DISPARITIES IN ENVIRONMENTAL EXPOSURES AND HEALTH IN THAILAND: MOLECULAR EFFECTS OF CADMIUM EXPOSURE AND TRENDS IN CHILDHOOD LEUKEMIA

By

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ABSTRACT

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Thailand has undergone rapid social and economic changes over the past fifty years, and prevalence and mortality of disease has decreased for infectious diseases and increased for chronic diseases across the age spectrum. This dissertation focuses on identifying disparities in childhood cancer and environmental health in Thailand by examining 1) childhood leukemia incidence and survival trends from 1990-2011 in the Songkhla Province in Thailand, and 2) high cadmium (Cd) exposure among northern Thai women from Mae Sot and its effects on biologic aging and co-exposure to toxic and essential metals. The first aim utilized cancer data from the Songkhla Cancer Registry and the United States (US). Leukemia incidence and survival was significantly lower in Songkhla compared to US but incidence and survival significantly increased annually from 1990-2011 in Thailand by approximately 2%.

In the second and third aims, epigenome-wide DNA methylation and blood and urine metal biomarkers of exposure were measured in two samples of women from Mae Sot. These women were exposed to Cd, a toxic metal, after ingesting water and rice contaminated by environmental pollution from nearby zinc mining. DNA methylation is a dynamic and sensitive epigenetic marker that changes during aging and is associated with Cd exposure. Biologic age, or the physiologic age of an individual, can be estimated from a collection of a subset of these changes. A greater difference between chronologic age and biologic age may indicate accelerated aging. In Aim 2, higher Cd exposure was associated with smaller difference between biologic and chronologic age, and Cd modified methylation at some age-associated sites included in predictors of biologic age. In Aim 3 co-exposure of metals were examined in blood and urine using multivariate methods. Blood lead and urinary arsenic were also elevated in this high Cd exposed sample. Unique patterns emerged among these metals, suggesting that lead and Cd exposures were independent. Further public health interventions are necessary to address pediatric cancer incidence and survival disparities in Thailand and to study sources of exposure to other metals and other biomarkers aging within the Mae Sot population.

CHAPTER 1. INTRODUCTION

BACKGROUND

The demographic and epidemiologic transitions are occurring at a faster rate in Southeast (SE) Asia than any other region in the world (1). The demographic transition is defined as the shift from a pre-industrialized economy with high birth and death rates to industrialized economy, where both birth and death rates decline. The epidemiologic transition is the shift in the prevalence of communicable diseases, e.g. infectious bacterial diseases and malaria, to non-communicable diseases, e.g. cardiovascular disease, diabetes, and cancer, as countries undergo social and economic development (2). SE Asia is comprised of countries in various states of the demographic and epidemiologic transitions. Thailand is a lower-middle income country (LMIC) undergoing these transitions (1, 3), where the prevalence of chronic diseases has increased, population structure has changed, and environmental exposures and toxicants have been introduced.

Thailand has experienced many of the effects of the demographic and epidemiologic transition since the1950s. The Thai population is one of the fastest aging populations in the world (4) and the fertility rate is below replacement. Almost half of the population resides in urban areas (5). Child mortality under age five has decreased from 87.9 per 1,000 in 1970 to 8.9 per 1,000 in 2010 (6). Thailand continues to be faced with a dual-burden of disease. Mortality from chronic disease has increased, but there is a persistent prevalence of infectious disease, including lower respiratory infections, diarrheal diseases, and tuberculosis, within this country

(7, 8). The most impoverished populations in Thailand are most vulnerable to mortality from both chronic and infectious disease. Like other countries in SE Asia, rapid and less restricted industrialization occurred in Thailand, exposing the population to both anthropometric and endogenous environmental toxicants.

To address the growing chronic disease problem within their population, Thailand introduced a system of universal health care in 2000. In addition, national disease registries were established to actively surveil cancer (9) and diabetes (10), and Thailand became a member of the WHO Children's Environmental Health research program in 2011. While diet, physical activity, and smoking are the primary modifiable risk factors for chronic diseases, environmental pollution is often an ignored but equally important risk factor for the development of chronic diseases in both adults and children (11).

This dissertation explored childhood cancer epidemiology and consequences of environmental exposures within Thailand, a model to examine chronic childhood disease and molecular effects of environmental exposures associated with the demographic and epidemiologic transition. Specifically, childhood leukemia incidence and survival were examined from 1990-2011 using cancer registry data from the Songkhla Province in southern Thailand. The effects of chronic cadmium (Cd) exposure in Northern Thai women, who were exposed to Cd after zinc mining polluted downstream water sources, were examined in relation to epigenetic aging and co-exposure to other toxic and essential metals.

Childhood cancer in developing countries. Childhood cancer is an emerging global public health problem in countries undergoing all stages of development. Disparities in incidence and mortality exist globally. In 2008, 175,000 cases of childhood cancer were diagnosed and 84% of

these diagnosed cases were in LMICs (12). Childhood cancer is the leading cause of death from disease in developed countries, and its incidence is increasing globally (13). Children diagnosed with cancer have a high burden of mortality, morbidity, and disability later in life compared to the general population (14), and this burden is greater in countries undergoing development. Changes in childhood cancer incidence may reflect changes in the healthcare infrastructure, which includes changes in access to diagnosis and treatment (15). Increasing childhood cancer incidence in countries undergoing rapid development may also be associated with changing environmental and infectious risk factors (12).

Childhood cancers have a unique distribution of cancers diagnosed compared to adult cancers with higher proportion of leukemias, lymphomas, and brain tumors and smaller proportion of carcinomas. Childhood cancers are unique cancers biologically and may have different origins and etiologies than adult cancers (16). Childhood cancer distributions also vary globally and by level of social-economic development (12). Since childhood cancers are different from adult cancers, they are classified using the International Classification of Childhood Cancer system, which includes twelve ICCC groups and 47 subgroups (**Figure 1.1**). This classifies cancers based on morphology rather than topology and balances homogeneity and heterogeneity within each group to enable standardized comparisons across population-based registries (17). For example, within Thailand (**Figure 1.1**), there is a similar proportion of leukemias diagnosed compared to the United States but the proportion of brain tumors and lymphomas diagnosed are lower. There is also a high proportion of other and not otherwise specified (NOS) tumors.

Population-based cancer registries document the burden of disease and in ideal conditions are an unbiased source of cases, and essential for childhood cancer surveillance. These

population-based registries capture the geographic variation, identify vulnerable subpopulations at risk, and provide a basis for generating hypotheses related to environmental or genetic risk factors (18). For childhood cancers, there is a high level of disparities in detection, diagnosis, survival, and treatment between LMICs and higher income countries, which make childhood cancer and population-based registries necessary for addressing these disparities and improving outcomes for children diagnosed in LMICs. Within Asia, only five percent of the population covered by high quality registries. Childhood cancer surveillance is problematic in developing countries because of this lack of registries, barriers of healthcare access, and difficulty diagnosing childhood cancers (19).

Etiology of childhood leukemia. Childhood leukemia is the most common childhood cancer diagnosed. Leukemia originates in the bone marrow, where blood cells are produced, and disease manifests as an abnormal proliferation of immature (blast) blood cells. There are two major subtypes of leukemia, acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), which account for approximately 75% and 20% of leukemias diagnosed, respectively. The peak age at diagnosis is 2-5 years for ALL and <4 and 15-19 years for AML (20) (**Table 1.1**). ALL affects the cells that form lymphocytes while AML affects the myeloid cells, which form red blood cells and platelets. A majority of ALL and AML cases are characterized by chromosomal structural abnormalities (21). These chromosomal abnormalities include hyperploidy, greater than 46 chromosomes in cell, and translocations, rearrangement between non-homologous chromosomes that usually results in a gene fusion, and inversions, rearrangements within the same chromosome.

The etiology of childhood cancers has been difficult to study for several reasons: there are few established risk factors, they are rare, and the latency period between onset of cancer and diagnosis is short compared to adult cancers. Most etiologic studies of childhood cancers are usually limited to traditional case-control study design, and the etiology of childhood leukemia is the most extensively studied since this is the most common childhood cancer diagnosed (22). A few established non-genetic risk factors for childhood leukemia (**Table 1.2**) include ionizing radiation (23, 24), chloramphenicol (an antibiotic) (25), and chemotherapeutics that include alkylating agents and epipodophyllotoxin (24, 26, 27). Several cancer predisposition syndromes increase the risk of leukemia up to 600-fold, and these syndromes can help identify important genetic drivers in leukemia. DNA repair defect syndromes, such as Fanconi anemia, Ataxia telangiectasia, and Bloom syndrome, implicate important DNA repair genes in leukemia etiology that include ATM Serine/Threonine Kinase (*ATM*), Bloom Syndrome RecQ Like Helicase (*BLM*), and several others (28). These predisposition syndromes enable the identification of key pathways in leukemiogenesis and contribute to understanding the etiology of leukemia.

Some maternal characteristics and environmental exposures are suspected risk factors for childhood leukemia, and these factors emphasize the importance of studying the prenatal period in childhood leukemia development. High birth weight is a potential risk factor for leukemia (29), however for AML, both low and high birth weight may increase AML risk (30). It is uncertain how birth weight influences childhood leukemia risk, but shared prenatal environmental risk factors and genetic pathways may contribute (22). There is some evidence that advanced maternal age (31) and smoking (32) increase childhood leukemia risk, and these are important to consider since advanced maternal age may influence the risk of a child being born with chromosomal hyperploidy (33) and the risk of miscarriages and stillbirths, which also

are commonly associated with chromosomal abnormalities (34, 35). Environmental studies have indicated that exposures such as benzene and pesticides elevate the risk of childhood leukemia (36-38). Protective risk factors include prenatal vitamin consumption (39) and daycare attendance (40). An additional limitation of environmental epidemiology studies of childhood leukemia is the exposure assessment. The exposure assessment usually depends on self-reported data from parental and patient interview surveys at time of diagnosis, which is subject to recall error (22), and it is unknown whether this measure of exposure is consistent with an internal biomarker of exposure or the exposure at the time of time of diagnosis or leukemia onset, which is suspected to occur during the prenatal period.

One established congenital risk factor for leukemia is Down Syndrome (DS). The risk for leukemia among children with DS is 20-40 fold greater than for children without DS (41). Among hyperploid leukemias, trisomy 21 is a feature among non-DS leukemia cases, suggesting this hyperploidy may be an initiating factor for leukemia (42). Risk factors for DS-leukemia include advanced maternal age, high birth weight, and exposure to pesticides and solvents (41). The development of leukemia, specifically AML, in DS children has been proposed as a multistage model for leukemogenesis (43). Studying leukemia in DS children is an ideal model for studying leukemia in non-DS children with leukemia in order to provide insight into shared maternal, environmental, genetic and epigenetic risk factors (41, 44).

The prenatal period is a key window of susceptibility for childhood leukemia, where environmental toxicants are known to affect development and cellular programming. Unfortunately, characterizing prenatal exposures is difficult when studying childhood cancers since obtaining biomarkers of exposure prenatally is expensive, invasive, and technically demanding. While childhood leukemias are likely initiated *in utero*, they are often diagnosed in

childhood, so obtaining prenatal biomarkers of exposures retroactively during pregnancy is impossible unless that child with leukemia was a member of a birth-cohort. Potential risk factors, such as birth weight, maternal age, and prenatal exposure to benzene and other toxicants, emphasize the importance of considering the prenatal period in leukemia etiology. Genomic alterations associated with childhood leukemia have been detected in cord blood (45) and newborn blood spots (NBS) (46) . New studies and birth cohorts are emerging that are utilizing NBS to assess prenatal exposures, epigenetics, and genetics (47), that ultimately can be applied to identify risk factors for childhood cancer globally.

Cadmium (Cd) in Mae Sot, Thailand, and co-exposure metal mixtures. Cd is a metal that is commonly extracted as by-product of processing zinc-ores from mining, and it is toxic to human health. Cd is used in batteries, plating, and pigments. The primary routes and sources of Cd exposure are 1) inhalation of cigarette smoke and ambient air and 2) ingestion of contaminated food and water (48). Cd binds to the metal-binding protein, metallothionein, and accumulates in the bones, liver, and kidneys. The kidneys accumulate up to half of the total body burden of Cd (49). In the kidney, the half-life of Cd is between ten and fourty years (50). Common biomarkers of Cd exposure are obtained from blood and urine. Blood Cd reflects recent and acute exposure while urine Cd reflects cumulative and chronic exposure from the kidney (48).

The Mae Sot district is in northeast Thailand, and this population was exposed to Cd after ingesting contaminated water and rice caused by downstream pollution from nearby zinc mining. Zinc was mined in this region for almost three decades, and many of the residents in this region were farmers, who grew and consumed their own rice. After identifying this widespread pollution crisis in the early 2000s, the Thai government implemented remediation efforts and

began health surveys of this population (51). These health surveys have occurred every three to five years in 2005, 2007, 2012, and most recently in 2014. In these surveys, this population had higher levels of urinary Cd and dietary intake of Cd than the general Thai population (52). Urinary Cd levels in the Mae Sot population were also highly associated with markers of kidney dysfunction that include beta-2-microglobulins (B2MG) and n-acetylglucosamine (NAG), and these biomarkers of kidney dysfunction dose-dependently increased with increasing Cd exposure. While urinary Cd and these kidney biomarkers did not differ by gender or smoking status, they did differ by age (53).

Cd exposure is associated with the risk of chronic disease at both low- and high-exposure. In **Table 1.3** these health risks are summarized for studies conducted within the Mae Sot population and meta-analyses examining the risk of chronic disease among low, dietary, and occupational exposures. Residents Mae Sot have higher prevalence of diabetes (54), hypertension (54-56), urinary stones (54, 56, 57), osteoporosis (56, 58), and chronic kidney disease (57). Several meta-analyses indicated Cd increased the risk of chronic disease with low and occupational exposure to Cd. Hypertension was associated with lower risk among all Cd studies (59) and higher risk among occupationally exposed groups (60). Cd exposure elevated the risk of cardiovascular disease (61). The risk of cancer increased with Cd exposure (62-69).

Often environmental pollution is associated with exposure to multiple environmental contaminants and toxicants. Zinc mining releases multiple metals into the environment often including lead (Pb), zinc, Cd, and arsenic (As) these may continue to be released after mining activity has ceased and represent a threat to environmental and human health (70). In environmentally contaminated regions and populations, rice uptakes these metals, especially Cd and As. It is unclear how rice uptakes Pb and mining activity may aerially deposit Pb and other

metals on the rice (71). Dietary exposure to these metals appears to be the primary source of exposure in non-occupationally exposed populations. Presently, the co-exposure patterns to other metals among the Mae Sot population have not been examined. A gold mining community in Ghana with high mercury exposure also was found to have urinary levels of other toxic metals including As and Cd (72). In rural Bangladesh, an As-exposed community also had elevated levels of Cd and Pb (73). Multiple toxic metal exposures interact to exacerbate the health effects associated with chronic Cd exposure (74).

Influence of Cd exposure on DNA methylation. While some environmental toxicants are genotoxic, capable of mutating DNA sequence, the mechanism behind many toxicants is unknown. Some toxicants are speculated to be non-genotoxic and affect the cellular oxidative and inflammatory environment, cellular signaling, chromatin and histone modifications, and DNA methylation. DNA methylation is dynamic and sensitive to cellular environmental changes that occur with aging, disease, and environmental exposures (75). Cd affects the epigenome by perturbing DNA methylation globally. In addition, other toxicants are also associated with DNA methylation changes (76).

Cd has been shown to affect global and gene-specific methylation. Cd may mediate this effect by perturbing DNA methyltransferase activity (77) and modifying the inflammatory and oxidative environment of the cell, which can also have downstream consequences on methylation. The methylation status of DNA methyltransferase and metallothionein encoding genes may mediate the effect of Cd exposure by changing the expression of these genes, which may have downstream effects on the extent to which Cd interferes with global DNA methylation and accumulates within cells, respectively. In **Table 1.4** studies of Cd exposure and DNA

methylation in human populations are summarized. Within an Argentinean population with low Cd exposure, Cd was associated with lower methylation at LINE-1 elements, a biomarker of global methylation (78). Within the Strong Heart Study in the US, Cd was associated with higher 5-hydroxymethylcytosine and 5-methylcytosine proportions (79). Within the Mae Sot population with high Cd exposure, Cd was associated with gene-specific hypomethylation in women (80).

Epigenome-wide studies assess DNA methylation across the entire genome by measuring methylation at selected CpG sites. To date, only the epigenomic effects of prenatal Cd exposure have been explored. An epigenome-wide study of mother-child pairs from Bangladesh found that prenatal Cd exposure was significantly associated with cord blood methylation, and these effects were sex-specific. Differentially methylated markers were found in genes associated with organ development, morphology, bone-mineralization, and cell death (81). Two studies from the US found that maternal Cd was associated with specifically, differentially methylated CpG sites that differed by gender (82, 83). The effect of Cd on the adult epigenome remains to be elucidated.

Measuring the influence of environment on aging through biomarkers of aging. Environmental and lifestyle factors influence the aging trajectory and chronic disease risk. Biologic age captures the physiologic and cellular state of an individual at a given chronologic age. Chronologic age may not accurately reflect our biologic age, and a greater biologic age relative to chronologic age may indicate accelerated aging within an individual. Biomarkers of aging are being developed from features of the human genome that change with age and include telomere shortening, age-associated DNA methylation changes, age-related gene expression changes, mitochondrial DNA copy number, and somatic chromosomal alterations such as loss of the Y chromosome. These

markers reflect cellular damage associated with the accumulation of DNA damaging exposures over the life course, as well as, responses to cellular damage associated with aging (84).

The expression of genes changes with aging, especially those in pathways related to mitochondria function, metabolism, and DNA replication and repair, and the collection of these changes can be used to estimate the transcriptomic age of an individual, which is a biomarker of aging (85). Telomeres are located at chromosome ends and preserve genomic stability by protecting the ends of the chromosome from degradation and being targeted by DNA repair, end-joining, and recombination processes, which promote chromosomal instability. During human aging, telomeres become shorter and correlate with aging, risk of diseases associated with aging, and mortality (86). Mitochondrial function declines with age, and mitochondrial DNA copy number is a biomarker of mitochondrial DNA quantity and functional capacity. Declines in mitochondrial DNA copy number are correlated with increasing age (87-89). The epigenetic clock describes consistent DNA methylation changes associated with aging across a population. It is hypothesized to capture common and progressive changes in methylation that occur during aging at specific CpG sites susceptible to the decline in maintaining methylation patterns (90, 91). The collection of these age-associated CpG sites can be used to estimate the epigenetic age of an individual and is a biomarker of biologic aging (90, 92). Biomarkers of aging may be essential for understanding chronic disease risk and progression and may mediate environmental exposure and disease relationships.

The epigenome changes throughout the life course. It is important to understand how the environment interacts with epigenome across the lifespan. Aging is associated with dynamic DNA methylation changes, commonly referred to as epigenetic drift, and the molecular mechanism behind epigenetic drift is multifactorial and unclear. Over the lifespan, promoter

regions of genes become more hypermethylated while gene bodies and intergenic regions become more hypomethylated with age (93). Many studies have investigated differential methylation at CpG sites measured in blood as a biomarker of age and used these markers to develop a predictor of biologic age. Methylation within *ELOVL2*, a transmembrane protein involved in polyunsaturated fatty acid synthesis and frequently identified age-related methylation marker, ranges from 7-91% and acts as an "on-off" switch depending on the stage of life, where it was hypomethylated in newborns and hypermethylated in older adults (94).

Horvath and Hannum developed biologic age calculators utilizing epigenome-wide age-associated changes in methylation (90, 92). The Hannum calculator identified a panel of 71 methylation markers that were highly predictive of age from a cohort of Hispanic and Caucasian individuals (92). Horvath developed a predictive model for aging utilizing data from 51 tissues and cell types, and he identified a panel of 353 methylation markers that predicted age (90). These calculators have been applied in several populations to examine the effect of biologic aging on mortality and chronic disease risk (95, 96), DS (97), air pollution (98), menopause (99), and fitness (100). These calculators have not been applied in populations from developing countries or with high exposure to environmental toxicants.

SPECIFIC AIMS

As countries undergo the demographic and epidemiologic transition, their populations experience new environmental exposures associated with unrestricted growth and industrialization, with a corresponding increase in the incidence of chronic diseases across the age spectrum. Among these countries, environmental toxicants, such as heavy metals, are often present at higher levels than we observe in the United States. Exposure to higher levels of these toxicants are associated with greater toxicity, and thus, expected to increase the prevalence of associated adverse health effects. The molecular mediators of these downstream adverse effects include DNA methylation, biologic aging, and co-exposure to multiple toxicants. Chronic disease incidence is often increasing at a greater rate during a shorter period of time within these countries, especially among children. In particular, increased childhood cancer incidence is strongly associated with social and economic development. Thailand is a country that has undergone rapid socio-economic development and has geographically distinct high exposure to environmental toxicants, such as Cd. It is unclear how increased exposure to environmental toxicants, such as Cd, may influence long-term chronic disease risk among adults and children.

Aim 1: *Identify distribution and trends of childhood hematologic malignancies incidence and survival in southern Thailand using 1990-2011 data from the Songkhla Provincial Cancer Registry.*

Childhood leukemia accounts for one third of cancers diagnosed in children and is an emerging global public health problem as disparities in leukemia survival, treatment and detection exist between developing, like Thailand, and developed countries. We analyzed ALL and AML incidence and survival trends from 1990-2011 in Songkhla, Thailand. For a point of reference,

we compared these results to childhood leukemia incidence and survival in the United States using Surveillance, Epidemiology, and End Results (SEER) data.

My *working hypothesis* is there are increasing trends in incidence and survival of childhood leukemia in Songkhla due to changes associated with socio-economic development and increased industrialization.

Aim 2*: Examine differential DNA age-associated methylation in highly exposed non-smoking women to Cd and its effects on biologic aging.*

Cd is a toxic heavy metal. The residents of Mae Sot were chronically exposed to Cd after ingesting contaminated water and food, caused by downstream environmental pollution from nearby zinc mining. Cd is known to disrupt DNA methylation. We analyzed the cross-sectional relationship among age, DNA methylation, and Cd exposure in forty non-smoking women and examined age-associated differential methylation and the relationship between methylation age and chronologic age by Cd exposure.

My *working hypothesis* is that chronic exposure to Cd globally deregulates DNA methylation, and this global disruption of DNA methylation accelerates aging, which may be associated with increased risk and earlier onset of chronic diseases.

Aim 3: *Examine the co-exposure to multiple toxic and essential metals in women chronically exposed to Cd in blood and urine from the Mae Sot District in northern Thailand*.

Chronic Cd exposure may be associated with co-exposure to toxic metals and may disrupt homeostasis of trace metals. The Mae Sot population in Thailand is exposed to Cd through

environmental contamination caused by nearby zinc mining. While this contamination is being remediated, it is unknown how the relationship among toxic and trace metals change longitudinally and as Cd exposure decreases in this population. We assessed changes in individual metals and exposure patterns from 2007 to 2012.

My *working hypothesis* is that women chronically exposed to high Cd are also exposed to higher levels of other toxic and essential metals that are expected to decrease longitudinally, and these patterns may reflect similar and different sources of exposure.

The successful completion of these aims will advance environmental and cancer epidemiology research in Thailand and rapidly developing countries. Studying vulnerable populations in Thailand are informative, and they represent an underutilized resource that can advance our understanding of the adverse molecular effects of toxic exposures and burden of emerging chronic diseases in a population undergoing the demographic transition.

CONCEPTUAL FRAMEWORK

Figure 1.2 presents the conceptual framework of this dissertation. The solid lines represent the pathways explored among the three aims in this dissertation while the dashed lines represent future avenues to be explored within these aims. As discussed in the introduction, these aims are explored in Thailand, a country that has undergone demographic and epidemiologic transitions. Both childhood cancer and environmental pollution in developing countries are emerging public health problems in Thailand, SE Asia, and globally.

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Table 1.1. *Summary of Childhood Leukemia*. Adapted from SEER*Cancer Review (20) and Pui et al 2011 (21).

	Incidence (per million)	**Proportion of Childhood Cancer**	**Peak Age at Diagnosis**	**5-year Survival**	**Common Chromosomal Aberrations**		
					Structural	Genes Affected	Frequency
Acute Lymphoblastic Leukemia (ALL)	35.5	20%	2-5	88%	Hyperploidy		25%
					t(12;21)	*ETV6-RUNX1*	25%
					t(4;22),t(11;19),t(9;11)	*MLL*	8%
					t(1;19)	*E2A-PBX1*	5%
					t(9;22)	*BCR-ABL1*	3%
					t(8;14), t(2;8), t(8;22)	*MYC*	2%
Acute Myeloid Leukemia (AML)	8.9	5%	<4 and 15-19	63%	t(9;11),t(10;11),t(11;19),t(6;11)	*MLL*	18%
					t(8;21)	*ETO-AML1*	12%
					t(15;17)	*PML-RARA*	12%
					inv(16)	*MYH11-CBF*	8%

Table 1.2. *Selected childhood leukemia risk factors*. Subscript refers to reference number.

