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CHRONIC ENVIRONMENTAL EXPOSURE TO METAL MIXTURES IN TRIBAL POPULATIONS AND POTENTIAL IMMUNE SYSTEM EFFECTS

by

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ABSTRACT

Hardrock mining in the United States (US) has left a legacy of mixed metal mine waste sites. Wastes may contain multiple metals of health concern, including arsenic, cadmium, lead, mercury, and uranium, among others. Mining waste sites are disproportionately located on or contiguous to the watersheds of tribal lands. Due to proximity, and because of reliance on natural resources to maintain traditional diets and customs, Native American communities' contact with multiple metals is often increased. Two impacted communities are the Cheyenne River Sioux Tribe (CRST) and Navajo Nation. Both tribes have expressed concerns that metals in mine waste adversely affect their communities' health and report an elevated prevalence of autoimmune diseases. To examine the effects of mixed metals, we measured metals and autoimmune-associated markers. We found that metals and metal mixtures are associated with alterations in certain autoimmune markers such as autoantibodies and cytokines.

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ABBREVIATIONS

AD	Autoimmune disease
AI/AN	American Indian/Alaskan Natives
AIC	Akaike information criterion
ANA	Antinuclear antibody
ANoA	Antinucleolar Antibody
As	Arsenic
As(III)	Arsenite
ATL	Adult T-cell Leukemia
BCD	Blood cadmium
BKMR	Bayesian Kernal Machine Regression
BMI	Body Mass Index
BMN	Blood manganese
BPB	Blood lead
BPR	Bayesian Profile Regression
BSE	Blood selenium
Cd	Cadmium
CD4	Cluster differentiation 4
CD8	Cluster differentiation 8
CDC	Centers for Disease Control
CENP	Centromere antigenic component
CI	Confidence Interval
CoETS	Composite ETS
CRST	Cheyenne River Sioux Tribe
Cu	Copper
dDNA	Denatured DNA
DENR	Department of Natural Resources
DMA	Dimethylarsinic Acid
DNA	Deoxyribonucleic acid
DP	Dirichlet Process
DPMM	Dirichlet Process Mixture Model
EC	Exposure Cluster
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assays
ETS	Environmental Tobacco Smoke
FC	Fish consumption
GSH-Px	Glutathione Peroxidase
HEp-2	Human epithelial type 2
Hg	Mercury
HPLC	High Performance Liquid Chromatrography
HSC	Health Sciences Center
I	Iodine
ICP-DRC-MS	Inductively coupled plasma dynamic reaction cell mass spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
IFNalpha	Interferon Alpha

IFNgamma	Interferon Gamma
Ig	Immunoglobulin
iHg	Inorganic Mercury
IHS	Indian Health Service
IIF	Indirect immunofluorescence
IIFA	Immunofluorescence Assays
IL-17	Interleukin-17
IL-1beta	Interleukin-1beta
IL-2	Interleukin-29
IL-29	Interleukin-29
IL-4	Interleukin-4
IL-7	Interleukin-7
IP3	1,4,5-triphospate
IQR	Interquartile range
K-12	Kindergarden Through 12th Grade
LOD	Limit of detection
M2 EP (MIT3)	Mitochondiral epitope 2 (combination of 3 mitochondrial antigens)
MBIRI	Missour Breaks Industries Research, Inc.
MCL	Maximum contaminant level
MCMC	Markov Chain Monte Carlo
MeHg	Methyl mercury
MMA	Monomethylarsonic Acid
MnSOD	Manganese Superoxide Dismutase
mRNA	Micro ribonucleic acid
n-3 PUFA	n-3 Poly-Unsaturated Fatty Acid
NBCS	Navajo Birth Cohort Study
NCEH	National Center for Environmental Health
nDNA	Native DNA
NHANES	National Health and Nutrition Examination Survey
NM	New Mexico
OD	Optical density
oHg	Organic Mercury
ONDIEH	Office of Noncommunicable Diseases, Injury and Environmental Health
OR	Odds Ratio
Pb	Lead
PBC	Primary biliary cirrhosis
PBMC	Peripheral Blood Mononuclear Cell
PBS-Tween	Phosphate buffered saline-Tween
Pol III	Polymerase III
PWS	Public Water Systems
QRM	Quantile Regression Model
RF	Rheumatoid Factors
Ribo-P	Ribosomal P
RNA	Ribonucleaic acid
RNA Pol III	RNA polymerase III
RNP	Ribonucleoprotein

rpm	rotations per minute
sAuAb	Specific autoantibody
SC	Serum cotinine
SCID	Severe Combined Immunodeficiency Disorder
Scl-70	Scleroderma 70 (topoisomerase 1)
SCU	Serum copper
SD	Standard Deviation
Se	Selenium
SLE	Systemic Lupus Erythematosus
Sm	Smith
SSA	Sjögren's antigen A
SSB	Sjögren's antigen B
SSE	Serum selenium
stepAIC	Stepwise Akaike information criterion
SZN	Serum zinc
TGF-beta	Transforming growth factor beta
Th1	T helper type 1
Th2	T helper type 2
THg	Total blood mercury
THG	Total blood mercury
TNF-alpha	Tumor Necrosis Factor alpha
U	Uranium
UAS3	Urine arsenite (As(III))
UAS5	Urine arsenate (As(V))
UBA	Urine barium
UCD	Urine cadmium
UCO	Urine cobalt
UCS	Urine cesium
UDMA	Urine dimethylarsinic acid
UDMA	Urine dimethylarsinic acid
UIO	Urine iodine
UMMA	Urine monomethylarsonic acid
UMMA	Urine monomethylarsonic acid
UMN	Urine manganese
UMO	Urine molybdenum
UNM CEHP	University of New Mexico Community Environmental Health Program
UPB	Urine lead
USB	Urine antimony
USEPA	United States Environmental Protection Agency
USN	Urine tin
USR	Urine strontium
UTAS	Urine total arsenic
UTL	Urine thallium
UTU	Urine tungsten
UUR	Urine uranium
WHO	World Health Organization

I. CHAPTER 1

BACKGROUND

History of mining in the United States (US) and proximity, exposure pathways, and exposure routes to Native American community members

Mining in the US has left a legacy of >500,000 abandoned mine waste sites. Wastes may contain geologic mixtures of primary mining minerals: uranium (U), vanadium, gold, silver, copper and lead, as well as metals that co-occur and/or remain after processing, including arsenic (As, metalloid), mercury (Hg), nickel, cadmium, selenium, and others. As a result, 40% of watersheds in the western US are contaminated by mine waste and related metals [1]. Mining waste sites are often located on or contiguous to the watersheds of tribal lands, and mobilized wastes may migrate through the environment. This is clear in Figure 1.1B, which shows the count of mining sites by distance to the nearest Native American reservation. Depending on the primary metal extracted, the number of mines varies from several hundred to several thousand sites within 100 kilometers (km) of Native American reservations. This is well within the distance over which environmental contaminants may be mobilized through air, water, and utilization of local natural resources to impact community members.

