Environmental Health Perspectives* 113(9):1153-1159.
- Tao SS-H, Bolger P. 1998. Dietary arsenic intakes in the United States: FDA total diet study, September 1991-December 1996. *Food Additives and Contamination* 16:465-472.
- Tseng C-H. 2007. Arsenic methylation, urinary arsenic metabolites, and human diseases: current perspectives. *Journal of Environmental Science and Health Part C* 25:1-22.
- Vahter M. 1994. Species differences in the metabolism of arsenic compounds. *Applied Organometallic Chemistry*:175-182.
- Vahter M. 1999. Variation of human metabolism of arsenic. In: Chappell W, editor. *Arsenic exposure and health effect*. Oxford: Elsevier. p 267-269.
- Wright J, Murphy P, Nieuwenhuijsen M, Savitz D. 2006. The impact of water consumption, point-of-use filtration and exposure categorization on exposure misclassification of ingested drinking water contaminants. *Science of the Total Environment* 366(65-73).
- Wuilloud R, Kannamkumarath S, Caruso J. 2004. Speciation of essential and toxic elements in edible mushrooms: Size-exclusion chromatography separation with on-line UV-inductively coupled plasma mass spectrometry detection *Applied Organometallic Chemistry* 18:156-165.

Chapter VI

Arsenic Methylation in Southeastern Michigan

I. Introduction

Arsenic has been associated with bladder cancer if it is ingested at high levels (Aposhian and Aposhian 2006; NRC 2001). Since the main excretion pathway of arsenic is through urine, bioactive arsenic metabolites present in urine may be in direct contact with the bladder mucosa (Silverman et al. 1996) increasing the risk of bladder cancer.

One way the relationship between arsenic and cancer has been investigated is through toxicological experiments (Basu et al. 2001; Styblo et al. 2002). The current view of arsenic carcinogenesis is that there are several possible ways in which arsenic can act as a carcinogen. Different mechanisms and specific arsenic species involved in those mechanisms have been studied, suggesting that arsenic does not react directly with DNA (Cohen et al. 2006). Therefore, research has been directed into the effects of arsenic on epigenetic apparatus involved in cell proliferation.

Several mechanisms have been proposed: oxidative damage, perturbation of DNA methylation, alteration of DNA repair, chromosomal damage, modulation of signal transduction pathways, and alteration in gene transcription (Schoen et al 2004; Rana 2008). A number of in vitro studies have provided evidence of arsenic toxicity through oxidative damage. MMA[III] and DMA[III] are cytotoxic at low concentrations in human cells (Cohen et al. 2002; Styblo et al. 2000). Oxidative damage has also been found in exposed populations as discussed in Chapter 2 (Pi et al. 2002; Wu et al. 2001) while a recent study suggests that reactive oxygen species are not required in the induction of anti-oxidative response or apoptosis (Morales et al. 2009). Another possible cytotoxic mechanism is changes in DNA methylation. Perturbation of DNA methylation reactions is usually associated with carcinogenesis. Global hypomethylation is related with tumors, while hypermethylation has been reported in different sites of promoter genes (Warnecke and Bestor 2000). How arsenic interferes in the DNA methylation process is not completely understood, but scientists consider the possibility of an interaction with SAM, since this enzyme is needed in the arsenic and DNA methylation processes (Schoen et al. 2004).

Arsenic may produce chromosomal damage such as chromosomal missegregation, aneuploidy, and tumorigenesis (Liao et al. 2007; Chao et al. 2006). In animal models and in vitro studies, formation of micro-nuclei, chromosome deletions and aneuploidy have been characterized (Hei et al. 1998; Vega et al. 1995). Likewise, epidemiological studies in highly exposed populations have reported an increase of micronuclei incidence and sister chromatid exchanges in bladder cells (Basu et al. 2002;

Mahata et al. 2003). Even though a great number of studies have tried to investigate arsenic's mode of carcinogenicity, an exact mechanism is not yet known. Recently, Singh and DuMond (2007) reported direct molecular evidence of arsenic-induced mutations at low doses of exposure, a mechanism not recognized before. The possibility of arsenic-induced mutations, however, needs further investigation. The role of biomethylation in the toxicity and carcinogenicity of arsenic is the apparent toxicity of trivalent species and to some extent pentavalent DMA. Cohen et al. (2007) proposed a carcinogenic mechanism for DMA[V] carcinogenicity in the rat urothelium as a non-linear process that involves 1) reduction to DMA[III]; 2) concentration in the urine; 3) urothelial cytotoxicity; 4) regenerative cell proliferation; and 5) tumor induction (Fig 6-1). In this pathway hemoglobin and protein sulfhydryl groups play important roles, binding to DMA[III] during the transport of the metabolite through the body. The reaction of DMA[III] with sulfhydryl groups together with its instability could be a reason why DMA[III] is not frequently found in urine. On the other hand, evidence suggests that arsenic may need a second genotoxic agent in order to cause carcinogenic effects (Rossman et al. 2004; Liao et al. 2007). These studies suggest that arsenic in drinking water may need a carcinogenic partner, such as sunlight, in the induction of cancer.

Epidemiological studies have provided evidence associating arsenic and bladder cancer. Ingestion of high-arsenic artesian well water and cancers of the bladder, lung and liver have been reported in Taiwan (Chen et al. 1985; Chen et al. 1986). Chiou et al. 2001 reported a dose-response relationship between arsenic $<1\mu\text{g/L}$ to $>3000\mu\text{g/L}$ and bladder cancer in northeastern Taiwan after correcting for age, gender and smoking. In

other countries such as Argentina, Chile and Japan, studies have shown an association between exposure to arsenic and bladder cancer (Hopenhayn-Rich et al. 1996; Smith et al. 1998; Tsuda et al. 1995; Wu et al. 1989). Bladder cancer evaluation using urine as a biomarker has been conducted by different groups in Taiwan and the USA. Chen et al. (2003) evaluated 49 cases and 224 controls in the northeast of Taiwan. The study suggests an interaction between arsenic exposure and arsenic methylation capacity with respect to the development of bladder cancer. In another Taiwanese study, Pu et al. (2007) found that smoking interacts with the urinary arsenic profile to modify the risk of urothelial carcinoma among 117 cases and 313 age-matched controls. They found that non-smokers with high PMI and low SMI had 6.6-fold (95% CI 2.5-17.4) higher risk than the referent group. Steinmaus et al. (2006) found data consistent with Chen and colleagues suggesting that some individuals who excrete a higher proportion of ingested arsenic as MMA[V] are more susceptible to bladder cancer. This analysis was performed with individuals from the USA (118 cases and 328 controls) and Argentina (114 cases and 114 controls).

Based on the amount of MMA[V] and MMA[III] in urine, epidemiological studies have been able to suggest an association between urinary arsenic metabolites and bladder cancer. However, studies have not been able to show clear significant differences in exposure between bladder cancer cases and controls, especially in populations exposed to low levels of arsenic. In addition, these studies and others using different biomarkers have been limited by sample size, exposure misclassification and variables such as smoking. In the Michigan Bladder Cancer Case-Control Study, cigarette

smoking information and lifetime exposures have been collected in order to assess the effect of these variables in the study population (Meliker 2007). Using variables such as smoking habits, age, gender, and amount of arsenic in water, the goal of this chapter is to determine if methylation capacity, based on urinary arsenic metabolites, is related to bladder cancer.

II. Methods

Study Subjects

Spot urine samples were collected from a sub-sample ($n=389$) of participants in southeastern Michigan enrolled in a case-control study of arsenic exposure and bladder cancer. Cases were obtained from the Michigan State Cancer Registry and were frequency-matched by age, race and gender with controls selected through a random-digit dialing procedure. Cases as well as controls were recruited on the basis that they had been living in the study area for at least five consecutive years prior to recruitment. The study area included the southeastern counties of Genesee, Huron, Ingham, Jackson, Lapeer, Livingston, Oakland, Salinac, Shiawassee, Tuscola, and Washtenaw. The sub-sample used in this study included 167 cases and 219 controls recruited from June 2005 through May 2007. Each participant signed an informed consent and random identification numbers were assigned to each participant to maintain confidentiality. This study was approved by the University of Michigan Health Sciences Institutional Review Board (IRB-Health). Further details on the selection of subjects and response rate are described elsewhere (Meliker 2007). Table 6-1 shows demographic

characteristics for the total population and the subsample population used in this study. In this chapter separate analyses were performed to address this issue. All cases and controls were administered phone interviews to obtain demographic information, medical history, life-style habits, and water consumption patterns (Slotnick et al. 2007).

Collection and Analysis of Water Samples for Lifetime Exposure Calculations

During home visits, research team members collected tap water samples. Participants identified their main drinking water sources and additional sources that they use for cooking, coffee, and other beverages if different. Water samples were collected in low-density polyethylene (LDPE) bottles and acid-washed for determination of trace metals (Nriagu et al. 1993). Samples were immediately stored on ice until arriving to the laboratory where they were acidified with 100µg/L trace-metal grade HNO₃ (Fisher Chemical) and stored at 4°F until analysis. Throughout water collection procedures, non-powdered vinyl gloves were worn and water samples were stored in plastic zippered bags to minimize contamination. In addition, field blanks and replicates were collected every day.

All samples were analyzed at the University of Michigan School of Public Health using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent Technologies Model 7500c). Additional details of the analytical protocol have been explained in Chapters 3 and 4. Details on exposure metrics calculation and individual lifetime exposure assessment has been previously described (Meliker et al. 2007). Briefly, inorganic arsenic concentrations in past residences were obtained from public suppliers,

the Michigan Department of Environmental Quality (MDEQ), or estimated from a geostatistically-derived dataset. Fluid and food consumption data obtained from the phone interview were integrated with mobility histories and arsenic water concentrations to calculate estimates of inorganic arsenic intake over the adult life-course.

Collection and Analysis of Urine Samples

The research team provided participants with materials and instructions for urine collection. Materials included a plastic bag packed with an acid-washed polyethylene urine cup, antibacterial wipes, gloves, and urine collection instructions. Participants were asked to collect a spot urine sample during field visits. Spot samples were immediately frozen in dry ice brought along by the research team and then transported to the laboratory. Spot samples were stored in the laboratory at -20°C until analysis. More details on sample collection and storage are explained in Chapter 3. Before analysis, samples were filtered using a 0.22µm syringe filter and no preservatives were added. Specific gravity measures were taken immediately after samples were thawed and reached room temperature; freezing a urine sample does not change its specific gravity (Nermell et al. 2008).

Urine samples were processed through a 5µm 250x4.6mm column (Phenomenex, Torrance, CA, USA). The mobile phase contained 4% (v/v) methanol, 5mM tetrabutylammonium hydroxide (TBAH), and 10 mM ammonium phosphate at pH 9.5. The HPLC system (Alltech) was coupled to the ICP-MS unit. Detection limits for

arsenic species by the method used were as follows: AsB, 0.06 μ g/L; As[III], 0.112 μ g/L; As[V], 0.147 μ g/L; MMA[V], 0.117 μ g/L; and DMA[V], 0.076 μ g/L. Urine certified reference material from the Japanese National Institute for Environmental Studies (NIES NO. 18, Tsukuba, Ibaraki, Japan) was used as reference standards. Details on this method are explained in Chapter 3.

Statistical Analysis

Since urinary arsenic values show a non-normal distribution, nonparametric analyses were used to compare differences in the urinary arsenic profile between cases and controls. For this paper, the sum of As[III], As[V], MMA[V] and DMA[V] was used to designate total arsenic species (*TotAs*). The sum of As[III] and As[V] was used as the total of inorganic arsenic (InAs) because it provides a more stable measure of inorganic arsenic in urine, since these two species may interconvert while in urine. Since MMA[III] and DMA[III] were not detected in any of the samples, MMA and DMA refer only to the respective pentavalent species, unless otherwise stated. Concentrations below the detection level (BDL) were set at one half the detection limit (Table 6-2). The relative proportion of arsenic in each species (%InAs, %MMA, and %DMA) was calculated by dividing the concentration of arsenic in each species by the concentration of InAs, MMA, and DMA combined. The primary methylation index (PMI) and secondary methylation index (SMI) were calculated by dividing absolute values of MMA by inorganic arsenic and absolute values of DMA by MMA, respectively. Relative proportions of arsenic species, PMI, and SMI are calculated to evaluate methylation capacity. In order to

compare studies and to evaluate urinary arsenic excretion in relation to ingested arsenic, the concentration of arsenic in urine and water was compared ($\text{TotAs}/[\text{As}]_{\text{water}}$). A ratio below one is indicating longer retention time of arsenic in the body (Lindberg et al. 2008).

Unconditional logistic regression models were used to estimate the multivariate adjusted and unadjusted odds ratios (OR) and the 95% confidence intervals (CI). Initially, the cutoff points for continuous variables were the respective tertiles of the controls. The joint effects of urinary arsenic species and cigarette smoking on the risk of bladder cancer were evaluated in both multiplicative and additive scales. However, no significant interactions were found between smoking and TotAs in logistic regression analyses and no multiplicative terms were included in the models. Separate analyses were carried out for ever- and never-smokers. The adjustment for smoking was performed using the average number of cigarettes smoked per day. Given the strong relationship of gender and bladder cancer, separate analyses were carried out for the male population. However, these results were very similar to those from the total sample.

Given the results of previous studies on arsenic methylation, we evaluated %MMA in further detail. The category cut off points for %MMA was set to the %MMA median (14.2%). Those cutoff values were the approximate averages of the cutoff points used in other studies of methylation and arsenic-related effects (Chen et al. 2003; Del Razo et al. 1997; Steinmaus et al. 2006). Lifetime exposure was stratified in three

categories, <1µg/L, 1-10µg/L, and >10µg/L to explore exposure in the low-to-moderate exposure level. Since 93 of the 386 individuals (24%) in the study had below detection levels of MMA, truncated data or censored data regression (CDR) analyses were performed. CDR evaluates the variance introduced to the model if individuals with arsenic levels below the level of detection were included in the regression models. Truncation reduces the variance compared with the variance of the untruncated distribution. Nevertheless, no difference was found in the results of the logistic regression models when individuals with levels below detection were included. Therefore, all individuals were included in the logistic regression analyses.

III. Results

The sociodemographic characteristics of cases and controls are shown in Table 6-3. No significant differences were detected between this sample and the total sample presented in Table 6-1 except for a relatively lower proportion of women in the parent study. Women were oversampled towards the end of the parent study when urine samples were also collected. Separate analyses were performed using only the male individuals to assess differences. However, no significant differences were found and females and males were evaluated together in this study.

Males had four times higher risk of having bladder cancer than females. This risk, however, only applies to the individuals in this sample since this is no longer a population-based study. Age, race and education did not significantly affect the risk of bladder cancer. In comparing cases and controls in term of smoking duration (years) and

daily smoking amount (cigarettes per day), cigarette smokers had a significantly higher bladder cancer risk than non-smokers in a dose dependent manner. Heavy smokers who smoked for more than 20 years, 0.5 packs per day or 1 pack per day had a 3.1-, 2.3- and 3.0-fold risk, respectively, compared to non-smokers. Modest smokers (>10≤20 years) however, did not have a significant risk compared to non-smokers. Odds ratios significantly increased for smokers when smoking status was evaluated among arsenic lifetime exposure categories (Table 6-4). The risk increased from 1.6 to 4.2 in the 1-10µg/L category whereas this risk decreased in the >10µg/L exposure group category (0.57). However, this category has a relatively small number of individuals when compared with the <1µg/L and 1-10µg/L categories.

Table 6-5 shows values for all species and TotAs stratified by disease status, gender, age, smoking status, race, and education. Average values for TotAs, InAs, MMA and DMA for all individuals were 7.11µg/L, 0.48µg/L, 1.00µg/L, and 5.63µg/L, respectively. No significant differences were found among absolute values in the different strata. Table 6-6 shows the mean relative proportions of each arsenic species stratified by the same variables. Bladder cancer cases and smokers had significantly higher %InAs. Smokers also had significantly lower %DMA. Men had a significantly higher %InAs and %MMA and lower %DMA. No differences were found among PMI or SMI values among different strata except for a significantly higher SMI in females.

To assess methylation capacity %MMA was explored. Lifetime exposure to arsenic was added to the models and %MMA was categorized as below and above the

median value of 14.2%. This analysis was also performed dichotomizing the sample by ever- or never-smokers. Significant associations were observed in individuals with a MMA% higher than the median value and lifetime exposure in the categories 1-10µg/L and >10µg/L, 0.92(0.84-0.99) and 0.70(0.49-0.98), respectively (Table 6-7). However, significant odds ratios below one suggest that an increase in urinary arsenic excretion decreases the risk of being a bladder cancer case in this population. An alternative explanation is that bladder cancer cases in this population excrete less arsenic than their respective controls. These results were similar for the smoker and non-smoker population (Tables 6-8-6-9). The median of the TotAs/[As]water ratio for individuals with arsenic concentrations <1µg/L was 16 while individuals with arsenic concentrations ≥1µg/L was 0.88. The TotAs/[As]water ratio was not associated with age, gender, race, education or smoking status.

IV. Discussion

We have identified significant associations between %MMA and bladder cancer in an area where individuals are exposed to elevated levels of arsenic in drinking water (10-50µg/L). Similar results have been found in other studies evaluating populations exposed to elevated levels of arsenic in drinking water and bladder cancer (Table 6-10). The study assessing arsenic exposure in Taiwan, (Chen et al. 2003), presents results for the MMA/DMA ratio and stratified by cumulative arsenic exposure (CAE) defined as $\sum[(\text{average arsenic concentration of artesian well water in mg/L}) * (\text{duration of consuming artesian well water in years})]$. Given that the interindividual variability in the MMA/DMA ratio is more dependent on %MMA than %DMA (Vahter 1999a), the relative

risks identified for MMA/DMA ratio are more likely caused by %MMA than %DMA (Steinmaus et al. 2006). These studies are too small in sample size to be able to make significant inferences from the data. Therefore, the evidence for possible associations between methylation patterns and arsenic-related bladder cancer has to be cautiously interpreted.