Known			Suspected		
Risk Factor		**Risk Ratio**	**Risk Factor**	**Cancer**	**Risk Ratio**
Ionizing Radiation	Leukemia	16.3[24] 20[23]	High Birth Weight	Leukemia	1.35[30] 1.24[29]
Down Syndrome	Leukemia	20-40[41]	Low Birth Weight	AML	1.50[30]
Bloom Syndrome	Leukemia	--[28]	Maternal Smoking	ALL	1.10[32]
Ataxia Telangiectasia	Leukemia	70[28]	Paternal Smoking	Leukemia	1.44[29]
Neurofibromatosis type I	Leukemia	5.4[28]	Maternal Age	Leukemia	1.08[31]
Fanconi Anemia	AML	600[28]	Daycare	ALL	0.76[40]
Chloramphenicol	ALL	12[25]	Pesticide	Leukemia	1.62[37] 2.09[38]
Akylating Agents	Leukemia	4.8[27] 4.8[26] 6.7[24]	Vitamin	Leukemia	0.61[39]
Epipodophyllotoxin	Leukemia	5.5[26] 17.1[24]	Benzene	AML	2.28[36]
			Air Pollution (NO_2)	ALL	1.21[36]

Table 1.3. Cadmium and chronic disease risk.

Health Effect	Mae Sot	Meta-Analysis			
	Summary	**Studies Included**	**Exposure**	**Risk (95% CI)**	**Reference**
Chronic Kidney Disease (CKD)	In a cross-sectional study, elevated Cd exposure increased CKD risk by 3.73 fold (95% CI: 2.50,5.57) after adjustment (57).	N/A	N/A	N/A	N/A
Diabetes	Among individuals (n=217) who had continued dietary exposure in 2010, the proportion with diabetes significantly increased from 6.9% in 2005 to 11.1% in 2010 (54). In survey of 5,273 people in 2009, urinary Cd was not associated with diabetes after adjustment in either women or men (55). Residents of contaminated areas did not have increased prevalence diabetes compared to those in non-contaminated (56).	N/A	N/A	N/A	N/A
Hypertension	Among individuals (n=217) who had continued dietary exposure in 2010, the proportion with hypertension significantly increased from 29.5% in 2005 to 35.5% in 2010 (54). In survey of 5,273 people in 2009, the prevalence of hypertension significantly increased from 25% in lowest-tertile of Cd exposure to 35% in highest tertile exposure (55).	Twelve (blood or urine Cd, blood pressure or hypertension, non-occupationally exposed, included smoking information, observational study design) Six (occupational, Cd, hypertension, observational studies	Blood or Urine Cd Occupation, blood, or urine Cd	Urine: 0.65 (0.45, 0.94) 1.81 (1.03, 3.20)	Gallagher and Meliker 2010 (57) Caciari et al 2013 (58)

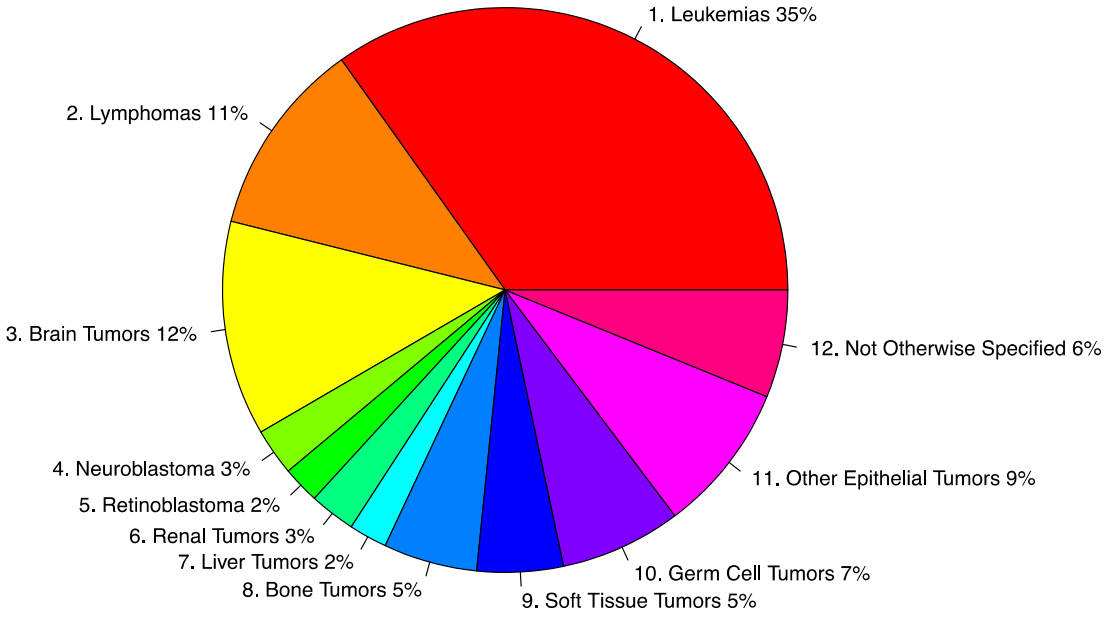
	Residents of contaminated areas had significantly increased prevalence of hypertension (32.5%) compared to those in non-contaminated (21.8%) (56).				
Cardiovascular Disease (CVD)	There was no increase in prevalence in hypercholesterolemia or hypertriglyceridemia among people living in contaminated areas vs non- contaminated (56). Further studies are needed to evaluate cardiovascular disease and Cd exposure in this population.	Twelve (CVD, blood or urine Cd, adjusted for age, smoking, and sex) Nine (all cause mortality, urine Cd, cohort study)	Blood or Urine Cd Urine Cd	General: 1.36 (1.11,1.66) Non-Smoking: 1.27 (0.97, 1.67) Women: 1.20 (0.92, 1.56) General: 1.57 (1.27, 1.95) Low-exposure: 1.50 (1.18, 1.91)	Tellez-Plaza et al 2013 (59) Larsson and Wolk 2015 (63)
Osteoporosis	Residents of contaminated areas had significantly increased prevalence of osteoporosis (54.4%) compared to residents of non-contaminated areas (45.3%) (56). Blood Cd was significantly associated with increased bone resorption markers after adjustment (58).				
Urinary Stones	The proportion with stones significantly increased from 8.9% in 2005 to 13.8% in 2010 (54). In a cross sectional study in 2009, the risk of urinary stones increase by 2.73 (1.16, 6.42) after adjustment (57). Residents of contaminated areas had significantly increased prevalence of stones (10.4%) compared to residents from non-	None Available			

	contaminated areas (5.0%) (56).				
Cancer	Not yet available.	Three (urinary Cd, cancer, adjustment for smoking and age, observational study design)	Urine Cd	1.22 (1.13, 1.31)	Nawrot et al 2015 (65)
		Nine (all cause mortality, urine Cd, cohort study)	Urine Cd	General: 1.39 (0.96, 1.99) LE: 1.56 (0.98, 2.47)	Larsson and Wolk 2015 (63)
		Eight (cancer, dietary Cd, cohort or case control study)	Dietary Cd	1.10 (0.99, 1.22)	Cho et al 2013 (61)
Breast		Six (breast cancer, dietary Cd, case-control)	Dietary Cd	1.03 (0.89,1.19)	Van Maele et al 2016 (67)
		Eleven (urine or dietary Cd, breast cancer, observational study design)	Dietary and Urine Cd	Urine: 2.24 (1.49, 3.35) Dietary: 1.01 (0.89, 1.15)	Lin et al 2016 (64)
		Eight (urine Cd, breast cancer, observational study design,	Urine Cd	2.24 (1.50, 3.34)	Larsson et al 2015 (62)
		Eight (cancer, dietary Cd, cohort or case control study)	Dietary Cd	1.15 (1.04, 1.28)	Cho et al 2013 (61)
Lung		Three (urinary Cd, cancer, adjustment for smoking and age, observational study design)	Urine Cd	General: 1.68 (1.47, 1.92)	Nawrot et al 2015 (65)
		Eleven (Cd exposure, lung cancer, observational study design)	Occupation and Urine Cd	General: 1.42 (0.91, 2.23) Occupation Cohort: 0.68 (0.33, 1.41) Occupation Case-Control: 1.61 (0.94, 2.75)	Chen et al 2016 (60)
Kidney		Nine (renal cancer, Cd exposure, observational study design)		1.47 (1.27, 1.71)	Song et al 2015 (66)

Table 1.4. Epigenomic effects of Cd in human populations.

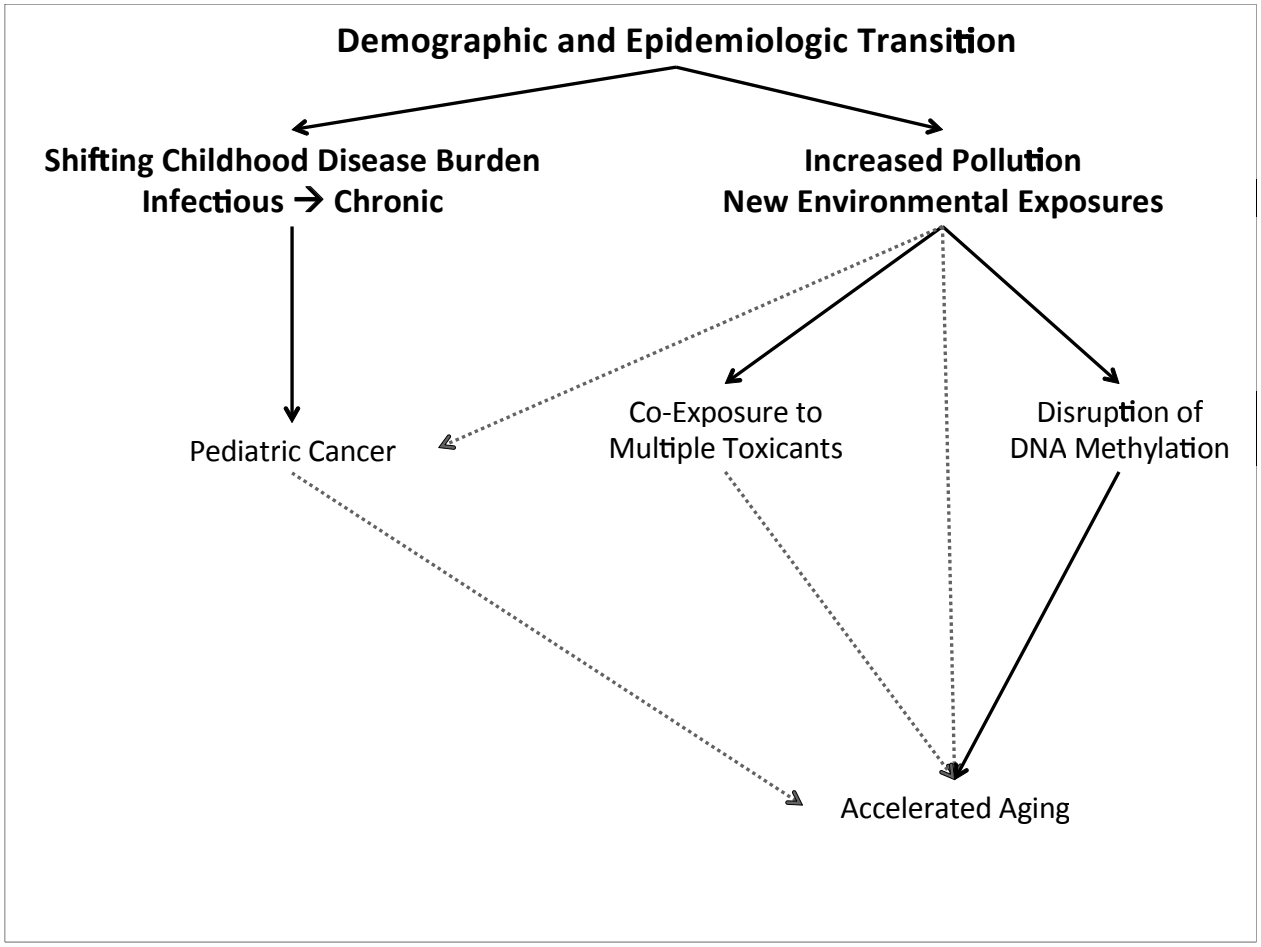
Cd Exposure	Population	Measurement	Summary	Reference
Maternal blood and Child urine	127 mother-child pairs from rural Bangladesh	450K array	Strong correlation between maternal Cd in cord blood, but none significant after multiple comparisons. Identified some sites that were correlated with both maternal Cd and child Cd at 4.5 years.	Kippler et al 2013 (81)
Placenta	24 mother-child pairs OMEGA study in Pacific Northwest		Methylation at ARL9 and MECOM in cord blood was associated with Cd in girls and boys, respectively.	Mohanty et al 2015 (82)
Maternal blood	17 mother-child pairs from the CEHI cohort in Durham County, NC		92 genes associated with Cd in maternal blood. 61 genes from fetal blood. These genes were enriched in pathways associated with regulation of transcription and apoptosis.	Sanders et al 2014 (83)
Urine		Bisulfite pyrosequencing of LINE-1, MGMT, MT2A, DNMT3B	Higher Cd was associated lower MGMT methylation. In women, MT2A and DNMT3B were also associated with lower methylation	Virani et al 2016 (80)
Blood and urine	202 women from northern Argentina	Bisulfite pyrosequencing of LINE-1, p16, MLH1	Urinary Cd was inversely associated with LINE-1 methylation. This association was modified by polymorphisms in DNMT1.	Hossain et al 2012 (78)
Urine	48 men and women from Strong Heart Study	Global DNA 5-methyl cytosine and 5-hydroxymethylcytosine quantification	Participants above the median Cd had significantly higher methylation.	Tellez-Plaza 2014 (79)
Maternal blood	319 mother-child pairs from the NEST study.	Bisulfite pyrosequencing of selected imprinted genes.	Maternal Cd associated with lower methylation in cord blood at PEF3.	Vidal et al 2015

Figure 1.1. Distribution of all pediatric cancers from five provincial registries in Thailand.



N=3,574

Figure 1.2. Conceptual Framework.



CHAPTER 2. DIFFERENCES IN CHILDHOOD LEUKEMIA INCIDENCE AND SURVIVAL BETWEEN SOUTHERN THAILAND AND THE UNITED STATES: A POPULATION-BASED ANALYSIS

ABSTRACT

BACKGROUND: Childhood leukemia incidence and survival varies globally, and this variation may be attributed to environmental risk factors, genetics, and/or disparities in diagnosis and treatment.

PROCEDURE: We analyzed childhood leukemia incidence and survival trends in children age 0-19 years from 1990 to 2011 in Songkhla, Thailand (n=316) and compared these results to US data from the Surveillance, Epidemiology, and End Results (SEER) registry (n=6,738). We computed relative survival using Ederer II and estimated survival functions using the Kaplan-Meier method. Changes in incidence and five-year survival by year of diagnosis were evaluated using joinpoint regression and are reported as annual percent changes (APC).

RESULTS: The age-standardized incidence of leukemia was 3.2 and 4.1 cases per 100,000 in Songkhla and SEER-9, respectively. In Songkhla, incidence from 1990-2011 significantly increased for leukemia (APC=1.7%, p=0.031) and acute lymphoblastic leukemia (ALL) (APC=1.8%, p=0.033). Acute myeloid leukemia (AML) incidence significantly increased (APC=4.2%, p=0.044) and was significantly different from the US (p=0.026), where incidence was stable during the same period (APC=0.3%, p=0.541). The overall five-year relative survival for leukemia was lower than that reported in the US (43% vs. 79%). Five-year survival significantly improved by at least 2% per year from 1990-2011 in Songkhla for leukemia , ALL,

and AML ($p<0.050$).

CONCLUSIONS: While leukemia and ALL incidence increased in Songkhla, differences in leukemia trends, particularly AML incidence, may suggest etiologic or diagnostic differences between Songkhla and the US. This work highlights the importance of evaluating childhood cancer trends in low- and middle-income countries.

INTRODUCTION

Leukemia is the most common malignancy in those under the age of 15 years, accounting for one out of three cases of childhood cancer. The two major subtypes of leukemia seen in children are acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), which account for 80% and 17% of leukemias diagnosed, respectively (1). Childhood leukemia continues to be a global public health problem as the incidence of this malignancy appears to be steadily increasing (2, 3), and disparities in survival and diagnosis persist in low- and middle-income countries (LMICs), like Thailand, when compared to high-income countries (HICs), like the United States (US). For instance, the age-standardized incidence of childhood leukemia in the US is 4.2 cases per 100,000 with a 5-year survival of 82% for the period 2003-2011 (2). While data from the Thai Pediatric Oncology Group indicates that the age-standardized incidence is 3.8 cases per 100,000 with a 5-year survival of 57% for the period 2003-2005 (4). Better characterization of incidence and survival are key to understanding why global disparities exist in order to improve childhood leukemia diagnosis and prognosis in LMICs.

As Thailand and many other LMICs undergo socio-economic development, childhood disease burden has shifted from infectious to chronic diseases, including childhood cancers. In 2008, 84%, or an estimated 148,000 cases, of childhood cancer were diagnosed in LMICs, where over 80% of children live. Furthermore, 94% of deaths from childhood cancer worldwide occurred in these regions (5). Rapid industrialization and economic development may increase exposure to undetermined risk factors that may be associated with childhood leukemia (6), but the increase in childhood leukemia incidence may also be attributable to improved reporting and reductions in competing causes of death (7). Nevertheless, leukemia survival rates continue to remain low in many LMICs (8). In fact, incidence and survival from childhood leukemia has

been proposed to serve as an indicator of economic and healthcare development (9). However, childhood cancer surveillance is a problem in many LMICs due to barriers in healthcare access and underdeveloped or nonexistent population-based cancer registries.

Within Asia, high-quality population-based cancer registries monitor less than 5% of the population (5). Surveillance for childhood leukemia in LMICs may pose additional challenges, as childhood leukemia is rare, symptoms are non-specific and resemble infections, and early death can occur before diagnosis (10). Precise statistics from cancer registries can identify gaps and outline directions to address the burden of childhood leukemia in LMICs, and therefore, resources can be allocated to improve childhood leukemia diagnosis, treatment, and survival (8, 11). Songkhla is located in southern Thailand, is a unique and ethnically diverse region within Southeast Asia, and has a long-standing cancer registry with standardized reporting and monitoring (12). Because there is limited information on childhood leukemia in Thailand and how its epidemiology differs when compared to HICs, we evaluated trends in childhood leukemia incidence and survival using data from the Songkhla Cancer Registry in Thailand and compared to data from the Surveillance, Epidemiology, and End Results (SEER-9) registry in the US.

METHODS

Study Population

Data on childhood leukemia cases were obtained from the Songkhla Cancer Registry. This registry actively collects information on cancer cases from all 16 districts located in the Songkhla Province. Details of the Songkhla Cancer Registry have been described previously (12-15). Briefly, the registry was established in 1989 and captures cancer cases from 23 sources that

include Songklanagarind Hospital, Hat Yai Hospital, Songkhla Hospital, and the population registration office of the Songkhla Province. Patients with a suspected diagnosis of any cancer were referred from community hospitals and other healthcare centers to the Songklanagarind, Hat Yai, or Songkhla hospitals. The Songkhla registry monitored a population of approximately 1.5 million in 2010 (16), and the Thai National Statistical Office estimates that Muslims and Buddhists comprise 25% and 75% of the population, respectively (17). From the Thai National Census, the proportion of the population in Songkhla under 19 years of age has decreased from 42% (n=457,200) in 1990 (18) to 29% (n=428,700) in 2010 (16).

In order to compare childhood leukemia trends between Thailand and the US, we obtained SEER research data from SEER*Stat (19). SEER is a cancer registry program of the National Cancer Institute that tracks cancer incidence and survival in the US. SEER-9 captures approximately 10% of population in the United States and has collected incidence and survival data since 1973. SEER-9 includes registries in Atlanta, Connecticut, Detroit, Hawaii, Iowa, New Mexico, San Francisco-Oakland, Seattle-Puget Sound, and Utah, and oversamples minority populations in the US that include Blacks, Asians, Pacific Islanders, Native Americans, and Hispanics. The proportion of children under age 19 in the US population covered by SEER-9 has declined slightly from 29% in 1990 to 27% in 2010 (20). We collected case listing and survival information from reported childhood leukemia cases diagnosed age 0-19 from SEER-9 registries from 1990-2011.

Data Extraction and Variables

Childhood leukemia cases were identified using their ICD-O-3 histology and site codes (21) and categorized into International Classification of Childhood Cancer (ICCC) groups. The ICCC groups included in this analysis were: I, leukemia, I(a), ALL, I(b), AML, I(c), chronic

myeloproliferative diseases (CML), and I(e), not otherwise specified (NOS) leukemia. No cases of ICCC group 1(d), myelodysplastic syndrome and other myeloproliferative diseases, were diagnosed in Songkhla (22). Denominator data for incidence rate calculations were obtained from the Thailand population censuses conducted in 1990, 2000, and 2010 (16, 18, 23). Annual inter-census populations in Songkhla were estimated by assuming an exponential change between each census by each 5-year age groups (i.e., 0-4 years, 5-9 years, 10-14 years, and 15-19 years) and sex. Mortality life tables for Thailand by sex and 5-year age groups in 1990, 2000, and 2012 were obtained from the Global Health Observatory (24), and we interpolated the probability of dying for the inter-census years. Variables included in the registry were age at diagnosis, year of diagnosis, vital status, date of diagnosis, date of last contact, histology, site, religion, sex, and location of diagnosis. The denominator data for incidence rate calculations for SEER-9 were obtained from population data provided by the US Census for SEER (20). All cause mortality data and life tables for SEER-9 were provided by the National Center of Health Statistics (NCHS) for SEER (25).

Statistical Analysis

Descriptive Analyses. Age, sex, and vital status were compared among the SEER-9 and Songkhla registries. As age was not normally distributed, median age was compared using the Mann-Whitney test. Pearson Chi-square tests were used to compare the distribution of leukemias diagnosed by 5-year age group at diagnosis, sex, and vital status among these registries. To analyze the incidence trends from 1990 to 2011, age-standardized incidence rates (ASR) were calculated using Songkhla or US populations by year of diagnosis, standardized using the WHO world standard 2000 population (26), and presented as cases per 100,000.

Analysis of Incidence Trends. Joinpoint regression was used to analyze trends under a log-linear model and to compute the annual percent change (APC) in age-standardized incidences using the Joinpoint Regression Program version 4.0.4 (27). Permutation tests determined number of joinpoints, slope of the trends, and their significance (28). When no cases were present, a half-case was added to enable computation on the log-linear scale (27). The trends between Songkhla and SEER-9 were compared for parallelism, which tests differences in slopes, and coincidence, which tests the similarity in incidence rates (29).

Analysis of Survival. Relative survival was computed using mortality and life tables obtained from the National Statistics Office from 1990-2011and from NCHS from 1990-2011 in US (25). Cases were excluded from the survival analysis if their basis for diagnosis was death certificate only (DCO) or unknown, did not have any follow-up, or unknown vital status. These rates were computed using the Ederer II method (30) and survival functions were generated using the Kaplan-Meier method (product limit) (31). We used the R package, *survival,* to analyze relative survival for Songkhla (32), and computed relative survival within the SEER*Stat software for SEER-9 (33). In Songkhla, we compared the relative survival by sex, religion, and year cohort (1990-1994, 1995-1998, 1999-2002, 2003-2006) using the log-rank test from 1990-2011. The 5-year relative survival rates from each year of diagnosis from 1990-2006 in Songkhla and SEER-9 were computed, and these trends were analyzed using joinpoint regression under a linear model (27), and the trends were tested for parallelism and coincidence between each registry.

RESULTS

Data Quality

Three hundred and sixteen cases of childhood leukemia were reported in the Songkhla Registry from 1990 to 2011. Of these leukemia cases, 304 (96%) and 273 (86%) were included in the incidence and survival analyses of childhood leukemia, respectively. Cases diagnosed based on unspecified methods (n=10) or whose follow-up was an extreme outlier were removed from the incidence analysis. Two cases of AML were removed from the incidence and survival analysis after examining initial descriptive statistics because their follow-up time was determined to be an extreme outlier (greater than 8,000 days). In addition to this criterion, leukemia cases were excluded from the survival analysis if their basis for diagnosis was DCO (n=10), did not have any follow-up (n=16), or unknown vital status (n=5). In the Songkhla registry, 85% of the leukemia cases were included in the survival analysis. Ninety-four percent of the total leukemia cases included in the registry were histologically confirmed by either positive histology or positive cytology of hematology. For both AML and ALL, over 95% of cases were diagnosed by positive histology or positive cytology based on hematology, and no cases were diagnosed based on DCO in these groups. The proportion of diagnoses based on positive cytology, DCO, and unknown decreased from 23%, 4%, 5% in 1990-1999, respectively, to 11%, 2%, 1% in 2000-2011, respectively ($p < 0.001$). No differences were observed based on age group at diagnosis.