Due to proximity, Native American/American Indian (NA/AI) community members are likely to be in contact with mines/mine waste sites, or metal mixtures that have migrated from these sites. This increases the likelihood of exposure through multiple routes including inhalation, absorption through the skin, and ingestion of

contaminated water or food. Because of reliance on natural resources to maintain traditional diets, lifestyles, customs and languages, Native American communities' contact with metal mixtures from mine sites is compounded, often leading to greater exposures than those predicted by US Environmental Protection Agency (USEPA) default parameters.

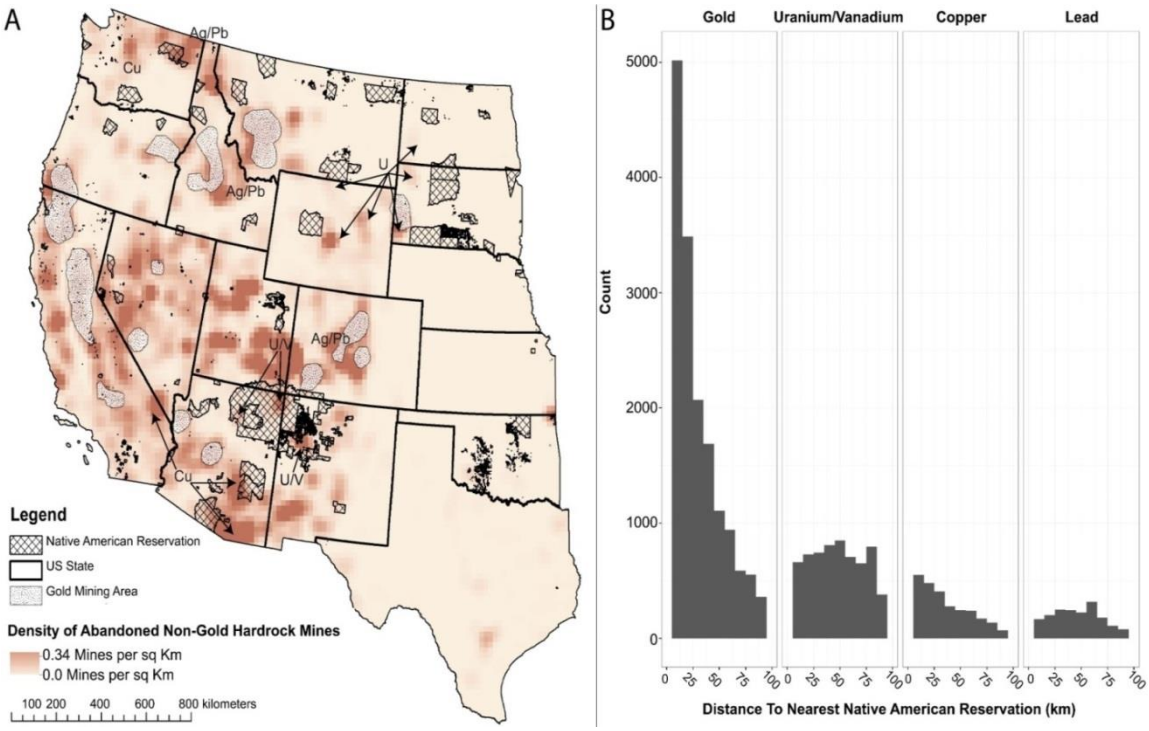

Figure 1.1. (A) Map of the western United States showing the locations of Native American reservations and the density of non-gold hard rock mines (B) Graphical data summary showing large number of mines in close proximity to Native American reservations in the US. Reprinted from "Mining and Environmental Health Disparities in Native American Communities," by J Lewis, 2017, *Curr Environ Heal Reports*, *4*(130), 41. Copyright [2017] by J Lewis.

Disparities contribute to potential sensitivity to toxicity and disease outcomes in tribal communities

Disparities in infrastructure, especially drinking water supplies, and unique social determinants of health from poverty in rural and isolated locations can exacerbate mine-related mixed-metal exposures in tribal communities. Fourteen percent of tribal communities lack access to regulated public drinking water as compared to 0.6% of the United States (US) population [2]. Even when public water systems are in place, limited resources, vast distances with sparse infrastructure, aging and antiquated systems, and highly mineralized aquifers often contribute to higher rates of violations to the Safe Drinking Water Act for many metal contaminants with health-based standards. Tribal populations are characterized by health disparities associated with lower socioeconomic status, lack of access to healthcare, and comorbidity. US Census Data [3] indicates that 28.4% of NA/AI live in poverty, in comparison with 15.3% the US as a whole. Although the US federal government is obligated by law to provide healthcare to NA/AI, and does so through Indian Health Service (IHS), health programs for NA/AI are chronically underfunded, with the 2016 IHS budget of $4.8 billion for 3.7 million AI/AN dividing out to $1297 per person, less than 20% of the amount allotted for healthcare per federal prison inmate [4]. While IHS hospitals and clinics are present on tribal lands, the remote conditions and poor transportation infrastructure can also be a major barrier to accessing care on larger reservations such as Navajo Nation and various Sioux lands. According to IHS data (2006-2008) [5], rates of infectious disease mortality are 40-60% higher than in "US All Races"; diabetes and liver disease mortality are 2.8-4.7 fold greater than compared to "US All Races"; and life expectancy is decreased by 4.2 years [5]. In spite of these disparities, tribal populations are often not included in epidemiologic studies of

toxicity, resulting in limited information concerning the impacts of environmental toxicants on tribal populations.

Two Specific Native American Communities Impacted by Legacy Mixed-Metal Mine Wastes — Cheyenne River Sioux Tribe (CRST) and Navajo Nation

As can be seen looking at a map of mine sites and Native American reservations (Figure 1.1A), the number of mine sites in proximity to Native American reservations is particularly striking in the western US, including CRST lands in South Dakota (SD), and Navajo Nation in the Four Corners Region (quadripoint of Utah, Colorado, Arizona, and New Mexico).