The main difference between our study and the studies shown in Table 6-10 is that urinary arsenic levels in our study were lower in bladder cancer cases than in controls. Few studies have reported this finding (Del Razo et al. 1997; Lindberg et al. 2008). One possible explanation for this finding is that elevated exposure to arsenic inhibits the methyltransferases involved in arsenic methylation and inhibition of methyltransferases has been showed to lead to longer retention times of arsenic in the body (Vahter 1999b). This observation may be supported in our study by the decreasing TotAs/[As]water ratio. However, this ratio could be affected by factors other than arsenic concentration in participant's drinking water. Other sources of arsenic such as consumption of water outside home, consumption of other beverages, cooking, and other sources of exposure may influence the appropriate interpretation of this ratio, incorporating random error in the measurement (Forssén et al. 2008). This error may also lead to confidence intervals that are too narrow (Höfler 2005).

Experimental studies on human subjects receiving specified doses of inorganic arsenic indicated that urinary MMA and InAs metabolites have similar associations with the overall arsenic elimination, while that of DMA is different (Hopenhayn-Rich et al.

1996). The urinary excretion of total arsenic metabolites decreased with increasing percents of both InAs and MMA in urine, but decreased with an increasing percent of DMA. In our study, bladder cancer cases at the upper tertile excrete significantly less %MMA than controls ($p=0.02$), suggesting that cases are retaining arsenic for a longer period of time. Most studies addressing arsenic methylation and arsenic-related diseases report that healthy individuals have lower %InAs and %MMA (Chen et al. 2003; Pu et al. 2007; Steinmaus et al. 2006; Yu et al. 2000).

Individuals with a lifetime exposure in the highest exposure category ($>10\mu\text{g/L}$) have lower odds ratios than the middle exposure ($1-10\mu\text{g/L}$) category (0.92 vs. 0.70). Sample size in this study, as well as other similar reported studies, becomes a limitation when interpreting results. The highest exposure category ($>10\mu\text{g/L}$) has a notably smaller amount of individuals ($n=37$) than other categories such as the $1-10\mu\text{g/L}$ category ($n=206$). Even though positive interaction between arsenic exposure and bladder cancer has been reported, studies are usually small in size and the recruitment after diagnosis may bring questions about the effect of the exposure before and after the disease development. Case-control studies use patients who already have a disease or other condition and look back to see if there are characteristics of these patients that differ from those who don't have the disease. Given that, cases and controls may differ in exposure periods, length of exposure, time of diagnosis, and stage of cancer. These differences may influence the interpretation of results. For example, individuals with higher exposure may have a more aggressive cancer and die before recruitment.

On the other hand, well designed case-control studies can offer important details about individual exposure and disease. Urine collection and analysis involves complex logistics and requires an extraordinary laboratory capacity. Although no significant inference can be drawn from our data, power calculations were performed. Power calculations depend on a pre-defined "high exposure" threshold that without analyzing all the data, is hard to determine (Meliker 2006; Xue and Hoover 2009). Using the 219 control subjects in the study we performed power calculations assuming an exposure rate in the controls ranging from 5% to 8% and using a goal of 80% power. From those 219 subjects, 5.5% drink water with arsenic $\geq 10\mu\text{g/L}$ and 7.8% drink water with arsenic $> 20\mu\text{g/L}$. If the relative risk (RR) was 2.2, 255 case-control pairs would be necessary to observe any difference between $10\mu\text{g/L}$ and $20\mu\text{g/L}$. If the relative risk was 2.0, 360 case-control pairs would be necessary to observe any difference between $10\mu\text{g/L}$ and $20\mu\text{g/L}$. If RR was 1.8, 525 case-control pairs would be necessary, and if $\text{RR}=1.5$, 1,150 case-control pairs would be necessary to observe any difference between $10\mu\text{g/L}$ and $20\mu\text{g/L}$.

One important factor influencing arsenic metabolism is genetic polymorphisms in genes coding for enzymes involved in arsenic metabolism. Several studies have reported differences in genes coding for arsenic(III)-methyltransferases (AS3MT) and genes involved in one-carbon metabolism affect arsenic metabolism (Lindberg et al. 2007; Sclawicke et al. 2009). DNA methyltransferases are responsible for most of the methylation of the human genome and is necessary and sufficient for maintaining global methylation (Robert et al. 2003). Arsenic has also been shown to induce DNA

hypomethylation by continuous methyl depletion through consuming S-adenosyl-methionine (SAM) (Sciandrello et al. 2004). Our findings suggest the importance of genetically determined inter-individual variability in the capacity of human arsenic methylation. However, since our study is not evaluating specific polymorphisms these findings require additional investigation. Another factor that may influence arsenic methylation is the nutritional status of participants. Lower intakes of dietary methyl groups can result in lower arsenic methylation (Vahter and Marafante, 1987). The evaluation of nutritional status and arsenic methylation in USA is an area of research that has to be addressed in future epidemiological studies.

Our data is consistent with other published work that smoking is associated with an increased risk of bladder cancer in a dose-dependent manner (Pu et al. 2007; Zeegers et al. 2004). Moreover, adding lifetime exposure to arsenic in the assessment of the risk of cancer among smokers increased the odds ratio from 2.6 to 4.2, in the middle category of 1-10 μ g/L. This increase in the odds ratio value suggests that smoking is not the only major environmental source of arsenic contamination since the risk increased when arsenic exposure was added to the model. This observation is also supported by a moderately significant odds ratio for the non-smoker population of our study. Pu et al. (2007) has reported that differential carcinogenic effects of the urinary arsenic profile were more prominent in non-smokers than in smokers based on differences between PMI and SMI among cases and controls.

On the other hand, even in the event of increasing sample size to adjust misclassification, the magnitude of the bias is dependent on the prevalence of the misclassified factor (Garcia-Closas et al. 1999), in this study, exposure to arsenic through drinking water. Misclassification in life-time exposure to arsenic calculations in the parent study was addressed by using stratification of fluid consumption estimates and time-aggregated analyses (Meliker 2007). The number of individuals in the sample used in this study does not provide us with enough power to stratify by fluid consumption. Exposure misclassification is still possible, but, is likely to be nondifferential and biased toward the null.

Nevertheless, active smoking is the strongest environmental risk for bladder cancer, contributing to more than 50% of the cases (Ferlay et al. 2007). Epigenetic gene methylation has been also linked to exposure to tobacco smoke (Satta et al. 2008; Walser et al. 2008). Smoking and inorganic arsenic have been associated with hypermethylation of gene-specific promoters in bladder cancer (Marsit et al. 2005). Accumulation of hypermethylation events may be related to the aging process and increased duration in carcinogen exposure (Crawford 2008). In addition, epigenetic alterations are not the only mechanism that arsenic and tobacco smoke share as carcinogens. Another possible carcinogenic mechanism for both toxics involves the formation of DNA adducts (Tezuka et al. 1993; Vineis et al. 1996). For the relationship of methylation and smoking status, we found associations at the univariate and multivariate level. Other studies have also found significant results in multivariate

analyses (Bates et al. 2004; Steinmaus et al. 2003). As a result, it is not surprising that cigarette smoking and arsenic exposure may interact in an additive and synergistic way.

Another important environmental factor when evaluating arsenic methylation and bladder cancer is gender (Vahter et al 2007). Gender may influence the risk of bladder cancer and the methylation capacity in our population. Males have a higher risk of bladder cancer (Crawford 2008). Although OR's did not change in our study when examining only the male population, there were significant differences in the urinary profile between males and females. Most studies suggest that women have a better methylation capacity than men (Chung et al. 2008; Lindberg et al. 2008). Women had a higher %DMA[V] and SMI, and a lower urinary total arsenic and %MMA[V] than men in a study in the Blackfoot Disease area (Tseng et al. 2005). Steinmaus et al. (2006) found that males had a higher %InAs and %MMA[V] and a lower %DMA[V] than women in a population exposed to moderate levels of arsenic (50-100µg/L) in USA. Our results suggest that women in our sample have a better methylation capacity than men based on significant metabolites percentages and SMI differences. Some studies suggest that sex hormones have an effect on arsenic methylation because women who are childbearing have higher arsenic methylation than men (Lindberg et al. 2007). Another possible mechanism is that choline, which is a methyl donor, can be derived from phosphatidylcholine. In a recent study, phosphatidylcholine is apparently up-regulated by estrogen, probably explaining the better methylation of arsenic in women (Fisher et al. 2007).

A common analytical challenge when doing arsenic speciation is the number of individuals with MMA values below the limit of detection. Most studies replace below-detection values with half of the detection level for each species (Christian et al. 2006; Steinmaus et al. 2006), while others exclude those samples (Karagas et al. 2002). In studies of intraindividual variability, where more than one sample was collected, mixed models that accommodate missing observations can be used (Calderon et al. 1999). Our analytical protocol provided a very low detection limit for MMA (0.1 μ g/L). Nonetheless, 24% of the individuals had below-detection levels of MMA. Variability in the MMA excretion among individuals and populations is a reflection of the arsenic metabolism. Therefore, excluding these individuals from the analysis will inflate the %MMA, biasing the results. However, assessing the data with uncensored data-analyses may help determine the amount of bias if these individuals are included. Recently, investigators have suggested Bayesian hierarchical analysis as a method that not only integrates different exposure pathways, but also accommodates large numbers of missing data (Santner et al. 2008). This method however, needs further development.

In conclusion, significant associations were found between urinary arsenic species and bladder cancer in our sample. The lower OR's values may suggest inhibition of the methyltransferases involved in arsenic methylation. However our study does not have adequate sample size to make conclusions. Our data agreed with previous studies which suggested that females have better methylation capacity and smokers have a higher risk of bladder cancer. It is important to assess misclassification before including lifetime exposure estimations when assessing arsenic methylation. These exposure estimates

and identification of exposure periods also influence the accuracy of estimating true risk, especially in populations exposed to low levels of arsenic in drinking water. Since the incidence of arsenic-related diseases is not just explained by the dosage of arsenic exposure, more studies in methylation patterns are needed, especially in populations exposed to elevated levels of arsenic in drinking water.

Fig 6-1. Proposed Mode of Action for Arsenic-Induced Urinary Bladder Carcinogenesis (Cohen et al. 2007).

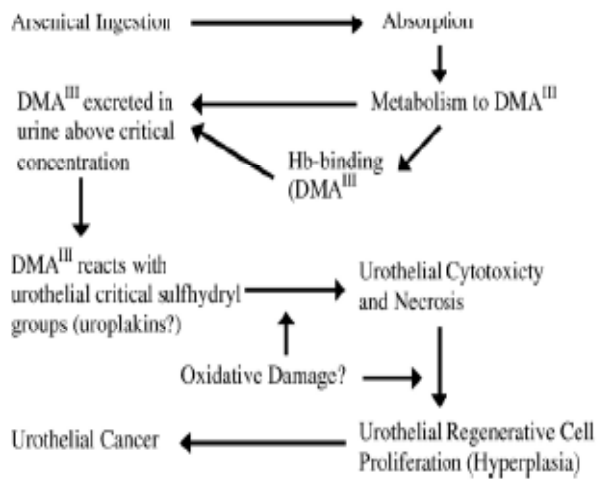


Table 6-1. Comparison of demographic characteristics between the total sample (parent project) and the subsample evaluated in this research.

Variable	Total Sample	Subsample
Age		
<55	156(16%)	29(7.5%)
55-64	271(27.7%)	123(31.9%)
64-74	325(33.3%)	115(29.8%)
>74	225(23%)	119(30.8%)
Gender		
Male	733(75%)	254(65.8%)
Females	244 (25%)	132(34.2%)
Race		
White	898(91.9%)	366(94.8%)
Other	79(8.1%)	20(5.2%)
Education		
High School or Less	309(31.6%)	111(28.8%)
Some College	268(27.4%)	108(28.0%)
College Graduate or More	400(40.9%)	166(43.0%)
Disease Status		
Cases	411(42.1%)	167(43.2%)
Controls	566(57.9%)	219(56.7%)
Smoking Status		
No	367(37.6%)	149(37.0%)
Yes	610(62.4%)	243(63.0%)
Lifetime exposure to arsenic	3.1µg/L ^a (5.1) ^b	3.7µg/L (5.4)

^aArithmetic Mean ^bStandard Deviation

Table 6-2. Amount of samples below detection limits by individual species.

Arsenic Species	Number of Samples below the Detection Level	%	Detection Limit (µg/L)
InAs	197	51.0	0.112
MMA	93	24.1	0.117
DMA	2	0.52	0.076
AsB	2	0.52	0.060

Table 6-3. Sociodemographic characteristics of 167 bladder cancer cases and 219 non-cancer controls.

Variable	Group	Control	Cases	Age and Gender Adjusted OR(95%)	p-value
Gender	Female	100	32	1.0	-
	Male	119	135	4.2(2.6-6.9)	<0.0001
Age(years)	<55	18	11	1.0	-
	55-64	60	63	1.1(0.45-2.8)	0.6751
	64-74	65	50	0.78(0.31-2.0)	0.7894
	>74	76	43	0.57(0.23-1.4)	0.2166
Race	White	238	128	1.0	-
	Others	13	7	0.92(0.30-2.8)	0.5203
Education	High School or Less	59	52	1.0	-
	Some College	59	49	0.96(0.54-1.7)	0.8979
	College Graduate or More	101	65	0.73(0.43-1.2)	0.2519
Smoking Status	No	105	38	1.0	-
	Yes	114	129	2.6(1.6-4.17)	<0.0001
Duration of cigarettes smoking (years)	0	105	42	1.0	-
	0.1-10	18	12	1.5(0.66-3.6)	0.3242
	>10≤20	28	19	1.5(0.71-3.0)	0.3030
	>20	67	94	3.1(1.9-5.2)	<0.0001
Amount of cigarettes per day	0	105	42	1.0	-
	1-10	26	13	1.3(0.60-3.0)	0.4779
	>10≤20	52	58	2.3(1.3-3.9)	0.0032
	>20	36	54	3.0(1.7-5.4)	0.0002

Table 6-4. Bladder cancer odds ratio for smoking status stratified by lifetime arsenic exposure.

Variable	Controls	Cases	OR(95%CI)	p-value
<1.0µg/L	82	61	1.63(0.79-3.43)	0.1816
1-10µg/L	118	88	4.2(2.1-8.5)	<0.0001
>10µg/L	19	18	0.57(0.09-3.6)	0.5524

Adjusted by age, gender and education

Table 6-5. Univariate analysis of the numeric values of each arsenic species ($\mu\text{g/L}$).

Variable	N	%	TotAs $\mu\text{g/L}$	InAs $\mu\text{g/L}$	MMA $\mu\text{g/L}$	DMA $\mu\text{g/L}$
All	386	100	7.11	0.48	1.0	5.63
Bladder Cancer						
Cases	167	43.3	6.55	0.48	1.05	6.01
Controls	219	56.7	7.54	0.49	0.94	5.13
Gender						
Women	132	33.9	6.97	0.51	1.09	*5.36
Men	254	66.1	7.40	0.43	0.82	6.15
Smoking						
No	142	33.2	6.80	0.45	0.95	5.40
Yes	241	65.8	7.33	0.51	1.04	5.78
Age						
<55	30	7.5	7.62	0.51	1.11	6.00
55-64	123	31.9	7.33	0.55	0.98	5.81
65-75	115	29.8	6.21	0.40	0.85	4.96
≥ 75	119	30.8	7.63	0.49	1.15	5.99
Race						
White	366	94.8	6.97	0.46	0.99	5.52
Other	17	4.2	10.31	1.07	1.24	8.00
Education						
High School or Less	111	28.8	8.19	0.54	1.37	6.28
Some College	108	28.0	7.40	0.63	1.20	5.57
College Graduate or More	166	43.0	6.90	0.44	0.90	5.56

*Significant at $\alpha=0.05$

Table 6-6. Univariate analysis of the proportions of each arsenic species.

Variable	N	%	%InAs ^b	%DMA[V]	%MMA[V]	PMI ^c	SMI ^d
All	386	100	8.1	78.0	13.9	3.0	27.0
Bladder Cancer							
Cases	167	43.3	*9.2	76.9	13.9	2.6	19.9
Controls	219	56.7	7.3	78.8	13.9	3.3	32.4
Gender							
Women	132	33.9	*6.9	*81.2	11.9	2.6	*45.8
Men	254	66.1	8.4	76.3	14.9	3.3	17.2
Smoking^a							
Never	142	33.2	*6.9	*79.5	13.6	2.9	25.5
Ever	241	65.8	8.9	76.9	14.2	3.1	27.8
Age							
<55	29	7.5	8.8	77.1	14.1	2.2	30.3
55-64	23	31.9	7.7	78.8	13.5	2.7	29.5
65-75	115	29.8	8.1	77.1	14.8	3.1	18.8
≥75	119	30.8	8.4	77.1	13.5	3.5	31.4
Lifetime Arsenic (µg/L)							
<1	143	37.0	7.8	80.1	12.3	2.5	34.3
1-10	206	53.4	8.6	76.5	14.9	3.3	23.7
>10	37	9.6	7.5	78.2	14.3	3.2	14.0

^aThree individuals with missing information

^bAs[III]+As[V]

^cPrimary Methylation Index =(MMA/InAs)

^dSecondary Methylation Index =(DMA/MMA)

*Significant values at $\alpha=0.05$

Table 6-7. Bladder cancer odds ratios for median levels of %MMA stratified by lifetime exposure including all individuals.