Demographic and Histologic Characteristics

In both Songkhla and SEER-9, males were more likely to be diagnosed with leukemia than females (**Table 2.1**). In Songkhla, the mean age at diagnosis for leukemia, ALL, and AML was 7.9, 6.4, and 10.3 years, respectively. Childhood leukemia was diagnosed at significantly older age in Songkhla compared to SEER-9 (mean age = 7.1 years, $p=0.018$). Significantly higher proportion of deaths from leukemia, ALL, and AML occurred among the cases of the Songkhla registry compared to the SEER-9 ($p < 0.001$).

The distribution of childhood leukemia subtypes was significantly different between SEER-9 and Songkhla ($p<0.001$) (**Table 2.2**). There was a greater proportion of NOS leukemia and lower proportion of ALL in Songkhla than observed in SEER-9. The proportion of CML diagnosed in Songkhla was double compared to SEER-9 (5.6% and 2.7%, respectively), and this proportion remained consistent by decade of diagnosis. In Songkhla, the distribution of leukemia subtypes was not significantly different between males and females ($p=0.530$) while the distribution of leukemia subtypes significantly differed by gender in SEER-9 ($p=0.032$). The distribution of leukemia subtypes by age group at diagnosis significantly differed within each registry ($p < 0.001$). In Songkhla, ALL accounted for 66% and 28% of cases diagnosed in ages 0-4 and 15-19, respectively, whereas the proportion of AML increased by age group at diagnosis from 16% in age 0-4 to 40% in age 15-19. This trend was also observed in SEER-9. In Songkhla, the childhood leukemia subtype distribution differed between those diagnosed prior to 2000 and after 2000 ($p=0.031$). The proportion of NOS leukemia decreased from 23% in 1990-1999 to 11% in 2000-2011. While ALL accounted for 55% of leukemia cases diagnosed in both 1990-1999 and 2000-2011, AML increased from 17% in 1990-1999 to 27% in 2000-2011.

Leukemia Incidence

From 1990 to 2011, the age-standardized incidence rates (ASR) for leukemia and ALL were lower in Songkhla, 3.2 and 1.8 cases per 100,000, respectively, compared to SEER-9, 4.1 and 3.1 cases per 100,000, respectively (**Table 2.1**). The ASR for AML was similar between Songkhla and SEER-9, 0.7 and 0.8 cases per 100,000, respectively. In joinpoint regression results, leukemia incidence significantly increased in Songkhla by 1.7% annually ($p=0.031$) and in SEER-9 by 0.8% annually ($p<0.001$) from 1990 to 2011. The leukemia incidence trends between Songkhla and US were determined to be parallel ($p=0.199$) but not coincident ($p <$

0.001) as incidence in SEER-9 was higher than in Songkhla (**Figure 2.1 A**). ALL incidence significantly increased in Songkhla by 1.8% annually (p=0.033) and in SEER-9 by 0.9% annually (p <0.001) (**Figure 2.1 B**). The ALL incidence trends between SEER-9 and Songkhla were parallel (p=0.386) but not coincident (p <0.001). In 1992, no cases of AML were diagnosed in Songkhla, and we applied a half-case correction (see **Methods**). AML incidence has been significantly increasing in Songkhla by 4.2% annually (p=0.036) while AML incidence has remained stable in SEER-9 (APC=0.3%, p=0.540) from 1990 to 2011 (**Figure 2.1 C**). The AML incidence trends were not parallel (p=0.026) but coincident (p=0.102). In a subgroup analysis within the SEER-9 Asian or Pacific Islander (API) population (data not shown), AML incidence has been significantly decreasing by 3.1% annually (p=0.015), and the annual ASR declined from 1.1 cases per 100,000 in 1990 to 0.5 cases per 100,000 in 2011.

Survival

The 5-year relative survival rates in Songkhla over the study period were 43%, 55%, 16%, for leukemia, ALL, and AML, respectively (**Table 2.3**). While there was no significant difference in survival by sex for childhood leukemia, ALL, and AML, females diagnosed with leukemia or ALL had better 5-year survival compared to males. For leukemia and ALL, the age group at time of diagnosis was significantly and inversely associated with survival (p<0.001), but this association was not observed for AML survival. Survival was significantly and positively associated with year of diagnosis in leukemia (p=0.007), ALL (p=0.015), and AML (p=0.015). Five-year survival improved between cases diagnosed from 1990-1994 and 2003-2006 for leukemia, ALL, and AML.

In Songkhla, 5-year survival for leukemia, ALL, and AML significantly improved annually by 1.9% (p=0.030), 2.3% (p=0.042) and 2.3% (p=0.041) from 1990 to 2006,

respectively (**Figure 2.2**). In SEER-9, 5-year survival from leukemia, ALL, and AML also significantly improved annually by 1.0% ($p < 0.001$), 0.7% ($p < 0.001$), and 1.8% ($p < 0.001$), respectively. While 5-year survival in Songkhla for leukemia, ALL, and AML were significantly lower when compared to SEER-9 ($p < 0.010$ for all comparisons), the gains in 5-year survival were greater in Songkhla. The trends in 5-year survival for leukemia and AML were parallel between SEER-9 and Songkhla. However, 5-year survival for ALL in Songkhla increased more dramatically compared to SEER-9 ($p=0.055$) from 1990 to 2006.

DISCUSSION

In this comprehensive descriptive analysis of childhood leukemia epidemiology in southern Thailand, our assessment of childhood leukemia trends in Songkhla, Thailand suggest that while 5-year survival has improved, the annual incidence has also increased from 1990 to 2011. ALL was less common in Songkhla compared to US and other HICs. NOS leukemia was more frequently reported in Songkhla, especially, during the first half of the study period from 1990-1999. However, in Songkhla from 2000-2011, NOS leukemia was less frequently reported while AML was more frequently reported after 2000, suggesting that the classification of leukemia cases improved in the Songkhla registry over time. The later age of diagnosis in childhood leukemia in Songkhla compared to the US may suggest a delay in diagnosis (34), which could have downstream consequences on leukemia progression, treatment efficacy, and survival. The ASR for childhood leukemia was lower in Songkhla compared to the US. This could be related to underreporting of childhood leukemia in Songkhla and/or population-specific differences related to childhood leukemia susceptibility and environmental exposures. When comparing childhood leukemia and ALL trends in Songkhla to the US, incidence was lower in

Songkhla, but in both of these regions, childhood leukemia incidence has been increasing. In contrast, AML incidence greatly increased in Songkhla while remaining stable in the US. This dramatic increase in AML incidence in Songkhla may be associated with the presence of population-specific risk factors, change in detection and diagnosis, or both.

Overall, survival from childhood leukemia improved in Songkhla, but was still lower than the US for the same period. In Songkhla, children diagnosed at a younger age had better survival than those diagnosed older for all leukemia and ALL, however, this effect was not observed for AML. Survival also improved by year of diagnosis for leukemia, ALL, and AML. Improvements in survival may be due to earlier diagnosis and better access to treatment possibly related to Thailand's shift to a universal healthcare system during the latter half of the study period. The five-year survival for leukemia, ALL, and AML has improved over time in Songkhla. Childhood leukemia survival by year of diagnosis was variable due to the small number of cases diagnosed per year. In spite of this, disparities in survival persisted between the US and Songkhla for leukemia, ALL, and AML during the study period.

Our results for childhood leukemia survival and incidence in Songkhla, Thailand were similar, yet distinct, from what has been reported in Khon Kaen, which is in the northern part of Thailand, and other Asian countries, including Indonesia, China, and India. Similar to Songkhla, in Khon Kaen from 1985-2002, the ASR for leukemia was 3.2 cases per 100,000, and childhood leukemia incidence was increasing (35). This may be due to common risk factors and other underlying population similarities. ALL and AML incidence in the Yogyakarta Special Province of Indonesia was 2.1 and 0.8 cases per 100,000, respectively, and childhood AML accounted for 27.7% of leukemia cases diagnosed from 1998-2009 (36). These incidences were similar to Songkhla, and the elevated proportion of AML was also observed from 2000-2011 in the

Songkhla registry. In Chennai, India, the absolute 5-year survival for childhood leukemia, ALL, and AML was reported to be 36%, 39%, and 31%, respectively, whereas 5-year survival for leukemia and ALL in Songkhla was higher (37). In urban Shanghai, China from 1973-2005, trends in ALL incidence non-significantly increased and AML incidence significantly decreased, in contrast to ALL and AML incidence trends in Songkhla from 1990-2011 (38).

Out study must be considered in the light of certain limitations. First, the small number of cases diagnosed during this period may have resulted in spurious incidence and survival trends and associations. Within the API population in SEER-9, incidence and survival was also variable by year of diagnosis, and trends within both API (data not shown) and Songkhla were strong enough to obtain statistical significance. Second, the analyses were descriptive in scope, and it was impossible to elucidate whether these changes in incidence were due to changes in risk factors or diagnostic changes. Third, it is possible that some of the leukemia cases included in this analysis were misdiagnosed. To reduce this effect, we removed two cases of AML with over 22 years of follow-up and cases with unknown basis for diagnosis since their survival was significantly higher compared to histologically confirmed cases. Additionally, all DCO cases were diagnosed as NOS leukemia. Finally, survival trends were difficult to interpret, as the number of cases per year was small especially for AML.

Compared to other studies examining childhood leukemia in LMICs, our analysis has numerous strengths. The Songkhla Cancer Registry actively ascertains cases from across regional hospitals, tertiary care, and healthcare centers in the region and the population denominators were estimated from high quality census data from the Thai National Statistical Office. The rate of histologic confirmation is high in this registry, and the proportion of cases histologically verified has increased over time, which may contribute to the increased incidence

of ALL and AML later in the study period. The Songkhla registry also actively follows each case, and takes advantage of universal health care in Thailand that allows for direct observation of long-term effects. The direct comparison between SEER-9 and Songkhla allowed us to observe differences in trends between HICs and LMICs. Population-based cancer registries that capture childhood cancers cases are crucial in LMICs, and by identifying trends in incidence and survival, we can determine if childhood cancer is being properly detected, diagnosed, and treated, and if necessary, develop interventions to improve diagnosis and prognosis in LMICs (39). Population-based registries, which can be considered an unbiased source of cases, can also examine geographic variation and identify regional, ethnic, and other subgroup differences that may be associated with unique environmental or genetic risk factors (40). The Songkhla registry is a rare and valuable data source for obtaining accurate estimates of childhood leukemia incidence and survival in a LMIC, and captures a unique and ethnically diverse population, where childhood leukemia has not been extensively studied.

In conclusion, childhood leukemia, ALL, and AML incidence and survival significantly increased in Songkhla, Thailand from 1990-2011, and both the incidence and survival were lower than in the US. While these increases in childhood leukemia incidence and survival suggest that detection, diagnosis, and treatment have improved in Songkhla, our results also expose the disparities in childhood leukemia detection, diagnosis, and treatment that persist between LMICs and HICs. Distinct differences in AML incidence between the US and Songkhla also suggested the presence of different etiologic and/or diagnostic factors. Additional studies are needed to characterize childhood leukemia trends in other regions of Thailand and other Southeast Asian countries such as Vietnam, Malaysia, and Indonesia, where population-based

registries have been and are being implemented. These assessments are critical to improve diagnosis and treatment in these LMICs and to develop prevention strategies.

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Table 2.1. *Descriptive characteristics and age-standardized leukemia incidences*. a) Age-standardized rates (ASR) per 100,000; b) Distributions compared using Pearson's chi-square test (categorical variables) and Wilcoxon rank sum test for age.

	Leukemia					ALL					AML				
	Songkhla		SEER9			Songkhla		SEER9			Songkhla		SEER9		
	n (%)	ASR	n (%)	ASR	p	n (%)	ASR	n (%)	ASR	p	n (%)	ASR	n (%)	ASR	p
n	304	3.2	6,738	4.1		169	1.8	5,080	3.1		69	0.7	1,267	0.8	
Sex					0.795					1					0.962
Male	167 (55%)	3.4	3,764 (56%)	4.5		96 (57%)	2.0	2,882 (57%)	3.5		37 (54%)	0.7	666 (53%)	0.8	
Female	137 (45%)	3.0	2,974 (44%)	3.7		73 (43%)	1.6	2,198 (43%)	2.8		32 (46%)	0.7	601 (47%)	0.8	
Age Group					0.148					0.436					0.581
0-4 years	121 (40%)	5.5	3,110 (46%)	7.5		80 (47%)	3.6	2,524 (50%)	6.1		19 (28%)	0.9	452 (36%)	1.1	
5-9 years	72 (24%)	3.0	1,510 (22%)	3.7		51 (30%)	2.1	1,258 (25%)	3.1		11 (16%)	0.5	190 (15%)	0.5	
10-14 years	54 (18%)	2.2	1,075 (16%)	2.6		22 (13%)	0.9	738 (15%)	1.8		16 (23%)	0.6	261 (21%)	0.6	
15-19 years	57 (19%)	2.2	1,043 (15%)	2.6		16 (9%)	0.6	560 (11%)	1.4		23 (33%)	0.9	364 (29%)	0.9	
Mean Age (sd)	7.9 (5.9)		7.1 (5.6)		**0.018**	6.4 (5.0)		6.5 (5.1)		0.903	10.3 (6.5)		9.0 (6.6)		0.113
Status					**<0.001**					**<0.001**					**<0.001**
Alive	117 (38%)		5,216 (77%)			81(48%)		4,277 (84%)			15 (22%)		671 (53%)		
Dead	182 (60%)		1,522 (23%)			85 (50%)		803 (16%)			52 (75%)		596 (47%)		
Unknown	5 (2%)		0 (0%)			3 (2%)		0 (0%)			2 (3%)		0 (0%)		

Table 2.2. *Descriptive characteristics by childhood leukemia subtype by registry.* a) Distributions compared using Pearson's chi-square test.

	Songkhla					SEER-9				
	ALL	**AML**	**CML**	**NOS**	**p**	**ALL**	**AML**	**CML**	**NOS**	**p**
n	169 (55.6%)	69 (22.7%)	17 (5.6%)	49 (16.1%)		5,080 (75.8%)	1,267 (18.9%)	179 (2.7%)	176 (2.6%)	
Sex					0.530					**0.032**
Male	96 (57.5%)	37 (22.2%)	11 (6.6%)	23 (13.8%)		2,882 (77.1%)	666 (17.8%)	94 (2.5%)	92 (2.5%)	
Female	73 (53.3%)	32 (23.4%)	6 (4.4%)	26 (19.0%)		2,198 (74.1%)	601 (20.2%)	85 (2.9%)	84 (2.8%)	
Age Group at Diagnosis					**<0.001**					**<0.001**
0-4 years	80 (66.1%)	19 (15.7%)	2 (1.7%)	20 (16.5%)		2,524 (81.9%)	452 (14.7%)	28 (0.9%)	77 (2.5%)	
5-9 years	51 (70.8%)	11 (15.3%)	3 (4.2%)	7 (9.7%)		1,258 (83.5%)	190 (12.6%)	23 (1.5%)	35 (2.3%)	
10-14 years	22 (40.7%)	16 (29.6%)	5 (9.3%)	11 (20.4%)		738 (68.8%)	261 (24.3%)	47 (4.4%)	27 (2.5%)	
15-19 years	16 (28.1%)	23 (40.4%)	7 (12.3%)	11 (19.3%)		560 (53.7%)	364 (34.9%)	81 (7.8%)	37 (3.6%)	
Decade of Diagnosis					**0.031**					0.841
1990-1999	70 (54.7%)	22 (17.2%)	7 (5.5%)	29 (22.7%)		2,131 (75.5%)	539 (19.1%)	73 (2.6%)	79 (2.8%)	
2000-2011	99 (56.3%)	47 (26.7%)	10 (5.7%)	20 (11.4%)		2,949 (76.0%)	728 (18.8%)	106 (2.7%)	97 (2.5%)	

Table 2.3. *Descriptive Statistics for Survival in Songkhla from 1990-2011*. a) Reported as relative survival proportion (standard error); b) Differences in survival were compared using log-rank test.

	Leukemia					ALL					AML				
	n	1-year	3-year	5-year	p	n	1-year	3-year	5-year	p	n	1-year	3-year	5-year	p
N	273	0.68 (0.03)	0.58 (0.03)	0.43 (0.03)		153	0.76 (0.03)	0.62 (0.04)	0.55 (0.04)		66	0.46 (0.06)	0.18 (0.05)	0.16 (0.05)	
Sex					0.274					0.154					0.799
Male	152	0.69 (0.04)	0.45 (0.04)	0.38 (0.04)		87	0.75 (0.05)	0.58 (0.06)	0.50 (0.06)		36	0.52 (0.08)	0.20 (0.07)	0.16 (0.06)	
Female	121	0.65 (0.04)	0.53 (0.05)	0.48 (0.05)		66	0.77 (0.05)	0.66 (0.06)	0.62 (0.06)		30	0.37 (0.09)	0.17 (0.07)	0.17 (0.07)	
Religion					0.303					0.342					0.570
Buddhist	220	0.69 (0.03)	0.50 (0.04)	0.44 (0.04)		122	0.78 (0.04)	0.64 (0.04)	0.58 (0.05)		55	0.48 (0.07)	0.18 (0.05)	0.18 (0.05)	
Islam	53	0.62 (0.07)	0.42 (0.07)	0.33 (0.07)		31	0.70 (0.08)	0.53 (0.10)	0.43 (0.10)		11	0.36 (0.13)	0.18 (0.10)	0.09 (0.07)	
Age Group					**<0.001**					**<0.001**					0.350
0-4 years	109	0.67 (0.05)	0.55 (0.05)	0.50 (0.05)		74	0.75 (0.05)	0.66 (0.06)	0.59 (0.06)		19	0.28 (0.10)	0.11 (0.07)	0.11 (0.07)	
5-9 years	67	0.83 (0.05)	0.59 (0.06)	0.50 (0.06)		47	0.85 (0.05)	0.68 (0.07)	0.59 (0.07)		10	0.78 (0.13)	0.11 (0.08)	0.11 (0.08)	
10-14 years	47	0.62 (0.07)	0.47 (0.08)	0.39 (0.08)		20	0.70 (0.10)	0.55 (0.11)	0.55 (0.11)		15	0.48 (0.13)	0.32 (0.12)	0.32 (0.12)	
15-19 years	50	0.52 (0.07)	0.20 (0.06)	0.17 (0.06)		12	0.50 (0.15)	0.13 (0.09)	0.13 (0.09)		22	0.46 (0.10)	0.23 (0.08)	0.18 (0.08)	
Year Group					**0.007**					**0.015**					**0.015**
1990-1994	53	0.67 (0.07)	0.45 (0.07)	0.35 (0.07)		32	0.83 (0.07)	0.62 (0.09)	0.52 (0.09)		9	0.25 (0.13)	0 (0)	0 (0)	
1995-1998	48	0.64 (0.07)	0.41 (0.07)	0.34 (0.07)		26	0.65 (0.09)	0.46 (0.10)	0.42 (0.09)		9	0.33 (0.14)	0.11 (0.08)	0.11 (0.08)	
1999-2002	46	0.75 (0.07)	0.57 (0.07)	0.57 (0.07)		25	0.84 (0.07)	0.72 (0.09)	0.72 (0.09)		10	0.45 (0.15)	0.11 (0.08)	0.11 (0.08)	
2003-2006	63	0.69 (0.06)	0.53 (0.06)	0.48 (0.06)		30	0.80 (0.07)	0.73 (0.08)	0.67 (0.08)		22	0.63 (0.10)	0.32 (0.10)	0.27 (0.10)	

Figure 2.1. *Age-Adjusted Incidence by Year of Diagnosis, 1990-2011*. a) Black denotes Songkhla; red denotes SEER-9, b) Significance levels for joinpoint regression trends indicated by 0.05 (*), 0.01(**), and 0.001 (***).

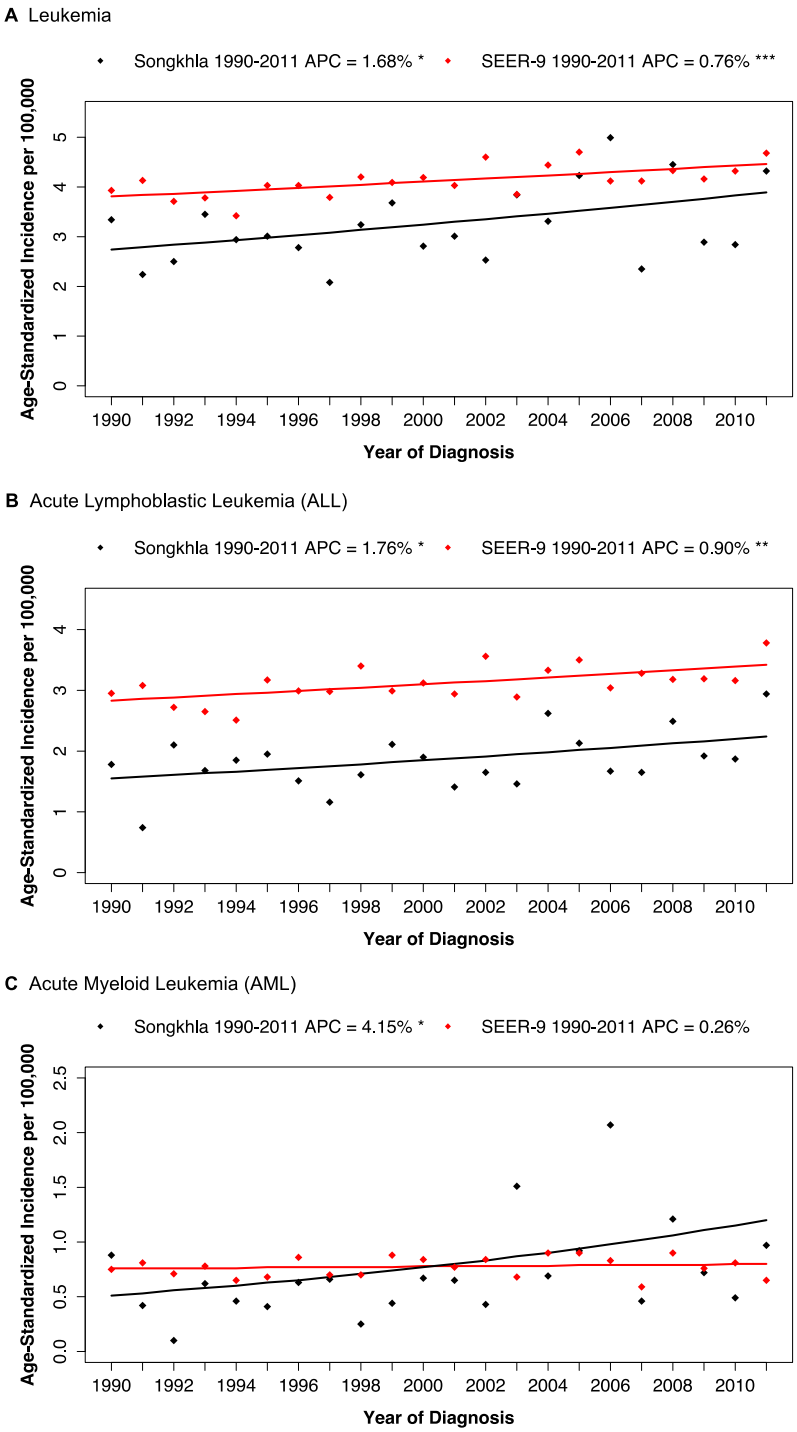
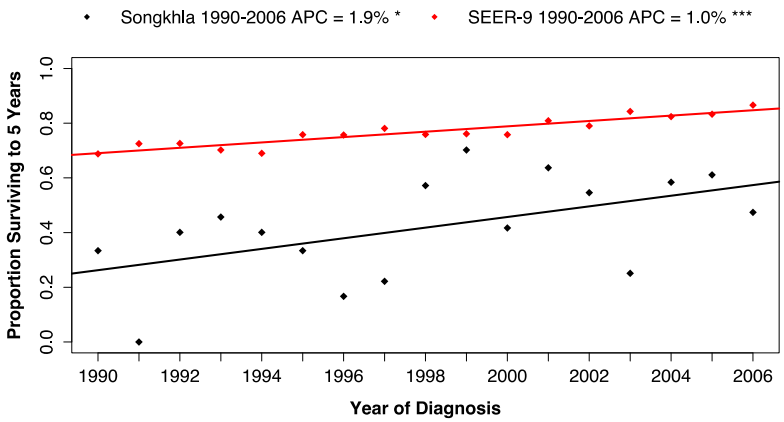
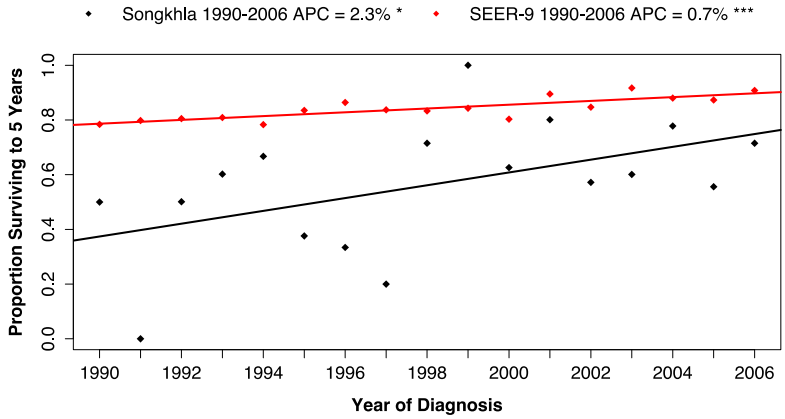


Figure 2.2. *5-Year Survival by Year of Diagnosis, 1990-2006*. a) Black denotes Songkhla; red denotes SEER-9; b) Significance levels for joinpoint regression trends indicated by 0.05 (*), 0.01(**), and 0.001 (***).