Cheyenne River Sioux Tribe (CRST)

The CRST reservation is located in north-central SD and is similar in size to Connecticut (Figure 1.2). The rural reservation consists primarily of rolling prairie bisected by the Moreau River and bounded by the Cheyenne River on the south (Figure 1.2). The Cheyenne River drains from the Black Hills in southwestern SD and provides the source of drinking water for the tribal water system serving CRST members. For more than a century, mining from >900 mines in the Black Hills, including gold mines in which Hg was used for amalgamation purposes, has released contaminants into watersheds draining onto CRST lands [6]. Additionally, approximately one ton of airborne Hg is emitted per year from coal power plants in Montana, Wyoming, North Dakota, and South Dakota [7], and carried downwind to CRST lands where precipitation and dust wash this Hg out of the air into water and soil. Thus, Hg is virtually ubiquitous throughout the CRST reservation. Studies over the last decade conducted by the tribal Department of

Environment and Natural Resources (DENR), United States Environmental Protection Agency (USEPA) and University of Colorado [8] have documented high Hg concentrations in mid-flow water samples [9], sediment [10] and fish [11–13]. Fishing and fish consumption are therefore significant potential exposure pathways for Hg and other metals. Fishing and fish consumption are not only important in Sioux culture, but high rates of poverty (~50%) [14] and unemployment (88%) [15] on the CRST reservation increase the community's likelihood of using fish to supplement household subsistence. More recently, high-concentration As sediment deposits along the Cheyenne River have been discovered in exposure pathway-relevant locations, drawing attention to potentially significant exposure pathways for As in addition to Hg and other environmental metals. These include local food consumption (corn, fruit, tea and radishes), horseback riding/roping which stirs up dust and dirt, and burning local wood for ceremonial practices such as sweats. This As is thought to result from more than 125 years of operation of the Homestake Gold Mine in Lead, SD (Figure 1.2), which closed in 2001, but historically discharged significant As into the Cheyenne River watershed as untreated waste.

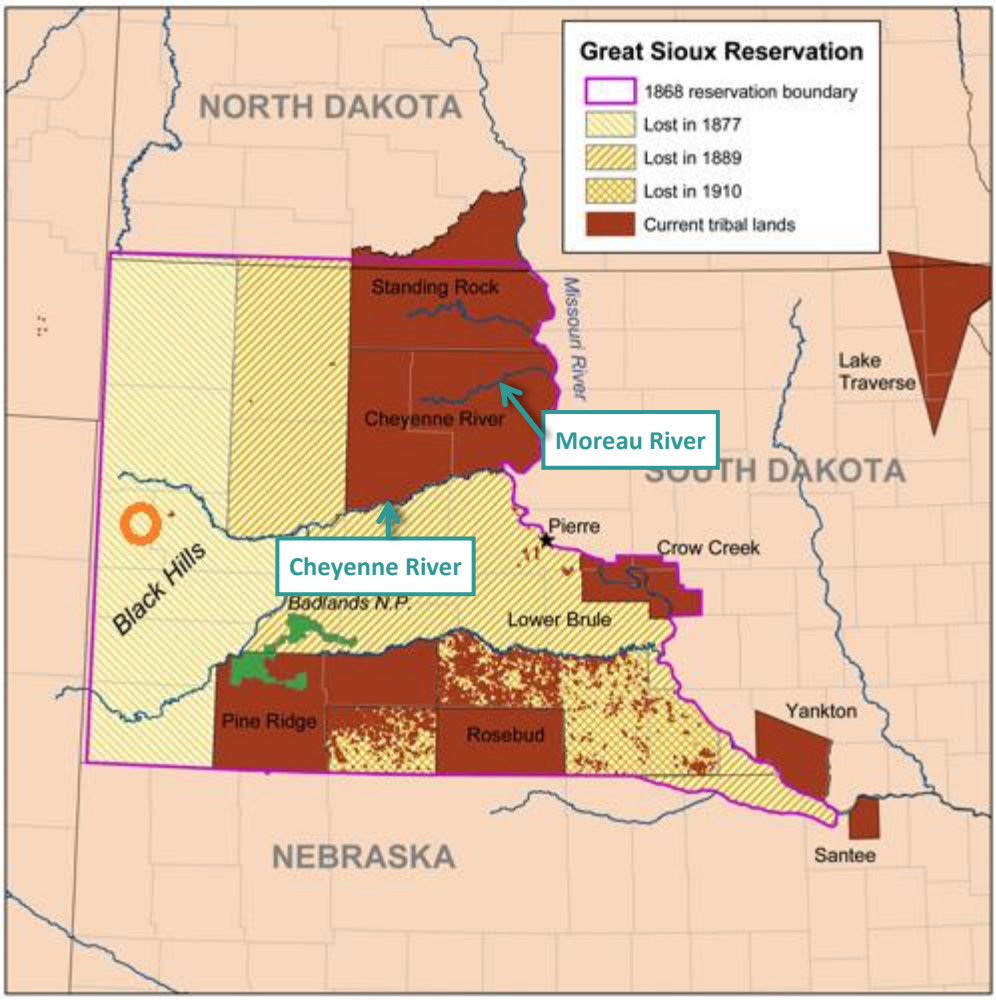

Figure 1.2. Map of the state of South Dakota. Cheyenne River and other Sioux tribal lands. An orange circle marks the approximate location of the Homestake Gold Mine. Adapted from Great Sioux Reservation, In Wikipedia, March 17, 2008, Retrieved November 11, 2019, from https://en.wikipedia.org/wiki/Great_Sioux_Reservation#/media/File:Siouxreservationmap.png. Copyright 2008 by K Musser.

Community forums and discussions with tribal leaders conducted by Dr. Johnnye Lewis, others on our team, and partners from the Cheyenne River Department of Environment and Natural Resources from 2002 to the present revealed the widespread frustration that actual health studies had not been conducted on CRST in spite of Hg warnings being posted for nearly 40 years. A major community concern identified was that a perceived increase in autoimmune disease (AD) prevalence in the CRST population might be related to Hg exposures through fish consumption. Although limited research has suggested a link between Hg exposure and autoimmunity, none has assessed chronic low-level environmental exposures in human populations as risk factors for AD. Some epidemiologic studies have investigated the role of mercury amalgam fillings in multiple sclerosis [16,17], and studies of ANA and cytokines in Hg-exposed Brazilian gold miners in the Amazon [18–21], but too few [22,23] have investigated the potential role of chronic environmental metal exposures, including Hg in mixtures, for relationships to AD. While relationships between metal exposure and immune dysfunction have been suggested in the studies cited, and demonstrated for single metals such as Hg [24,25] in animal studies, limited data in humans make it difficult to understand the potential role of metal exposures as risk factors for AD, either as single metals or in the metal mixtures likely to occur in community-level environmental exposures.

Navajo Nation

Navajo Nation is located in the Four Corners Region of the Southwestern US with a land area equivalent to the state of West Virginia (Figure 1.3). It is the largest NA/AI reservation in the US, covering parts of Arizona, New Mexico and Utah. Although active mining and milling on Navajo Nation ended in 1986, the legacy on Navajo lands from the atomic bomb and Cold War Era production of uranium for weapons includes 521 abandoned U mines and >1100 of the 10,400 U waste sites identified in the western US (Figure 1.4). The wastes associated with these sites contain *multiple* metals and metalloids. Navajo Nation community members may be chronically exposed to these metal-mixture wastes through multiple pathways: consumption of local water and crops, direct contact with or inhalation of contaminated soil and dust from mine features, and inhalation of metals released from combustion for home heating. Drinking water is of primary concern, because 8-10% of unregulated water sources serving the >30% of Navajos without access to public water systems (PWS) exceeded the U maximum contaminant level (MCL) (Figure 1.5B), while nearly 15% had elevated As (Figure 1.5A). Many of these water sources exceeded both U and As MCLs (Figure 1.5). Additionally, major public water systems on Navajo Nation are known to have been repeatedly out of compliance with one or more water standards for metals [26]. Traditionally Navajos have consumed locally-grown crops, locally-grazed cattle, and locally-foraged tea, all contributing potential exposure pathways due to direct uptake by plants of metal contaminants in soil or water, secondary consumption of animals consuming these plants, or consumption of livestock drinking contaminated water.