Subjects	Cases	Controls	OR(95%CI)	p-value
Median %MMA				
<14.2	83	110	1	-
≥14.2	84	109	0.93 (0.87-0.99)	0.0171
<1.0µg/L				
<14.2 %MMA	33	49	1	-
≥14.2 %MMA	28	33	0.99(0.901-1.2)	0.9128
1-10 µg/L				
<14.2 %MMA	43	51	1	-
≥14.2 %MMA	45	67	0.92(0.84-0.99)	0.0557
≥10 µg/L				
<14.2 %MMA	7	10	1	-
≥14.2 %MMA	11	9	0.70(0.49-0.98)	0.0398

14.2 =%MMA Median

Adjusted by age, gender, and education

Table 6-8. Bladder cancer odds Ratios for median levels of %MMA stratified by lifetime exposure in the non-smokers group.

Subjects	Cases	Controls	OR(95%CI)	p-value
Median %MMA				
<14.2	17	53	1	-
≥14.2	21	51	0.92(0.82-1.0)	0.1657
<1.0µg/L				
<14.2 %MMA	8	21	1	-
≥14.2 %MMA	10	16	1.0(0.91-1.2)	0.6290
1-10 µg/L				
<14.2 %MMA	9	25	1	-
≥14.2 %MMA	6	33	0.61(0.40-0.93)	0.0240
≥10 µg/L				
<14.2 %MMA	5	7	-	-
≥14.2 %MMA	-	2	-	-

14.2 =%MMA Median

Adjusted by age, gender, and education

Table 6-9. Bladder cancer odds ratios for median levels of %MMA stratified by lifetime exposure in the smokers group.

Subjects	Cases	Controls	OR(95%CI)	p-value
Median %MMA				
<14.2	64	56	1	-
≥14.2	63	58	0.92(0.85-0.99)	0.0350
<1.0µg/L				
<14.2 %MMA	25	28	1	-
≥14.2 %MMA	18	17	0.94(0.81-1.1)	0.4551
1-10 µg/L				
<14.2 %MMA	25	32	1	-
≥14.2 %MMA	34	39	0.92(0.84-1.0)	0.0772
≥10 µg/L				
<14.2 %MMA	7	3	1	-
≥14.2 %MMA	6	7	0.73(0.49-1.1)	0.1211

14.2 =%MMA Median

Adjusted by age, gender, and education, and average years smoking

Table 6-10. Studies in populations exposed to low-to-moderate levels of arsenic in drinking water (<150µg/L).

Study	Population	Finding
Chen et al. 2003 49 cases/224 controls	Taiwan	MMA/DMA>0.21 CAE≤2 ^a Reference CAE>2-12 0.54(0.09-3.33) CAE>12 1.12(1.12-16.01)
Steinmaus et al. 2006 118 cases/328 controls	California and Nevada, US	Arsenic Intake>100µg/d ^b Ave. %MMA <14.9 Reference Ave. %MMA ≥14.9 2.70 (0.39-18.6) ^c High %MMA<17.7 Reference High %MMA≥17.7 6.24(0.89-43.7)
Pu et al. 2007 177 cases/328 controls	Taiwan	%MMA ≤3.0 1.0 %MMA 3.1-9.2 0.9 (0.5-1.9) %MMA >9.2 2.8 (1.6-4.8)
Southeastern Michigan 167 cases/254 controls	Michigan, US	%MMA>14.2 and lifetime exposure 1-10µg/L 0.918(0.84-0.99) %MMA>14.2 and lifetime exposure >10µg/L 0.696(0.49-0.98)

^aCumulative arsenic exposure in mg/l-yr

^bOdds ratios are for the average %MMA recorded for each subject

^cOdds ratios are for the highest %MMA recorded for each subject

References

- Aposhian H, Aposhian M. 2006. Arsenic toxicology: five questions. *Chemical Research in Toxicology* 19:1-15.
- Basu A, Mahata J, Gupta S, Giri A. 2001. Genetic toxicology of paradoxical human carcinogen, arsenic: a review. . *Mutation Research* 488:171-194.
- Basu A, Mahata J, Gupta S, Giri A, Sarkar J, Poddar G, Nandy N, Sarkar P, Dutta O, Banerjee A and others. 2002. Enhanced frequency of micronuclei in individuals exposed to arsenic through drinking water in West Bengal, India. *Mutation Research* 516:29-40.
- Bates M, Rey O, Biggs M, Hopenhayn C, Moore L, Kalman D, Steinmaus C, Smith A. 2004. Case-control study of bladder cancer and exposure to arsenic in Argentina. *American Journal of Epidemiology* 159(4):381-389.
- Calderon R, Hudgens E, Le X, Schreinemachers D, Thomas D. 1999. Excretion of arsenic in urine as a function of exposure to arsenic in drinking water. *Environmental Health Perspectives* 107:663-667.
- Chao JI, Hsu SH, Tsou TC. 2006. Depletion of selenium increases arsenite-induced chromosome instability and apoptosis via a p53-independent pathway. *Toxicology Science* 90:73-86.
- Chen C-J, Chuang Y-C, Lin T-M, Wu H-Y. 1985. Malignant neoplasm among resident of Blackfoot disease-endemic area in Taiwan: high arsenic artesian well water and cancers. *Cancer Research* 45:5895-5899.
- Chen C-J, Chuang Y-C, You S-L, Lin T-M, Wu H-Y. 1986. A retrospective study on malignant neoplasm of bladder, lung and liver in black-foot disease endemic area in Taiwan. *British Journal of Cancer* 53:339-405.
- Chen C-J, Su H-J, Leon Guo Y, Hsueh Y-M, Smith T, Ryan L, Lee M-S, Christiani D. 2003. Arsenic methylation and bladder cancer risk in Taiwan. *Cancer Causes and Control* 14:303-310.
- Christian W, Hopenhayn C, Centeno J, Todorov T. 2006. Distribution of urinary selenium and arsenic among pregnant women exposed to arsenic in drinking water. *Environmental Research* 100:115-122.

- Chung C-J, Huang C, Pu Y-S, Su C, Huang Y, Chen Y, Hsueh Y-M. 2008. Urinary 8-hydroxydeoxyguanosine and urothelial carcinoma risk in low arsenic exposure data. *Toxicology and Applied Pharmacology* 226:14-21.
- Cohen S, Arnold L, Eldan M, Lewis A, Beck B. 2006. Methylated arsenicals: the implications of metabolism of arsenic and carcinogenicity studies in rodents to human risk assessment. *Critical Reviews in Toxicology* 36:99-133.
- Cohen S, Arnold L, Uzvolgyi E, Cano M, St. John M, Yamamoto S, Lu X, Le X. 2002. Possible role of dimethylarsinous acid in dimethylarsinic acid-induced urothelial toxicity and regeneration in the rat. *Toxicology* 15:1150-1157.
- Crawford J. 2008. The origins of bladder cancer. *Laboratory Investigation* 88:686-693.
- Del Razo L, Garcia-Vargas G, Vargas H, Albores A, Gonsbatt M, Montero R, Ostrosky-Wegman P, Kelsh M, Cebrian M. 1997. Altered profile of urinary arsenic metabolites in adults with chronic arsenicism. *Toxicokinetics* 71:211-217.
- Ferlay J, Randi G, Bossetti C. 2007. Declining mortality from bladder cancer in Europe. *British Journal of Urology*(101):11-19.
- Fisher L, daCosta K, Kwok L, Steward P, Lu T, Stabler S, Allen R, Zeisel S. 2007. Sex and menopausal status influence human dietary requirements for the nutrient choline. *American Journal of Clinical Nutrition* 85:1275-1285.
- Garcia-Closas M, Rothman N, Lubin J. 1999. Misclassification in case-control studies of gene-environment interactions: assessment of bias and sample size 8:1043-1050.
- Hei T, Liu S, Waldren C. Mutagenicity of arsenic in mammalian cells: role of reactive species. In: *Sciences NAO*, editor; 1998. p 8103-8107.
- Höfler M. 2005. The effect of misclassification on the estimation of association: a review. *International Journal of Methods in Psychiatric Research* 14:92-101.
- Hopenhayn-Rich C, Biggs M, Kalman D, Moore L, Smith A. 1996. Arsenic methylation patterns before and after changing from high to lower concentrations of arsenic in drinking water. *Environmental Health Perspectives* 1200-1207.
- Karagas M, Nelsom H, Kelsey K, Morris S, Blum J, Tosteson T, Carey M, Lu X, Le X. 2002. Urinary arsenic species in relation to drinking water, toenail arsenic concentrations and genetic polymorphisms in GSTM1 in New Hampshire. In: Wilson S, Suk W, editors. *Biomarkers of Environmentally Associated Disease: Technologies Concepts and Perspectives*. NY: Lewis Publisher.

- Liao WT, Lin P, Cheng TS, Yu HS, Chang LW. 2007. Arsenic promotes centrosome abnormalities and cell colony formation in p53 compromised humn lung cells. *Toxicology and Applied Pharmacology* 225:162-170
- Lindberg A-L, Ekstrom E-C, Nermell B, Rahman M, Lonnerdal B, Persson L-A, Vahter M. 2008. Gender and age differences in the metabolism of inorganic arsenic in a highly exposed population in Bangladesh. *Environmental Research* 106:110-120.
- Lindberg A-L, Kumar R, Goessler W, Thirumaran R, Gurzau E, Koppova K, Rudnai O, Leonardi G, Fletcher T, Vahter M. 2007. Metabolism of low dose inorganic arsenic in a Central European population-influence of gender and genetic polymorphism. *Environmental Health Perspectives* 115:1081-1086.
- Mahata J, Basu A, Ghosahal S, Sarkar J, Roy A, Poddar G, Nandy N, Barnerjee A, Nataranjan A, Nisson R and others. 2003. Chromosomal aberrations and sister chromatid exchanges in individuals exposed to arsenic through drinking water in West Bengal, India. . *Mutation Research* 534:133-143.
- Marsit C, Karagas M, Andrews A. 2005. Epigenetic activation of SFRP genes and TP53 alteration act jointly as markers of invasive bladder cancer. *Cancer Research* 65:7081-7085.
- Marsit C, Karagas M, Danaee H, Liu M, Andrews A, Schned A, Nelson H. 2007. Carcinogen exposure and gene promoter hypermethylation in bladder cancer. *Carcinogenesis* 27:112-116.
- Meliker J. 2006. Lifetime exposure to arsenic in drinking water in southeastern Michigan
Ann Arbor: University of Michigan
- Meliker J, Slotnick M, AvRuskin G, Kaufman AF, SA, Goovaerts P, Jacquez G, Nriagu J. 2007. Individual lifetime exposure to inorganic arsenic using a space-time information system. *International Archives of Occupational and Environmental Health* 80:184-197.
- Morales AA, Gutman D, Cejas PJ, Lee KP, Boise LH. 2009. Reactive oxygen species are not required for an arsenic trioxide-induced anti-oxidant response or apoptosis. *The Journal of Biological Chemistry* Mar 11 [Epub ahead of print]
- Nermell B, Lindberg A, Rahman M, Berglund M, Persson L, Arifeen S, Vahter M. 2008. Urinary arsenic concentration adjustment factors and malnutrition. *Environmental Research* 106(2):212-218.

NRC. 2001. Arsenic in drinking water 2001 Update. Washington, DC: National Academy Press. 15-23 p.

Nriagu J, Lawson G, Wong H, Aczue J. 1993. A protocol for minimizing contamination in the analysis of trace metals in Great Lakes waters. *Journal of Great Lakes Research* 19:175-182.

Pi J, Yamauchi H, Kumagai Y, Sun G, Yoshida T, Aikawa H, Hopenhayn-Rich C, Shimojo N. 2002. Induction of oxidative stress caused by chronic exposure of Chinese residents to arsenic contained in drinking water. *Environmental Health Perspectives* 110:331-336.

Pu Y-S, Yang S-M, Huang Y-K, Chung C-J, Huang S, Chiu A, Yang M-H, Chen C-J, Hsueh Y-M. 2007. Urinary arsenic profile affects the risk of urothelial carcinoma even at low arsenic exposure. *Toxicology and Applied Pharmacology* 218:99-106.

Rana SV. 2008. Metals and apoptosis: recent developments. *Journal of Trace Element in Medical Biology* 22:262-284.

Robert MF, Morin S, Beaulieu N. 2003. DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nature Genetics* 33:61-65.

Rossman TG, Uddin AN, Burns FJ. 2004. Evidence that arsenite acts as a cocarcinogen in skin cancer. *Toxicology and Applied Pharmacology* 198:394-404.

Santner T, Craigmile P, Calder C, Paul R. 2008. Demographic and behavioral modifiers of arsenic exposure pathway: a Bayesian Hierarchical analysis of NHEXAS data. *Environmental Science Technology* 42:5607-5614.

Satta R, Maloku E, Zhubi A, Pibiri F, Hajos M, Costa E, Guidotti A. 2008. Nicotine decreases DNA methyltransferase 1 expression and glutamic acid decarboxylase 67 promoter methylation in GABAergic interneurons. 16356-16361.

Schoen A, Beck B, Sharma R, Dube E. 2004. Arsenic toxicity at low doses: epidemiological and mode of action considerations. *Toxicology and Applied Pharmacology* 198:253-267.

Sciandrello G, Caradonna F, Mauro M, Barbate G. 2004. Arsenic-induced DNA hypomethylation affects chromosomal instability in mammalian cells. *Carcinogenesis* 25:413-417

Sclawicke E, Nermell B, Concha G, Stromberg U, Vahter M, Broberg K. 2009. Arsenic metabolism is influenced by polymorphisms in genes involved in one-carbon metabolism and reduction reactions. *Mutation Research Epub ahead of print.*

- Silverman D, Mossion A, Devesa S. 1996. Bladder Cancer. In: Schottenfield D, Fraumeni J, editors. *Cancer Epidemiology and Prevention*. NY: Oxford University Press. p 1156-1179.
- Slotnick MJ, Meliker J, AvRuskin GA, Ghosh D, Nriagu JO. 2007. Toenails as a biomarker of inorganic arsenic intake from drinking water and foods. *Journal of Toxicology and Environmental Health, Part A* 70:148-158.
- Smith A, Goycolea M, Haque R, Biggs M. 1998. Marked increased in bladder and lung cancer mortality in a region of Northern Chile due to arsenic in drinking water. *American Journal of Epidemiology* 147:660-669.
- Steinmaus C, Bates M, Yuan Y, Kalman D, Atallah R, Rey O, Biggs M, Hopenhayn C, Moore L, Hoang B and others. 2006. Arsenic methylation and bladder cancer risk in case-control studies in Argentina and the United States. *Journal of Occupational and Environmental Medicine* 48:478-488.
- Steinmaus C, Yuan Y, Bates M, Smith A. 2003. Case-control study of bladder cancer and drinking water arsenic in the western United States. *American Journal of Epidemiology* 158:1193-1201.
- Styblo M, del Razo L, Vega L, Gemolec D, LeCluyse E, Hamilton G, Reed W, Wang C, Cullen W, Thomas D. 2000. Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. *Archives of Toxicology* 74:289-299.
- Styblo M, Drobna Z, Jasper I, Lin S, Thomas D. 2002. The role of biomethylation in toxicity and carcinogenicity of arsenic: a research update. *Metals Toxicity* 110:767-771.
- Tezuka M, Hanioka K, Yamanaka K, Okada S. 1993. Gene damage induced in human alveolar type II (L-132) cells by exposure to dimethylarsinic acid. *Biochemical and Biophysical Research Communications* 191:1178-1183.
- Tseng C, Huang Y, Huang Y, Chung C-J, TYang M, Chen C-J. 2005. Arsenic exposure, urinary arsenic speciation, and peripheral vascular disease in Blackfoot disease-hyperendemic villages in Taiwan. *Toxicology and Applied Pharmacology* 206:299-308.
- Tsuda T, Bbazono A, Yamamoyo E, Kurumatani N, Mino Y, Ogana T, al e. 1995. *American Journal of Epidemiology*. 141:198-209.
- Vahter M. 1999a. Methylation of inorganic arsenic in different mammalian species and population groups. *Science in Progress* 1999:69-88.
- Vahter M. 1999b. Variation of human metabolism of arsenic. In: Chappell W, editor. *Arsenic exposure and health effect*. Oxford: Elsevier. p 267-269.

Vahter M, Marafante E. 1987. Effects of low dietary intake of methionine, choline or proteins on the biotransformation of arsenite in the rabbit. *Toxicological Letters* 37:41-46.

Vahter M, Akesson A, Liden C, Ceccatelli S, Berglund M. 2007. Gender differences in the disposition and toxicity of metals. *Environmental Research* 104:85-95.

Vega L, Gonshebbatt M, Ostrosky-Wegman P. 1995. A neogenic effect of sodium arsenite in human lymphocytes in vitro: and individual susceptibility effect detected. *Mutation Research* 334:365-373.

Vineis P, Talaska G, Malaveille C, Bartsch H, Martone T, Sithisarankul P, Strickland P. 1996. DNA adducts in urothelial cells: relationship with biomarkers of exposure to arylamines and polycyclic aromatic hydrocarbons from tobacco smoke. *International Journal of Cancer* 65:314-316.

Walser T, Cui X, Yanagawa J, Lee J, Heinrich E, Sharma S, Dubinette S. 2008. Smoking and lung cancer: the role of inflammation. *Proceedings of the American Thoracic Society* 5(8):811-815.

Warnecke O, Bestor T. 2000. Cytosine methylation and human cancer. *Currents Opinion in Oncology* 3:223-229.

Wu M, Chiou H, Wang T, Hsueh Y-M, Wang I, Chen C-J, Lee T. 2001. Association of blood arsenic levels with increased reactive oxidants and decreased antioxidant capacity in a human population of Northeastern Taiwan. *Environmental Health Perspectives* 109:1011-1017.