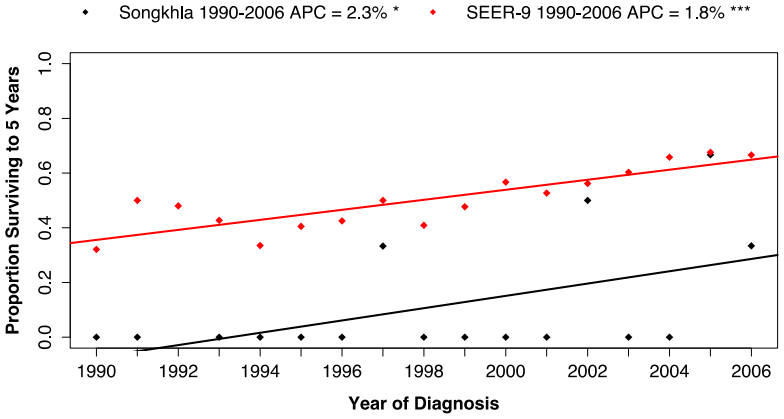
A Leukemia



B Acute Lymphoblastic Leukemia (ALL)



C Acute Myeloid Leukemia (AML)



CHAPTER 3. CADMIUM EXPOSURE AND AGE-ASSOCIATED DNA METHYLATION CHANGES IN NON-SMOKING WOMEN FROM NORTHERN THAILAND

ABSTRACT

DNA methylation changes with age, which may serve as a biomarker of biologic aging. Cadmium (Cd) modifies cellular processes that promote aging and disrupts methylation globally. Whether Cd modifies aging processes by influencing establishment of age-associated methylation marks is currently unknown. We characterized methylation profiles in >450,000 CpG sites in 40 non-smoking women from Thailand age 40-80. We classified them as high (HE) and low (LE) exposed using a cutoff of 2 ug/L urinary Cd and age-matched within five years. We predicted methylation age using two published methods by Horvath and Hannum. We assessed differences by Cd exposure using paired t-tests and linear mixed models adjusted for estimated white blood cell proportions, BMI, and urinary creatinine. We identified 213 age-associated methylation sites in our population ($p < 10^{-4}$). The mean Δage was smaller in HE vs. LE (Hannum: 3.6 vs. 7.6 years, p=0.0093; Horvath: 2.4 vs. 4.5 years, p=0.1308). The Cd exposed group was associated with changes in methylation ($p < 0.05$) at 12, 8, and 20 age-associated sites identified in our population, Hannum, and Horvath. Elevated Cd exposure is associated with methylation changes at age-associated sites and smaller differences between predicted and chronologic age. Further work is needed to understand the complex association between Cd, methylation, and biologic aging.

INTRODUCTION

The epigenetic clock is hypothesized to capture common and progressive changes in methylation that occur during aging at specific CpG sites that are susceptible to the decline in maintaining methylation patterns (1, 2). DNA methylation may affect downstream gene expression, and it is dynamic and sensitive to internal and external environmental changes that result from aging, lifestyle factors, disease, and exposure to environmental toxicants (3). DNA methylation becomes increasingly dysregulated with age, and aging is associated with loss of global methylation and preferential gains in methylation at island-associated promoters (4-6). Using human methylation arrays, several studies have shown that a combination of methylation at CpG sites measured in whole blood can describe an epigenetic clock and predict biologic age (1, 4, 7, 8). Multiple studies have characterized that greater difference between chronologic age and epigenetic age is associated with health and mortality (9, 10) and adults with Down syndrome have accelerated epigenetic aging in multiple tissue types (11). Environmental exposures that are known to alter the epigenome may modify the rate at which epigenetic alterations are accrued during aging.

Cadmium (Cd) is an environmental exposure that is associated with changes in the cell, including the epigenome, and potentially, with health in later life. Cadmium initiates aging-related cellular processes within cells, including perturbation of gene-specific and global methylation. CpG methylation may be sensitive to disruptions in Cd-associated changes in the oxidative (12) and inflammatory environment of the cell (13). Cd is also associated with altered DNA methyltransferase activity as suggested by in vivo and in vitro studies (14-16).

The relationship between Cd exposure and age-associated methylation changes has not been thoroughly investigated in an environmentally exposed population. This is partially due to

the lack of studies in high Cd exposed populations. We have described epigenetic changes in a population in northern Thailand that was exposed to Cd through contamination from mining activities (17). This population has documented levels of urinary Cd levels that exceed the WHO standard level (18) and associated chronic health problems such as diabetes, hypertension, and osteoporosis (19, 20). We took advantage of a pilot study of non-smoking women from northern Thailand (with high and low levels of Cd) with genome-wide epigenetic data to evaluate the cross-sectional relationship between age, DNA methylation, and Cd exposure. We observe that higher Cd is associated with a smaller difference between epigenetic age and chronologic age, we also identify several age-associated CpG sites that are more variable with Cd exposure.

METHODS

Study Population. Forty non-smoking women between ages 40-80 years were selected from a Cd-exposed cohort from Mae Sot, Thailand. These women participated in a follow-up health impact survey in 2012 and provided whole blood and urine at the time of the survey (21). This Cd-exposed cohort has been previously described (18). We selected non-smoking women based on their urinary Cd adjusted by specific gravity and identified twenty women with high Cd exposure (HE), and twenty women with low Cd exposure (LE), using a cutoff of 2 ug/L. The women in each group were matched by age within five years. Study approval for this study was obtained from the research and ethics committee from the Faculty of Medicine, Chiang Mai University. (Approval No. 004/2012).

Cadmium Exposure Assessment. Twenty-five mL of morning urine were collected in Cd-free polyethylene containers. Specific gravity (SG) was measured for each sample using a

refractometer (PALS-10S), shortly after collection. The median specific gravity in the entire study population in 2012 was 1.015, and we used the following adjustment, unadjusted Cd (ug/L) * ((1.015-1)/(SG-1)), in order to account for urine density at time of collection (22). After transportation to the University of Michigan on dry ice, samples were stored at -20C until analysis. Urinary Cd was measured at the Michigan Department of Community Health. The samples were diluted 1:10 with an extraction solution of 2.0% nitric acid, 0.05% Triton X, and internal standards. Cd concentrations were measured using an inductively coupled plasma mass spectrometer (ICP-MS). The analytical accuracy using Cd urinary standard reference material (QMEQAS08U, Institut National de Santé Publique du Québec, INSPQ) was 101.1%, and all samples were above the detection limit of 0.15 ug/L.

DNA Extraction and Bisulfite Conversion. Five mL of fasting venous whole blood was collected in EDTA-coated tubes. After transportation to the University of Michigan on dry ice, samples were stored at -80C until DNA extraction. Genomic DNA was extracted from 300uL of whole blood using QiaAMP DNA Mini Kit (Qiagen). Extracted DNA concentrations and quality were quantified using the Nanodrop spectrophotometer (Thermoscientific). We bisulfite converted 500ng of genomic DNA using EpiTect Bisulfite Kit (Qiagen) per manufacturer's protocol.

DNA Methylation Measurement. DNA methylation was measured using the Illumina Infinium HumanMethylation450 BeadChip (23). Human Illumina 450K Methylation arrays were processed by the University of Michigan Sequencing Core Facility according to manufacturer protocol. The proportion of methylation at each site is reported as the beta-value, which is

computed as beta = methylated signal intensity / (methylated signal intensity + unmethylated signal intensity + a), where a is an adjustment constant.

Normalization Summary. We filtered 1,977 probes with detection p-values above 0.01 and 3,198 probes with bead counts less than three in two or more samples from the analysis. We restricted our analysis to probes corresponding to CpG sites located on autosomal chromosomes and removed 11,270 probes on the sex chromosomes. No samples in our analysis performed poorly, as defined by having more than 5% of probes above the detection p-value. We performed background and dye-bias correction using the normal-exponential model (24) implemented in methylumi (25). We also removed probes identified by Chen et al (26), where the CpG site or single-base extension site corresponded to a SNP with a minor allele frequency greater than or equal to 0.01, which removed 24,720 probes. Additionally, we removed 29,233 cross-reactive probes. We then applied subset-quantile within array normalization (SWAN) (27). We selected SWAN normalization method after evaluating 99^{th} quantile absolute deviations and correlations among our MCF7 replicates as proposed by Wu et al (28). We removed these replicates before our final normalization. We then used ComBat to correct for plate and technical effects (29, 30). The final number of probes included in our analysis was 419,995, which covers 80% of the 450K Illumina array.

Statistical Analysis. All statistical analyses were performed on the M-values from the beta-values using a logit2 transformation. We reported beta-values for easier interpretation. We examined the unadjusted age associations by computing the Spearman correlation for each CpG site in our entire study population, among the LE group, and among the HE group. We assessed

age-associated markers in our study population using linear multivariate regression performed in limma. We selected *a priori* and adjusted for BMI, urinary creatinine, and estimated white blood cell proportions using the Houseman method (31). After identifying age-related markers in our study population, we analyzed differences in methylation between Cd exposure groups and by log-transformed continuous Cd exposure using linear mixed models among the age-related markers in our study population, reported by Hannum et al (4) and Horvath (1) using lmerTest in R. The false discovery rate (FDR) was computed using the Benjamini-Hochberg method. All analyses were implemented in R version 3.2.3.

Age Prediction. For the age prediction analyses, we generated background corrected betas. To estimate predicted methylation age, we applied two published methods for methylation age predictors from Hannum et al and Horvath. In Caucasian and Hispanic individuals, the combination of 71 age-associated methylation markers predicted the age of individuals ages 9-101, and additionally, identified individuals whose age predicted by methylation significantly deviated from their chronologic age (4). Horvath developed a predictive model for aging utilizing data from 51 tissues and cell types and identified a panel of 353 age-associated methylation markers that he proposed estimated the epigenetic clock and methylation age within an individual (1). The predicted methylation age using the Horvath method was computed using his online age calculator. The predicted biologic age using the Hannum method was computed by extracting their coefficients for their age-related markers and multiplying our background corrected betas with these. The sum of these products yielded the Hannum predicted methylation age. The predicted methylation ages were adjusted by chronologic age, array and estimated Houseman white blood cell composition using linear regression (1, 31) and the residuals from

this model were added to the mean predicted methylation age to give an adjusted methylation age (9). We analyzed the adjusted predicted methylation age and chronologic age difference (Δage) by Cd exposure using paired t-tests and linear mixed models.

RESULTS

Study Population Characteristics. The median and range of adjusted Cd was 0.7 ug/L (0.3-1.9 ug/L) in the LE group and 10.8 ug/L (7.0-48.3 ug/L) in the HE group. The age range of our study population was 43-80 years and the mean ages in the LE and HE groups were 58.8 (sd=9.4) and 60.4 (sd=10.3) years, respectively, and these were not significantly different by exposure group. The low and high groups had similar anthropometric measures (**Table 3.1**). The LE group had higher BMI than the HE group, 26.4 vs 22.2, respectively (p=0.0028).

The urine specific gravity did not significantly differ by exposure group, and all values were within normal range. Urinary specific gravity was positively associated with continuous unadjusted Cd (Spearman's rho=0.3683, p=0.0194). The mean urinary creatinine was significantly higher in the HE group compared to the LE group, 1.29 vs 0.91 ug/L, respectively ($p < 0.0001$). Both urinary beta-2-microglobulin, a biomarker of chronic kidney disease, and N-acetyl-beta-D-glucosaminidase (NAG), a biomarker of renal tubular impairment were significantly elevated in the HE compared to LE (Wilcoxon sign rank, $p <0.0001$ for both). Urinary citrate, a biomarker of renal stone formation, was significantly lower in the HE compared to LE (Wilcoxon sign rank, p=0.0073). These results among our study population are consistent with chronic kidney disease and damage identified with chronic Cd exposure in Mae Sot **(32)**.

While the estimated proportion of granulocytes, CD8T and CD4T cells, and natural killer cells were similar between both exposure groups, the mean monocyte proportion was greater in the HE group compared to LE group, 6.5% vs. 4.5%, respectively (paired t-test, p=0.0105), and the mean B-cell proportion was smaller in the HE compared to the LE, 3.8% vs. 6.0%, respectively, (paired t-test, p=0.0263). While none of the white blood cell proportions were significantly correlated with age, monocyte proportion was positively correlated with increasing Cd exposure (Spearman's rho=0.414, p=0.0079).

Predicted methylation age and association with Cd exposure. The predicted methylation ages from both Hannum and Horvath were positively and significantly correlated with chronologic age, 0.76 (p <0.0001) and 0.73 (<0.0001), respectively (**Figure 3.1 A and B**). The predicted methylation ages from Hannum and Horvath were also positively and significantly correlated with each other, 0.88 (p < 0.0001) (**Figure 3.1 C**). Hannum predicted methylation age was on average 5.56 (sd=6.37) years greater than the chronologic age while Horvath predicted methylation age was on average 3.49 (sd=6.70) years greater than actual age.

The mean predicted methylation Hannum and Horvath ages did not significantly differ by exposure group (Hannum: LE=65.9 (sd=8.4) and HE=64.4 (sd=8.0) years; Horvath: LE=62.4 (sd=8.0) and HE=63.7 (sd=7.8) years. Among the LE group, the correlation between chronologic age and methylation age predicted by Hannum and Horvath was 0.65 (0.0021) and 0.61 (p=0.0042), respectively, while among the HE group, the correlations were 0.90 (p < 0.0001) and 0.84 (p < 0.0001), respectively (**Figure 3.1 A and B**). The proportion of variance of methylation age explained by chronologic age is much greater within the HE group compared to the LE group. Within the HE group, the Hannum methylation age increased by 0.72 years per year in

chronologic age ($p < 0.0001$), and the r^2 for this model was 0.87; the Horvath methylation age increased by 0.64 years per year in chronologic age ($p < 0.0001$) and the r^2 for this model was 0.76. Within the LE group, the Hannum methylation age increased by 0.73 years per year in chronologic age and the r^2 for this model was 0.54; the Horvath methylation age increased by 0.75 years per year in chronologic age and the r^2 for this model was 0.63.

After adjusting for plate and estimated white blood cell composition, mean difference between predicted methylation age and chronologic age (Δage) using the Hannum predictor was 7.6 years (sd=10.9 years) in the LE group and 3.6 years (sd=9.9 years) in the HE group. The Δage was significantly smaller in the HE group compared to LE group (difference=-3.98 years, p=0.0093) (**Figure 3.2 A**). The mean Δage using the Horvath predictor was 4.5 years (sd=11.2 years) in the LE group and 2.4 years (sd=10.0 years) in the HE group. The Δage for Horvath was also smaller in the HE group compared to LE group (difference=-2.10 years, p=0.1308) (**Figure 3.2 C**). In addition, Hannum Δage significantly decreased per percent increase in continuous Cd exposure (p=0.0311) after adjustment by BMI and urinary creatinine. The Horvath Δage was not significantly associated with continuous Cd exposure (p=0.1035) after adjustment. No associations were found between Δage and our urinary biomarkers of kidney dysfunction after adjustment.

Methylation among sites included in Hannum and Horvath methylation age predictors. At a nominal significance level ($p < 0.05$), fifty-six percent of CpG sites included in the Hannum methylation age predictor were correlated (n=40) with age in our study population (not shown). After adjustment by estimated blood cells, BMI, and urinary creatinine, 61% of these sites were associated (n=43) with age in our study population. Only 14% of sites included in the Horvath

methylation age predictor were correlated (n=51) with age in our study population, and after adjustment, 15% of sites were associated with age (n=53) in our study population. Among the markers included in Hannum and Horvath, only six sites overlapped.

Among the Hannum CpG sites, we identified eight sites significantly associated ($p < 0.05$) with Cd exposure group after adjustment (**Table 3.2**). The difference in methylation among these sites was between 1.1% and 3.1% lower in the HE group compared to the LE group after adjustment. Among the Horvath CpG sites, we identified twenty sites significantly associated ($p < 0.05$) with Cd exposure group after adjustment (**Table 3.3**). These sites were bi-directionally associated with high Cd exposure group. Fourteen sites were associated with decreased methylation (range: -0.8% to -3.4%) in HE group and eleven sites were associated with increased methylation (range: 0.2% to 3.5%) in HE group.

Age-Associated methylation in study population. We then took an agnostic approach to identify age-associated CpG sites in our study population. We first examined the unadjusted association between epigenome-wide methylation and age by examining the Spearman correlation for each site (not shown). One hundred fourteen sites were significantly correlated at epigenome-wide relaxed significance ($p < 1e\text{-}4$). The absolute values of the Spearman correlations ranged from 0.58 to 0.77. Three of these sites had an FDR less than 0.05 and Spearman correlation greater than 0.7, and these sites were annotated to *TRIM59*, *ELOVL2*, and *SIX1*. Of the 114 significantly correlated sites, 92% (n=105) were positively correlated with age.

We then examined the adjusted association between epigenome-wide methylation and age (**Figure 3.3 and 3.4**). After adjusting for BMI, creatinine, and estimate white blood cell proportion, 213 sites were associated with methylation at relaxed epigenome-wide significance

($p < 10e-4$), and ten of these sites had an FDR less of 0.05. Of these sites, 38% (n=43) of them were among the identified correlated sites ($p < 10e-4$) suggesting that some of these age-correlated sites are confounded by BMI, urinary creatinine, or white blood cell composition. Ninety-four percent of these sites were positively associated with age. Fifty-four percent of these sites were located within CpG islands and 23% were located within the open sea (**Figure 3.5**). This distribution significantly differed from the locational distribution of the entire array ($p < 0.0001$). One hundred sixty sites annotated to a gene. Among these age-associated sites in our population, ten sites were located in the *PRRT1* gene, three sites were located in each of the following genes: *KLF14*, *ELOVL2*, and *FBLN2*.

Among our age-associated sites identified in our population, twelve sites were nominally significant ($p <0.05$) with Cd exposure group after adjustment (**Table 3.4**). Eighty-three percent of these sites were associated with decreased methylation in the HE group (range: -1.1% to -2.8%). Two sites were associated with increased methylation in the HE group compare to LE group, and one hemi-methylated site in *PBSM9/TAP1* was associated with an increase of 6.4% in the HE group compared to LE group after adjustment. Seven sites were associated with continuous Cd exposure.

DISCUSSION

We determined that the Hannum and Horvath predicted methylation ages were correlated with chronologic age in our study population and the difference between chronologic age and predicted methylation age was smaller in the high exposed Cd group. We identified a unique set of age-associated CpG sites in our population from Thailand, suggesting that age-associated methylation patterns vary between populations or by environmental exposures, like Cd. Cd may

modify methylation at age-associated sites included in the Hannum and Horvath predictors and our study population. The association between Cd and methylation at these CpG sites included in these predictors may affect the estimation of predicted methylation age.

The epigenomic effects of Cd have not been extensively studied among individuals with moderately high chronic exposure, as seen in this population, and among adults. Epigenome-wide studies of Cd have examined cord blood methylation of infants from Bangladesh (33) and United States (34), and both of these studies observed sex-specific associations with maternal and placental Cd exposure, respectively. Low-Cd exposure levels were associated with hypomethylation of LINE-1, a surrogate measure of global methylation (35). Cd exposure may be a crucial modifier of methylation, which could have important consequences on age-associated methylation. Cd is also associated with other age-associated processes. Cd induces oxidative stress within cells and inflammation by promoting cytokine production (36), which both perturbs signaling within the cell and may initiate cellular senescence and aging. Low Cd exposure has been shown to be associated in a dose-response manner with shortened telomere length in leukocytes, a marker of biologic aging and cellular senescence (37).

Both the Hannum and Horvath predicted methylation ages were correlated with chronologic age in our population and were more strongly correlated with age in the HE compared to the LE Cd group. The Hannum predicted methylation age correlated more strongly with chronologic age. This may be expected because the Hannum predictor was generated from DNA methylation analysis of whole blood samples using 450K array, while the Horvath predictor was generated from DNA methylation analysis of 51 different tissues and cell types using the previous generation 27K array. The Δage was smaller in the high exposed Cd group, and increasing Cd exposure was associated with decreasing Δage after adjustment. However, in

both the LE and HE Cd groups, the mean difference between predicted age and chronologic age was positive, suggesting biologic age was greater than chronologic age in these women. Alternatively, these predictors may not work optimally in this population. A few other studies have examined infectious and environmental exposures and their effects on accelerated aging. Smoking was not shown to be associated with Δage in blood in four birth cohorts (9). Using methylation biomarkers to estimate smoking exposure and the Hannum predictor, smoking was shown to accelerate biologic aging, however, alcohol consumption was shown to be associated with decelerated aging (38). Chronic cytomegalovirus infection was associated with a higher epigenetic age in young adults (39). There may be a complex relationship between Cd exposure as an epigenetic modifier, disease related processes, and their combined effect on epigenome-wide methylation. Notably, we observed substantial increased methylation at an age-associated site annotated to the *TAP1/PSMB9* gene in the high Cd exposure group and by increasing Cd exposure. This locus is located within MHC region, and methylation in *TAP1* or *PSMB9* is associated with prognosis in head and neck cancer (40) and ovarian cancer (41, 42). Cd exposure may modify epigenetic aging, and further studies will explore whether this is predictor of chronic disease onset and mortality in our study population.

Within the constraints of our small sample size, we identified a unique set of age-associated sites in our study population. A limited number of age-associated sites identified in our population overlapped with those included in Horvath predictor, and there was more similarity between age-associated sites in our population and those identified in Hannum, providing support that our results are consistent with age-associated sites identified in the literature (4, 7, 43). Unique to our population, we observed that ten age-associated sites were located within *proline-rich transmembrane protein 1* (*PRRT1*) gene. *PRRT1* lies within the gene

dense region of 6p12.32 and is near the major histocompatibility complex (MHC). This chromosomal region is a source of epigenetic heritability (44) and encodes numerous immune-response genes and CpG sites within these genes may be susceptible to changes in methylation (45, 46). These unique age-associated sites may be associated with aging in this population, but these sites may reflect changes inflicted by both Cd exposure and aging.

Our sample size is limited, and a larger sample size would verify our results and allow us to investigate developing an age-prediction model this population. We also used a 450K array, which, while the most comprehensive array available at the time, only covers a fraction of the total CpG sites in the genome. This platform is biased towards sites located in promoter and intergenic regions. We can only assess age-associated methylation changes cross-sectionally in this population, and it might be more informative to examine these changes temporally across the exposure and age trajectory. We only included women because there was a large enough sample of non-smokers, and a large proportion of men in this population are smokers. Given that men and women age differently, it is important to examine the effect of biologic aging in men exposed to Cd in future studies. Finally, our study would be benefit to have non-exposed population of women from this region since even our low-exposed women have relatively high exposed to Cd.

Our study population has been chronically and highly exposed to Cd, which makes it an ideal population to understand the Cd may have on methylation, and includes self-reported non-smoking women. Smoking may confound many analyses of cadmium and is associated with extensive and specific methylation changes (47), Urine is also a good marker of long term Cd exposure as it captures between 10 and 30 years of exposure (48). A concern our study addresses, despite our limitations, is that there appears to be some population-level heterogeneity in age-

associated methylation sites, and in fact, the environment may shape aging in a population-specific manner, but this cannot be necessarily drawn from our study. These calculators may not optimally predict biologic aging using epigenome-wide methylation in this population since it is genetically and ethnically different from the US and age-associated epigenetic patterns may be influenced by a combination of different genetic, dietary, lifestyle and environmental factors.