Combustion of local wood and coal for home heating and cooking must also be factored into metal exposure characterization.

Although Navajo communities have long been concerned that environmental exposure to mine waste contributes to poor health outcomes among tribal members, no comprehensive characterization of metal body burden of this population has been conducted. Tribal populations are not well-represented in the National Health and Nutrition Examination Survey (NHANES); tribal populations are aggregated with various racial/ethnic groups into the "Other" group which only comprises 5.3% of the most recent NHANES study population [27], making it difficult to impossible to identify data representative of tribal populations as a whole, let alone those relevant to a specific tribe. The first step in in addressing Navajo Nation environmental health concerns is to understand the underlying structure of the complex body burden of multiple metals (biomonitoring), demographics, and exposure routes, and the relationship among these aspects in Navajo Nation community members.

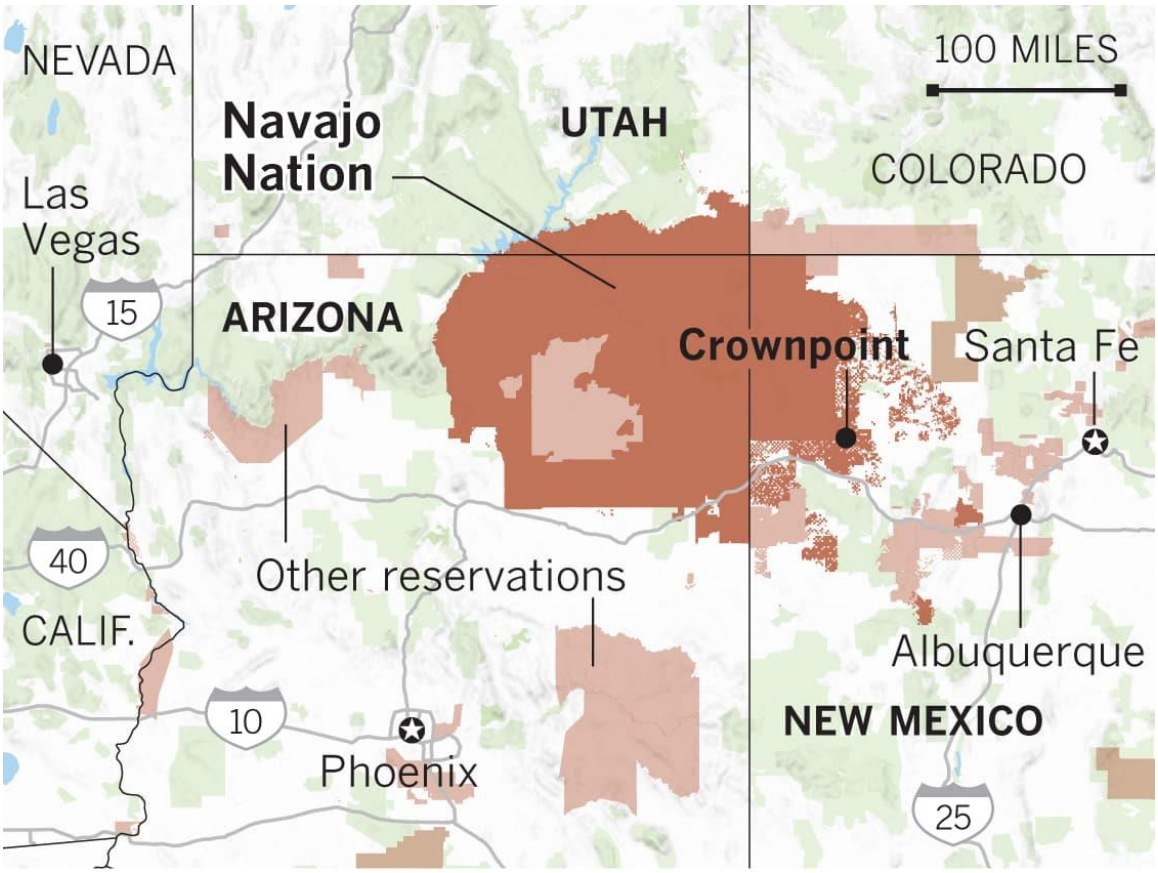

Figure 1.3. US Census Bureau map of Four Corners Region with Navajo Nation marked in dark brown. *U.S. Census Bureau, Nextzen, OpenStreetMap.*