Wu M, Kuo T, Hwang Y, Chen C-J. 1989. Dose-response relationship between arsenic concentration in well water and mortality from cancers and vascular diseases. *American Journal of Epidemiology* 130:1123-1132.

Xue X, Hoover D. 2009. Statistical methods in cancer epidemiological studies. *Methods in Molecular Biology* 471:239-272.

Yu R, Hsu K, Chen C-J, JR F. 2000. Arsenic methylation capacity and skin cancer. *Cancer Epidemiology, Biomarkers, and Prevention* 9:1259-1262.

Zeegers M, Kellen E, Buntix F, van den Brandt P. 2004. The association between smoking, beverage consumption, diet and bladder cancer: a systematic literature review. *World Journal of Urology* 21:392-401.

Chapter VII

Conclusions

As a result of the substantial evidence of the human exposure to arsenic around the world, it is essential to improve the ability to accurately characterize exposures. Exposure characterization becomes significantly challenging when individuals are exposed to low concentrations (10-50 μ g/L) of arsenic in drinking water. Environmental epidemiology needs tools to accurately characterize those exposures. The process of biomarker validation is difficult but continuous. Urinary arsenic metabolites have emerged as the most frequently-used biomarker of exposure for arsenic exposure. However, the validation process still requires substantial work due to the many aspects that may influence their characterization and interpretation. On the other hand, urinary arsenic metabolites offer not only the exposure assessment component but the potential to elucidate arsenic metabolism pathways in the human body. The research described in this dissertation provides insights to advance the exposure assessment process and the biomarker-disease relationship. Likewise, it refines the analytical chemistry involved in the detection and measurement protocols that usually interferes with the correct interpretation of the biomarker value.

The major findings from this research support the use of urinary arsenic biomarkers as measures of recent exposure to arsenic. The results presented demonstrate that arsenic intake, as estimated from recent consumption of plain water, is a predictor of urinary arsenic. The prediction became stronger when continuous variables are categorized to better characterize exposure. Intakes from food such as fish, seafood, and chicken are reflected in the biomarker, adding evidence to the current literature that consumption of these food increase arsenic concentrations in urine. Urinary arsenic metabolites were also associated with the risk of bladder cancer. However, a protective effect was revealed where cases excreted less urinary arsenic than controls. This may be evidence for a possible inhibition of the methyltransferases involved in the arsenic methylation pathway. Lastly, the developed arsenic speciation protocol presents an excellent resolution capacity scarcely reported in previous studies. This is the first study reporting differences between first-morning void urine samples with spot urine samples. As a result, FMV and spot samples can be used without preferences in environmental epidemiology studies.

Biomonitoring has become a fundamental tool in exposure assessment; however, even with the latest analytical and exposure techniques exposure assessment is still in an early stage. Therefore, results obtained from this dissertation have to be carefully considered. In terms of analytical procedures, there are a variety of available methods to identify and quantify urinary arsenic metabolites. Urine is the most common biological sample analyzed for arsenic (Hughes et al. 2007). Arsenic speciation is the basis of a reliable biomarker interpretation in the utilization of urinary arsenic

metabolites as biomarkers of arsenic exposure. Equally, sampling, storage, and preservation are critical in this process.

There are several arsenic metabolites in urine and they all have different chemical characteristics which make the analytical process difficult and challenging (Pages 31-37). This becomes even more complex when applied to epidemiological studies where a large number of samples are collected over a relatively long period of time. One of the biggest issues in this area is the detection of the trivalent metabolites MMA[III] and DMA[III]. MMA[III] and DMA[III] are claimed to be detectable in arsenic-exposed subjects (Valenzuela et al. 2005). Although the validity of the analytical methods is questionable, the possibility of correctly identifying the trivalent species represents an advance not only in arsenic exposure but arsenic metabolism as well. During this decade, studies have shown that MMA[III] and DMA[III] may be more toxic than inorganic As[III] and As[V] (Petrick et al. 2000; Styblo et al. 2000). After an immediate analysis (<6hours after collection), we did not detect methylated arsenic trivalent species in 100 samples analyzed in this study (Page 65).

FMV and spot samples were included in this random selection. We collected FMV and spot urine samples in order to compare them and establish a possible relationship with the biomarker interpretation. Different factors may affect the biomarker variability. The selection of sampling strategies reflects some exposures better than others. The proper selection of a sampling method, more than a logistic or budgetary issue, may lead to better understanding of arsenic exposures and to

elucidating the arsenic metabolic pathway. To our knowledge this is the first study evaluating the biomarker value between two different sampling strategies with enough power to responsibly provide a recommendation (Chapter 4). Similar studies need to be performed in different populations with lower-to-moderate arsenic exposure levels.

In populations with low-to-moderate arsenic exposure levels, factors such as age, gender, and food and water intakes affect the biomarker interpretation. This research does not provide enough data to establish serious intra- or inter-variability associations. However, 90% of the variability in our sample comes from between individuals not within individuals (Page 90, 93). Individual exposures to arsenic may influence the intake estimate. When exposure assessment is carried through self-reported questionnaires, this estimate may be biased due to a misclassification of exposure. This research incorporated categorical stratification of exposure in order to account for volume of water intake, which has been reported as a very influential modifier of ingestion exposures (Wright et al. 2006). The correct classification of exposure is critical since one of the major concerns to establish disease-biomarker relationship in populations exposed to low levels of arsenic is exposure misclassification (Cantor and Lubin 2007).

Another issue that comes up when estimating arsenic exposure through water or food intakes is the consumption of food with higher amounts of arsenosugars such as arsenobetaine or arsenocholine. Although they are not metabolized in human and are known to be non-toxic, their consumption may increase levels of DMA[V] and other

unidentified urinary arsenic species found in urine (Ma and Le 1998). Even when the analytical protocol can separate and identify arsenobetaine or arsenocholine, their influence in the other metabolites is unknown yet. This research presented evidence that arsenosugars are present in other food besides fish or seafood (Page 127-128). Restraining individuals to consume fish, seafood, mushrooms, rice, and even chicken is very challenging in large epidemiological studies. Therefore, separate analyses should be performed to account for these foods and their influence in the biomarker.

Evidence suggests that toenail may integrate exposure better than arsenic metabolites in urine since toenails may reflect longer period of exposure (Slotnick and Nriagu 2006). Given that arsenosugars are excreted immediately after 24-72 hours of ingestion, toenails probably reflect inorganic arsenic better (NRC 1999). However, when the exposure is categorized by arsenic concentrations in water and water intake, urinary arsenic explains more variability than toenail arsenic concentrations in this population. Toenail arsenic concentrations may be affected by longer-term temporal variation in exposure, such as participant mobility and variation in drinking-water arsenic concentrations among others (Slotnick et al. 2007). On the other hand, urinary arsenic metabolites, in this study, are measures of recent exposure.

Finally, the relationship between the biomarker and disease is the main objective in the process of correct interpretation of biomonitoring data. However, even when perfect exposure assessment techniques are applied, the interpretation of urinary arsenic metabolites as biomarkers of arsenic exposure will depend on our knowledge of

arsenic metabolism in the human body. The current literature on arsenic metabolism presents two different pathways of how the arsenic metabolism occurs in the human body as explained in Chapter 2 (Fig 2-1-2-2). Likewise, several possible modes of action have been proposed. This research suggests alterations in the methylation profiles. However, due to sample size limitations and study design these results should be carefully interpreted. It is likely that no carcinogenic mechanism acts in isolation and that several of the proposed mechanism are interdependent and influenced by factors in the cellular environment. As an example of that, epigenetic mechanisms have been related to bladder cancer and smoking. In this research cigarette smoking and arsenic exposure may interact in an additive and synergistic way.

It is worth mentioning that this research is framed under a case-control study. The appropriate identification of the relevant exposure period together with exposure estimates are the starting point to correctly assess the degree to which risk estimates reflect true risk (Cantor and Lubin 2007). In this study, an effort was made to estimate historical levels of drinking water arsenic for each study subject. However, characterizing past exposures in detail is challenging and individuals participating in case-control studies have been diagnosed before the exposure assessment evaluation.

The work presented in this dissertation improves the knowledge surrounding the validation of urinary arsenic metabolites as biomarkers of arsenic exposure. This research improve our knowledge in the characterization of the contribution of diet and drinking water to inorganic arsenic exposure, developed a method to distinguish and

quantify trivalent MMA, determined the correlation between urinary arsenic and toenail arsenic and approached a population exposed to environmentally relevant-low levels of arsenic. Overall, this investigation supports exposure assessment efforts in epidemiological research addressing the risk associated with low-level arsenic exposure, which have critical implications for risk assessment and public health.

References

Cantor K, Lubin J. 2007. Arsenic, internal cancers, and issues in inference from studies of low-level exposure in human populations. *Toxicology and Applied Pharmacology* 222:252-257.

Hughes M, Kenyon E, Kitchin K. 2007. Research approaches to address uncertainties in the risk assessment of arsenic in drinking water. *Toxicology and Applied Pharmacology* 222:399-404.

Ma M, Le X. 1998. Effect of arsenosugar ingestion on urinary arsenic speciation. *Clinical Chemistry* 44:539-550.

NRC. 1999. Arsenic in drinking water. Washington, DC. National Academy Press.

Petrick J, Ayala-Fierro F, Cullen W, Carter D, Aposhian H. 2000. Monomethylarsonous acid (MMAIII) is more toxic than arsenite in Chang human hepatocytes. *Toxicology and Applied Pharmacology* 163:203-207.

Slotnick M, Meliker J, AvRuskin G, Ghosh D, Nriagu J. 2007. Toenails as a biomarker of inorganic arsenic intake from drinking water and foods. *Journal of Toxicology and Environmental Health, Part A* 70:148-158.

Slotnick M, Nriagu J. 2006. Validity of human nails as biomarker of arsenic and selenium exposure: A review. *Environmental Research* 102:125-139.

Styblo M, Del Razo L, Vega L, Germolec D, LeCluyse E, Hamilton G, Reed W, Wang C, Cullen W, Thomas D. 2000. Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. *Archives of Toxicology* 74:289-299.

Valenzuela O, Borja-Aburto H, Garcia-Vargas G, Cruz-González M, Garcia-Montalvo E, Calderón-Aranda E, Del Razo L. 2005. Urinary trivalent methylated species in a population exposed to inorganic arsenic *Environmental Health Perspectives* 113:250-254.

Wright M, Murphy P, Nieuwenhuijsen M, Savitz D. 2006. The impact of water consumption, point-of-use filtration and exposure categorization on exposure misclassification of ingested drinking water contaminants. *Science of the Total Environment* 366:65-73.

APPENDICES

Appendix A

Questionnaire for Computer-Aided Telephone Interview

Question title

ARSENIC BLADDER CANCER STUDY
[PRESS 1 TO RECORD CALL OUTCOME]

Question CALL_ST

[IWER: WHAT IS THE CURRENT STATUS OF THE CALL?]

- [1. PERSON ANSWERS THE PHONE]
- [2. NO ANSWER]
- [3. BUSY]
- [4. ANSWERING MACHINE-NO MESSAGE]
- [5. ANSWERING MACHINE-MESSAGE]
- [6. FAX/DATA LINE]
- [7. DISCONNECTED/NON-WORKING NUMBER]
- [8. WRONG # / # CHANGED: IWER CONFIRM #] I'm sorry is this ###-###-####?
[PRESS 8 ONLY AFTER VERIFYING YOU HAVE DIALED CORRECTLY]
- [9. CELLULAR PHONE]
- [10. TECHNICAL PHONE BARRIERS: CALL BLOCKING]
- [11. TECHNICAL PHONE PROBLEMS: STATIC, NO VOLUME, ETC.]
- [12. OTHER: Interviewer select disposition]

Question CANHLL01 (For Cancer R only)

Hello, may I please speak to [Case name] ?

- [1. R IS ON THE PHONE]
- [2. PERSON ON PHONE WILL GET R]
- [3. R IS NOT HOME OR AVAILABLE] (Skip to CALLBAK)
- [4. R ASKED FOR CALLBACK] (Skip to CALLBAK2)
- [5. R REFUSES OR CANNOT DO STUDY OVER THE PHONE] (Skip to ENDREF)

Question CANHLL02 (For Cancer R only)

Hello, my name is _____. I am calling on behalf of
the University of Michigan, School of Public Health.

You were recently sent a letter informing you of a project sponsored by the National
Cancer Institute. The purpose of the project is to study arsenic in drinking water
and associated health effects for residents in your area. As a benefit to participating in the study you
will receive \$40.

Question CONHLL01 (For Control R only)

Hello, my name is _____. I am calling on behalf of
the University of Michigan, School of Public Health.

We are conducting a study on arsenic in drinking water and associated
health effects in your area. The project is sponsored by the National Cancer
Institute and your phone number was selected at random. As a benefit to participating in the study you
will receive \$40.

Question CONSELCT (For Control R only)

May I speak to the (male/female) living in the house who had the most recent birthday?

- [1. YES, PERSON IS ON PHONE] (Skip to HELLO2)
- [2. YES, PERSON ON PHONE WILL GET R]
- [3. NO, R IS UNAVAILABLE] (Skip to CALLBAK)
- [4. PERSON ON PHONE REFUSED TO GET R] (Skip to ENDREF)
- [5. NO, NO ONE IS ELIGIBLE] (Skip to ENDREF)

*** (Since the gender ratio in the cancer group is roughly 3 males to 1 female, we can set up a weighted list behind the scenes that randomly selects "males" to fill in the question text 75% of the time. If we examine the data and find that males are not selected often enough, we can change the weighting of the list.)***
*** (Genesys can send sample based on household age and gender, so we can load a weighted age sample to match the cancer group.)***

Question CONHLL02 (For Control R only)

Hello, my name is _____. I am calling on behalf of the University of Michigan, School of Public Health.

We are conducting a study on arsenic in drinking water and associated health effects in your area. The project is sponsored by the National Cancer Institute and your phone number was selected at random. As a benefit to participating in the study you will receive \$40.

Question HELLO2

I will start by asking you a few questions, and then tell you more about the study.

Question GENDER

[IWER: CODE THE RESPONDENTS GENDER. ASK IF YOU ARE NOT SURE.]

- [1. Male]
- [2. Female]

Question CANAGE (For Cancer R only)

According to our records, you were born on [xx/xx/xxx] and are now [xx] years old.

Is that correct?

- 1. YES (Skip to COUNTY1)
- 2. NO
- [9. DK/REFUSED]

Question DOB

What is your date of birth?

[ENTER "01/01/9999" FOR DK/REFUSED]

Question AGE

That would make you [xx] years old. Is that correct?

1. YES (Skip to COUNTY1)
2. NO
- [9. DK/REFUSED]

Question NEWAGE

Could you tell me your age?

[ENTER "999" FOR DK/REFUSED]

Question COUNTY1

What county do you live in?

- [1. Genesee]
- [2. Huron]
- [3. Ingham]
- [4. Jackson]
- [5. Lapeer]
- [6. Livingston]
- [7. Oakland]
- [8. Sanilac]
- [9. Shiawassee]
- [10. Tuscola]
- [11. Washtenaw]
- [12. OTHER] (Skip to ENDREF2)
- [99. DK/REFUSED] (Skip to ENDREF)

Question CNTYYRS

Have you lived in this county for the past 5 years?

1. YES (Skip to CANCDIAG)
2. NO
- [9. DK/REFUSED] (Skip to ENDREF)

Question COUNTY2

What other counties have you lived in during the past 5 years?

- [1. Genesee]
- [2. Huron]
- [3. Ingham]
- [4. Jackson]
- [5. Lapeer]
- [6. Livingston]
- [7. Oakland]
- [8. Sanilac]
- [9. Shiawassee]
- [10. Tuscola]
- [11. Washtenaw]
- [12. OTHER] (Skip to ENDREF2)
- [99. DK/REFUSED] (Skip to ENDREF)

*** (this question is a "check all that apply").***

Question CANCDIAG

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...

did a doctor ever tell you that you had a malignant tumor or cancer?

1. YES

2. NO [SKIP TO HELLO3]

[9. DK/REFUSED] (**Maybe a new ENDREF will be required for people who don't know. If they refuse—hopefully we can convert them... **)

Question CANCLOC

Where did the cancer or malignant tumor start?

1. Skin

2. Any Other [SKIP TO ENDREF4]

Question SKINTYPE

Did a doctor tell you that you had:

1. Squamous cell carcinoma

2. Basal cell carcinoma

3. Melanoma (or some other) [SKIP TO ENDREF4]

Question HELLO3

During the phone interview today, we will ask some questions about your drinking water, food and vitamin intake, tobacco use, and medical and family history.

You will also be sent a short written questionnaire about where you have lived and worked.

Lastly, we would like to send a member of the research team to your home to get a water sample and a toenail sample. These samples will be analyzed for arsenic, at no charge to you, and the results will be sent to you at a later date.

Question HELLO4

As I have already said, you would receive \$40 for participating in all of these research activities.

SOME participants will be contacted at a later date and asked to provide additional water and toenail samples, and a urine sample. These people would receive additional compensation for these samples.

Question HELLO5

This survey is strictly for research purposes. Your name will not appear in any of the research reports. Your participation in this study is voluntary. You may choose not to answer any question and you may stop the interview at any time. Any information you provide will be kept confidential. You will not be penalized if you choose not to participate or if you decide not to complete the survey. This call may be monitored or recorded by a supervisor as part of our quality control efforts.

Would you like to participate in this study?