It is difficult to separate the effects of aging and Cd exposure on methylation since these factors both change temporally and age is associated with Cd exposure. In this study, age-associated CpG sites in the Horvath and Hannum were not strongly associated with age in our study population; however, Cd was associated with differential methylation at some of the sites in these predictors. The predicted methylation ages in this population may not reflect the biologic age but the epigenomic effects of Cd on these identified age-associated sites in the genome. In order to decipher whether Cd may affect biologic aging, it is necessary to examine other non-methylation based biomarkers of aging in this population. Two such biomarkers of aging include transcriptomic age, which estimates biologic age using gene expression patterns associated with aging (49), and composite biologic age constructed from biomarkers associated with health and function of different organ systems (50). While the relationship among biomarkers of aging needs to be examined, different biomarkers of age are expected to reflect a similar predicted biologic age but may be based on different aging biologic mechanisms. The relationship observed between Cd exposure and biologic age should be confirmed using biomarkers of age that reflect a mechanism of aging independent of Cd exposure.

In conclusion, our results suggest that Cd exposure is associated with age-associated methylation changes, although the consequences of these changes are not clear. The complex effects of Cd exposure and aging may promote a complex chronic disease phenotype that may

have long-term consequences throughout the lifetime. These associations between Cd and age-related diseases and cellular processes warrant exploring whether Cd exposure modifies age-associated methylation and methylation age, which may be potential mediators between chronic Cd exposure and increased risk of these age-related chronic diseases or predictors of these age-related effects. These age-associated methylation changes and their potential modification by Cd exposure suggest that adulthood is an additional critical window of vulnerability.

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Table 3.1. *Descriptive Statistics for Study Population*.

	Low Cd (n=20)	High Cd (n=20)	t-test/ WSR p	Spearman Correlation w/Age	p	Spearman Correlation w/Cd	p
Adjusted Cd	0.71 (0.26, 1.94)	10.76 (6.97, 48.3)	**<0.0001**	0.2042	0.2061		
Age (years)	58.8 (9.4)	60.4 (10.3)	0.6196			0.0596	0.7150
BMI (kg/m^2)	26.4 (2.9)	22.2 (4.9)	**0.0028**	-0.2458	0.1263	-0.3324	**0.0361**
Adjusted Urinary Markers							
Urinary Creatinine (ug/L)	0.91 (0.47, 1.67)	1.29 (0.65, 2.00)	**<0.0001**	-0.2017	0.2119	0.3981	**0.0115**
Urinary beta-2 microglobulin (ug/L)	91.03 (20.25, 17,222.73)	5263.64 (146.25, 40,500.00)	**<0.0001**	0.2735	0.0920	0.6737	**<0.0001**
Urinary NAG (ug/L)	4.00 (1.31, 26.10)	9.25 (2.84, 30.50)	**<0.0001**	0.1867	0.2486	0.4438	**0.0041**
Urinary citrate (ug/L)	0.31 (0.09, 0.55)	0.09 (0.02, 0.28)	**0.0073**	-0.1117	0.4926	-0.6277	**<0.0001**
Blood Counts							
White Blood Cell Count (10^6 cells /uL)	9.47 (3.87)	7.80 (1.89)	0.0725	0.0110	0.9465	-0.2667	0.0962
Hemoglobin (g/dL)	12.94 (1.02)	12.43 (1.13)	0.0936	-0.0560	0.7316	-0.2566	0.1100
Hematocrit (%)	39.85 (3.32)	37.57 (3.20)	**0.0165**	-0.0725	0.6567	-0.3528	**0.0256**
Red Blood Cell Count (10^6 cells /uL)	4.85 (0.66)	4.44 (0.57)	**0.0298**	-0.1910	0.2379	-0.4010	**0.0103**
Estimated White Blood Cell Proportions							
CD8T (%)	8.28 (4.26)	7.15 (4.84)	0.4694	0.0129	0.9372	-0.2517	0.1171
Granulocyte (%)	51.92 (9.24)	51.43 (7.81)	0.8562	-0.2729	0.0884	0.0692	0.6716
Monocyte (%)	4.52 (2.09)	6.48 (2.88)	**0.0105**	-0.0928	0.5689	0.4139	**0.0079**
CD4T (%)	10.19 (4.13)	9.81 (4.87)	0.8022	0.0757	0.6423	0.0077	0.9624
Natural Killer (%)	17.11 (6.76)	18.39 (7.23)	0.5653	0.2773	0.0833	0.1080	0.5071
B Cells (%)	6.01 (3.24)	3.82 (3.03)	**0.0263**	0.1633	0.3141	-0.2946	0.0650

Table 3.2. *Results of Age-Associated Methylation by Exposure Group among Hannum CpG sites.* Models adjusted for age, urinary creatinine, BMI, and estimated white blood cell proportions.

	Chr	Location	Relation to CpG Island	Mean (SD) % Methylation: Low	Mean (SD) % Methylation: High	Change in Methylation by Exposure Group	p	Change in Methylation per percent Change in Cd	p
cg04474832	3	First Exon, Body, 5'UTR, Distal Promoter	North Shore	26.7% (2.2%)	26.0% (2.6%)	-2.16%	0.0104	-0.006%	0.0421
cg07927379	7	Body, Distal Promoter	Island	3.7% (1.1%)	3.6% (1.0%)	-1.01%	0.0144	-0.004%	0.0027
cg02867102	17		Open Sea	8.3% (2.7%)	6.9% (1.7%)	-2.13%	0.0165	-0.006%	0.0452
cg23500537	5		Open Sea	45.5% (3.3%)	44.6% (4.1%)	-2.79%	0.0168	-0.009%	0.0229
cg02085953	2	Distal Promoter	North Shore	34.9% (2.7%)	34.9% (3.3%)	-2.07%	0.0255	-0.007%	0.0290
cg02650266	4		Island	11.0% (3.4%)	10.3% (3.7%)	-2.17%	0.0272	-0.007%	0.0433
cg16054275	1	Distal Promoter	Open Sea	41.1% (3.1%)	40.4% (3.6%)	-3.12%	0.0346	-0.010%	0.0491
cg14692377	17	First Exon, 5'UTR	Island	16.7% (2.9%)	15.2% (3.3%)	-2.18%	0.0431	-0.007%	0.0743

Table 3.3. *Results of age-associated methylation by exposure group among Horvath CpG sites*. Models adjusted for age, urinary creatinine, BMI, and estimated white blood cell proportions.

	Chr	Location	Relation to CpG Island	Mean (SD) % Methylation: Low	Mean (SD) % Methylation: High	Change in Methylation by Exposure Group	p	Change in Methylation per percent Change in Cd	p
cg24058132	14	First Exon, 5'UTR	South Shore	44.1% (3.6%)	43.7% (3.6%)	-3.40%	0.0045	-0.010%	0.0120
cg02580606	17	Distal Promoter	Open Sea	90.0% (1.6%)	89.8% (1.3%)	-1.49%	0.0058	-0.005%	0.0057
cg19478743	17	Proximal Promoter, First Exon	Island	6.2% (1.9%)	5.4% (1.0%)	-1.65%	0.0082	0.002%	0.1106
cg04474832	3	First Exon, Body, 5'UTR, Distal Promoter	North Shore	26.7% (2.1%)	26.0% (2.6%)	-2.16%	0.0104	-0.006%	0.0421
cg25928579	17	Distal Promoter	South Shore	7.0%(1.4%)	7.6% (1.5%)	1.38%	0.0133	0.004%	0.0233
cg23941599	5	Distal Promoter	South Shore	13.5% (2.9%)	11.9% (3.9%)	-2.36%	0.0137	-0.007%	0.0315
cg10486998	18	Distal Promoter	Island	13.8% (3.2%)	15.0% (3.1%)	3.14%	0.0192	0.007%	0.1368
cg22432269	15	First Exon, 5'UTR	Island	2.8% (0.3%)	2.9% (0.4%)	0.37%	0.0232	0.001%	0.0556
cg14992253	1	Body, Distal Promoter	Island	16.3% (3.1%)	16.4% (3.4%)	3.13%	0.0272	0.012%	0.0106
cg16150435	6	Proximal Promoter	Open Sea	81.9% (3.2%)	81.7% (3.4%)	-2.70%	0.0304	-0.008%	0.0710
cg19692710	11	First Exon, 5'UTR	Open Sea	87.4% (2.6%)	87.5% (2.7%)	-1.91%	0.0306	-0.006%	0.0590
cg13547237	11	Body, Distal Promoter	South Shore	29.8% (2.8%)	28.7% (2.8%)	-2.71%	0.0330	-0.008%	0.0600
cg13899108	19	5'UTR	South Shore	45.6% (3.9%)	49.2% (5.4%)	3.52%	0.0339	0.009%	0.1061
cg02275294	1	Distal Promoter	North Shore	10.1% (2.4%)	9.7% (2.4%)	-1.48%	0.0390	-0.005%	0.0605
cg11932564	22	Body	Island	2.5% (0.8%)	2.6% (1.1%)	-0.78%	0.0394	-0.002%	0.0930
cg01407797	22	Proximal Promoter	North Shore	5.9% (1.2%)	5.5% (1.4%)	-1.18%	0.0456	-0.004%	0.0542
cg27015931	16	First Exon, 5'UTR	Open Sea	11.1% (1.1%)	10.7% (1.2%)	-0.82%	0.0467	-0.002%	0.0959
cg02654291	9	Distal Promoter	Island	51.0% (5.0%)	49.5% (2.3%)	-3.10%	0.0476	-0.011%	0.0517
cg14163776	3	Distal Promoter	South Shore	20.9% (4.0%)	20.5% (3.2%)	-2.54%	0.0491	-0.009%	0.0352
cg23092072	4	Body, Distal Promoter	North Shore	2.9% (0.3%)	2.8% (0.3%)	0.23%	0.0495	0.001%	0.0842

Table 3.4. *Results of age-associated methylation by exposure group among age-associated sites in study population*. Models adjusted for age, urinary creatinine, BMI, and estimated white blood cell proportions.

	Chr	Location	Relation to CpG Island	Mean (SD) % Methylation: Low	Mean (SD) % Methylation: High	Change in Methylation by Exposure Group	p	Change in Methylation per percent Change in Cd	p
cg23350274	14	5'UTR	Open Sea	88.6% (1.5%)	88.3% (1.7%)	-1.48%	0.0039	-0.005%	0.0095
cg01695225	2	Body	South Shore	12.1% (2.2%)	11.1% (2.3%)	-1.65%	0.0160	-0.006%	0.0091
cg23500537	5		Open Sea	45.5% (3.3%)	44.6% (4.1%)	-2.79%	0.0168	-0.009%	0.0229
cg05837727	11		Island	9.3% (2.1%)	8.7% (1.8%)	-1.10%	0.0173	-0.004%	0.0116
cg03465320	6	Body, Distal Promoter	South Shore	49.4% (9.9%)	54.6% (8.0%)	6.32%	0.0181	0.019%	0.0427
cg21516291	20	Body	Open Sea	55.7% (4.3%)	53.9% (4.7%)	-2.35%	0.0246	-0.007%	0.0525
cg18239431	8	Body	North Shore	16.9% (4.7%)	14.7% (3.6%)	-2.49%	0.0296	-0.006%	0.1752
cg16001722	6	Body	Island	13.8% (2.9%)	12.9% (2.9%)	-1.30%	0.0315	-0.005%	0.0187
cg26985289	1	Proximal Promoter	Island	7.3% (3.9%)	6.7% (2.6%)	-1.52%	0.0362	-0.005%	0.0723
cg04562589	4		Open Sea	23.1% (2.8%)	21.6% (2.3%)	-1.68%	0.0391	-0.005%	0.1036
cg02044219	4	Proximal Promoter	South Shore	6.6% (4.0%)	5.1% (1.7%)	-2.41%	0.0464	-0.007%	0.1695
cg02259914	20		Open Sea	27.6% (3.5%)	28.1% (4.6%)	1.99%	0.0496	0.006%	0.0668

Figure 3.1. *Hannum and Horvath predicted methylation age.* Blue and red denotes LE and HE groups, respectively. Pearson correlation coefficients (r) for entire study population ($p < 0.0001$ for each r).

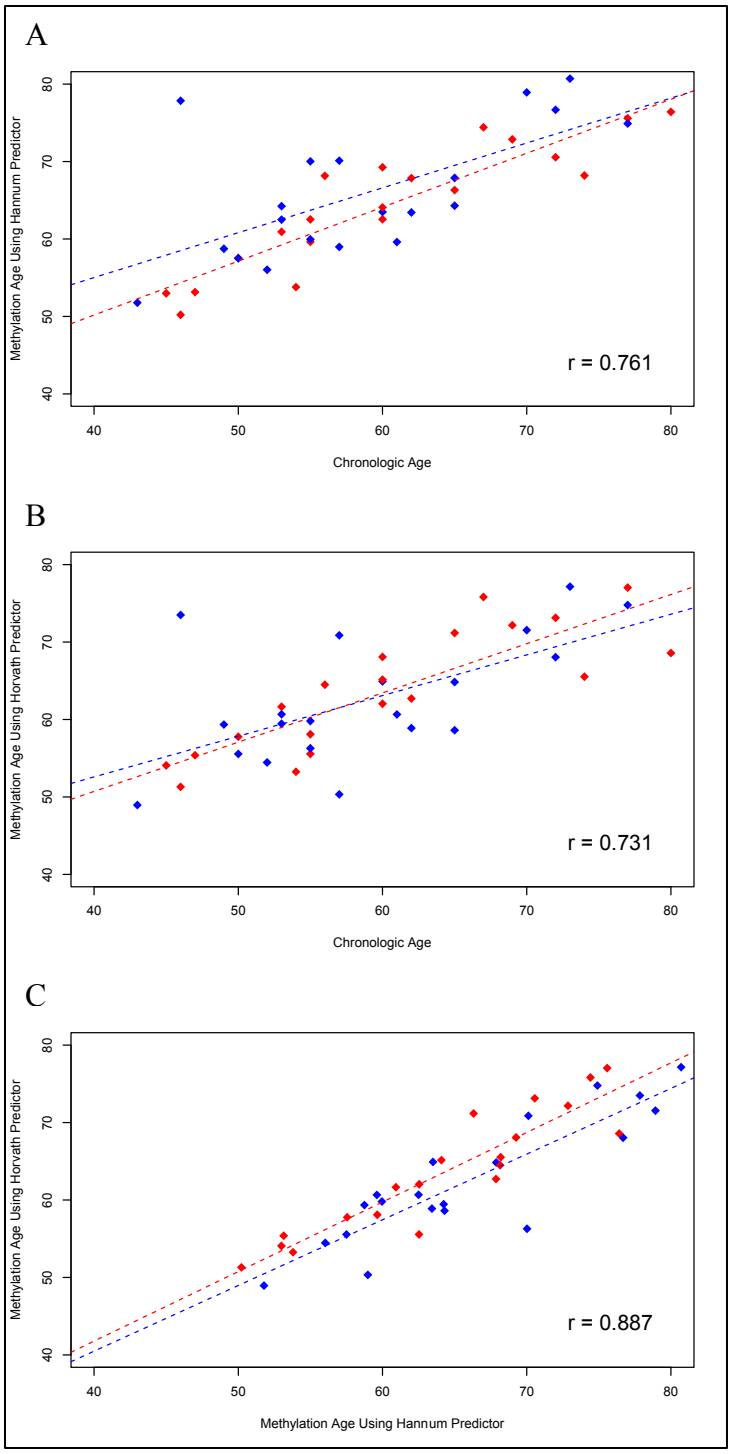


Figure 3.2. *Difference between predicted methylation age and chronologic age (Δage).* Boxplots of Δage for Hannum (A) and Horvath (C). Relationship between log-transformed Cd and Δage for Hannum (B) and Horvath (D). Blue and red denote LE and HE group, respectively.

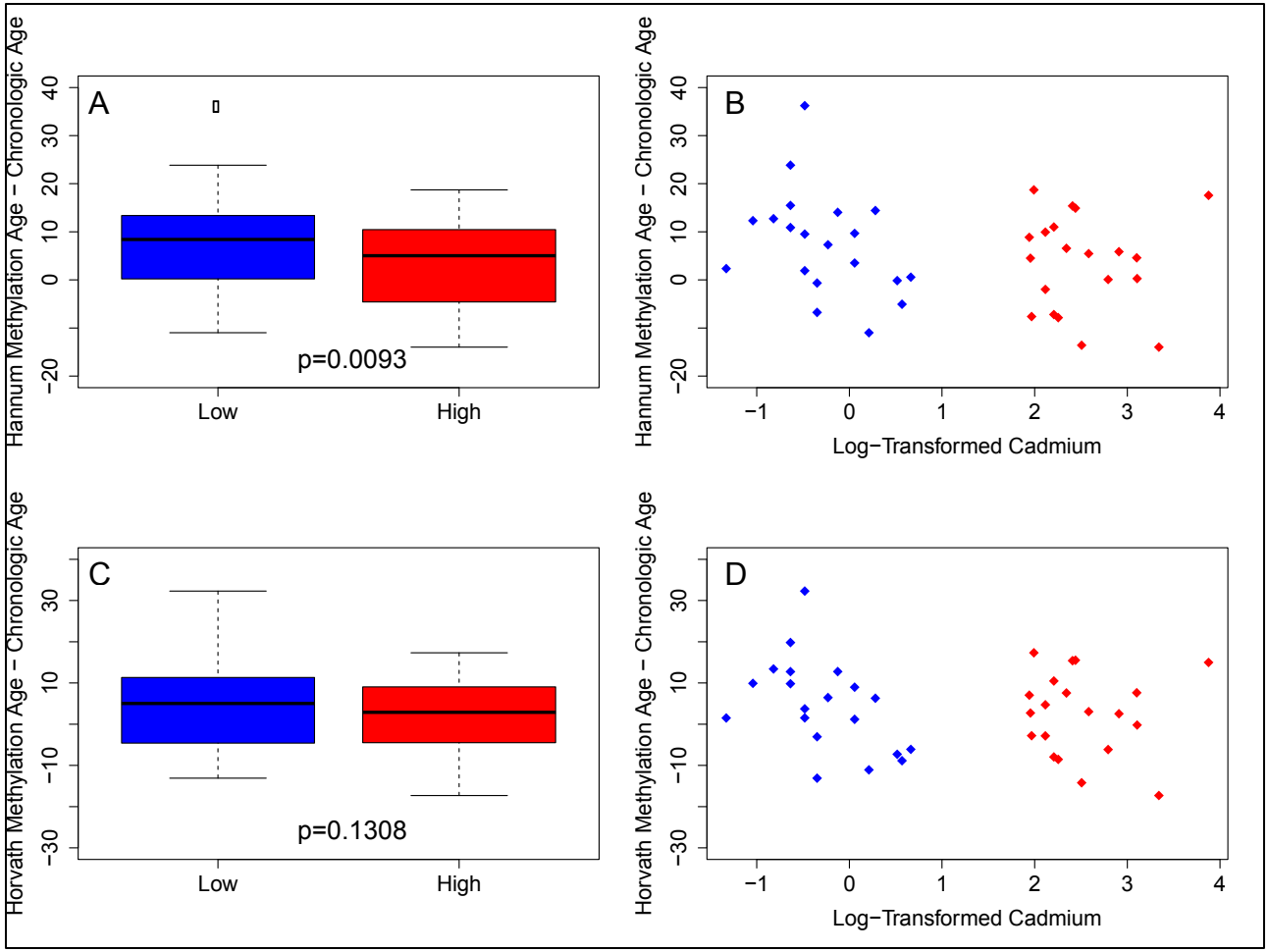


Figure 3.3. *Manhattan Plot of Age-Associated Methylation Markers in study population*. Adjusted for BMI, urinary creatinine, and estimated white blood cell composition. Solid line denotes experimental epigenome-wide significance (p < 1e-7). Dashed line denotes relaxed epigenome-wide significance (p < 1e-4).

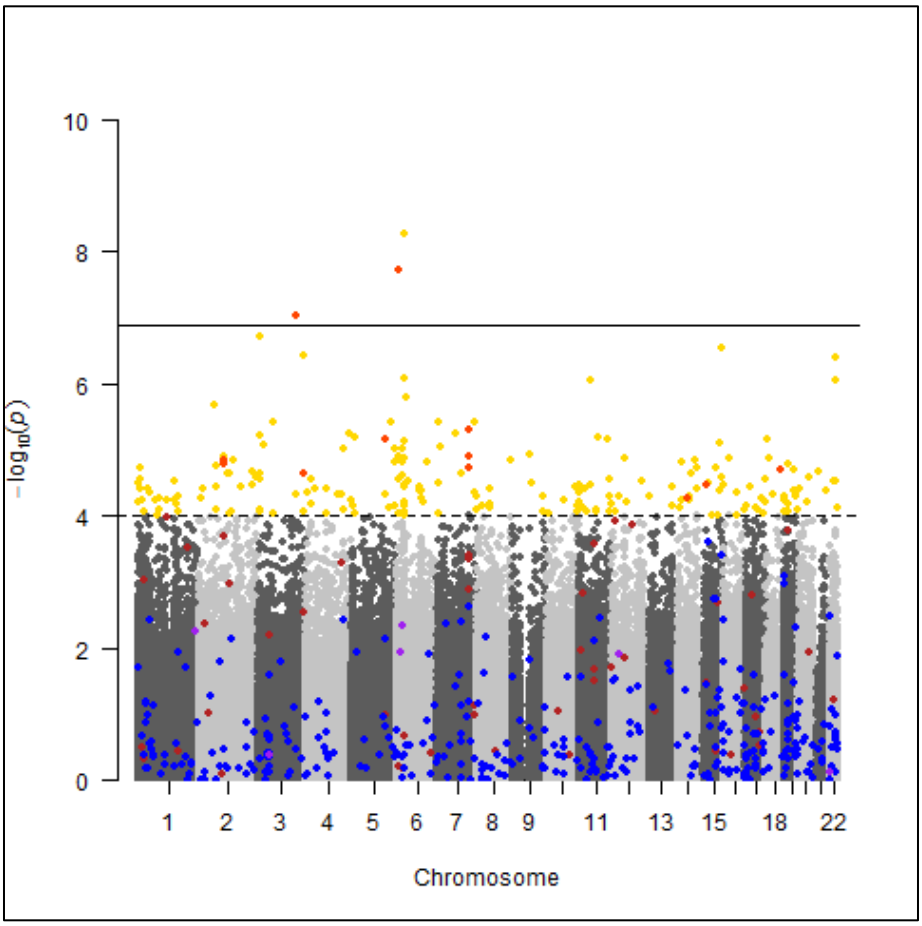


Figure 3.4. *Volcano plot of adjusted age-associated markers in study population*. For both plots, blue denotes markers included in Horvath predictor, red denotes markers in Hannum predictor, yellow are those significant in our population, orange is the overlap between Hannum and our markers, and purple is the overlap between Horvath and Hannum.

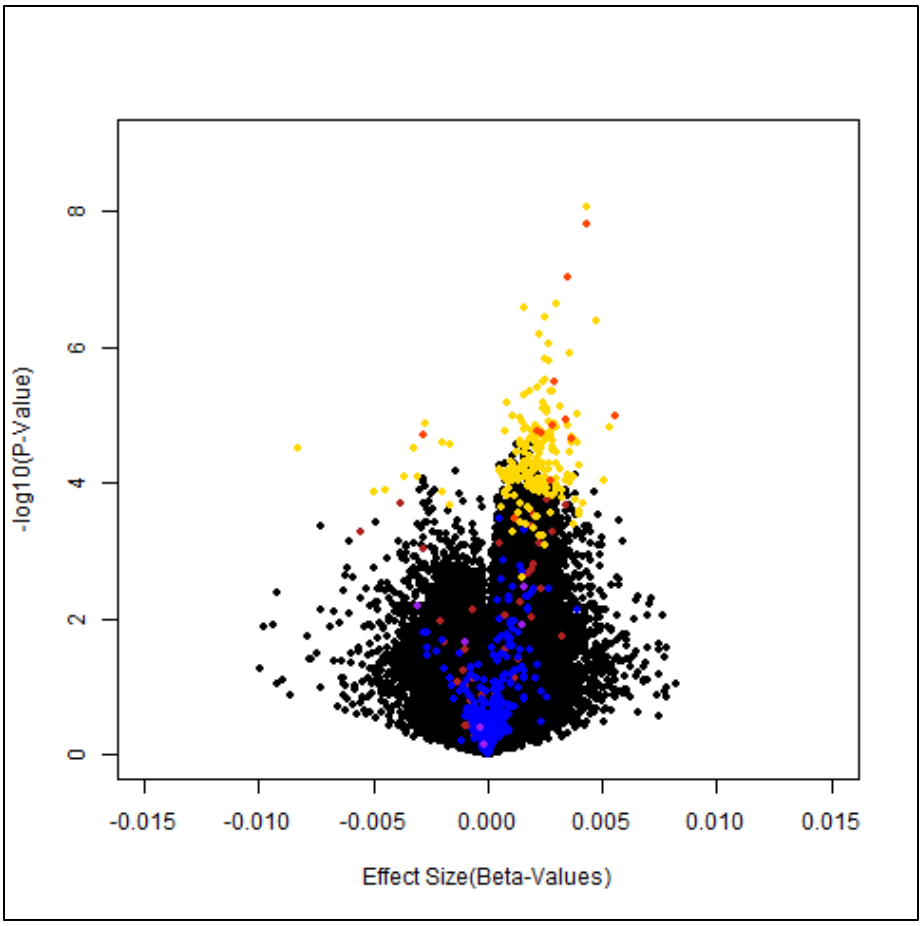
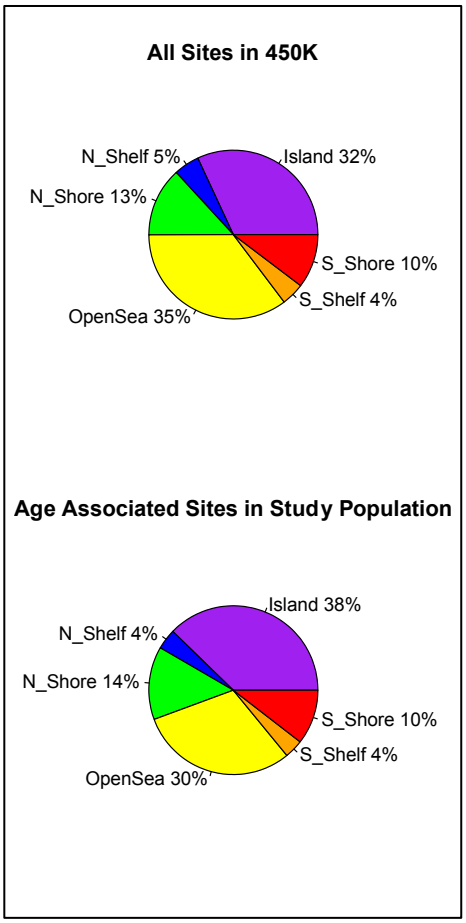


Figure 3.5. *Relation to CpG island among age associated markers in study population*. Age-associated sites included for those with $p < 0.05$ in study population compared to entire array ($p < 0.0001$).