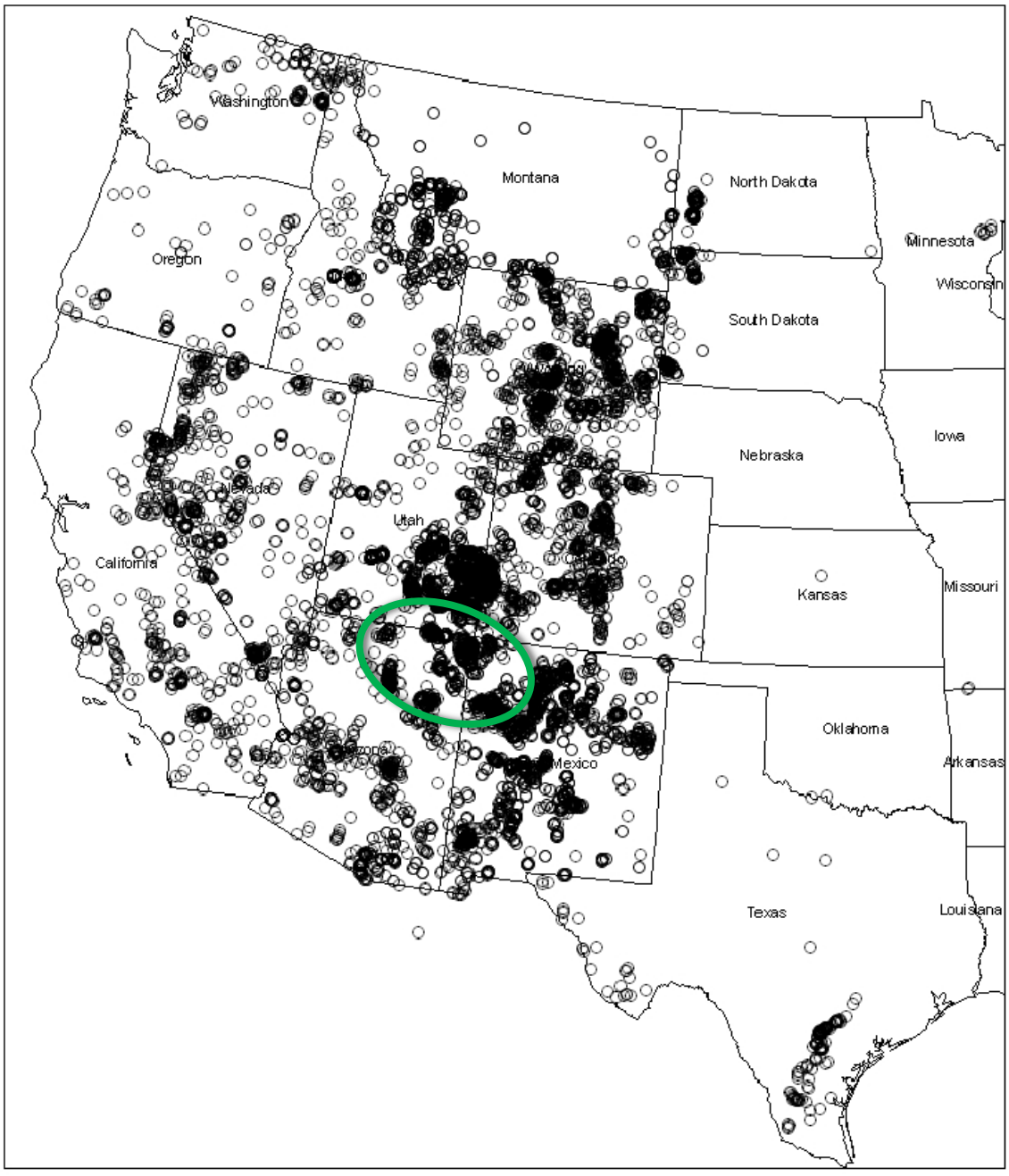

Figure 1.4. Map of EPA-identified U locations in the western US with approximate location of Navajo Nation circled. Adapted from *Uranium Location Database Compilation* (Report EPA 402-R-05-009). by Office of Radiation & Indoor Air Radiation Protection Division, 2006, Retrieved from https://www.epa.gov/sites/production/files/2015-05/documents/402-r-05-009.pdf. Copyright [2006] by UESPA. Adapted with permission.

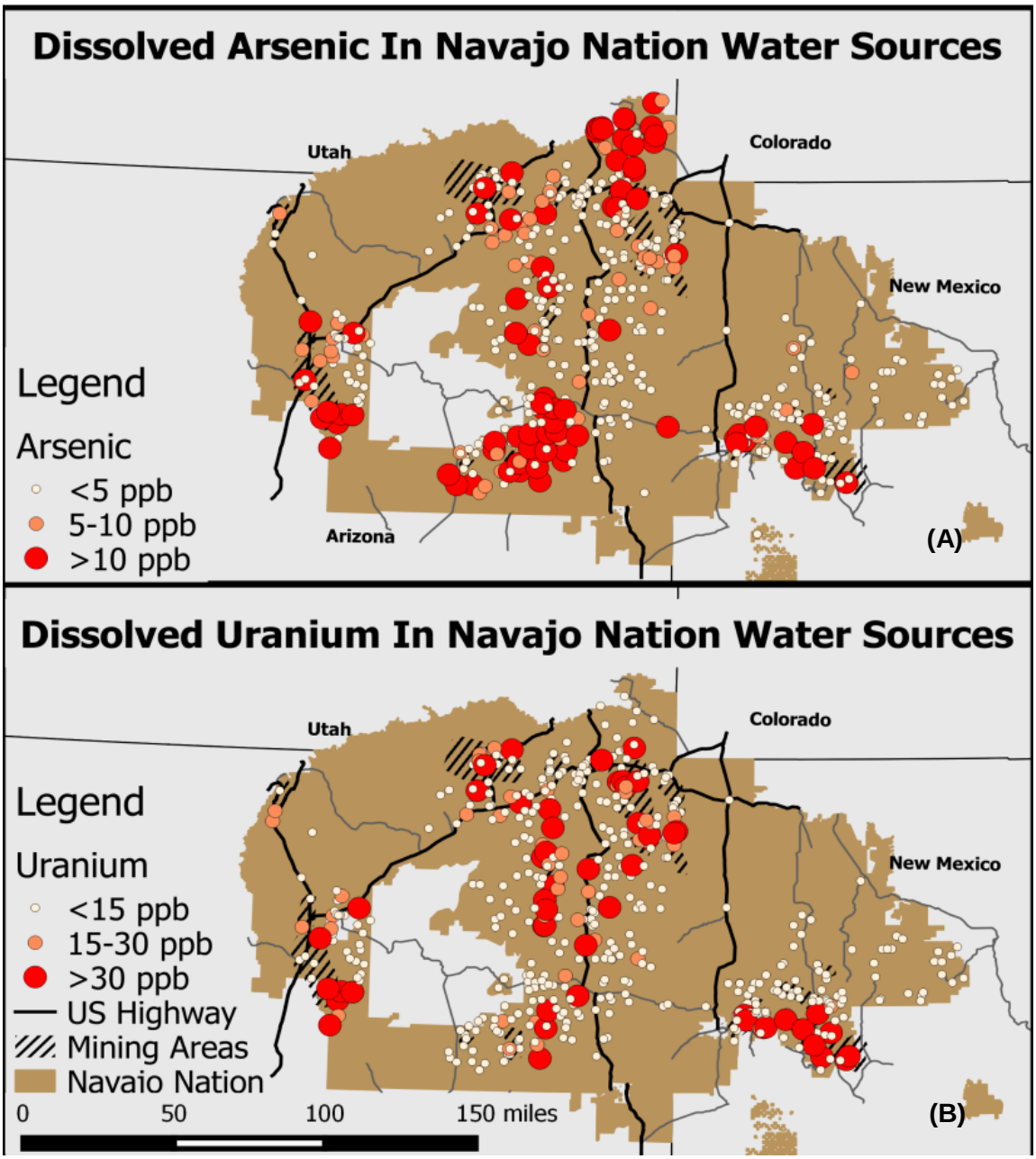

Figure 1.5. Map of arsenic (A) and uranium (B) concentrations in Navajo Nation water sources and their proximity to mining areas. Adapted from "Elevated Arsenic and Uranium Concentrations in Unregulated Water Sources on the Navajo Nation, USA," by J Hoover, 2017, *Expo Heal*, *9*:113. Copyright [2017] by J Hoover.