- [1. YES, R WILL PARTICIPATE NOW]
- [2. YES, R WILL PARTICIPATE, BUT CALL BACK LATER] (Skip to CALLBAK)
- [3. NO, R REFUSES TO PARTICIPATE] (Skip to ENDREF)

Question HELLO6

Please ask for a break at any time and let me know if you don't understand any questions. Do you have any questions before we begin?

Question AINTRO

I will begin by asking you about your use of drinking water. By drinking water I mean bottled water, tap-water from community supply or private well, or any other drinking water source. We are interested in knowing how much water you drink AT HOME, AT WORK, OR OUTSIDE HOME AND WORK.

Please remember to consider that people may drink more water during different times of the year (such as summertime when it's hot).

If you have had a major illness recently, please think about the amount of water you drank before you became ill.

Question WATERHOM

During the year prior to diagnosis of bladder cancer, in an average month(CASES)...

During the past year, in an average month(CONTROLS)...

about how many glasses of water did you drink at HOME during an

average day? Include glasses you drank at home on a weekday and on the weekend.

Water can be tap or bottled water, but does not include flavored or carbonated water (with bubbles). By glass, I mean 8 ounces or 2/3 of a coke can. Would you say,

- 1. Less than 2 glasses [SKIP to WATERWRK]
- 2. 2-3 glasses [SKIP to WATERWRK]
- 3. 4-5 glasses [SKIP to WATERWRK]
- 4. 6-7 glasses [SKIP to WATERWRK]
- 5. 8 or more glasses
- [9. DK/REFUSED]

Question WTRHOMSP

Could you be more specific. How many glasses of water did you drink?

[ENTER "999" FOR DK/REFUSED]

Question WATERWRK

During the year prior to diagnosis of bladder cancer, in an average month(CASES)...

During the past year, in an average month(CONTROLS)...

about how many glasses of water did you drink at WORK during an

average day?

1. Less than 2 glasses [SKIP to WATEROTH]
2. 2-3 glasses [SKIP to WATEROTH]
3. 4-5 glasses [SKIP to WATEROTH]
4. 6-7 glasses [SKIP to WATEROTH]
5. 8 or more glasses
- [9. DK/REFUSED]

Question WTRWRKSP

Could you be more specific. How many glasses of water did you drink at work?

[ENTER "999" FOR DK/REFUSED]

Question WATEROTH

During the year prior to diagnosis of bladder cancer, in an average month(CASES)...

During the past year, in an average month(CONTROLS)...

about how many glasses of water did you drink in an average day, at SOME PLACE OTHER THAN HOME OR WORK?

1. Less than 2 glasses [SKIP to WTRHOM10]
2. 2-3 glasses [SKIP to WTRHOM10]
3. 4-5 glasses [SKIP to WTRHOM10]
4. 6-7 glasses [SKIP to WTRHOM10]
5. 8 or more glasses
- [9. DK/REFUSED]

Question WTROTHSP

Could you be more specific. How many glasses of water did you drink at some place other than home or work?

[ENTER "999" FOR DK/REFUSED]

Question WTRHOM10

Over the past ten years, about how many glasses of water did you drink at HOME during an average day?

1. Less than 2 glasses [SKIP to WTRWRK10]
2. 2-3 glasses [SKIP to WTRWRK10]
3. 4-5 glasses [SKIP to WTRWRK10]
4. 6-7 glasses [SKIP to WTRWRK10]
5. 8 or more glasses
- [9. DK/REFUSED]

Question WTRH10SP

Could you be more specific. How many glasses of water did you drink at home?

[ENTER "999" FOR DK/REFUSED]

Question WTRWRK10

Over the past ten years, about how many glasses of water did you drink at WORK during an average day?

1. Less than 2 glasses [SKIP to WTROTH10]
2. 2-3 glasses [SKIP to WTROTH10]
3. 4-5 glasses [SKIP to WTROTH10]
4. 6-7 glasses [SKIP to WTROTH10]
5. 8 or more glasses
- [9. DK/REFUSED]

Question WTRW10SP

Could you be more specific. How many glasses of water did you drink at work?

[ENTER "999" FOR DK/REFUSED]

Question WTROTH10

Over the past ten years, about how many glasses of water did you drink in an average day, at SOME PLACE OTHER THAN HOME OR WORK?

1. Less than 2 glasses [SKIP to WTRCHNG]
2. 2-3 glasses [SKIP to WTRCHNG]
3. 4-5 glasses [SKIP to WTRCHNG]
4. 6-7 glasses [SKIP to WTRCHNG]
5. 8 or more glasses
- [9. DK/REFUSED]

Question WTRO10SP

Could you be more specific. How many glasses of water did you drink at some place other than home or work?

[ENTER "999" FOR DK/REFUSED]

Question WTRCHNG

Compared with your average water consumption over the past 10 years, have you made any major changes in the total amount of water that you drank during your adult life?

1. YES
2. NO [SKIP TO Question DRINKINT]
- [9. DK/REFUSED]

Question WTRCHNUM

How many different changes have you made during your adult life?

[ENTER "999" FOR DK/REFUSED]

[Repeat Question WTRCHGYR-Question WTRBFORE if R indicates more than 1 change occurred]

Question WTRCHGYR

For the [Xth change],
around what year did this change in water consumption take place?

[ENTER "9999" FOR DK/REFUSED]

Question WTRINC

Did you increase or decrease your overall consumption of water?

1. Increase
2. Decrease
- [9. DK/REFUSED]

Question WTRBFORE

Before [year], about how many total glasses of water did you
drink in a day?

[ENTER "999" FOR DK/REFUSED]

Question DRINKINT

Now I'd like to ask you about beverages made with tap water.

Examples of such beverages include fruit drinks from concentrate,
iced tea or hot tea, and beverages from powder, such as kool-aid or hot chocolate. I will
not be asking you about coffee, soft drinks, beer, or wine right
now, but will ask about them later.

Again, If you have had a major illness recently, please think about the
amount of water you drank before you became ill.

Question TAPHOME

During the year prior to diagnosis of bladder cancer, in an average
month(CASES)...

During the past year, in an average month(CONTROLS)...

about how often did you drink a glass of a cold or hot beverage at HOME
that you made with tap water? Include glasses of beverages you drank at home
on a weekday and on the weekend.

Again, by glass, I mean 8 ounces or 2/3 of a can of coke. Would you say,

1. Never [SKIP to TAPHCHNG]
2. Less than one time per week [SKIP to TAPHCHNG]
3. 1-3 times per week [SKIP to TAPHCHNG]
4. 4-6 times per week [SKIP to TAPHCHNG]
5. 1 or more times per day
- [9. DK/REFUSED]

Question TAPHOMSP

Could you be more specific. How many times a day did you drink
beverages made from tap water at HOME?

[ENTER "999" FOR DK/REFUSED]

Question TAPHCHNG

Compared with your average recent consumption, have you made any major changes in the total amount of cold or hot beverages made from tap water that you drank at HOME during your adult life?

1. YES
2. NO [SKIP to TAPWORK]
- [9. DK/REFUSED]

Question TAPHMNUM

How many different changes have you made during your adult life?

[ENTER "999" FOR DK/REFUSED]

[Repeat Question TAPHCGYR – Question TAPHBFSP if R indicates more than 1 change occurred]

Question TAPHCGYR

For the [Xth time],
around what year did this change in consumption take place?

[ENTER "9999" FOR DK/REFUSED]

Question TAPHINC

Did you increase or decrease your overall consumption of cold or hot beverages?

1. Increase
2. Decrease
- [9. DK/REFUSED]

Question TAPHBFRE

Before the change, about how often did you drink a glass of a cold or hot beverage at HOME that you made with tap water?

1. Never [SKIP to TAPWORK]
2. Less than one time per week [SKIP to TAPWORK]
3. 1-3 times per week [SKIP to TAPWORK]
4. 4-6 times per week [SKIP to TAPWORK]
5. 1 or more times per day
- [9. DK/REFUSED]

Question TAPHBFSP

Could you be more specific. How many times a day did you drink cold or hot beverages made from tap water at HOME?

[ENTER "999" FOR DK/REFUSED]

Question TAPWORK

During the year prior to diagnosis of bladder cancer, in an average month(CASES)...

During the past year, in an average month(CONTROLS)...

about how often did you drink a glass of a cold or hot beverage at WORK that you made with tap water?

Again, please do not include coffee in your answer.

1. Never [SKIP to TAPWCHNG]
2. Less than one time per week [SKIP to TAPWCHNG]
3. 1-3 times per week [SKIP to TAPWCHNG]
4. 4-6 times per week [SKIP to TAPWCHNG]
5. 1 or more times per day
- [9. DK/REFUSED]

Question TAPWRKSP

Could you be more specific. How many times a day did you drink cold or hot beverages made from tap water at WORK?

[ENTER "999" FOR DK/REFUSED]

Question TAPWCHNG

Compared with your average recent consumption, have you made any major changes in the total amount of cold or hot beverages made from tap water that you drank at WORK during your adult life?

1. YES
2. NO [SKIP to TAPOTHER]
- [9. DK/REFUSED]

Question TAPWKNUM

How many different changes have you made during your adult life?

[ENTER "999" FOR DK/REFUSED]

[Repeat Question TAPWCGYR -Question TAPWBFSP if R indicates more than 1 change occurred]

Question TAPWCGYR

For the [Xth time],
around what year did this change in consumption take place?

[ENTER "9999" FOR DK/REFUSED]

Question TAPWINC

Did you increase or decrease your overall consumption of cold or hot beverages?

1. Increase
2. Decrease

[9. DK/REFUSED]

Question TAPWBFRE

Before the change, about how often did you drink a glass of a cold or hot beverage at WORK that you made with tap water?

1. Never [SKIP to TAPOTHER]
 2. Less than one time per week [SKIP to TAPOTHER]
 3. 1-3 times per week [SKIP to TAPOTHER]
 4. 4-6 times per week [SKIP to TAPOTHER]
 5. 1 or more times per day
- [9. DK/REFUSED]

Question TAPWBFSP

Could you be more specific. How many times a day did you drink cold or hot beverages made from tap water at WORK?

[ENTER "999" FOR DK/REFUSED]

Question TAPOTHER

(During the year prior to diagnosis of bladder cancer, in an average month(CASES)...

During the past year, in an average month(CONTROLS)...

about how often did you drink a glass of a cold or hot beverage at SOME PLACE OTHER THAN HOME OR WORK that you made with tap water?

1. Never [SKIP to TAPOCHNG]
 2. Less than one time per week [SKIP to TAPOCHNG]
 3. 1-3 times per week [SKIP to TAPOCHNG]
 4. 4-6 times per week [SKIP to TAPOCHNG]
 5. 1 or more times per day
- [9. DK/REFUSED]

Question TAPOTHSP

Could you be more specific. How many times a day did you drink beverages made from tap water at SOME PLACE OTHER THAN HOME OR WORK?

[ENTER "999" FOR DK/REFUSED]

Question TAPOCHNG

Compared with your average recent consumption, have you made any major changes in the total amount of cold or hot beverages made from tap water that you drank at SOME PLACE OTHER THAN HOME OR WORK during your adult life?

1. YES
 2. NO [SKIP to WFOODINT]
- [9. DK/REFUSED]

Question TAPOTNUM

How many different changes have you made during your adult life?

[ENTER "999" FOR DK/REFUSED]

[Repeat Question TAPOCGYR -Question TAPOBFSP if R indicates more than 1 change occurred]

Question TAPOCGYR

For the [Xth time],
around what year did this change in consumption take place?

[ENTER "9999" FOR DK/REFUSED]

Question TAPOINC

Did you increase or decrease your overall consumption of cold or hot
beverages made from tap water at SOME PLACE OTHER THAN HOME
OR WORK?

1. Increase
2. Decrease
- [9. DK/REFUSED]

Question TAPOBFRE

Before the change, about how often did you drink a glass of
a cold or hot beverage at SOME PLACE OTHER THAN HOME OR WORK?

1. Never [SKIP to WFOODINT]
2. Less than one time per week [SKIP to WFOODINT]
3. 1-3 times per week [SKIP to WFOODINT]
4. 4-6 times per week [SKIP to WFOODINT]
5. 1 or more times per day
- [9. DK/REFUSED]

Question TAPOBFSP

Could you be more specific. How many times a day did you drink
cold or hot beverages at SOME PLACE OTHER THAN HOME OR WORK?

[ENTER "999" FOR DK/REFUSED]

Question WFOODINT

Now think about foods that you add water to:

Question SOUPHOME

(During the year prior to diagnosis of bladder cancer, in an average
month(CASES)...

During the past year, in an average month(CONTROLS)...

about how often did you eat a bowl of hot breakfast cereal (such as
oatmeal), home-made soup, or canned soup made from concentrate or dry
mix at HOME?

Would you say,

1. Never [SKIP to SOUPHCHG]
 2. Less than one time per week [SKIP to SOUPHCHG]
 3. 1-3 times per week [SKIP to SOUPHCHG]
 4. 4-6 times per week [SKIP to SOUPHCHG]
 5. 1 or more times per day
- [9. DK/REFUSED]

Question SOUPHMSP

Could you be more specific. How many times a day did you eat a bowl of hot breakfast cereal, home-made soup, or canned soup made from concentrate or dry mix at HOME?

[ENTER "999" FOR DK/REFUSED]

Question SOUPHCHG

Compared with your average recent consumption, have you made any major changes in the total amount of hot bowls of breakfast cereal, home-made soup, or canned soup made from concentrate or dry mix at HOME during your adult life?

1. YES
 2. NO [SKIP to PASTA]
- [9. DK/REFUSED]

Question SOUPHNUM

How many different changes have you made during your adult life?

[ENTER "999" FOR DK/REFUSED]

[Repeat Question SOUPCGYR-Question SOUPBFSP if R indicates more than 1 change occurred]

Question SOUPCGYR

For the [Xth time],
around what year did this change in consumption take place?

[ENTER "9999" FOR DK/REFUSED]

Question SOUPINC

Did you increase or decrease your overall consumption of hot bowls of breakfast cereal, home-made soup, or canned soup made from concentrate or dry mix at HOME?

1. Increase
 2. Decrease
- [9. DK/REFUSED]

Question SOUPBFRE

Before the change, about how often did you eat a bowl of hot

breakfast cereal, home-made soup, or canned soup made from concentrate or dry mix at HOME?

1. Never [SKIP to PASTA]
 2. Less than one time per week [SKIP to PASTA]
 3. 1-3 times per week [SKIP to PASTA]
 4. 4-6 times per week [SKIP to PASTA]
 5. 1 or more times per day
- [9. DK/REFUSED]

Question SOUPBFSP

Could you be more specific. How many times a day did you eat a bowl of hot breakfast cereal, home-made soup, or canned soup made from concentrate or dry mix at HOME?

[ENTER "999" FOR DK/REFUSED]

Question PASTA

(During the year prior to diagnosis of bladder cancer, in an average month(CASES)...

During the past year, in an average month(CONTROLS)...)
about how often did you eat a serving of pasta?

One serving is 1 measuring cup.

1. Never [SKIP to PSTACHNG]
 2. Less than one time per week [SKIP to PSTACHNG]
 3. 1-3 times per week [SKIP to PSTACHNG]
 4. 4-6 times per week [SKIP to PSTACHNG]
 5. 1 or more times per day
- [9. DK/REFUSED]

Question PASTASP

Could you be more specific. How many times a day did you eat a serving of pasta?

[ENTER "999" FOR DK/REFUSED]

Question PSTACHNG

Compared with your average recent consumption, have you made any major changes in the total amount of servings of pasta that you ate, during your adult life?

1. YES
 2. NO [SKIP to RICE]
- [9. DK/REFUSED]

Question PASTANUM

How many different changes have you made during your adult life?

[ENTER "999" FOR DK/REFUSED]

[Repeat Question PSTACGYR -Question PASTABSP if R indicates more than 1 change occurred]

Question PSTACGYR

For the [Xth time],
around what year did this change in consumption take place?

[ENTER "9999" FOR DK/REFUSED]

Question PASTAINC

Did you increase or decrease your consumption of servings of pasta?

1. Increase
2. Decrease
- [9. DK/REFUSED]

Question PASTABFR

Before the change, about how often did you eat a serving of pasta?

1. Never [SKIP to RICE]
2. Less than one time per week [SKIP to RICE]
3. 1-3 times per week [SKIP to RICE]
4. 4-6 times per week [SKIP to RICE]
5. 1 or more times per day
- [9. DK/REFUSED]

Question PASTABSP

Could you be more specific. How many times a day did you eat a serving of pasta?

[ENTER "999" FOR DK/REFUSED]

Question RICE

(During the year prior to diagnosis of bladder cancer, in an average month(CASES)...

During the past year, in an average month(CONTROLS)...
about how often did you eat a serving of rice?

One serving is 1 measuring cup.

1. Never [SKIP to RICECHNG]
2. Less than one time per week [SKIP to RICECHNG]
3. 1-3 times per week [SKIP to RICECHNG]
4. 4-6 times per week [SKIP to RICECHNG]
5. 1 or more times per day
- [9. DK/REFUSED]

Question RICESP

Could you be more specific. How many times a day did you eat a serving of rice?

[ENTER "999" FOR DK/REFUSED]

Question RICECHNG

Compared with your average recent consumption, have you made any major changes in the total amount of servings of rice that you ate, during your adult life?

1. YES
2. NO [SKIP to COFFEINT]
- [9. DK/REFUSED]

Question RICENUM

How many different changes have you made during your adult life?

[ENTER "999" FOR DK/REFUSED]

[Repeat Question RICECGYR-Question RICEBSP if R indicates more than 1 change occurred]

Question RICECGYR

For the [Xth time],
around what year did this change in consumption take place?

[ENTER "9999" FOR DK/REFUSED]

Question RICEINC

Did you increase or decrease your consumption of servings of rice?

1. Increase
2. Decrease
- [9. DK/REFUSED]

Question RICEBFR

Before the change, about how often did you eat a serving of rice?