CHAPTER 4. EVALUATING METAL MIXTURES IN BLOOD AND URINE IN WOMEN FROM A CADMIUM EXPOSED POPULATION RESIDING IN NORTHERN THAILAND

ABSTRACT

Introduction. Chronic cadmium (Cd) exposure may be associated with co-exposure to toxic and environmental trace metals and may disrupt homeostasis of trace metals. The Mae Sot population in northern Thailand is exposed to Cd through environmental contamination caused by nearby zinc (Zn) mining. While this contamination is being remediated by the Thai government, it is unknown how the relationship among toxic and trace metals changes longitudinally and what the relationship is between metal biomarkers measured in urine and blood in this population. We assessed changes in individual metals and exposure patterns from 2007 to 2012 in both urine and blood of women from Mae Sot.

Methods. We selected 38 women surveyed in 2007 and 2012. We measured trace metals and toxic metals in blood (Cd, lead [Pb]), copper [Cu], manganese [Mn], molybdenum [Mo], selenium [Se]) and urine (cobalt [Co], Cu, Mo, nickel [Ni], Zn, antimony [Sb], arsenic [As], Cd, Pb). Principle components analysis (PCA) and hierarchical clustering was used to evaluate the mixture of metals.

Results. Median blood Cd decreased from 3.6 ug/L (range: 1.2-15.6 ug/L) in 2007 to 2.4 ug/L (range: 0.6-17.0 ug/L) in 2012 ($p=0.008$). Median urine Cd was 4.9 ug/L (range: 0.2–59.1 ug/L). In 2007 Cd was not correlated with any metals; however, in 2012 Cd was positively correlated with all metals ($p < 0.05$) except Pb. In 2007 the first three PCA components captured 75% of the variance among these metals and were described by Mn-Pb-Se (37%), Mo-Cd (22%), and

Cu-Se (16%). In 2012 the first three PCA components captured 88% of the variance and were described by Cu-Se-Mo-Cd (57%), Mn-Pb (18%), and Pb (13%). Urine Cd was positively correlated with As, Cu, Mo, Zn ($p < 0.100$). The first three PCA components captured 70% of the variance among these metals and were described by Ni-Zn-Pb (36%), Mo-As-Cd (23%), and Cd-Co (11%).

Conclusion. The relationship of Cd to other blood and urine metals is complex and may change over time. Further work is needed to decipher the biologic and health consequences of co-exposure to metals and biomarkers of metal exposure in this chronic Cd exposed population. This study emphasizes the need to establish better toxicologic and reference ranges to assess consequences of chronic Cd exposure in environmentally exposed populations.

INTRODUCTION

Cadmium (Cd) is a potent toxic metal and common environmental pollutant especially in regions, where there is heavy metal mining and industrialization. Cd and other metals are environmentally introduced as mixtures (1). Cd bioaccumulates and targets the kidneys and bones, and many of the health effects associated with chronic cadmium exposure affect these organs and include an increased risk of osteoporosis (2) renal tubular dysfunction (3). Cd may interact synergistically or additively with other toxic metals, such as lead (Pb) and arsenic (As) (4), and these interactions may exacerbate downstream health effects associated with Cd exposure, notably, renal dysfunction (5-7). Cadmium may affect endogenous essential metals levels by disrupting metal homeostasis (8, 9). Exposure to toxic and essential metal mixtures may have important environmental, toxicological, and health implications for highly and chronically Cd exposed populations. Co-exposure to toxic and essential metals has not been evaluated in the context of a highly and chronically Cd exposed population.

Within other communities, important co-exposure patterns have emerged that emphasize the importance of considering exposure to multiple toxic and essential metals. In a study of a small-scale gold mining community in Ghana, mercury (Hg) was the primary exposure of concern, but men in this community also had elevated levels of urinary chromium and As. Exposure to other toxic and essential metals was independent from Hg exposure (10). Within a rural community with elevated groundwater As in Bangladesh, there were elevated levels of urinary Cd and Pb as well as Zn deficiencies, and these metal levels varied by age and gender (11). Within the United States (US), urban and rural population-based cohorts exhibited distinct metal co-exposure patterns, where uranium, tungsten, As, and Zn were elevated in the rural cohort, possibly reflecting different sources of dietary and groundwater exposures (12). Exposure

to other toxic metals is often coupled, and the shared molecular targets and downstream health effects further emphasizes the importance of considering co-exposure.

Cd environmental pollution is a global and understudied problem that disproportionally affects regions that have undergone rapid and unrestricted industrialization, such as the Mae Sot District in northern Thailand. The residents of Mae Sot were chronically exposed to Cd after ingesting Cd-contaminated water and food, especially rice, caused by downstream environmental pollution of the Mae Tao Creek from active zinc mining (13). In this chronically Cd-exposed population, the urinary Cd levels in Mae Sot residents were elevated and increasing levels of urinary Cd were dose-dependently associated with urinary biomarkers of renal dysfunction (14). The residents of Mae Sot experienced numerous adverse health effects that were associated with elevated Cd exposure, which included increased rates of bone resorption, diabetes, and hypertension (15, 16). Co-exposure to other metals is a public health concern in this population since residents may be exposed to other metals associated with environmental contamination caused by zinc mining that could additional health effects or exacerbate those associated with Cd exposure (17). Presently, co-exposure to toxic and essential metals in this population has not been extensively examined.

The objective of this analysis was to measure multiple essential and toxic metals in both blood and urine from 38 women from the Mae Sot district in northern Thailand. First, we examined the temporal changes in blood biomarkers of metal exposure between 2007 and 2012. Second, we assessed the co-exposure patterns among urinary biomarkers of metal exposure in 2012. Finally, we compared biomarkers of metal exposure in blood and urine between matching samples in 2012. These analyses have advanced our understanding of the co-exposure to other metals and their longitudinal shift over time in this chronically exposed Cd population.

METHODS

Study Population. Thirty-eight women between ages 35-70 years at baseline in 2007 were randomly selected from a Cd-exposed cohort from Mae Sot, Thailand. These women resided in the polluted areas of Mae Sot: Mae Ku, Phra Thad, and Mae Tao (13). These women participated in a follow-up health impact survey in 2007 and 2012 and provided whole blood and urine at the time of the survey. This Cd-exposed cohort has been previously described (16, 18). Study approval for this study was obtained from the research and ethics committee from the Faculty of Medicine, Chiang Mai University.

Metal Measurement. Twenty-five mL of morning urine were collected in Cd-free polyethylene containers. Specific gravity (SG) was measured for each sample using a refractometer (PALS-10S), shortly after collection. Fasting venous whole blood was collected and stored in EDTA-coated 5-ml tubes. After transportation to the University of Michigan on dry ice, all samples were stored at -80C until analysis.

Urinary elements analyzed included aluminum (Al), antimony (Sb), As, Cd, cobalt (Co), Cu, molybdenum (Mo), nickel (Ni), Pb, and Zn. Blood elements analyzed included Cd, Pb, Mo, Cu, selenium (Se), and manganese (Mn). Urine samples were digested overnight with 70% nitric acid at a 1:1 volume ratio. The following day, the acid concentration was adjusted with Milli-Q water to 4.4% and then introduced into the Inductively Coupled Plasma Mass Spectrometer (ICPMS). Blood samples (0.5 mL) were digested overnight with 70% nitric acid (1 mL). The following day 0.5 mL hydrogen peroxide was added to the digest. The digest was next heated for 3 hrs at 70°C and allowed to cool overnight. The following day, the acid concentration was adjusted with Milli-Q water to 4.4% and then introduced into the ICPMS.

All elements were detected using Agilent 7500c ICPMS (Agilent Technologies, Palo Alto, CA) equipped with a quadrupole analyzer and octopole collision/reaction cell pressurized with hydrogen or helium reaction gas as previously described (19). Sample uptake was 0.4 mL/min from a peristaltic pump with 1.2 L/min Ar carrier gas through a Babbington-style nebulizer into a Peltier-cooled double-pass spray-chamber at 2°C; 1.0 L/min auxiliary Ar and 12.0 L/min plasma gas Ar were added for a total of 14.2 L/min separated from nickel cones by a sampling depth of 8.5 mm. The ICPMS was tuned using a tuning solution of 10ppb of Li, Y, Ce, Tl, and Co (Agilent internal standard mix). Interference levels were reduced by optimizing plasma conditions to produce low oxide and doubly charged ions (formation ratio of <1.0 %) and residual matrix interferences were removed using the collision/reaction processes in the Octopole Reaction System.

Several analytical quality control measures were used. All laboratory glassware and plastic was acid-washed (cleaned, soaked overnight in 6M nitric acid followed by another overnight soak with 2M nitric acid) prior to use. Accuracy and precision were measured by use of urinary (QMEQAS09U-03) and blood (QMEQAS09-B08) reference standards obtained from the Institut National de Santé Publique du Québec (INSPQ). In addition, each batch run contained procedural blanks and replicate runs. For each element analyzed, the analytical detection limit was calculated as three times the standard deviation of the mean blank value.

The accuracy, precision, and limit of detection (LOD) are summarized in **Table 4.1**. The LOD for each metal was calculated and deemed to be acceptable. For nearly all samples (8/76 blood molybdenum, 3/26 urinary Co, 1/26 urinary Cd, and 12/26 urinary Sb were below detection limits and thus assigned ½ LOD values) quantifiable results were obtained. The average recovery of elements (except for urinary Mo, urinary Zn, and blood Se) from the

standard reference material was within 20% of expected value for all elements and were not adjusted based on these recoveries. Analytical precision was generally less than 5% for each element except for blood Se, blood Mo, and urinary Co.

Statistical Analysis. Unadjusted urinary metals were adjusted by the median specific gravity in the study population in 2012, 1.015, and we used the following adjustment: unadjusted metal (ug/L) * ((1.015-1)/(SG-1)) in order to adjust for urine density (20). We computed the summary descriptives and checked for normality using histograms and Shapiro Wilks test for each urine and blood metal. Any non-normal and right-skewed variables were log-transformed, and this included the following elements in blood (Cd, Pb, Se, and Mn) and in urine (Cd, Mo, Pb, Sb, Ni, Zn, and Cu). We used these transformed variables in downstream principle components analysis (PCA) and hierarchical clustering analysis (HC).

We computed the Spearman correlations to analyze the bivariate associations among and between metals. When analyzing the longitudinal change in blood levels, we used Wilcoxon sign rank (WSR) test. We applied the Kruskal-Wallis test (KW) and Kolmogorov-Smirnov test (KS) to compare non-parametrically the distribution of metals by covariate categorical factors (i.e. smoking status) and the shape of metal distributions, respectively. To examine the variation among these mixed metals, we applied principle components analysis (PCA) and agglomerative hierarchical clustering (HC). PCA is a multivariate method that describes the underlying variation among a set of variables and constructs independent linear combinations of the original variables that account for the most variation (21). HC can uncover groups of subjects in our population that may have similar metal exposure patterns by determining the dissimilarity between pairs of subjects and using a linkage function of this dissimilarity to create clusters (22).

Prior to implementing both PCA and HC, we normalized each metal to unit variance one and mean zero. We ran our PCA using the prcomp function in R, which executes PCA using singular value decomposition of the data matrix (23). For our HC analysis, we used euclidean distances as a measure of dissimilarity among our observations and the Ward's criterion for our linkage function to form clusters. To evaluate the stability of our clusters, we used multiscale bootstrap resampling and examined the approximately unbiased (AU) p-values (24). We extracted clusters with AU p-values < 0.05, which may suggest that the cluster was stable and existed, and created heatmaps of the normalized metals for each cluster. We implemented our HC analysis using the pvclust package in R (25). Due to our concern that smoking might be an important confounding factor underlying our results and exposure patterns, we conducted a sensitivity analysis with self-reported smokers removed from the analysis and these results are included.

RESULTS

Descriptive summary of study population. In this longitudinal study of 38 northern Thai women from the polluted areas of the Mae Sot district, their mean age at the start of the study period was 54.3 (sd=10.3) years (**Table 4.2**). Among these women, 66% were non-smokers (n=25), 18% were former smokers (n=7), and 16% were current smokers (n=6). All of these women had blood metal biomarker measurements in both 2007 and 2012; however, only 26 women had urinary metal biomarker measurements in 2012. Mean body mass index (BMI) significantly increased by 0.9 kg/m^3 between 2007 and 2012 among these women (paired t-test, p=0.005). Among the urinary biomarkers of kidney function, only beta-2-microglobulin significantly increased between 2007 and 2012, where the median increased by 3.2-fold (WSR, p=0.0014).

Longitudinal change among individual blood metals between 2007-2012. Median blood Cd levels significantly decreased from 3.6 ug/L in 2007 to 2.4 ug/L in 2012 (WSR, p=0.0083), and these Cd levels were significantly positively correlated between 2007 and 2012 (Spearman's rho=0.716, p < 0.0001) (**Figure 4.1**). The proportion above the US reference range for blood Cd decreased from 38.9% in 2007 to 30.6% in 2012 (McNemar's $\mathbf{X^2}$, p=0.3173) (**Table 4.3**).

In addition to decreasing blood Cd levels during our study period, median blood Se levels decreased from 135.6 ug/L in 2007 to 105.7 ug/L (WSR, p <0.0001) and blood Cu levels decreased from 827.3 ug/L to 766.4 ug/L (WSR, p=0.0050); however, these metals were weakly, positively correlated in 2007 and 2012. The proportion of individuals below the US reference range for blood Se significantly increased from 0% in 2007 to 22.2% in 2012 (McNemar's $\mathbf{X^2}$, p=0.0047), and similarly, the proportion below the reference range for blood Cu significantly increased from 44.4% in 2007 to 66.7% in 2012 (McNemar's $\mathbf{X^2}$, p=0.0455).

Both blood Mn and Pb levels did not significantly change between 2007 and 2012 (p > 0.05). While Pb levels in 2007 and 2012 were uncorrelated in our population, Mn levels were modestly positively correlated (rho=0.280, p=0.0882). For blood Pb, the proportion above the reference range increased from 41.7% in 2007 to 55.6% in 2012 (McNemar's $\mathbf{X^2}$, p=0.1967), and 27.8% were above the reference range in 2007 and 2012. Approximately 40% of our study population had blood Mn levels above the reference range in 2007 and 2012; however, only seven individuals had levels above the reference range in both years. While blood Mo levels decreased from 2.5 ug/L in 2007 to 2.0 ug/L in 2012 (WSR, p=0.0884), no other differences were observed during the study period.

Relationship among blood metals in 2007 and 2012. In 2007, blood Cd moderately negatively correlated with blood Mn (rho=-0.313, p=0.0556) and blood Pb (rho=-0.304, p=0.0636) (**Table**

4.4). Mn significantly positively correlated with Pb (rho=0.586, p=0.0001) and Mo (rho=0.386, p=0.0167) and negatively correlated with Se (rho=-0.363, p=0.0250). Pb negatively correlated with Se (rho=-0.501, p=0.0014). Cu and Se were uncorrelated.

In 2012, Cd significantly positively correlated with all metals except Pb: Mo (rho=0.745, $p < 0.0001$), Cu (rho=0.422, p=0.0082), and Se (rho=0.376, p=0.0201). While Cd and Mn were inversely correlated in 2007, Cd positively correlated with Mn (rho=0.561, p=0.0003). Mo was also significantly positively correlated with these metals as well ($p < 0.05$ for all). Pb only significantly correlated with Mn (rho=0.385, p=0.0170). Interestingly, it was uncorrelated with Cd (rho=0.099, p=0.5537) in this year. Cu and Se were also significantly positively correlated (rho=0.616, $p< 0.0001$).

After applying PCA to examine the variation among these metals in 2007 and 2012, the results revealed distinct patterns between these study periods that are supported by our correlation analysis (**Figure 4.2 A**). In 2007 the first three components explained 75.3% of the variation among these metals and were characterized by an inverse relationship between Mn-Pb and Se (37.4% variance explained), Cd-Mo (21.6%), and Cu-Se (16.3%), respectively (**Table 4.5**). To support our PCA results, HC identified seven stable clusters ($p <0.05$) in 2007. The third (n=4) and fifth cluster (n=4) were characterized by higher blood Pb and Cd, respectively. Interestingly, low and high Se described the second (n=3) and fourth cluster (n=4), respectively (**Figure 4.2 A and C**). Seven women did not fall into one of the seven stable clusters.

In 2012 the first three components explained 88.1% of the variation and were characterized by Cd-Cu-Mo-Se (57.4%), Mn-Pb (18.1%), and Pb (12.6%), respectively (**Figure 4.2 B**). HC revealed four stable clusters identified ($p < 0.05$). Low Se and Cu characterized the

first cluster (n=4). Low Pb described the second cluster (n=10) while high Cd and Pb described the fourth cluster (n=11). Eight women were not assigned to a cluster (**Figure 4.2 B and D**).

Urinary Metals in Cd-exposed population. We measured urinary metals in twenty-six women of our study population in 2012. The descriptive characteristics of this subset did not significantly differ from the entire study population (data not shown). Twenty women (76.9%) had urinary Cd above the US reference range (**Table 4.3**). For toxic metals, As and Pb, 88.5% and 15.4% of women were above the reference range. Among these women, 19.2%, 50%, and 57.7% were above the reference range for Cu, Zn, and Ni, respectively.

A majority of the correlations among the urinary metals were moderately or strongly positive. Cd positively correlated with Mo (rho=0.671, p=0.0005), Zn (rho=0.392, p=0.0476), As (rho=0.386, p=0.0515), and Cu (rho=0.366, p=0.0658) (**Table 4.6**). Pb positively correlated with Ni (rho=0.849, p< 0.0001) and Zn (rho=0.739, p<0.0001) while As correlated with Co (rho=0.471, p=0.0152) and Mo (rho=0.405, p=0.0401). Zn correlated with the other trace metals: Co (rho=0.404, p=0.0408), Cu (rho=0.440, p=0.0245), and Ni (rho=0.734, p<0.0001). This suggests a complex correlation structure among these metals, and their correlations are stronger among each other than with Cd, especially, for Zn and Pb.

The first three components explained 70.3% of the variance among these metals in our population (**Table 4.7**). The first component indicated that these metals load in the same direction and was characterized by Ni-Pb-Zn (35.5%) while the second component was characterized by Cd-As-Mo (23.8%) (**Figure 4.3 A**). The third component captured an inverse relationship between Cd and Co (11.0%). To support our PCA results, HC analysis identified two stable clusters. The first cluster (n=9) was described by lower levels all metals except Pb

while the second cluster (n=17) was described by higher levels of all metals except Pb (**Figure 4.3 A and B**).

Relationship between blood and urine metals in 2012. We measured Cd, Pb, Cu, and Mo in both blood and urine. After generating kernel density estimated distributions, the shapes of the distribution for urine and blood Cd were not significantly different (KS, p=0.0887), and both indicate right skewed density distributions (**Figure 4.4**). The distributions of blood and urine metals for Pb, Cu, and Mo were significantly different (KS, $p < 0.0001$). Blood Mo and Pb were normally distributed while urine Mo and Pb were right-skewed. Urine Cu was normally distributed while blood Cu appeared to be bimodally distributed. To further support this notion of differently shaped distributions between blood and urine metals, urine and blood Cd levels significantly positively correlated in this population (rho=0.593, p=0.0014) (**Figure 4.5**). Additionally, blood and urine Mo levels positively correlated (rho=0.372, p=0.0616). For both Pb and Cu, their urine and blood measurements were not correlated.

Sensitivity analysis with smokers removed. Smoking is a known confounder of environmental toxicant exposure, especially heavy metals, including As, Pb, and Cd. Our study population included six self-reported current smokers. The blood and urine metals did not significantly differ by smoking status (KW, $p > 0.05$) except for blood Se in 2012 (KW, p=0.0473) (**Table 4.8**). After we removed the current smokers from our analysis, blood Cd in 2007 and 2012 significantly correlated (rho=0.745, $p<0.0001$) (**Table 4.9**), as well as, blood and urine Cd in 2012 (rho=0.635, p=0.0020). Median blood Cd was different between 2007 and 2012 but was not significant (WSR, p=0.0622). After log-transforming blood Cd in 2007 and 2012, the

difference in blood Cd was significantly different (paired t-test, p=0.0208). Median blood Se significantly differed between 2007 and 2012 (WSR, p<0.0001).

The correlation analysis also revealed similar patterns for blood metals measured in 2007 and 2012. Notably, blood Cd was more negatively correlated with Mn (rho=-0.414, p=0.0186) and Pb (rho=-0.493, p=0.0041) in this subset in 2007 (**Table 4.4**). The PCA analysis revealed similar patterns for both 2007 and 2012 that were observed in the entire study population, suggesting that these patterns are shaped by environmental Cd exposure rather than smoking (**Table 4.5**). While the correlation coefficients among the urinary metals were similar in this subset of the population, the significance levels associated with these coefficients were weaker (**Table 4.6**). When we examine the PCA, a similar relationship among the urinary metals emerged that we observed in the entire study population (**Table 4.7**). Overall, the results from this sensitivity analysis revealed that the co-exposure patterns in this population were not strongly affected by smoking.

DISCUSSION

Among these women from a highly exposed Cd population residing in Mae Sot, higher levels of other toxic and essential metals were present in this population when compared to a clinical reference range (26). Over half of these women had elevated levels of blood Pb, urinary As, and urinary Cd, and among those with complete blood and urine measurements, 42% had elevated levels of all three toxic metals. In addition to Cd exposure, co-exposure to other toxic metals is a public health concern in this population. Notably, Cd was not correlated with any blood metals in 2007 but was strongly correlated with all metals except Pb in 2012, indicating that the correlations among these metals changed longitudinally. Stronger correlations between

urinary Zn were present compared to urinary Cd in 2012. The results from our PCA indicated that Pb exposure in both blood and urine was distinct from Cd exposure. Among the urinary metals, both Cd and As loaded together in the PCA, and these patterns may be explained by similar exposure sources for Cd and As and a different source for Pb. Our study emphasized the importance of studying metal mixtures in the context of a highly exposed Cd population and justifies exploring the interactions among these metals, their sources, and the health effects associated with co-exposure.

The women in our study were not likely occupationally exposed to Cd or these metals since they were farmers, homemakers, government workers or merchants. Their source of exposure to these metals was dietary via consumption of food and water. Plants readily uptake both Cd and As from the soil, but this uptake is less and different for Pb (27), and this could explain the independent patterns between Pb and Cd/As exposure. While Pb exposure has not been extensively examined in the Mae Sot population, it has been studied in the nearby zinc-mining region of Thong Pha Phum District in northwestern Thailand. In this region, soil Pb concentrations increased with decreased distance from the mine. Compared to rice, other plants, such as cassava, had greater uptake of Pb (28). The observed patterns in our study population may be explained by variations in dietary consumption.

We observe that the patterns of exposure among toxic metal may be dependent on the population under study and their potential risk for co-exposure. In occupationally and environmentally exposed gold-mining community, positive correlations were observed for among the urinary metals studied in this population. While Cd significantly positively correlated with Zn, Cu, and Cd, it also correlated with Pb in contrast to our study population, where we observe no correlation between Pb and Cd. This may be due to different sources of exposure and

gender differences between the two populations (10). Within two population-based cohorts in the US with low-exposure to toxic metals, urinary Cd, Pb, and As loaded to different PCs, indicating potentially independent sources of exposure (12). Among pregnant women in rural Norway with low-exposure, blood Cd and Pb were associated with the same component (29). The patterns observed among metal mixtures may be dependent on the population studied, exposure level, and principle environmental toxicant.