The immune system as a potential link between environmental metal exposures and adverse health effects in tribal populations

Community members voiced concerns about the adverse health effects of chronic environmental exposure to metal mixtures in general, and autoimmune disease in particular. In 2000, members of 20 communities in eastern Navajo Nation approached University of New Mexico Community Environmental Health Program (UNM CEHP) researchers with concerns about metals exposure through drinking water and the development of a number of chronic diseases including autoimmune disease, diabetes, kidney disease, and cardiovascular disease, all at higher than expected prevalence on Navajo Nation. In 2002 the CRST tribal members expressed similar concerns, reaching out to CEHP researchers to partner with them in an environmental justice investigation of exposure and health on Cheyenne River. CRST and Navajo Nation members were joined by IHS clinicians in expressing concerns about immune dysfunction linked to an inability to fight infectious disease, and their perceived elevated prevalence of several clinical autoimmune diseases in their communities: severe combined immunodeficiency (SCID), systemic lupus erythematosus (SLE), and idiopathic liver disease (personal communications, IHS clinicians, 2000-2016). In this way, the community's environmental health concerns converged with existing scientific evidence indicating a possible link between metal exposure and autoimmunity. Further, it underscored the need for additional research on metals and autoimmunity in these specific populations, such as is included in this dissertation.

Autoimmunity and Autoimmune Disease

Autoimmune diseases arise when an individual's immune system attacks his/her own tissues and organs. There are more than 80 different autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), inflammatory bowel disease (IBD), type 1 diabetes (T1D), Sjogren's syndrome, and systemic sclerosis. Autoimmune diseases are found in 5-7% of the population, and disease prevalence is skewed towards females and increases with age [28]. Autoimmune diseases are thought to be a result of gene-environment interactions, which may be exacerbated by hormones [29]. Autoimmune diseases present differently depending on disease and patient, and multiple tests and reported symptoms are required to diagnose an autoimmune disease. Common tests include screens for the presence of antinuclear antibodies (ANA) followed by specific autoantibodies (e.g. antibodies to mitochondrial proteins or deoxyribonucleic acid (DNA)). Approximately 13.8% of the US population is positive for ANA [30], but not all people who are positive for ANA or specific autoantibodies have an autoimmune disease, and conversely, not all people diagnosed with autoimmune disease are ANA positive. However, ANA positivity may be an early biomarker for autoimmune disease development, and a potential indicator of increased general autoimmune processes (autoimmunity) in an individual, and therefore is a routine first-tier screen clinically. ANA also may be an indicator of dysfunction in the immune system expressed through production of antibodies to "self" without progressing to frank clinical disease.

Autoimmunity is defined as an immune response leading to reaction with self-antigen (autoantigen), any molecule that is normally found in the animal mounting the immune response [31]. Autoimmunity is manifested by a combination of antibodies and/or activated immune cells; autoantigens can trigger immune cells directly through

receptors or by virtue of cross-reaction between foreign and self-antigens [31]. Metals, which have documented immunotoxic effects [32], may exacerbate autoimmune responses by perturbing the immune system's complex interplay of immune cells (T cells, B cells, natural killer (NK) cells, macrophages, and granulocytes) and immune signaling molecules (cytokines). Specific pathways by which metals may perturb immune function are described below in the "Metals and Autoimmunity" section. To gain insight into which of these pathways may be involved in our study populations in these initial investigations of associations between metal exposures and immune dysfunction, verified metal exposure was modelled against clinical biomarkers of autoimmunity (ANA and specific autoantibodies), and circulating cytokines (proteins released by immune cells that have been implicated in multiple health endpoints associated with chronic inflammation).

Brief summaries about what is known about 1) indigenous populations and autoimmunity, 2) metals and mechanisms of autoimmunity, and 3) and our primary metals of concern (Hg, As, and U) and mechanisms of autoimmunity can be found below.

Indigenous Populations and Autoimmunity

There is epidemiological evidence of autoimmune diseases occurring at higher-than-expected rates in indigenous populations. The 2014 lupus registry estimates that the prevalence and incidence of SLE for NA/AI served by IHS sites in Alaska, Arizona, and Oklahoma is as high, or higher than, those for the black female population in the US, the group clinically acknowledged to have the highest rates of SLE in the US. Other studies indicate a genetic susceptibility to systemic sclerosis [33] and RA [34] in specific tribes.

Though these population studies support CRST and Navajo Nation concerns about levels of autoimmune disease in their communities, as well as the possibility of a genetically susceptible background in our study populations, prior studies did not include exposure information, and most often lack estimates of polymorphism prevalence within a tribe from which to interpret observed polymorphisms in those with the disease. Genomic data on tribal populations is limited due to abuses in genetic research in tribal populations in the past which have led to bans or moratoriums on this category of research [35]. Therefore, there are no data on which to determine whether a genetic susceptibility in a particular tribe is directly responsible for autoimmune disease, or creates a sensitivity to susceptibility that may be triggered by environmental exposures. Yet understanding this difference is essential in developing prevention strategies.

Previous and ongoing work by our group indicates a link between environmental exposure to metals and autoimmune markers. In the Diné Network for Environmental Health (DiNEH) Project, with an average age in the mid-50s, specific autoantibodies to denatured DNA and chromatin were linked to uranium consumption in drinking water [36]. In a subsequent study of younger generation residents with an average age in the mid-20s, a greater proportion of Navajo Birth Cohort Study (NBCS) participants were positive for ANA, 18% versus the national average of 13.8%, and ANA positivity was associated with increased urine uranium levels in males (work in progress). Our previous work, combined with epidemiological studies on autoimmune disease in indigenous populations, supports the need for a better understanding of the mechanisms through which metal exposure may lead to autoimmunity.

Metals and Autoimmunity – Mechanisms

In conjunction with a genetically-susceptible background, metals may act through multiple mechanisms to exacerbate autoimmunity. The immune effects of metals are potentially mediated through enzyme inhibition [37,38], cell membrane disturbance [39], and free radical formation/oxidative stress [40]. For example, metals may interact, or compete, with nutritionally essential metals such as calcium and zinc. Aberrant calcium signaling has been implicated in autoimmunity due to its major role in normal B cell development, particularly the elimination of autoreactive B cells [41], and zinc deficiency has been associated with overproduction of inflammatory cytokines, TH1/TH2 imbalance, increased TH17, and decreased Treg cells [42]. Certain metals can serve as adjuvants, promoting dendritic cell activation and migration as well as antigen presentation [43]. Metals may directly bind to major histocompatibility complex class II (MHC II) molecules or T-cell receptors (TCRs), or modify autoantigens to reveal higher-affinity hidden epitopes [44]. Metals may bind to sulfhydryl groups, potentially precipitating autoimmunity by modulating membrane-bound thiols, increasing T and B cell responsiveness, and altering downstream lymphocyte signaling and function [44]. By modifying cytokine production, metals may induce autoimmune response by dysregulating the balance between TH1 and TH2 phenotypes, enhancing autoantibody production [45]. Research on metal-associated autoimmunity in populations, animal studies, and in vitro studies has focused primarily on single metals, or metal co-exposure; information about multiple metal exposure and autoimmunity is sparse.