1. Never [SKIP Question RICEBSP]
2. Less than one time per week [SKIP Question RICEBSP]
3. 1-3 times per week [SKIP Question RICEBSP]
4. 4-6 times per week [SKIP Question RICEBSP]
5. 1 or more times per day
- [9. DK/REFUSED]

Question RICEBSP

Could you be more specific. How many times a day did you eat a serving of rice?

[ENTER "999" FOR DK/REFUSED]

Question COFFEINT

Now I'm going to ask you about your coffee drinking habits.

Question EVERCOFF

Did you ever drink coffee?

1. YES
2. NO [SKIP TO Question BEVINTRO]
- [9. DK/REFUSED]

Question DRNKCOFF

Do you still drink coffee?

1. YES
2. NO
- [9. DK/REFUSED]

Question LASTCOFF

How old were you when you last drank at least one cup of coffee per week?

[ENTER "999" FOR DK/REFUSED]

Question AGECOFF

How old were you when you first had at least one cup of coffee a week?

[ENTER "999" FOR DK/REFUSED]

Question COFFEE

During the year prior to diagnosis of bladder cancer, in an average month(CASES)...

During the past year, in an average month(CONTROLS)...
how often did you drink a cup of coffee?

By cup, I mean 6 ounces or 1/2 of a can of coke.

1. Never [SKIP to COFFCHNG]
2. Less than one time per week [SKIP to COFFCHNG]
3. 1-3 times per week [SKIP to COFFCHNG]
4. 4-6 times per week [SKIP to COFFCHNG]
5. 1 or more times per day
- [9. DK/REFUSED]

Question COFFEESP

Could you be more specific. How many times a day did you drink a cup of coffee?

[ENTER "999" FOR DK/REFUSED]

Question COFFHOME

What proportion of your coffee consumption was at HOME?

[ENTER "999" FOR DK/REFUSED]

Question COFFCHNG

During your adult life, have you made any major changes in the number of cups of coffee that you drank?

1. YES
2. NO [SKIP to BEVINTRO]
- [9. DK/REFUSED]

Question COFFENUM

How many different changes have you made during your adult life?

[ENTER "999" FOR DK/REFUSED]

[Repeat Question COFFCGYR -Question COFFEBSP if R indicates more than 1 change occurred]

Question COFFCGYR

For the [Xth time],
around what year did this change in consumption take place?

[ENTER "9999" FOR DK/REFUSED]

Question COFFINC

Did you increase or decrease your overall consumption of coffee?

1. Increase
2. Decrease
- [9. DK/REFUSED]

Question COFFEBFR

Before the change, about how often did you drink a glass of coffee?

1. Never [SKIP to BEVINTRO]
2. Less than one time per week [SKIP to BEVINTRO]
3. 1-3 times per week [SKIP to BEVINTRO]
4. 4-6 times per week [SKIP to BEVINTRO]
5. 1 or more times per day
- [9. DK/REFUSED]

Question COFFEBSP

Could you be more specific. How many times a day did you drink a glass of coffee?

[ENTER "999" FOR DK/REFUSED]

Question BEVINTRO

Now I'm going to ask you about your consumption of other beverages.

Question BOTJUICE

During the year prior to diagnosis, (CASES)...

During the past year, (CONTROLS)...

how often did you drink a glass of bottled fruit juice (not from a frozen can)?

By glass, I mean 8 ounces or 2/3 of a can of coke.

1. Never [SKIP to MINWATER]
 2. Less than one time per week [SKIP to MINWATER]
 3. 1-3 times per week [SKIP to MINWATER]
 4. 4-6 times per week [SKIP to MINWATER]
 5. 1 or more times per day
- [9. DK/REFUSED]

Question JUICESP

Could you be more specific. How many times a day did you drink a glass of bottled fruit juice?

[ENTER "999" FOR DK/REFUSED]

Question MINWATER

During the year prior to diagnosis, (CASES)...

During the past year, (CONTROLS)...

how often did you drink a glass of mineral water?

1. Never [SKIP to MILK]
 2. Less than one time per week [SKIP to MILK]
 3. 1-3 times per week [SKIP to MILK]
 4. 4-6 times per week [SKIP to MILK]
 5. 1 or more times per day
- [9. DK/REFUSED]

Question MWATERSP

Could you be more specific. How many times a day did you drink a glass of mineral water?

[ENTER "999" FOR DK/REFUSED]

Question MILK

During the year prior to diagnosis, (CASES)...

During the past year, (CONTROLS)...

how often did you drink a glass of milk?

Again, by glass, I mean 8 ounces or 2/3 of a can of coke.

1. Never [SKIP to MILKCHNG]
2. Less than one time per week [SKIP to MILKCHNG]

3. 1-3 times per week [SKIP to MILKCHNG]
 4. 4-6 times per week [SKIP to MILKCHNG]
 5. 1 or more times per day
- [9. DK/REFUSED]

Question MILKSP

Could you be more specific. How many times a day did you drink a glass of milk?

[ENTER "999" FOR DK/REFUSED]

Question MILKCHNG

Compared with your average recent consumption, have you made any major changes in the total amount of milk that you drank during your adult life?

1. YES
 2. NO [SKIP to SODAPOP]
- [9. DK/REFUSED]

Question MILKNUM

How many different changes have you made during your adult life?

[ENTER "999" FOR DK/REFUSED]

[Repeat Question MILKCGYR - Question MILKBSP if R indicates more than 1 change occurred]

Question MILKCGYR

For the [Xth time],
around what year did this change in consumption take place?

[ENTER "9999" FOR DK/REFUSED]

Question MILKINC

Did you increase or decrease your overall consumption of milk?

1. Increase
 2. Decrease
- [9. DK/REFUSED]

Question MILKYR

Around what year did this change in consumption take place?

[ENTER "9999" FOR DK/REFUSED]

Question MILKBFR

Before the change, about how often did you drink a glass of milk?

1. Never [SKIP to SODAPOP]
 2. Less than one time per week [SKIP to SODAPOP]
 3. 1-3 times per week [SKIP to SODAPOP]
 4. 4-6 times per week [SKIP to SODAPOP]
 5. 1 or more times per day
- [9. DK/REFUSED]

Question MILKBSP

Could you be more specific. How many times a day did you drink a glass of milk?

[ENTER "999" FOR DK/REFUSED]

Question SODAPOP

During the year prior to diagnosis, (CASES)...

During the past year, (CONTROLS)...

how often did you drink a glass of soda, pop, or soft drink?

1. Never [SKIP to SODACHNG]
 2. Less than one time per week [SKIP to SODACHNG]
 3. 1-3 times per week [SKIP to SODACHNG]
 4. 4-6 times per week [SKIP to SODACHNG]
 5. 1 or more times per day
- [9. DK/REFUSED]

Question SODASP

Could you be more specific. How many times a day did you drink a glass of soda, pop, or soft drink?

[ENTER "999" FOR DK/REFUSED]

Question SODACHNG

Compared with your average recent consumption, have you made any major changes in the total amount of soda, pop, or soft drink that you drank during your adult life?

1. YES
 2. NO [SKIP to BEER]
- [9. DK/REFUSED]

Question SODANUM

How many different changes have you made during your adult life?

[ENTER "999" FOR DK/REFUSED]

[Repeat Question SODACGYR -Question SODABSP if R indicates more than 1 change occurred]

Question SODACGYR

For the [Xth time],
around what year did this change in consumption take place?

[ENTER "9999" FOR DK/REFUSED]

Question SODAINC

Did you increase or decrease your consumption of soda, pop, or soft drink?

1. Increase
2. Decrease
- [9. DK/REFUSED]

Question SODABFR

Before the change, about how often did you drink a glass of soda, pop, or soft drink?

1. Never [SKIP to BEER]
2. Less than one time per week [SKIP to BEER]
3. 1-3 times per week [SKIP to BEER]
4. 4-6 times per week [SKIP to BEER]
5. 1 or more times per day
- [9. DK/REFUSED]

Question SODABSP

Could you be more specific. How many times a day did you drink a glass of soda, pop, or soft drink?

[ENTER "999" FOR DK/REFUSED]

Question BEER

During the year prior to diagnosis, (CASES)...

During the past year, (CONTROLS)...

how often did you drink a glass of beer?

1. Never [SKIP to BEERCHNG]
2. Less than one time per week [SKIP to BEERCHNG]
3. 1-3 times per week [SKIP to BEERCHNG]
4. 4-6 times per week [SKIP to BEERCHNG]
5. 1 or more times per day
- [9. DK/REFUSED]

Question BEERSP

Could you be more specific. How many times a day did you drink a glass of beer?

[ENTER "999" FOR DK/REFUSED]

Question BEERCHNG

Compared with your average recent consumption, have you made any major changes in the total amount of beer that you drank during your adult life?

1. YES
2. NO [SKIP to WINE]
- [9. DK/REFUSED]

Question BEERNUM

How many different changes have you made during your adult life?

[ENTER "999" FOR DK/REFUSED]

[Repeat Question BEERCGYR -Question BEERBSP if R indicates more than 1 change occurred]

Question BEERCGYR

For the [Xth time],
around what year did this change in consumption take place?

[ENTER "9999" FOR DK/REFUSED]

Question BEERINC

Did you increase or decrease your consumption of beer?

1. Increase
2. Decrease
- [9. DK/REFUSED]

Question BEERBFR

Before the change, about how often did you drink a glass of beer?

1. Never [SKIP to WINE]
2. Less than one time per week [SKIP to WINE]
3. 1-3 times per week [SKIP to WINE]
4. 4-6 times per week [SKIP to WINE]
5. 1 or more times per day
- [9. DK/REFUSED]

Question BEERBSP

Could you be more specific. How many times a day did you drink a glass of beer?

[ENTER "999" FOR DK/REFUSED]

Question WINE

During the year prior to diagnosis, (CASES)...

During the past year, (CONTROLS)...

how often did you drink a glass of wine?

1. Never [SKIP to WINECHNG]
2. Less than one time per week [SKIP to WINECHNG]
3. 1-3 times per week [SKIP to WINECHNG]
4. 4-6 times per week [SKIP to WINECHNG]

5. 1 or more times per day
- [9. DK/REFUSED]

Question WINESP

Could you be more specific. How many times a day did you drink a glass of wine?

[ENTER "999" FOR DK/REFUSED]

Question WINECHNG

Compared with your average recent consumption, have you made any major changes in the total amount of wine that you drank during your adult life?

1. YES
 2. NO [SKIP to OTHERBEV]
- [9. DK/REFUSED]

Question WINENUM

How many different changes have you made during your adult life?

[ENTER "999" FOR DK/REFUSED]

[Repeat Question WINECGYR -Question WINEBSP if R indicates more than 1 change occurred]

Question WINECGYR

For the [Xth time],
around what year did this change in consumption take place?

[ENTER "9999" FOR DK/REFUSED]

Question WINEINC

Did you increase or decrease your consumption of wine?

1. Increase
 2. Decrease
- [9. DK/REFUSED]

Question WINEBFR

Before the change, about how often did you drink a glass of wine?

1. Never [SKIP to OTHERBEV]
 2. Less than one time per week [SKIP to OTHERBEV]
 3. 1-3 times per week [SKIP to OTHERBEV]
 4. 4-6 times per week [SKIP to OTHERBEV]
 5. 1 or more times per day
- [9. DK/REFUSED]

Question WINEBSP

Could you be more specific. How many times a day did you drink a glass of wine?

[ENTER "999" FOR DK/REFUSED]

Question OTHERBEV

During the year prior to diagnosis, (CASES)...
During the past year, (CONTROLS)...
how often did you drink a glass of other beverages?

Some examples of other beverages include a can of iced tea, tonic water, or a martini.

1. Never [SKIP to BATHINT]
 2. Less than one time per week [SKIP to BATHINT]
 3. 1-3 times per week [SKIP to BATHINT]
 4. 4-6 times per week [SKIP to BATHINT]
 5. 1 or more times per day
- [9. DK/REFUSED]

Question OTHBEVSP

Could you be more specific. How many times a day did you drink a glass of other beverages?

[ENTER "999" FOR DK/REFUSED]

Question BATHINT

Now I am going to ask you some questions about your use of water when bathing, and your use of swimming pools.

Question BATHNUM

On average, how many baths did you take a week? This question is not specific to a particular home and refers to your overall adult life.

[ENTER "999" FOR DK/REFUSED]

Question SWIMMING

Over your entire lifetime, did you swim, play, or exercise in a swimming pool at least 100 times?

1. YES
 2. NO
- [9. DK/REFUSED]

Question BINTRO

This portion of the interview will ask about your typical food and vitamin intake during your adult life.

Again, if you have had a major illness recently, please think about your typical food and vitamin intake before you became ill.

Question VIT

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...

did you ever regularly take any vitamin or mineral supplement, in a pill or liquid form, for a period of 30 days or more?

1. YES
2. NO [SKIP TO Question FISHCTCH]
- [9. DK/REFUSED]

[REGULARLY: AT LEAST 4 TIMES A WEEK.]

Question MULTIVIT

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...

did you regularly take a multi-vitamin supplement, such as One-A-Day, Centrum, or Theragram?

1. YES
2. NO [SKIP TO Question SNGLVIT]
- [9. DK/REFUSED]

[REGULARLY: AT LEAST 4 TIMES A WEEK.]

Question YRSMULTI

For how many years have you taken multivitamins?

[ENTER "999" FOR DK/REFUSED]

Question SNGLVIT

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...

did you regularly take any single vitamin or mineral supplements?

1. YES
2. NO [SKIP TO Question FISHCTCH]
- [9. DK/REFUSED]

[REGULARLY: AT LEAST 4 TIMES A WEEK.]

[Questions SPPLMNTS to YRSSNGL are asked for each item in the list]

Question SPPLMNTS

Did you take any of the following supplements?

Zinc?

Iron?

Selenium?

Beta-Carotene?

Other Vitamins?

1. YES
2. NO
- [9. DK/REFUSED]

Question SPPLOTH

Please specify the other vitamins you took.

Question YRSSNGL

For how many years have you taken

[ENTER "999" FOR DK/REFUSED]

Question FISHCTCH

On average, over a typical week of your adult life, how often do you eat fish that you catch?

1. Never [SKIP Question FISHOTH]
2. 1 meal per week, or less [SKIP Question FISHOTH]
3. More than 1 meal per week
- [9. DK/REFUSED]

Question FISHOTH

Please specify the number of meals per week.

[ENTER "999" FOR DK/REFUSED]

Question FOODINT

Now I will ask you about a series of foods. Please tell me how often you ate this food. To help you, I will list a serving size.

[Questions EATFOOD to OFTOTHSP are asked for each item in the list]

Question EATFOOD

During the year prior to diagnosis, (CASES)...

During the past year, (CONTROLS)...

how often did you eat
fish (fried, canned, broiled, etc) ?
mussels, oysters, crab, or lobster ?
mushrooms ?
sushi or seaweed ?

1. Never [SKIP Question EATOTHSP]
2. Less than one time per week [SKIP Question EATOTHSP]
3. 1-3 times per week [SKIP Question EATOTHSP]
4. 4-6 times per week [SKIP Question EATOTHSP]
5. 1 or more times per day
- [9. DK/REFUSED]

Question EATOTHSP

Please specify how many times per day.

[ENTER "999" FOR DK/REFUSED]

Question SERVING

Was your typical serving size of [food]
small, medium, or large?

1. Small
2. Medium
3. Large
- [9. DK/REFUSED]

Question FOODCHNG

Have you made any major changes in your consumption of
[food] during your adult life, compared
with your eating habits...(during the year prior to diagnosis (CASES)? (during the past year
(CONTROLS)?)

1. YES
2. NO [SKIP TO Question EATHERB]
- [9. DK/REFUSED]

Question OFTEAT

Over your adult life, how often did you eat [food] ?

1. Less than one time per week [SKIP Question OFTOTHSP]
2. 1-3 times per week [SKIP Question OFTOTHSP]
3. 4-6 times per week [SKIP Question OFTOTHSP]
4. 1 or more times per day
- [9. DK/REFUSED]

Question OFTOTHSP

Please specify how many times per day.

[ENTER "999" FOR DK/REFUSED]

Question EATHERB

have you ever consumed Asian herbal medicines or Asian medicinal
teas, which could be bought at an Asian Grocery or a Natural Foods
grocery store?

Please do not include ginkgo or echinacea in your answer.

1. YES
2. NO [SKIP TO Question CINTRO]
- [9. DK/REFUSED]

Question OFTHERB

How often did you use Asian herbal medicines or Asian medicinal teas?

Question HERBAGE

At what age did you start using Asian herbal medicines or Asian medicinal teas?

[ENTER "999" FOR DK/REFUSED]

Question HERBLAST

At what age did you last use Asian herbal medicines or Asian medicinal teas?

[ENTER "999" FOR DK/REFUSED]

Question HERBLONG

How long have you used Asian herbal medicines or Asian medicinal teas?

[IF DK/REFUSED, CODE AS 99 YEARS AND 99 MONTHS]

YEARS
MONTHS

Question CINTRO

Now I have some questions about your smoking habits.

Again, if you have had a major illness recently, please think about your tobacco use before you became ill.

Question LIFESMK

During your entire lifetime, have you ever smoked a total of 100 cigarettes or more?

1. YES
2. NO [SKIP TO Question CHEWTOB]
[9. DK/REFUSED]

[100 CIGARETTES = 5 PACKS]

Question REGSMK

Did you ever smoke cigarettes regularly, that is, at least one per day for six months or longer?

1. YES
2. NO [SKIP TO Question CHEWTOB]
[9. DK/REFUSED]

Question CIGNUM

Thinking about all the years that you smoked cigarettes,
how many cigarettes per day did you usually smoke?