Women are more vulnerable to the adverse health effects associated with high Cd exposure. These health effects may become exacerbated with co-exposure to other metals, especially as these women age. Cd is associated with an increased risk of bone resorption in women for both low exposure (30, 31) and high exposure (16), and bone resorption rates increase after menopause. Within an environmentally exposed Chinese population, the interaction between Cd and Pb may be associated with decreased bone mineral density in women (32). Additionally, Cd and other metals were associated with cardiovascular disease risk in elderly women, and women had higher levels of Cd, Cu, and Mn (33). Most importantly, the interaction among toxic metals with Cd exacerbate the nephrotoxic effects of chronic and high Cd exposure, especially in women. The interaction between As and Cd increased the levels of biomarkers of renal tubular damage in a Chinese environmentally exposed population (34). Co-exposure to Cd and Pb increase biomarkers of renal response in an occupational exposed cohort (35). Notably, co-exposure to Cd and Pb decreased blood Se (6), and Se among children exposed to As and Cd may protect against kidney dysfunction (36).

Traditional paradigms of biomarkers of exposure exist for metals in blood and urine; however, in the context of highly exposed population these biomarkers may reflect other aspects of exposure. Blood Pb is the gold standard biomarker of exposure to Pb (37). Blood Cd captured

acute exposures while urine Cd captures chronic Cd exposure (38). This paradigm optimally applies to a non-chronically exposed population because blood Cd might also reflect endogenous Cd levels even after exposure has ceased rather than continued acute exposure. It is important to consider what this exposure might mean in the context of our population and consider both the endogenous and exogenous sources of exposure. Reference ranges are typically based within a healthy, unexposed population, and levels found above or below the reference range may not be the level at which adverse health effects occur but rather these provide a clinical point of reference for further examination (39). Additionally, reference ranges may be population-specific as diets, lifestyles, and ambient exposures may differ by population. Within a suburb of Beijing, the range of blood metals measured differed from the reference range extracted from US (40). While the toxic levels for As, Cd, and Pb are clear, the level for adverse health effects of essential metals is not.

The results of our study must be considered in the context of its limitations. The sample size included in our analysis is small. The small sample size can statistically influence the proportion of variance explained in our PCA, the significance of the correlations, and increase variability within our biomarkers of metal exposure. Our study population included current smokers. While the general patterns from the PCA and clustering analysis were consistent when smokers were removed from the analysis and there were no significant differences in metal levels by smoking status, the co-exposure profiles should be investigated further within smoking and non-smoking groups. We also restricted our sample to women. Women may be more vulnerable to the toxic effects of Cd and other metals (41), and exposure profiles may be different in men, given increased prevalence of smoking and occupational exposures, and their responses to the biologic and health effects associated with Cd and other toxic metals. While we

can assess the co-exposure of these metals through biomarkers, it is difficult to discern whether they are reflecting exogenous or endogenous changes in metal levels. We cannot determine whether urine metals change longitudinally, and urinary biomarkers of metal exposure may be more reflective of body burden and chronic exposure.

In conclusion, within this highly and chronically exposed Cd population from the Mae Sot district in northwestern Thailand, we observed unique metal exposure profiles in women that demonstrate different relationships among the metals. These results warrant further investigation into the co-exposure metal profiles within this population. Relationship between environmental levels of metals and biomarkers of metals needs to be examined to better determine the source of exposure.

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Table 4.1. *Overview of quality control parameters*. The limit of detection (LOD; values reported as µg/L) was calculated as the mean value of several blank samples plus 3x the standard deviation of the mean. Accuracy (closeness to actual value) and precision (reproducibility) of each element was determined by use of urine standard reference materials (SRM) obtained from the Institut National de Santé Publique du Québec (INSPQ). Chromium (Cr) results were normalized to 100% recovery values for each participant.

	LOD (ug/L)	Accuracy	Precision
Blood			
Cd	0.013	103%	3%
Cu	0.530	85%	4%
Mn	0.014	118%	4%
Mo	0.024	105%	14%
Pb	0.071	94%	3%
Se	0.098	76%	10%
Urine			
Mo	0.025	130%	3%
Ni	0.011	111%	2%
Pb	0.033	116%	4%
Sb	0.001	114%	2%
Zn	0.300	132%	2%
As	0.010	102%	1%
Cd	0.002	106%	1%
Co	0.002	105%	20%
Cu	0.040	104%	2%

Table 4.2. *Descriptive summary of study population.* Expressed in mean and standard deviation (sd) and p-value obtain from paired t-test or median and interquartile range (IQR) and p-value obtained from Wilcoxon sign rank (WSR) test.

	2007	2012	p
Mean Age (sd)	54.3 (10.3)	58.5 (10.6)	**<0.0001**
Mean BMI (sd)	23.0 (3.7)	23.9 (4.8)	**0.005**
Median Blood Creatinine (IQR)	0.95 (0.80, 1.1)	0.90 (0.8, 1.1)	0.332
Median Urine Creatinine (IQR)	1.14 (0.87, 1.58)	1.17 (0.90, 1.34)	0.2551
Median Urine Beta-2-microglobulin (IQR)	160.2 (60.0, 1692.0)	514.3 (111.8,5928.0)	**0.0014**
Median Urine N-acetyl-glucosamide (IQR)	5.5 (3.9, 10.9)	5.3 (3.0, 9.4)	0.2044

Table 4.3. *Descriptive summary of blood metals in 2007 and 2012*. All measures are in ug/L. Reference range values obtained from (A) Tietz Clinical Guide to Laboratory Tests, 2004 (27) and (B) Harrington et al. (40).

	2007								2012								Reference Range
				Percentile								Percentile					
BLOOD	**GM**	**mean**	**sd**	**min**	**25**	**50**	**75**	**max**	**GM**	**mean**	**sd**	**min**	**25**	**50**	**75**	**max**	
Mn	16.2	17.9	8.9	7.1	12.3	15.6	21.0	55.0	15.6	16.8	6.8	6.2	12.3	16.4	18.6	34.6	4.2-16.5[a]
Cu	813.5	824.7	138.3	587.9	710.9	827.3	918.9	1151.2	637.9	690.1	229.9	261.1	676.5	766.4	821.3	1011.8	800.0-1300.0[b]
Se	135.3	139.0	34.9	90.0	115.4	135.6	150.3	250.6	81.5	94.2	38.2	19.2	95.4	105.7	117.2	147.2	58.0-234.0[b]
Mo	2.2	2.4	0.9	0.4	1.8	2.5	2.9	4.6	1.8	2.1	1.2	0.4	1.5	2.0	2.6	4.8	0.8-3.3[b]
Cd	3.7	4.8	3.7	1.2	2.0	3.6	5.9	15.6	2.7	3.9	3.9	0.6	1.4	2.4	5.3	17.0	< 5.0[a]
Pb	42.2	53.0	39.9	12.0	26.9	42.2	61.0	179.2	45.2	50.6	24.1	16.4	34.7	49.7	62.2	124.8	< 49.0[a]
URINE									**GM**	**mean**	**sd**	**min**	**25**	**50**	**75**	**max**	
Co									0.3	0.5	0.4	0.0	0.2	0.4	0.5	1.6	0.1-2.0[a]
Ni									7.6	9.4	6.8	2.6	4.6	5.8	12.8	28.3	< 5.2[a]
Cu									36.4	47.7	36.3	9.4	21.0	36.9	58.7	165.0	2.0-80.0[a]
Zn									1265.6	1572.9	1106.5	443.8	768.1	1159.7	2281.6	4708.6	150.0-1200.0[a]
As									67.0	78.0	37.4	9.7	47.6	75.9	101.0	154.5	< 35.0[a]
Mo									93.4	109.0	74.7	37.2	62.0	87.8	118.5	335.2	
Cd									5.8	10.4	12.1	0.2	2.7	4.9	13.6	59.1	< 2.6[a]
Sb									0.1	0.8	2.0	0.0	0.0	0.1	0.3	7.6	< 2.0[a]
Pb									8.3	12.3	12.2	1.5	4.0	5.4	17.1	44.1	< 23.0[a]

Table 4.4. *Spearman correlation matrix among blood metals in 2007 and 2012*. Values above the diagonal indicate Spearman correlations and below the diagonal are corresponding p-values. Bolded and italicized indicate significant correlation at significance levels, 0.05 and 0.10, respectively.

		Entire Study Population								**Smokers Removed**					
		2007								**2007**					
		Spearman Correlations								**Spearman Correlations**					
		Mn	**Cu**	**Se**	**Mo**	**Cd**	**Pb**			**Mn**	**Cu**	**Se**	**Mo**	**Cd**	**Pb**
P-Values	**Mn**		0.262	**-0.363**	**0.386**	*-0.313*	**0.586**		**Mn**		*0.326*	-0.287	0.293	**-0.414**	**0.574**
	Cu	0.1123		0.020	0.155	-0.006	0.204		**Cu**	*0.0682*		-0.003	0.102	-0.084	0.184
	Se	**0.0250**	0.9071		**-0.340**	0.129	-0.501		**Se**	0.1112	0.9873		-0.262	0.087	**-0.502**
	Mo	**0.0167**	0.3543	**0.0366**		0.177	0.200		**Mo**	0.1039	0.5768	0.1470		0.192	0.116
	Cd	*0.0556*	0.9735	0.4394	0.2864		*-0.304*		**Cd**	**0.0186**	0.6464	0.6378	0.2936		**-0.493**
	Pb	**0.0001**	0.2191	**0.0014**	0.2275	*0.0636*			**Pb**	**0.0006**	0.3124	**0.0034**	0.5258	**0.0041**	
		2012								**2012**					
		Spearman Correlations								**Spearman Correlations**					
		Mn	**Cu**	**Se**	**Mo**	**Cd**	**Pb**			**Mn**	**Cu**	**Se**	**Mo**	**Cd**	**Pb**
P-Values	**Mn**		0.270	0.186	**0.625**	**0.561**	**0.385**		**Mn**		0.272	0.167	**0.651**	**0.665**	**0.400**
	Cu	0.1012		**0.616**	**0.525**	**0.422**	0.167		**Cu**	0.1315		**0.599**	**0.462**	**0.374**	0.076
	Se	0.2626	**<0.0001**		**0.379**	**0.376**	0.011		**Se**	0.3605	**0.0003**		*0.315*	*0.322*	-0.098
	Mo	**<0.0001**	**0.0007**	**0.0191**		**0.745**	0.246		**Mo**	**<0.0001**	**0.0078**	*0.0794*		**0.758**	0.236
	Cd	**0.0003**	**0.0082**	**0.0201**	**<0.0001**		0.099		**Cd**	**<0.0001**	**0.0347**	*0.0719*	**<0.0001**		0.111
	Pb	**0.0170**	0.3154	0.9475	0.1370	0.5537			**Pb**	**0.0233**	0.6812	0.5955	0.1932	0.5470	

Table 4.5. *Variable factor loadings from PCA Analysis for blood metals in 2007 and 2012*. Loadings with an absolute value 0.400 are bolded.

	ENTIRE STUDY POPULATION								SMOKERS REMOVED							
	2007				2012				2007				2012			
Component	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Cu	0.256	-0.076	**-0.865**	-0.423	**-0.466**	0.278	-0.399	0.182	0.277	-0.075	**-0.765**	**0.572**	**-0.463**	0.310	**-0.408**	0.101
Mo	0.368	**-0.611**	0.107	0.071	**-0.454**	0.081	0.337	0.126	0.285	**-0.673**	0.060	-0.212	**-0.458**	0.040	0.373	-0.201
Mn	**0.521**	-0.148	-0.121	**0.632**	-0.368	**-0.491**	0.397	**0.545**	**0.494**	-0.205	-0.254	**-0.536**	-0.383	**-0.462**	0.297	**0.726**
Se	**-0.468**	-0.07	**-0.447**	**0.611**	**-0.451**	0.337	-0.384	0.103	**-0.440**	0.127	**-0.532**	**-0.536**	**-0.448**	0.357	-0.399	0.146
Cd	-0.199	**-0.717**	0.114	-0.207	**-0.441**	0.037	0.38	**-0.756**	-0.327	**-0.626**	0.112	0.232	**-0.454**	-0.072	0.367	**-0.561**
Pb	**0.519**	0.282	0.112	-0.006	-0.206	**-0.748**	**-0.526**	-0.268	**0.545**	0.303	0.225	0.012	-0.150	**-0.746**	**-0.559**	-0.294
Eigenvalue	2.244	1.295	0.976	0.642	3.444	1.084	0.757	0.340	2.246	1.369	1.010	0.695	3.394	1.235	0.647	0.328
Total Variance	37.4%	21.6%	16.3%	10.7%	57.4%	18.1%	12.6%	5.7%	37.4%	22.8%	16.8%	11.6%	56.6%	20.6%	10.8%	5.5%
Cumulative Variance	37.4%	59.0%	75.3%	85.9%	57.4%	75.5%	88.1%	93.8%	37.4%	60.3%	77.1%	88.7%	56.6%	77.2%	87.9%	93.4%

Table 4.6. *Spearman correlation matrix among urine metals in 2012*. Values above the diagonal indicate Spearman correlations and below the diagonal are corresponding p-values. Bolded and italicized indicate significant correlation at significance levels, 0.05 and 0.10, respectively.

		Spearman Correlations																			
		Entire Study Population										**Smokers Removed**									
		Co	**Ni**	**Cu**	**Zn**	**As**	**Mo**	**Cd**	**Sb**	**Pb**			**Co**	**Ni**	**Cu**	**Zn**	**As**	**Mo**	**Cd**	**Sb**	**Pb**
P-Value	**Co**		*0.340*	0.222	**0.404**	**0.471**	0.329	0.322	0.221	0.284		**Co**		0.344	0.079	0.339	*0.418*	0.235	0.165	0.087	0.286
	Ni	*0.0891*		*0.352*	**0.734**	-0.098	0.030	0.169	0.326	**0.849**		**Ni**	0.1266		*0.413*	**0.777**	-0.127	0.114	0.177	0.329	**0.899**
	Cu	0.2760	*0.0774*		**0.440**	0.164	0.275	*0.366*	0.314	0.197		**Cu**	0.7328	*0.0628*		*0.409*	-0.034	0.165	0.249	0.193	0.264
	Zn	**0.0408**	**<0.0001**	**0.0245**		0.048	0.000	**0.392**	0.311	**0.739**		**Zn**	0.1328	**<0.0001**	*0.0656*		-0.061	-0.016	0.336	0.252	**0.813**
	As	**0.0152**	0.6335	0.4241	0.8151		**0.405**	*0.386*	*0.385*	-0.130		**As**	*0.0592*	0.5825	0.8845	0.7927		0.332	0.295	0.210	-0.101
	Mo	0.1005	0.8827	0.1736	0.9987	**0.0401**		**0.672**	**0.423**	-0.132		**Mo**	0.3050	0.6218	0.4749	0.9465	0.1409		**0.642**	*0.370*	-0.110
	Cd	0.1082	0.4105	*0.0658*	**0.0476**	*0.0515*	**0.0002**		0.188	0.037		**Cd**	0.4749	0.4437	0.2757	0.1360	0.1945	**0.0017**		0.004	0.029
	Sb	0.2773	0.1046	0.1183	0.1217	*0.0518*	**0.0314**	0.3566		*0.331*		**Sb**	0.7075	0.1457	0.4021	0.2704	0.3598	*0.0985*	0.9866		*0.383*
	Pb	0.1596	**<0.0001**	0.3358	**<0.0001**	0.5281	0.5194	0.8592	*0.0982*			**Pb**	0.2093	**<0.0001**	0.2482	**<0.0001**	0.6622	0.6338	0.9022	*0.0869*	

Table 4.7. *Variable factor loadings from PCA Analysis for urine metals in 2012*. Loadings with an absolute value 0.400 are bolded.

	ENTIRE STUDY POPULATION (N=26)				SMOKERS REMOVED (N=21)			
Component	1	2	3	4	1	2	3	4
Co	0.302	-0.036	**-0.727**	0.305	0.284	0.037	-0.263	**-0.737**
As	0.069	**-0.492**	-0.304	-0.276	-0.033	**0.468**	**-0.523**	-0.073
Ni	**0.506**	0.171	0.054	0.009	**0.536**	-0.025	-0.005	0.020
Cu	0.343	-0.199	-0.086	0.225	0.307	0.179	0.272	-0.250
Zn	**0.501**	0.057	0.220	0.098	**0.498**	0.034	0.213	0.133
Mo	0.063	**-0.561**	0.002	0.162	0.008	**0.610**	0.073	-0.131
Cd	0.065	**-0.484**	**0.531**	0.342	-0.041	**0.547**	**0.496**	0.192
Sb	0.250	-0.245	0.039	**-0.787**	0.205	0.218	**-0.536**	**0.518**
Pb	**0.458**	0.275	0.193	-0.085	**0.494**	-0.163	0.000	0.222
Eigenvalue	3.197	2.142	0.993	0.924	3.173	1.871	1.059	1.039
Total Variance	35.5%	23.8%	11.0%	10.3%	35.3%	20.8%	11.8%	11.6%
Cumulative Variance	35.5%	59.3%	70.3%	80.6%	35.3%	56.0%	67.8%	79.4%

Table 4.8. *Individual metals by smoking status.* Presented as median (min, max). All metals expressed in ug/L. Kruskal-Wallis test (KW) p-value reported.

	Year	Non-Smokers	Former Smokers	Smokers	p
BLOOD					
n		25	7	6	
Mn	2007	16.4 (7.5, 56.0)	12.6 (7.1, 20.9)	14.3 (8.1, 28.9)	0.1587
Cu	2007	803.8 (604.2, 1114.0)	819.1 (587.9, 923.1)	928.7 (728.1, 1151.0)	0.1215
Se	2007	135.1 (95.6, 250.6)	130.9 (90.0, 168.0)	140.6 (107.9, 216.7)	0.5544
Mo	2007	2.5 (0.4, 4.1)	2.7 (1.7, 4.6)	2.4 (1.5, 3.5)	0.6677
Cd	2007	2.6 (1.2, 13.9)	5.3 (1.3, 9.6)	5.7 (1.5, 15.6)	0.3734
Pb	2007	42.2 (12.0, 133.9)	42.2 (17.2, 170.5)	42.9 (25.6, 179.2)	0.877
Mn	2012	16.4 (6.2, 34.6)	14.8 (7.9, 32.9)	16.5 (9.6, 22.2)	0.8067
Cu	2012	738.9 (261.1, 992.4)	808.3 (696.4, 1012.0)	766.4 (279.0, 816.1)	0.3093
Se	2012	108.1 (19.2, 137.6)	111.4 (96.8, 147.2)	93.0 (19.6, 100.6)	**0.0473**
Mo	2012	1.9 (0.4, 4.7)	2.1 (1.6, 4.8)	2.0 (1.1, 2.8)	0.472
Cd	2012	1.6 (0.6, 17.0)	4.7 (1.4, 6.9)	2.0 (1.0, 5.5)	0.3582
Pb	2012	45.3 (16.4, 124.8)	53.2 (39.6, 76.5)	41.1 (16.7, 101.0)	0.4295
URINE					
n	2012	15	6	5	
Co	2012	411.7 (5.0,1553.0)	302.5 (4.5,1080.0)	361.6 (160.7, 557.5)	0.7224
Ni	2012	5.1 (2.6, 28.4)	11.8 (5.2, 23.3)	5.8 (2.6, 16.9)	0.2205
Cu	2012	33.6 (9.5, 165.0)	47.1 (19.9, 85.7)	39.9 (9.4, 47.5)	0.5109
Zn	2012	932.3 (454.9, 3146.0)	1965.0 (1077.0, 4709.0)	1109.0 (443.8, 1790.0)	0.2011
As	2012	75.9 (27.5, 136.9)	78.6 (36.5, 154.5)	65.4 (9.7, 102.7)	0.6101
Mo	2012	95.7 (37.6, 335.2)	83.3 (59.8, 179.3)	82.7 (37.2, 199.2)	0.9467
Cd	2012	4.4 (0.2, 59.1)	10.0 (2.7, 11.8)	3.7 (1.7, 17.1)	0.8931
Sb	2012	95.0 (13.0, 7617.0)	45.2 (25.9, 7265.0)	170.9 (15.0, 282.0)	0.3237
Pb	2012	4.7 (1.5, 30.2)	17.7 (3.8, 44.1)	4.9 (3.8, 17.4)	0.1937

Table 4.9. *Descriptive summary of blood metals in 2007 and 2012 with smokers removed*. All measures are in ug/L. Reference range values obtained from (A) Tietz Clinical Guide to Laboratory Tests, 2004 (27) or (B) Harrington et al (40) .

	2007								2012										
				Percentile								Percentile							
BLOOD	**GM**	**mean**	**sd**	**min**	**25**	**50**	**75**	**max**	**GM**	**mean**	**sd**	**min**	**25**	**50**	**75**	**max**	**WSR p**	**rho**	**p**
Mn	16.6	18.3	9.3	7.1	12.5	16.0	21.4	56.0	15.6	17.0	7.1	6.2	12.4	16.0	18.5	34.6	0.3498	0.227	0.2120
Cu	795.6	806.0	131.9	587.9	696.8	811.5	892.0	1113.8	654.0	703.7	225.7	261.1	682.8	766.0	827.0	1011.8	0.0565	0.246	0.1749
Se	133.9	137.5	34.7	90.0	114.4	133.0	147.5	250.6	85.6	98.0	37.8	19.2	98.0	108.5	118.8	147.2	<0.0001	0.321	0.0739
Mo	2.2	2.5	0.9	0.4	1.8	2.5	3.0	4.6	1.7	2.2	1.2	0.4	1.6	2.0	2.7	4.8	0.2035	0.123	0.5023
Cd	3.5	4.4	3.2	1.2	2.0	3.6	5.5	13.9	2.7	4.1	4.1	0.6	1.4	2.5	5.4	17.0	0.0622	0.745	<0.0001
Pb	40.9	51.0	36.6	12.0	25.7	42.2	63.9	170.5	46.1	50.9	22.9	16.4	37.5	49.9	61.1	124.8	0.8034	0.190	0.2976
URINE									**GM**	**mean**	**sd**	**min**	**25**	**50**	**75**	**max**			
Co									0.2	0.5	0.4	0.0	0.2	0.4	0.6	1.6			
Ni									7.9	10.0	7.2	2.5	4.5	6.1	13.3	28.3			
Cu									39.3	52.0	39.5	9.4	24.2	36.2	77.4	165.0			
Zn									1335.2	1710.4	1201.9	454.9	757.7	1548.0	2405.7	4708.6			
As									74.0	82.2	37.2	27.5	48.5	75.9	107.3	154.5			
Mo									94.4	112.4	80.2	37.6	60.3	93.0	119.0	335.2			
Cd									5.9	10.7	13.3	0.2	2.9	4.9	11.8	59.1			
Sb									0.1	0.9	2.2	0.0	0.0	0.1	0.5	7.6			
Pb									8.6	13.5	13.3	1.5	3.8	5.6	22.1	44.1			

Figure 4.1. *Longitudinal change in blood metal levels from 2007 to 2012*. Red and blue indicate individuals above and below median blood Cd (3.6 ug/L) in 2007. P-value obtained from Wilcoxon sign rank (WSR) test and Spearman's rho are also noted.