Selection of Primary Metals of Interest

The list of metals to which community members are potentially exposed through environmental mobility of abandoned mine waste is long. To focus the work in this

dissertation, several sources of information were reviewed to identify key metals of interest, and a somewhat longer list of potentially modulating metals based on 1) the history of mining and the prevalence of associated metals, 2) associations suggested between specific environmental metal exposures and immune function in previous animal and human research, and 3) likely exposure pathways identified in discussions with communities on CRST and Navajo Nation. The of metals of primary health interest extracted from the confluence of information from these sources were identified to be Hg, As, and U.

Mercury is known to elicit a wide range of inflammatory and autoimmune responses in humans and animals [25], and epidemiologic studies have linked chronic low-dose drinking-water exposures to As *and*/*or* U through drinking water to adverse health effects including kidney damage, various cancers, cardiovascular diseases and hypertension [46–54]. The associations between Hg, As, and U exposures and this list of adverse health effects parallel the list of health concerns on CRST and Navajo Nation: cancer, autoimmunity, kidney disease, diabetes and hypertension, all of which may be mediated or modulated by the immune system. While it is known from animal studies that As suppresses immune function [55,56], little is known about the impacts of chronic U exposures, although it has been shown to cause immune suppression [57]. Notably, although two or all of these metals have been shown to co-occur in sources such as mine waste and coal combustion emissions, few previous studies have been conducted to examine the combined effect of chronic multiple metal exposure to Hg, As, and U on the immune system, much less the broader suite of metals contained in mixed mine wastes.

Primary Metals of Interest and Autoimmunity (Hg, As, U)

Mercury's associations with autoimmune disease and autoimmunity in populations, cells systems, and animal models have recently been reviewed extensively [25]. The model for the molecular and cellular mechanisms of mercury-induced immune activation and autoimmunity begins with inflammation at the site of exposure followed by activation and expansion of CD4 T cells, production of immunoglobulin G (IgG), generation of IgG ANAs, and deposition of antigen-antibody immune complexes in blood vessels and subsequently at symptomatic sites [25]. It is also posited that tissue damage following mercury exposure leads to the availability of damage-associated molecular patterns (DAMPs) that engage toll-like receptors (TLRs), stimulate inflammatory cytokine production, and induce chronic inflammation with the ultimate result of sustained autoimmune response [58] . Arsenic exposure has been implicated in immunologic imbalance and immunotoxicity through multiple mechanisms [59]. Notably, As causes oxidative stress, inflammation, and cell injury, which may lead to autoimmunity through post-damage mechanisms similar to those of mercury although studies in animals [55,56] and humans [36] implicate immunosuppressive effects. Research concerning mechanistic effects of uranium on autoimmunity is sparse, though human studies show associations between uranium consumption and autoantibodies [36], disease [60,61], and cytokine alterations [62]. A study of macrophages and CD4+ T cells exposed to depleted uranium showed changes in lymphoproliferation, differences in gene expression of cytokines, and polarization of T cells to TH2 phenotypes, suggesting ways in which uranium may contribute to autoimmunity [63].

As described in the previous sections, the presence of mixed metals, from mine wastes and other sources, has been confirmed on both CRST lands and Navajo Nation.

Community members of these tribes may experience increased environmental exposure from pathways related to land-use practices influenced by cultural, rural, and socioeconomic factors. Unique exposure pathways, disparities in healthcare, and dearth of toxicity knowledge about mixed metals and outcomes may exacerbate disparities in adverse health outcomes such as cancer [64], cardiovascular disease [65], diabetes [66,67], kidney disease [68], and autoimmunity [69] that have been observed in indigenous populations. The immune system is complex with wide-ranging effects, and metals have been shown to have immunotoxic effects [32]. The immune system's role in tumor surveillance [70–72], as well as the role of immune system elements (e.g. cytokines) [73–75] in producing and maintaining conditions such as chronic inflammation that have been implicated in development of multiple diseases prevalent in indigenous communities, suggests that disruption of normal immune function by chronic metal exposures could be contributing to a range of observed health disparities in these populations. Thus, it is feasible that dysregulation or maladaptation of the immune system due to environmental metal exposure plays a role in adverse health outcomes observed in AN/AI populations.

RATIONALE

Chronic low level environmental exposure to metals toxicants is a widespread concern of scientific, societal and environmental importance. Due to factors including proximity to hard rock mines, socioeconomic circumstances, and traditional land-use practices, tribal populations in particular are disproportionately exposed to mixtures of metal contaminants. These may travel through various exposure pathways to enter the body by varying routes, contributing to physiological changes in susceptible populations that ultimately favor disease development and progression. Tribal populations are subject to environmental health disparities [64,65,76–78]. We posit that the immune system is a major link between environmental metal exposures and adverse health outcomes. By characterizing and linking environmental exposures and biomonitoring in tribal populations, and modeling them with health endpoints, we endeavor to identify risk factors and prevention strategies beneficial to the community. Mining will continue worldwide as long as it is economically lucrative, and the metals released by mining cannot be immobilized without enormous resources spent over a protracted time. Due to the complexity of mine/mine waste site remediation and the sheer number of sites, clean-up costs are prohibitive. Longer-term goals of this research include aiding in the prioritization of mine waste remediation, as well as developing environmental exposure prevention strategies and early health interventions. Thus, this research will be a stepping stone to pursuing environmental restoration, empowering environmental health decision-making, and ameliorating tribal health disparities.

OVERARCHING SCIENTIFIC RESEARCH GOALS

Goal 1 (Exposure): Understand the measurable multiple metals (biomonitoring), demographics, and exposure routes, and the relationship among these aspects, in two tribal populations chronically exposed to metals

Goal 2 (Immune outcomes): Examine the relationships among metal biomonitoring, metal exposure routes, and potential immune system alterations in two tribal populations chronically exposed to metals

Central Hypothesis:

Chronic low-level environmental exposure to metal mixtures results in measurable metals in exposed tribal populations, which is associated with immune dysregulation.

AIM 1: Explore the relationship between environmental metal exposure and immune markers in Cheyenne River Sioux Tribe (CRST) community members.

AIM 2: Explore the relationship between environmental metal exposure and immune markers in Navajo Nation community members.

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II. CHAPTER 2

Mercury in Fish as a Potential Environmental Factor in the Development of Autoimmunity: A Mini-review with a Focus on Human Population Studies

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This mini-review illustrates a specific case of how chronic environmental exposure to a single metal may contribute to immune system alterations and potentially exacerbate disease.