Would you say:

1. Less than 10
2. 11 to 20
3. 21 to 30
4. More than 31
- [9. DK/REFUSED]

Question AGEICIG

How old were you when you first started smoking at least
one cigarette per day?

[ENTER "999" FOR DK/REFUSED]

Question LASTCIG

How old were you when you last smoked cigarettes?

[ENTER "999" FOR DK/REFUSED]

Question QUITSMK

Thinking about the years from when you began smoking until
your last cigarette, for how many total months or years did you **NOT** smoke cigarettes ?

YEARS
MONTHS

Question CIGTYPE

Were your cigarettes usually:

1. Filtered
2. Unfiltered
3. Both filtered and unfiltered
- [9. DK/REFUSED]

Question CHEWTOB

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...

did you ever use smokeless tobacco (such as chewing tobacco) for
more than six months?

1. YES
2. NO [SKIP TO Question SMKCIGAR]
- [9. DK/REFUSED]

Question AGECHEW

How old were you when you first started using smokeless tobacco?

[ENTER "999" FOR DK/REFUSED]

Question LASTCHEW

How old were you when you last used smokeless tobacco?

[ENTER "999" FOR DK/REFUSED]

Question LONGCHEW

For how long altogether have you used smokeless tobacco?

Please do not include any periods during which you may have quit.

[IF DK/REFUSED, CODE AS 99 YEARS AND 99 MONTHS]

YEARS
MONTHS

Question SMK CIGAR

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...

have you ever smoked at least one cigar per week for six months or longer?

1. YES
2. NO [SKIP TO Question SMKPIPE]
- [9. DK/REFUSED]

Question AGE CIGAR

How old were you when you first started smoking at least one cigar per week?

[ENTER "999" FOR DK/REFUSED]

Question LAST CIGR

How old were you when you last smoked a cigar?

[ENTER "999" FOR DK/REFUSED]

Question LONG CIGR

For how long altogether have you smoked cigars?

Please do not include any periods during which you may have quit.

[IF DK/REFUSED, CODE AS 99 YEARS AND 99 MONTHS]

YEARS
MONTHS

Question CIGARNUM

Thinking about all the years that you smoked cigars, how many cigars did you usually smoke in a week?

Would you say,

1. Less than 10
2. 11 to 20
3. 21 to 30
4. More than 31
- [9. DK/REFUSED]

Question SMKPIPE

Before you were diagnosed with bladder cancer, (CASES)...
Before 1 year ago, (CONTROLS)...

have you ever smoked at least one pipeful of tobacco per week for six months or longer?

1. YES
2. NO [SKIP TO Question DINTRO]
- [9. DK/REFUSED]

Question AGEPIPE

How old were you when you first started smoking at least one pipeful of tobacco per week?

[ENTER "999" FOR DK/REFUSED]

Question LASTPIPE

How old were you when you last smoked a pipe?

[ENTER "999" FOR DK/REFUSED]

Question LONGPIPE

For how long altogether have you smoked a pipe?

Please do not include any periods during which you may have quit.

[IF DK/REFUSED, CODE AS 99 YEARS AND 99 MONTHS]

YEARS
MONTHS

Question AMTPIPE

Thinking about all the years that you smoked a pipe, how many pipefuls of tobacco did you usually smoke in a week?

Would you say:

1. Less than 10
2. 11 to 20
3. 21 to 30
4. More than 31
- [9. DK/REFUSED]

Question DINTRO

In the next series of questions, I'm going to ask about a variety of diseases, and other medical conditions. Please consider the time period from your birth up until 1 year ago.

If you have had a major illness recently, please consider the time period before you became ill.

There may be some questions that you can't answer precisely, but please give your best estimate.

Question HEIGHT

What has been your usual height as an adult?

[IF DK/REFUSED, CODE AS 99 FEET AND 99 INCHES]

FEET
INCHES

Question WGHTAVE

Before you were diagnosed with bladder cancer, (CASES)...
Before 1 year ago, (CONTROLS)...

What was been you usual weight as an adult?

[ENTER "999" FOR DK/REFUSED]

POUNDS

Question WEIGHTLO

What was your lowest weight as an adult?

[ENTER "999" FOR DK/REFUSED]

POUNDS

Question WEIGHTHI

What was your maximum weight as an adult?

[ENTER "999" FOR DK/REFUSED]

POUNDS

Question HVYEXER

during an average WEEK how many days did you perform STRENUOUS exercise for at least 30 minutes? Strenuous exercise is exercise you do that makes you breathe heavily and your heart beat fast (e.g. hiking, jogging, aerobics)

Would you say:

1. None
 2. 1 day only
 3. 2 to 3 days
 4. 4 to 5 days
 5. More than 5 days a week
- [9. DK/REFUSED]

Question LGHTEXER

during an average WEEK how many days did you perform LIGHT exercise for at least 30 minutes? Light exercise is exercise that does not make you breathe heavily or your heart beat fast (e.g. walking at a normal pace or slow bicycling)

Would you say:

1. None
 2. 1 day only
 3. 2 to 3 days
 4. 4 to 5 days
 5. More than 5 days a week
- [9. DK/REFUSED]

Question SITTING

During a normal week of your adult life, how many hours a DAY do you watch television, or, sit and read a newspaper or book, or work sitting at a desk or table?

Would you say:

1. None
 2. 1 hour or less
 3. 2 to 3 hours
 4. 4 to 5 hours
 5. 6 or more hours
- [9. DK/REFUSED]

Question WALKPACE

Over the past 10 years, how would you describe on average your usual walking pace outdoors?

Would you say:

1. Slow or casual
 2. Normal to average
 3. Brisk or hurried
 4. Unable to walk, except with difficulty
- [9. DK/REFUSED]

Question STAIRCLM

On average over the past 10 years, how many flights of stairs (not individual steps) did you climb each day?

Would you say:

1. 2 flights or less
2. 3-4 flights
3. 5-9 flights
4. 10 or more flights
- [9. DK/REFUSED]

Question DIALYSIS

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...
did you ever have kidney dialysis?

1. YES
2. NO
- [9. DK/REFUSED]

Question HAVEBLAD

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...
did you ever have a bladder infection with at least one of the following symptoms: frequent urination, or pain or burning when urinating?

1. YES
2. NO [SKIP TO Question KIDDIAG]
- [9. DK/REFUSED]

Question TIMSBLAD

How many times did you have this kind of infection?

Would you say:

[IWER NOTE: BLADDER INFECTION]

1. 1 or 2 times
2. 3 to 5 times
3. 6 to 10 times
4. 11 or more times
- [9. DK/REFUSED]

Question AGEBLAD

How old were you when you first had this type of infection?

[IWER NOTE: BLADDER INFECTION]

[ENTER "999" FOR DK/REFUSED]

Question LASTBLAD

How old were you when you last had this type of infection?

[IWER NOTE: BLADDER INFECTION]

[ENTER "999" FOR DK/REFUSED]

Question BLADDIAG

Were any of these bladder infections diagnosed by a physician?

1. YES
2. NO
- [9. DK/REFUSED]

Question KIDDIAG

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...

did you ever have a kidney infection diagnosed by a physician?

1. YES
2. NO [SKIP TO Question RENSTONE]
- [9. DK/REFUSED]

Question AGEKID

How old were you when you first had this type of infection?

[IWER NOTE: KIDNEY INFECTION]

[ENTER "999" FOR DK/REFUSED]

Question TIMESKID

How many times did you have this kind of infection?

Would you say:

[IWER NOTE: KIDNEY INFECTION]

1. 1 or 2 times
2. 3 to 5 times
3. 6 to 10 times
4. 11 or more times
- [9. DK/REFUSED]

Question RENSTONE

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...

did you ever have nephritic colic, kidney or renal stones?

1. YES

- 2. NO
- [9. DK/REFUSED]

Question BLADSTON

Before you were diagnosed with bladder cancer, (CASES)...
Before 1 year ago, (CONTROLS)...
did you ever have urinary bladder stones?

- 1. YES
- 2. NO
- [9. DK/REFUSED]

Question BLADGROW

Before you were diagnosed with bladder cancer, (CASES)...
Before 1 year ago, (CONTROLS)...
did you ever have a growth removed from your bladder?

- 1. YES
- 2. NO
- [9. DK/REFUSED]

Question CONFINT

The next questions may seem personal in nature.
Please remember that all your answers are confidential.

Question DIFFURIN

Did you ever have any of the following symptoms when urinating:
difficulty starting, difficulty stopping, or increased frequency
during night?

- 1. YES
- 2. NO [SKIP TO Question FREQINT]
- [9. DK/REFUSED]

Question AGEDIFF

At what age did you first start to experience any of these symptoms?

[ENTER "999" FOR DK/REFUSED]

Question FREQINT

Now I'm going to ask you about your frequency of urination during
your adult life. I'm interested in your usual habits as an adult,
before 2 years ago.

Again, if you have had a major illness recently, please consider
the time period before you became ill.

Question URINE1

After waking up but before breakfast, how many times on the average did you urinate?

1. Never
2. Once
3. 2 to 3 times
4. 4 or more times
- [9. DK/REFUSED]

Question URINE2

Between breakfast and lunch time, how many times on the average did you urinate?

1. Never
2. Once
3. 2 to 3 times
4. 4 or more times
- [9. DK/REFUSED]

Question URINE3

Between lunch and dinner time, how many times on the average did you urinate?

1. Never
2. Once
3. 2 to 3 times
4. 4 or more times
- [9. DK/REFUSED]

Question URINE4

After dinner time, how many times on the average did you urinate?

1. Never
2. Once
3. 2 to 3 times
4. 4 or more times
- [9. DK/REFUSED]

Question URINE5

While you were sleeping during a typical night, how many times did you usually wake up to urinate?

1. Never
2. Once
3. 2 to 3 times
4. 4 or more times
- [9. DK/REFUSED]

Question HOLDURIN

Before you were diagnosed with bladder cancer, (CASES)...
Before 1 year ago, (CONTROLS)...
did you ever regularly have to postpone your urination for at

least 1 hour, before being able to go to the bathroom? For example, because of work commitments, or being stuck in traffic, etc.

1. YES
2. NO [SKIP TO Question CANCEINT]
- [9. DK/REFUSED]

Question HOURHOLD

On average, how many hours passed between when you first wanted to urinate and when you were able to urinate?

1. 1 hour
2. 2 hours
3. 3 hours
4. 4 or more hours
- [9. DK/REFUSED]

Question OFTHOLD

On average during your adult life, how often did you have to "hold-it" without being able to urinate?

1. Every day
2. Once a week
3. Once a month
4. Less than once a month
- [9. DK/REFUSED]

Question AGEHOLD

At what age did you begin to regularly have to hold your urinations without being able to urinate?

[ENTER "999" FOR DK/REFUSED]

Question DIAGINT

Now, I will ask you about health conditions you may have been diagnosed with.

Question SKINDIAG

Did you ever have a skin condition diagnosed by a doctor?

1. YES
2. NO [SKIP TO Question NAILDIAG]
- [9. DK/REFUSED]

[Questions SKINCOND to AGESKIN are asked for each item on the list]

Question SKINCOND

Did a doctor tell you that you had:
eczema?
psoriasis?

keratosis?
hypopigmentation?
hyperpigmentation?
vitiligo?
other skin ailment?

1. YES
2. NO [SKIP TO Question NAILDIAG]
- [9. DK/REFUSED]

Question SKCONDOT

Please specify the other skin ailment.

Question AGESKIN

How old were you when you were first diagnosed with [skin condition] ?

[ENTER "999" FOR DK/REFUSED]

Question NAILDIAG

Did you ever have a nail condition diagnosed by a doctor?

1. YES
2. NO [SKIP TO Question ACETAMIN]
- [9. DK/REFUSED]

[Questions NAILCOND to AGENAIL are asked for each item on the list]

Question NAILCOND

Did a doctor tell you that you had:
pityriasis rubra pilaris?
idiopathic onycholysis?
bullous ichthyosiform erythroderma?
Yellow nail syndrome?
Beau's lines
other nail ailment?

1. YES
2. NO [SKIP TO Question AGENAIL]
- [9. DK/REFUSED]

Question NLCONDOT

Please specify the other nail ailment.

Question AGENAIL

How old were you when you were first diagnosed with [nail condition] ?

[ENTER "999" FOR DK/REFUSED]

Question ACETAMIN

Before you were diagnosed with bladder cancer, (CASES)...
Before 1 year ago, (CONTROLS)...
did you regularly take any medications with acetaminophen as the active ingredient, such as tylenol? By regularly, I mean at least 4 times a week for one month or longer.

1. YES
2. NO [SKIP TO Question FOWLER]
- [9. DK/REFUSED]

Question AGEACET

How old were you when you first took this medicine, regularly?

[ENTER "999" FOR DK/REFUSED]

Question LASTACET

How old were you when you last took this medicine, regularly?

[ENTER "999" FOR DK/REFUSED]

Question LONGACET

For how long altogether did you take this medicine, regularly?

[IF DK/REFUSED, CODE AS 99 YEARS AND 99 MONTHS]

YEARS
MONTHS

Question FOWLER

Were you ever treated with arsenic or Fowler's solution for a skin or other condition?

1. YES
2. NO [SKIP TO Question COALOINT]
- [9. DK/REFUSED]

Question LONGFOWL

For how long were you treated?

[IF DK/REFUSED, CODE AS 99 YEARS AND 99 MONTHS]

YEARS
MONTHS

Question COALOINT

Were you ever treated with coal tar ointment for a skin or other condition?

1. YES

2. NO [SKIP TO Question DIABETES]
[9. DK/REFUSED]

Question LONGCOAL

For how long were you treated?

[IF DK/REFUSED, CODE AS 99 YEARS AND 99 MONTHS]

YEARS
MONTHS

Question DIABETES

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...

were you ever told by a doctor that you had diabetes?

1. YES
2. NO [SKIP TO Question CRAMP]
- [9. DK/REFUSED]

Question AGEDIBT

How old were you when you were first told you had diabetes?

[ENTER "999" FOR DK/REFUSED]

Question CRAMP

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...

did you ever have a cramp or pain in the leg, calf, or
buttocks while walking that is relieved by rest?

1. YES
2. NO [SKIP TO Question HRTATTCK]
- [9. DK/REFUSED]

Question CIRCPROB

Did the doctor tell you that you had a circulation problem
that contributed to these symptoms?

1. YES
2. NO [SKIP TO Question HRTATTCK]
- [9. DK/REFUSED]

Question AGE CIRC

How old were you when you were first told you had this
circulation problem?

[ENTER "999" FOR DK/REFUSED]

Question HRTATTCK

Before you were diagnosed with bladder cancer, (CASES)...
Before 1 year ago, (CONTROLS)...
did you ever have a heart attack?

1. YES
2. NO [SKIP TO Question HYPERTEN]
- [9. DK/REFUSED]

Question AGEHRTAT

How old were you when you had your first heart attack?

[ENTER "999" FOR DK/REFUSED]

Question HYPERTEN

Before you were diagnosed with bladder cancer, (CASES)...
Before 1 year ago, (CONTROLS)...
were you ever told by a doctor that you had high blood
pressure or hypertension?

1. YES
2. NO [SKIP TO Question ARTERO]
- [9. DK/REFUSED]

Question AGEHYPE

How old were you when you were first told you had high blood
pressure or hypertension?

[ENTER "999" FOR DK/REFUSED]

Question ARTERO

Before you were diagnosed with bladder cancer, (CASES)...
Before 1 year ago, (CONTROLS)...
were you ever told by a doctor that you had arterosclerosis?

1. YES
2. NO [SKIP TO Question KIDCOMP]
- [9. DK/REFUSED]

Question AGEARTH

How old were you when you were first told you had arterosclerosis?

[ENTER "999" FOR DK/REFUSED]

Question KIDCOMP

Before you were diagnosed with bladder cancer, (CASES)...
Before 1 year ago, (CONTROLS)...
were you ever told by a doctor that you had a kidney
complication such as hematuria, leukocyturia, or glycosuria?

1. YES
2. NO [SKIP TO Question LIVCOMP]
- [9. DK/REFUSED]

Question AGEKDCMP

How old were you when you were first told you had a kidney complication?

[ENTER "999" FOR DK/REFUSED]

Question LIVCOMP

Before you were diagnosed with bladder cancer, (CASES)...
Before 1 year ago, (CONTROLS)...
were you ever told by a doctor that you had a liver complication,
such as cirrhosis of the liver or non-cirrhotic portal fibrosis?

1. YES
2. NO [SKIP TO Question EXSSFATS]
- [9. DK/REFUSED]

Question AGELVCMP

How old were you when you were first told you had a liver ailment?

[ENTER "999" FOR DK/REFUSED]

Question EXSSFATS

Before you were diagnosed with bladder cancer, (CASES)...
Before 1 year ago, (CONTROLS)...
were you ever told by a doctor that you had hyperlipidemia
(the presence of excess fats or lipids in the blood)?

1. YES
2. NO [SKIP TO Question STROKE]
- [9. DK/REFUSED]

Question AGEEXFAT

How old were you when you were first told you had hyperlipidemia?

[ENTER "999" FOR DK/REFUSED]

Question STROKE

Before you were diagnosed with bladder cancer, (CASES)...
Before 1 year ago, (CONTROLS)...
were you ever told by a doctor that you had a stroke,
cerebrovascular accident, blood clot or bleeding in the brain,
or transient ischemic attack?

1. YES
2. NO [SKIP TO Question THYROID]

[9. DK/REFUSED]

[Questions STRKTYPE to STRKAGE are asked for each item on the list]

Question STRKTYPE

Did you have a:

Stroke
Transient ischemic attack
Another cerebrovascular event

1. YES
 2. NO [SKIP TO Question THYROID]
- [9. DK/REFUSED]

Question STRKOTH

Please specify the other cerebrovascular event.