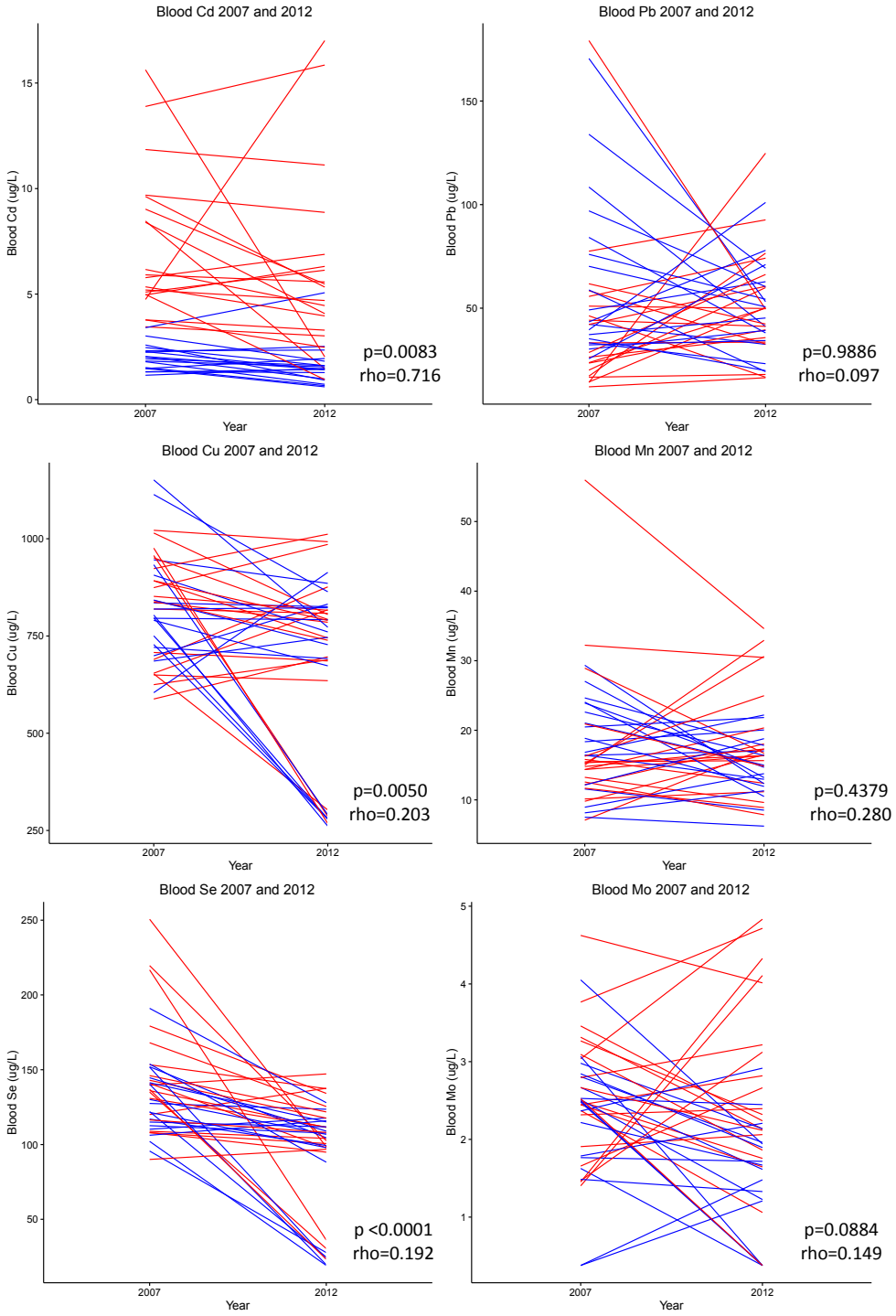


Figure 4.2. *Results from PCA and Hierarchical Clustering Analysis for blood levels in 2007 and 2012.* PCA biplots with clusters from hierarchical clustering analysis overlaid for blood levels in 2007 (A) and in 2012 (B). Heatmap of normalized blood metal levels of clustered groups in 2007 (C) and in 2012 (D).

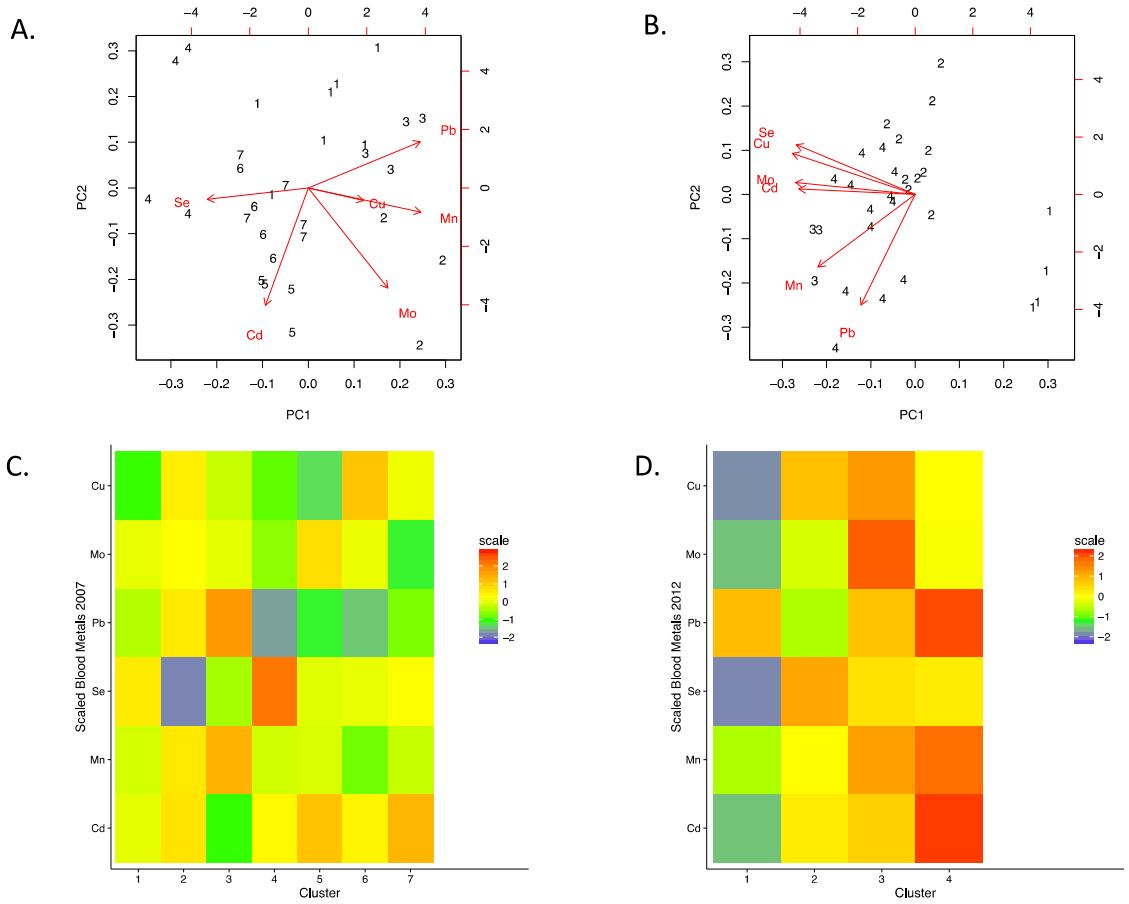


Figure 4.3. *Results from PCA and Hierarchical Clustering Analysis for urine metal levels in 2012.* PCA biplots with clusters from hierarchical clustering analysis for urine metal levels (A). Heatmap of normalized urine metal levels of clustered groups in 2012 (B).

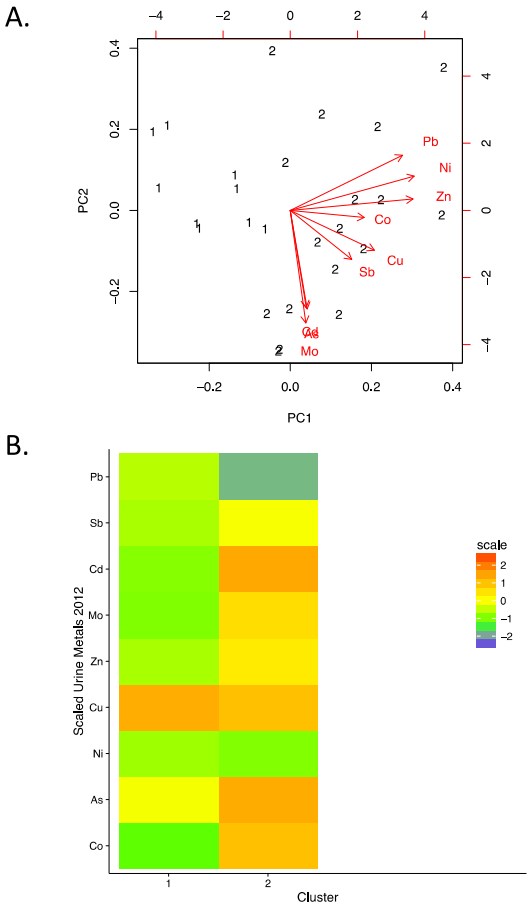


Figure 4.4. *Kernel density distribution plots of urine and blood metal levels 2012*. Metal level (ug/L) on x-axis and density proportion on y-axis.

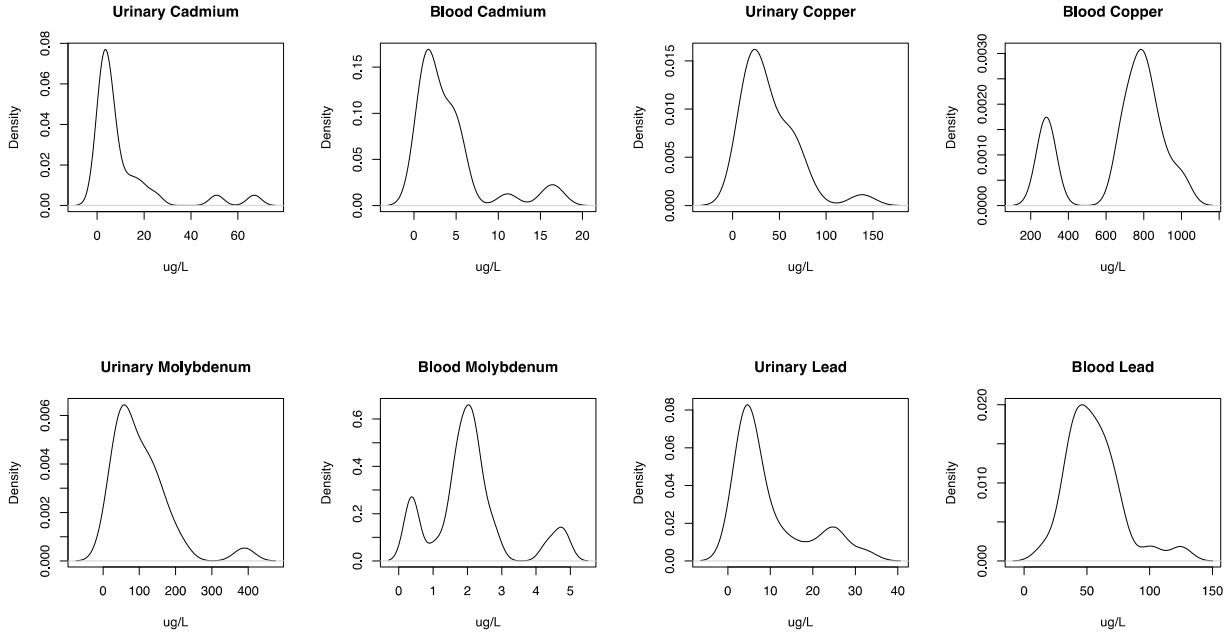
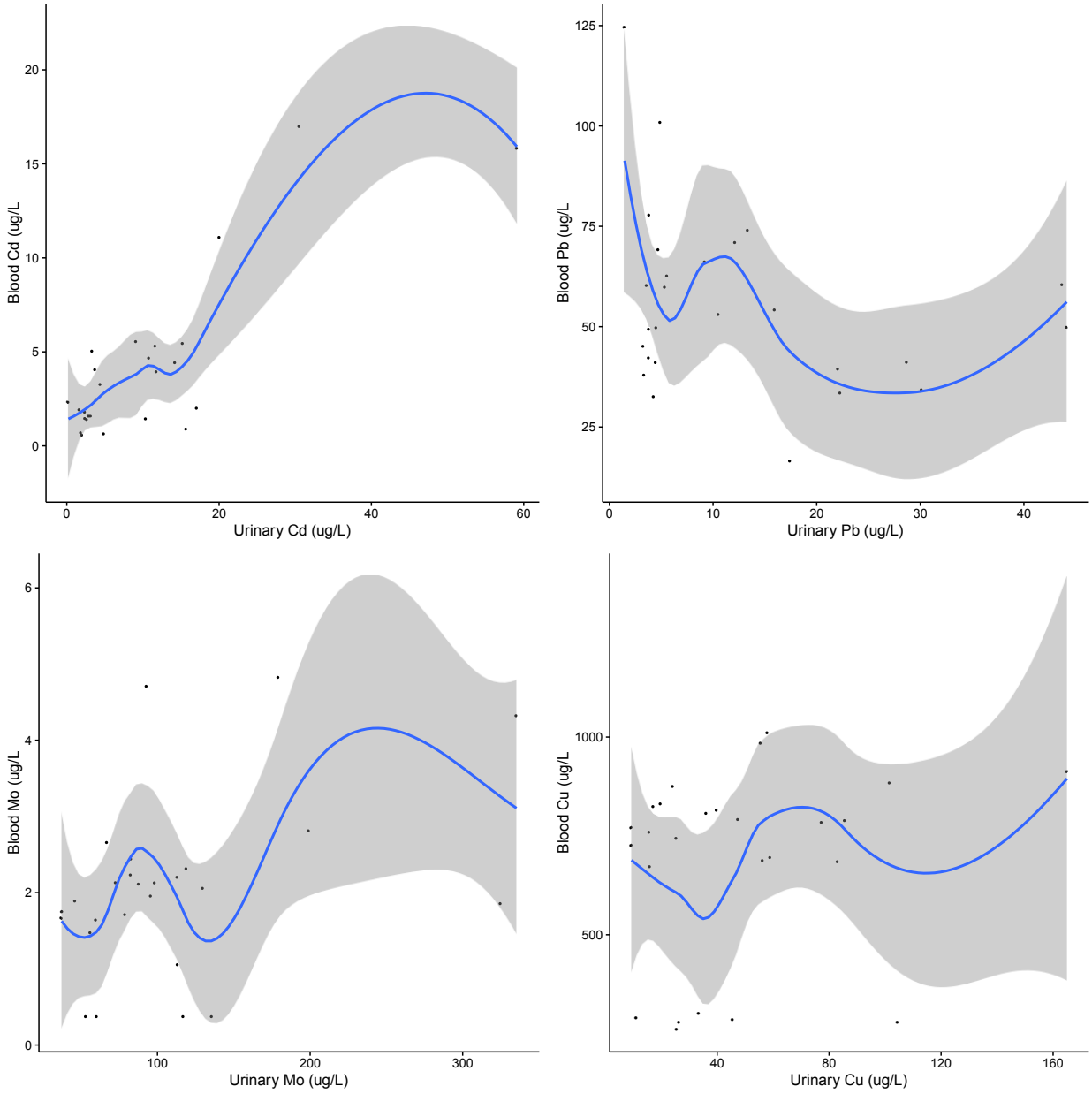


Figure 4.5. *Plots of urine and blood metal levels in 2012.* Urine metal level (ug/L) on x-axis and blood metal level (ug/L) on y-axis. Blue line is fitted loess curve and gray is 95% confidence band for curve.



CHAPTER 5. CONCLUSIONS

This dissertation presented results from three studies that consider emerging problems in childhood cancer and environmental health in Thailand. The findings in this dissertation have immediate and important implications for public health in Thailand and globally. The analytical and study design frameworks established among these studies in Thailand can be applied to other environmental exposures, diseases, and developing countries. Most importantly, these studies reveal several health disparities and critical environmental health issues that are unique to Thailand and other countries undergoing rapid social and economic development. The results in this dissertation emphasize that pediatric cancers are important diseases to consider among children in rapidly developing countries and high exposure to environmental toxicants can influence biomarkers of aging and co-exposure to other toxicants.

Childhood leukemia in Songkhla, Thailand, 1990-2011

The first aim addressed pediatric leukemia incidence and survival disparities between Songkhla and the US. In Songkhla, the subtype distribution of pediatric leukemia differed from the US, where ALL and AML accounted for 56% and 23% of cases diagnosed in Songkhla. ALL and AML accounted for 76% and 19% of cases diagnosed in US. While both leukemia incidence and survival trends increased in Songkhla to indicate that detection, diagnosis, and treatment of these cancers are improving over time, survival disparities persist between Songkhla and the US. The five-year survival was 43% and 16% for ALL and AML in Songkhla while it is 86% and

54% in the US. Leukemia may be an environmentally susceptible cancer, and the presence of environmental risk factors for these cancers may be different or more prevalent in Thailand than in the US. Childhood cancer patients and survivors encounter cancer- and treatment-associated risks in both developing and developed countries. Many chemotherapeutic treatments are associated with acute toxicities and these can be especially life-threatening when access to specialized pediatric oncologists or care is limited (1). Childhood cancer survivors have an increased risk of early mortality and chronic disease later in life (2).

This analysis of childhood leukemia and lymphoma presented a clear framework for understanding childhood cancer epidemiology in Songkhla and Thailand. However, there are a few limitations that should be considered when interpreting these data. While there was a high proportion of histologic and microscopic verification among childhood leukemias included in this analysis, there were a relatively high proportion of not otherwise specified (NOS) cases, 16%, compared to US, where 3% of cases were NOS. The precise classification of these NOS cancers would provide clearer trends for leukemia subtypes, like ALL and AML. Finally, compared to the US, the number of cases included in the analyses was small, and with this limited number of cases, there was increased variability, especially when analyzing trends. These trends were descriptive, and clinical information related to treatment, side effects, stage at diagnosis, and health at follow-up was not available. With supporting clinical information, the underlying cause of disparities in incidence and survival potentially could be elucidated.

Thailand is one of the few LMICs that implemented high quality provincial cancer registries, which have actively ascertained cases since the late 1980s (3). The data quality and classification among childhood cancers is exceptional for this region. One of the major limitations of studying childhood leukemia is that there are very few known risk factors for

leukemia, and for both developed and developing countries, and this descriptive analysis of leukemia incidence and survival provides a foundation to study both known and unknown environmental and genetic risk factors. The results from this aim are also important for addressing the burden of childhood leukemia in Thailand and clearly presents the disparities in incidence and survival that can be mitigated by allocating resources to improve detection, diagnosis, and treatment.

These results highlight the disparities in incidence and survival between the United States, a developed country, and Songkhla, a region in Thailand, undergoing rapid social and economic development. The immediate public health implications include applying these results to better understanding diagnostic and survival disparities between Songkhla and the US. While we lack the data to quantify the delay in diagnosis, it is clear that time to diagnosis is an important predictor of prognosis. This study in Songkhla justifies studying pediatric cancers in other LMICs since the distribution and trends among pediatric cancers appears to be different than what is observed in the US. While these results lend themselves to immediate clinical applications, the long-term public health implications are largely related to understanding the etiology of these cancers. Notably, AML is significantly and rapidly increasing in Songkhla. This cancer is suspected to be associated with environmental risk factors (4), and there may be some underlying environmental or population-specific risk factor that should be investigated in Songkhla. Notably, AML may be associated with risk factors associated with the demographic transition that include advancing maternal age during pregnancy, Down Syndrome, and increased environmental pollution. Population based registries should be implemented in other SE Asian countries in order to assess the emerging burden of pediatric leukemia in this rapidly developing region.

Cadmium, biologic aging, and DNA age-associated methylation in women from Mae Sot

The second aim addressed Cd's effect on biologic aging by examining DNA methylation-based epigenetic age calculators (5, 6) and age-associated methylation changes in non-smoking women from Mae Sot. Biomarkers of biologic aging are becoming increasingly important to consider in the context of environmental, dietary, disease and lifestyle risk factors as these risk factors may modify the aging trajectory (7-12). The Cd-exposed population from Mae Sot provides an opportunity to study the effect of an exposure that influences the risk of age-related chronic diseases (13-17), exacerbates renal dysfunction with aging (18), and perturbs DNA methylation (19) in this population. The results from this study demonstrated that Cd affects age-associated DNA methylation among the CpG sites included in the epigenetic age calculators and in our study population. Higher Cd exposure was associated with a smaller difference between biologic and chronologic age compared to low exposure. Environmental exposures may influence aging biologically, and it is important to consider whether these age-associated effects differ by exposure level and duration, age, and gender.

Cd may affect biologic aging, however, a few limitations should be considered when interpreting these results. The Cd exposure spectrum affects both aging and methylation, and distinguishing between the two may be challenging. This study of Cd and its effects on biologic aging measured by methylation was cross-sectional, and thus biologic aging could not be assessed longitudinally. Additionally, the effects of Cd may be different in women (20) and women have a lower risk for accelerated aging compared to men (5), and study included only women since most of the men in this population smoked. Both epigenetic age calculators were constructed from healthy Caucasian and Hispanic populations in the US, and population-wide

age-associated methylation may be influenced ambient environmental exposures, diet, and lifestyle factors and these may differ from the US. Applying these epigenetic age calculators in a Thai unexposed comparison group would establish a baseline for biologic aging in the Thai population and enable comparisons with the healthy US population and Cd-exposed population from Mae Sot. The relationship between other non-methylation based biomarkers of aging and Cd exposure needs to be examined since it is difficult to determine whether Cd is affecting biologic age as predicted by methylation or simply the methylation of the sites included in methylation age predictors. Future studies should examine the relationship among biologic aging, Cd exposure, and chronic disease in the Mae Sot population.

This aim presents preliminary evidence that Cd modifies age-associated methylation and epigenetic age. The use of urinary Cd and inclusion of only non-smokers strengthened the results from this study. The next steps for this analysis include increasing the sample size, adding men to the study population, identifying a non-exposed comparison group, and examining longitudinal changes in age-associated methylation and epigenetic age in this population. This aim justifies examining biologic aging among highly and chronically exposed populations and further studies should examine environmental exposures and their relationship with biologic aging.

Biomarkers of aging are becoming an important component for understanding chronic disease risk for those diseases associated with aging. It is clear that a combination of lifestyle, dietary, and environmental factors underpin both biologic aging and chronic disease. Biologic aging, quantified by biomarkers of aging, may be an important mediator in environmental exposure and chronic disease relationships. The burden of Cd exposure changes as we age. Applying biomarkers of biologic aging has immediate public health implications in the context

of chronic and high Cd exposure. While Cd may disrupt DNA methylation age-associated changes, it appears to be acting in a different way on methylation than aging. These biologic age calculators were still highly correlated with chronologic age in our population. Other measures of biologic aging should be explored in this Cd exposed population.

Biomarkers of aging also have important long-term public health implications. From these analyses, middle-age is an important window of vulnerability, and applications of these biomarkers may work best in this population to predict future chronic disease risk. Methylation biomarkers of aging may allow us to examine the effect of prevention for reducing Cd exposure and chronic disease risk. Within the Mae Sot population, there is an expected increase of disability and morbidity burden associated with chronic disease risk and high Cd exposure (21).

Co-exposure to toxic and essential metals in Cd-exposed women from Mae Sot

The final aim assessed the co-exposure to other toxic and essential metals in a random sample of women from the Cd-exposed regions of Mae Sot. Co-exposure to other metals is important to consider in the context of studying environmental exposures and their health effects in communities highly exposed to environmental toxicants (22, 23) and low exposed (24). Almost half of the women included in this study had elevated levels of toxic metals, Pb, As, and Cd, and essential metal Zn that were above the clinical reference range (25). Blood Cd, Cu, and Se significantly decreased from 2007 and 2012, and the exposure patterns among the blood metals changed between these time points. Twenty percent of the women were below the reference level for Se in 2012 but none were below in 2007. The study highlights the importance of considering of longitudinal changes of individual metal exposures as well as metal mixtures. Further, the biospecimen, blood or urine, used to assess metal biomarkers of exposure was

important for considering chronic, recent, or ongoing exposure for each metal. The half-lives for total body burden for Pb and Cd reflect multiple decades of cumulative exposure (26, 27).

This aim uncovered unique co-exposure patterns in this preliminary study of Cd-exposed women from Mae Sot, but some limitations should be noted. Metals measured in urine may be reflect biomarkers of recent exposure for most metals except Cd (28), and longitudinal changes of recent exposure to metals in urine cannot be assessed in this study since urine was not available in 2007. It is expensive to measure multiple metals using ICPMS, and the study included a small sample size. The small sample size may influence statistical methods that depend on the variance, such as PCA. The study only measured these metals in women, and men may have different metal exposure levels and patterns.

This study presented a preliminary representation of metal exposure patterns among Cd-exposed women in the Mae Sot population. The statistical methods applied revealed consistent relationships among the metals measured in blood and urine. Smoking status did not influence these metals. The study presented clear evidence of longitudinal changes in blood Cd, suggesting remediation may be reducing acute Cd exposure (29). Future studies should consider examining metal co-exposure patterns in a larger study sample, men, and source of individual metal exposures. Our study population included non-occupationally exposed women, and sources of dietary exposure are important to consider for these metals. Co-exposure patterns need to be assessed in any highly exposed population since the exposure to other toxicants may potentiate and interact with the main toxicant, which may lead to additional health effects.

This aim emphasizes the importance of studying metal mixtures in the Cd-exposed Mae Sot population since mining is associated with exposure to other metals and toxicants. There are immediate public health implications associated with these co-exposure patterns. Elevated levels

of Pb and As are associated with independent adverse health effects including neurocognitive impairment (26) and cancer (30). These metals may also interact with Cd to exacerbate renal damage (31-33). Elevated levels of essential metals should also be studied to determine potential adverse health effects. The possible selenium deficiency among the study population should be further studied since there is evidence of protective inverse interactions between selenium sufficiency and toxic metal exposure (31). Long-term results indicate that further remediation is necessary for Cd and other toxic metals in this population. Accounting for mixtures in adverse health effects and chronic disease risk is important among highly environmentally exposed populations.

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