Question STRKAGE

How old were you when you were diagnosed with:

[cerebrovascular event] ?

[ENTER "999" FOR DK/REFUSED]

Question THYROID

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...

did you ever have a thyroid disorder diagnosed by a doctor?

1. YES
 2. NO [SKIP TO Question EINTRO]
- [9. DK/REFUSED]

[Questions THYRTYPE to THYROTH are asked for each item on the list]

Question THYRTYPE

Did a doctor tell you that you had:

Hyperthyroidism? (over-active thyroid)
Hypothyroidism? (under-active thyroid)
Other thyroid disorder?

1. YES
 2. NO [SKIP TO Question EINTRO]
- [9. DK/REFUSED]

Question THYROTH

Please specify the other thyroid disorder.

Question EINTRO

Now we will be asking you about your family history. For these questions, we are interested in your immediate blood relatives. That is, your mother, father, siblings, or children.

Question SURVIVE

How many brothers, sisters, and children did you have who survived past age 18?

Question FAMDIAG

Were any of your brother, sisters, or children ever diagnosed with cancer?

1. YES
2. NO
- [9. DK/REFUSED]

Question FAMURCAN

Were any of your immediate blood relatives, ever diagnosed as having urinary tract cancer?

1. YES
2. NO [SKIP TO Question FAMDIAB]
- [9. DK/REFUSED]

[IMMEDIATE BLOOD RELATIVE IS MOTHER, FATHER, SIBLINGS, OR CHILDREN]

Question RELTYPE

Which relative was diagnosed?

1. Mother
2. Father
3. Brother
4. Sister
5. Child
- [9. DK/REFUSED]

Question RELAGE

How old was he/she when the cancer was first diagnosed?

Question RELMORE

Do you have any more immediate blood relatives that have been diagnosed as having urinary tract cancer?

1. YES
2. NO
- [9. DK/REFUSED]

[IMMEDIATE BLOOD RELATIVE IS MOTHER, FATHER, SIBLINGS, OR CHILDREN]

Question FAMDIAB

Were any of your immediate blood relatives ever diagnosed with diabetes?

1. YES
2. NO
- [9. DK/REFUSED]

[IMMEDIATE BLOOD RELATIVE IS MOTHER, FATHER, SIBLINGS, OR CHILDREN]

Question FAMHYPER

Were any of your immediate blood relatives ever diagnosed with hypertension or high blood pressure?

1. YES
2. NO
- [9. DK/REFUSED]

[IMMEDIATE BLOOD RELATIVE IS MOTHER, FATHER, SIBLINGS, OR CHILDREN]

Question FAMSTRKE

Did any of your immediate blood relatives ever suffer a stroke?

1. YES
2. NO
- [9. DK/REFUSED]

[IMMEDIATE BLOOD RELATIVE IS MOTHER, FATHER, SIBLINGS, OR CHILDREN]

Question FAMHEART

Did any of your immediate blood relatives ever suffer a heart attack?

1. YES
2. NO
- [9. DK/REFUSED]

[IMMEDIATE BLOOD RELATIVE IS MOTHER, FATHER, SIBLINGS, OR CHILDREN]

Question GINTRO

We are almost done with the interview now. I just need to ask some basic questions about you.

Question ETHNICTY

Are you Hispanic or Latino?

1. YES
2. NO
- [9. DK/REFUSED]

Question RACE

Could you tell me about your race?

- [1. Black or African American]
- [2. White]
- [3. American Indian or Alaskan Native]
- [4. Asian]
- [5. Native Hawaiian or Other Pacific Islander]
- [6. Multiracial (specify)]
- [7. Other (specify)]
- [9. DK/REFUSED]

*** (Since Census figures document that the racial composition of the study area is similar to the composition of the cancer group, random sampling of the study area should theoretically produce the same frequencies.)***

*** (If kept in back, can remain "check all that apply". If moved to front, must be mutually exclusive answers for quota.)***

Question EDUCATE

What is the highest grade or level of schooling you have completed?

[IWER NOTE: DO NOT READ RESPONSES, CODE BASED ON R'S ANSWER]

- [1. Less than 8 years]
- [2. 8-11 years]
- [3. 12 years or high school graduate]
- [4. Post-high school training (vocational or technical training)]
- [5. Some college]
- [6. College graduate]
- [7. Postgraduate education]
- [8. Other]
- [9. DK/REFUSED]

Question MARSTAT

What is your current marital status?

Are you...

- 1. Married/Living with a partner
- 2. Widowed
- 3. Separated
- 4. Divorced
- 5. Never married
- 6. Other
- [9. DK/REFUSED]

Question RATEHLTH

Before you were diagnosed with bladder cancer, (CASES)...

Before 1 year ago, (CONTROLS)...

how would you rate your health?

- 1. Excellent
- 2. Very Good
- 3. Good

- 4. Fair
- 5. Poor
- [9. DK/REFUSED]

Question DIAGHLTH (If CONTROL then skip DIAGHLTH)

Since you were diagnosed with bladder cancer, how would you rate your health?

- 1. Excellent
- 2. Very Good
- 3. Good
- 4. Fair
- 5. Poor
- [9. DK/REFUSED]

Question VERPHONE

That was our last question. Thank you very much for your assistance. A member of the research team at the University of Michigan, School of Public Health will be calling you within the next month to make an appointment to collect a water sample, toenail clippings, and your residential and occupational histories.

Would you like to be called at this number, _____ ?

- 1. YES [SKIP TO Question TERM1]
- 2. NO
- [9. DK/REFUSED]

Question NEWPHONE

At what phone number would you like us to call you back at?

Question TERM1

Do you have any questions for us?

Question CALLBAK

Okay, when would be a good time to call back?

Question CALLBAK2

When would be a better time to call back?

Question QALNOT

I'm sorry, from the information you have given me, the computer says that we have filled the interviews needed for either your age group or county.

So, we do not need to ask you any questions today.

Thank you for your time. Goodbye.

Question ENDREF

Okay, I'm sorry to have bothered you.

Thank you. Goodbye.

Question ENDREF2

I'm sorry, that county is not in the study area.

Thank you for your time today. Goodbye.

Question ENDREF3

I'm sorry, this study focuses on individuals who have lived in the area for 5 or more years.

Thank you for your time today. Goodbye.

Question ENDREF4

I'm sorry, since this a scientific study, individuals with cancer have already been selected, so we will not be able to include you in the study.

Thank you for your time today. Goodbye.

Appendix B

Food Frequency Questionnaire

Participant ID: _____



The University of Michigan School of Public Health

Arsenic and Bladder Cancer in Michigan

Food Frequency & Behavior Questionnaire

The following questionnaire accompanies collection of the urine sample. We are interested in certain behaviors and eating habits up to two days before you collect the urine sample. Therefore, this questionnaire is divided into two different days.

Instructions:

- Please collect your urine in the morning, following the instructions in the urine kit.
- Please complete the following questionnaire after you have finished collecting your urine or the night before collecting your urine.
- When finished, please place this questionnaire in the envelope provided and put it with the Styrofoam cooler.

If you have any questions, please call us (toll free) 866-418-0028.

Thank you!

DAY 1

Participant ID: _____ **Day of Week:** _____ **Date:** _____

In this section, we are interested in certain foods that you ate on the day indicated above. In the table below, please tell us the number of servings you ate on that day. If you did not eat a food, indicate that you ate "0" (zero) servings. Use the serving sizes in the right column as a reference.

FOODS	NUMBER OF SERVINGS	SERVING SIZE
Rice		1 cup cooked (8oz)
Rice cereal		1 cup (8oz)
Mushrooms		1 cup fresh (8oz) or ½ cup cooked (4oz)
Clam/fish chowder		1 bowl
Chicken		2 small pieces (e.g., drumstick and thigh) or 1 large piece (e.g., breast)
Canned fish (e.g., tuna)		½ can (3oz)
Fish - fresh or frozen: a. Cod b. Haddock c. Salmon d. Scup e. Swordfish f. Sole g. Sea bass h. Tuna i. Other (specify): _____	_____ _____ _____ _____ _____ _____ _____ _____ _____	4 ounces or one sandwich

Sushi		2 rolls
Seaweed		2 sheets
Shellfish		
a. Shrimp	_____	8 pieces
b. Clams	_____	8 pieces
c. Oysters	_____	8 pieces
d. Scallops	_____	8 pieces
e. Mussels	_____	8 pieces
f. Crabs	_____	3 crabs
g. Lobster	_____	1 lobster
h. Other (specify):		
_____	_____	

DAY 1 (continued)	Day of Week: _____	Date: _____
--------------------------	--------------------	-------------

Below, please tell us the number of glasses of water, cups of coffee or tea, and amount of other beverages that you drank **using your tap water** on the day indicated above. If you did not drink the beverage listed please indicate that you drank "0" (zero) servings. Use the serving size in the right column as a reference.

BEVERAGES	NUMBER OF SERVINGS	SERVING SIZE
Water from your home tap water		8 ounce glass
Cold drinks made with your tap water (e.g., ice tea, lemonade, tang, orange juice made from concentrate)		8 ounce glass
Coffee made with your tap water		1 cup (8oz)
Hot tea made with your tap water		1 cup (8oz)
Other hot drinks made with your tap water		1 cup (8oz)

Below, please tell us what dietary supplements you took on the day indicated above. If you did not take the supplement listed, please write "0" (zero) in the "number of pills taken" column.

DIETARY SUPPLEMENTS	NUMBER OF PILLS TAKEN	BRAND
Multivitamin		_____ _____
Single Vitamin, Mineral & Herbal Supplements		
Iron	_____	_____
Selenium	_____	_____
Zinc	_____	_____
Vitamin E	_____	_____

Vitamin C	_____	_____
Calcium	_____	_____
Others (please specify):		
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Day 2

Participant ID: _____ Day of the Week: _____ Date: _____

In this section, we are interested in certain foods that you ate on the day indicated above. In the table below, please tell us the number of servings you ate on that day. If you did not eat a food, indicate that you ate "0" (zero) servings. Use the serving sizes in the right column as a reference.

FOODS	NUMBER OF SERVINGS	SERVING SIZE
Rice		1 cup cooked (8oz)
Rice cereal		1 cup (8oz)
Mushrooms		1 cup fresh (8oz) or ½ cup cooked (4oz)
Clam/fish chowder		1 bowl
Chicken		2 small pieces (e.g., drumstick and thigh) or 1 large piece (e.g., breast)
Canned fish (e.g., tuna)		½ can (3oz)
Fish - fresh or frozen: a. Cod b. Haddock c. Salmon d. Scup e. Swordfish f. Sole g. Sea bass h. Tuna i. Other (specify): _____	_____ _____ _____ _____ _____ _____ _____ _____ _____	4 ounces or one sandwich

Sushi		2 rolls
Seaweed		2 sheets
Shellfish		
a. Shrimp	_____	8 pieces
b. Clams	_____	8 pieces
c. Oysters	_____	8 pieces
d. Scallops	_____	8 pieces
e. Mussels	_____	8 pieces
f. Crabs	_____	3 crabs
g. Lobster	_____	1 lobster
h. Other (specify):		
_____	_____	

DAY 2 (continued) Day of Week: _____ Date: _____
--

Below, please tell us the number of glasses of water, cups of coffee or tea, and amount of other beverages that you drank **using your tap water** on the day indicated above. If you did not drink the beverage listed please indicate that you drank "0" (zero) servings. Use the serving size in the right column as a reference.

BEVERAGES	NUMBER OF SERVINGS	SERVING SIZE
Water from your home tap water		8 ounce glass
Cold drinks made with your tap water (e.g., ice tea, lemonade, tang, orange juice made from concentrate)		8 ounce glass
Coffee made with your tap water		1 cup (8oz)
Hot tea made with your tap water		1 cup (8oz)
Other hot drinks made with your tap water		1 cup (8oz)

Below, please tell us what dietary supplements you took on the day indicated above. If you did not take the supplement listed, please write "0" (zero) in the "number of pills taken" column.

DIETARY SUPPLEMENTS	NUMBER OF PILLS TAKEN	BRAND
Multivitamin		_____ _____
Single Vitamin, Mineral & Herbal Supplements		
Iron	_____	_____
Selenium	_____	_____
Zinc	_____	_____
Vitamin E	_____	_____

Vitamin C	_____	_____
Calcium	_____	_____
Others (please specify):		
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

DAY 2 (continued) Day of Week: _____ **Date:** _____

Please answer the following questions about tobacco use on the date indicated above.

TOBACCO USE	
Did you smoke any cigarettes?	<input type="checkbox"/> Yes How many? _____ <input type="checkbox"/> No
Yesterday, did you smoke a cigar?	<input type="checkbox"/> Yes How many? _____ <input type="checkbox"/> No
Yesterday, did you smoke a pipe?	<input type="checkbox"/> Yes How many? _____ <input type="checkbox"/> No
Yesterday, did you chew tobacco?	<input type="checkbox"/> Yes How many times? _____ <input type="checkbox"/> No
Yesterday, did someone smoke in your home?	<input type="checkbox"/> Yes <input type="checkbox"/> No

Please indicate if you drank any of the following alcoholic beverages on the day indicated above. If you did not drink the beverage listed please indicate that you drank "0" (zero) servings. Use the serving sizes in the right column as reference.

ALCOHOLIC BEVERAGES	NUMBER OF DRINKS	SERVING SIZE
Beer		1 beer = 12oz bottle or can
Wine		1 glass = 4oz of wine
Other alcoholic beverages		1 drink = 1oz of liquor

DAY 3

Participant ID: _____ Day of Week: _____ Date: _____

In this section, we are interested in certain foods that you ate on the day indicated above. In the table below, please tell us the number of servings you ate on that day. If you did not eat a food, indicate that you ate "0" (zero) servings. Use the serving sizes in the right column as a reference.

FOODS	NUMBER OF SERVINGS	SERVING SIZE
Rice		1 cup cooked (8oz)
Rice cereal		1 cup (8oz)
Mushrooms		1 cup fresh (8oz) or ½ cup cooked (4oz)
Clam/fish chowder		1 bowl
Chicken		2 small pieces (e.g., drumstick and thigh) or 1 large piece (e.g., breast)
Canned fish (e.g., tuna)		½ can (3oz)
Fish - fresh or frozen: a. Cod b. Haddock c. Salmon d. Scup e. Swordfish f. Sole g. Sea bass h. Tuna i. Other (specify): _____	_____ _____ _____ _____ _____ _____ _____ _____ _____	4 ounces or one sandwich
Sushi		2 rolls
Seaweed		2 sheets

Shellfish		
a. Shrimp	_____	8 pieces
b. Clams	_____	8 pieces
c. Oysters	_____	8 pieces
d. Scallops	_____	8 pieces
e. Mussels	_____	8 pieces
f. Crabs	_____	3 crabs
g. Lobster	_____	1 lobster
h. Other (specify):	_____	
_____	_____	

DAY 3 (continued)**Day of Week:** _____ **Date:** _____

Below, please tell us the number of glasses of water, cups of coffee or tea, and amount of other beverages that you drank **using your tap water** on the day indicated above. If you did not drink the beverage listed please indicate that you drank “0” (zero) servings. Use the serving size in the right column as a reference.

BEVERAGES	NUMBER OF SERVINGS	SERVING SIZE
Water from your home tap water		8 ounce glass
Cold drinks made with your tap water (e.g., ice tea, lemonade, tang, orange juice made from concentrate)		8 ounce glass
Coffee made with your tap water		1 cup (8oz)
Hot tea made with your tap water		1 cup (8oz)
Other hot drinks made with your tap water		1 cup (8oz)

Below, please tell us what dietary supplements you took on the day indicated above. If you did not take the supplement indicated, please write “0” (zero) in the “number of pills taken” column.

DIETARY SUPPLEMENTS	NUMBER OF PILLS TAKEN	BRAND
Multivitamin		_____ _____
Single Vitamin, Mineral & Herbal Supplements		
Iron	_____	_____
Selenium	_____	_____
Zinc	_____	_____
Vitamin E	_____	_____

Vitamin C	_____	_____
Calcium	_____	_____
Others (please specify):		
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

DAY 3 (continued) Day of Week: _____ Date: _____

Please answer the following questions about tobacco use on the date indicated above.

TOBACCO USE	
Did you smoke any cigarettes?	<input type="checkbox"/> Yes How many? _____ <input type="checkbox"/> No
Yesterday, did you smoke a cigar?	<input type="checkbox"/> Yes How many? _____ <input type="checkbox"/> No
Yesterday, did you smoke a pipe?	<input type="checkbox"/> Yes How many? _____ <input type="checkbox"/> No
Yesterday, did you chew tobacco?	<input type="checkbox"/> Yes How many times? _____ <input type="checkbox"/> No
Yesterday, did someone smoke in your home?	<input type="checkbox"/> Yes <input type="checkbox"/> No

Please indicate if you drank any of the following alcoholic beverages on the day indicated above. If you did not drink the beverage listed please indicate that you drank "0" (zero) servings. Use the serving sizes in the right column as reference.

ALCOHOLIC BEVERAGES	NUMBER OF DRINKS	SERVING SIZE
Beer		1 beer = 12oz bottle or can
Wine		1 glass = 4oz of wine
Other alcoholic beverages		1 drink = 1oz of liquor

THANK YOU FOR COMPLETING THIS FORM.

PLEASE PLACE IT IN THE ENVELOPE PROVIDED AND PUT IT WITH THE COOLER THAT CONTAINS YOUR COLLECTED URINE SAMPLE.

PLEASE LEAVE THE COOLER BY THE FRONT DOOR WHERE A MEMBER OF THE STUDY TEAM WILL PICK UP THE URINE SAMPLE AND THIS FORM.