Abstract

Autoimmune diseases develop due to the interaction between genetic susceptibility and additional factors, such as environmental exposure to toxicants. Mercury (Hg), a well-established neurotoxin, has more recently been studied as an immunotoxin linked with biomarkers of autoimmunity, including the presence of antinuclear antibodies (ANA) and distinct cytokine profiles. Mercury (Hg) is virtually ubiquitous in the environment, and concerns about the potential health impacts of Hg exposure through fish consumption exist. A few studies have specifically examined the relationships among mercury, fish consumption, and autoimmune biomarkers in human populations. The findings of these studies are conflicting; this may be due to confounding exposures and opposing mechanisms of action. Additional studies are necessary to clarify the role of Hg through seafood consumption in autoimmunity.

Introduction

Autoimmune diseases develop due to interactions between genetic susceptibility and additional factors, including environmental exposure to toxicants [1]. Mercury (Hg) has been implicated as an environmental factor that contributes to the development and exacerbation of autoimmune disease [2]. Hg, a ubiquitous pollutant known to affect ecosystems and human health [3], exists in several chemical forms including inorganic mercury (iHg) and organic mercury (oHg). Microorganisms transform iHg present in sediment or water into oHg by methylation, yielding methyl mercury (MeHg). Plankton and algae absorb MeHg and are consumed by small fish, which are subsequently eaten by predators, ultimately resulting in bio-magnification of MeHg up the food chain [4].

Humans are thus exposed to Hg through ingestion when they consume seafood into which Hg has bioaccumulated, particularly because the form of MeHg in fish tissue is not removed through cooking or cleaning processes [4]. This mini-review discusses the potential autoimmune effects of MeHg with a focus on human MeHg exposure through fish consumption. This question of dietary MeHg exposure is significant because Hg is a global toxicant [3], and billions of people worldwide risk increased exposure to MeHg through reliance on fish as a major source of dietary protein and nutrition [5,6].

MeHg immune effects in animal and in vitro studies

Animal models provide evidence for Hg's role in inducing autoimmune effects. Exposing genetically-susceptible mouse strains to Hg leads to the development and/or exacerbation of lupus-like symptoms [7–12], including increased antinuclear autoantibodies (ANA) [7,8]. MeHg exposure in mice led to an initial immunosuppression via reduction in T- and B-cell populations [13], followed by an increase in ANA and IL-4 mRNA expression [13–15]. *In vitro* studies in human peripheral blood mononuclear cells (PBMCs) treated with sub-toxic MeHg resulted in increased concentrations of cytokine IL-1β [16] and suppression of cytokines IL-2 and TGF-β [17]. These results support that MeHg, the form of Hg in human dietary sources, leads to immune dysregulation and autoimmunity.

MeHg, fish consumption, and immune system effects in human population studies

Few human studies explicitly examine the role of Hg exposure through fish consumption with biomarkers of autoimmunity. Silva et al. [18] reports increased prevalence of ANA (10.7%) and antinucleolar antibodies (ANoA) (18%) in a population exposed to MeHg

through fish consumption versus the reference site (ANA 7.1%, ANoA 2.0%), though the prevalence was not as elevated as those measured in occupationally exposed miners (54.1% ANA, 40.8% ANoA). Another study of Amazonian communites [19] observed positive serum ANA more frequently in riverines who consumed fish daily (including species with confirmed high MeHg) than in controls (12.4% vs 2.9%), and mean hair Hg of riverines (34.5 ppm) was significantly higher than controls (1.0 ppm). Despite the significant differences in both ANA and mean hair Hg in riverine versus control communities, there was no significant association between hair Hg and ANAs [19]. In a similar trend, our study [23] of participants residing on Cheyenne River Sioux Tribe (CRST) Lands, a known area of MeHg contamination, found a relationship between fish consumption and elevated levels of ANA and specific autoantibodies, yet blood Hg itself was not associated with autoantibodies. In both the Amazonian Brazil and CRST studies, fish consumption, but not Hg measure in biological matrices, is associated with increased autoantibodies.

In seeming contrast, more recent studies published on Hg and autoimmune biomarkers in Hg-exposed populations in Columbia [24] and the Middle Atlantic Coast of the United States (Long Island) [25] show that fish consumption is significantly associated with increased levels of Hg in biological matrices, yet these Hg measures are not associated with altered levels of ANAs [24,25], rheumatoid factors (RF) [24], or cytokines [25]. It is difficult to isolate the effects of Hg exposure from eating fish with the effects of Hg exposure from other sources because Hg is an environmentally pervasive contaminant, and people who regularly consume Hg-contaminated local fish likely also encounter Hg through occupational or other enviromental exposures. In the

case of the studies in Amazonian Brazil [18,19] and Columbia [24], additional iHg exposures result from gold mining, while the studies of CRST [23] and Long Island [25] cite iHg exposures from emissions and industry. Although all studies discussed in this mini-review [18,19,23–25] show an increase in total Hg in biological blood and/or hair correlated specifically to reported fish consumption, only the Silva et al. Amazoninan Brazil [18] implies a full linkage from fish consumption to increased concentration of bodily Hg, and Hg body burden with increased autoimmune markers.

Discussion

The fact that fish consumption is associated with autoimmune markers in Amazonian Brazil [18,19] and CRST [23] studies may be due to additional exposures to contaminants implicated in immune dysregulation. Fish consumption likely serves as an exposure surrogate or composite exposure predictor. Participants in these studies reside in environments impacted by mine wastes that include other metals (gold, cadmium, arsenic) known to play a role in autoimmunity [28]. Additionally, pesticide exposure was not adjusted for in the Amazonian Brazil [18,19] and CRST [23] studies. Like Hg, pesticides are persistent environmental contaminants capable of bioaccumulating in fish and have been implicated in immune alterations [29]. The adjustment for pesticide exposure may explain the lack of autoantibody induction observed in the Columbia study [24] in spite of the fact that this population also resides in a gold mining setting.

Differences in genetic, metabolic, lifestyle, and total environmental exposure across populations are also likely contributors to the discrepeancies in findings. A notable difference among these human studies is the total body burden of Hg in the study

populations. The CRST, Columbian, and Long Island population studies all measured low levels of total blood Hg in comparison to the Amazonian Brazil studies, and no signifcant associations between total Hg and autoimmune markers were observed. This suggests that chronically high total body burden of Hg, rather than MeHg from fish, is associated with increased autoimmune markers. This idea is supported by additional studies published on Amazonian Brazil mining communities without reported fish consumption that showed positive associations between high total hair Hg and ANA, ANoA, and cytokines (IL-1β, TNF-α, IFN-γ) [16,22]. An alternative possibility to a minimum total Hg exposure, or an additional requirement, may be that effective induction of autoimmune markers requires the presence of both iHg and MeHg. iHg and MeHg have been shown in mice [26] and human PBMCs [16] to elicit differential immune responses with iHg favoring a Th2 response whereas MeHg favors a Th1 reponse. Furthermore, studies of Amazonian Brazil populations reported a high prevalence of malaria [16,18,22], which has been shown in mouse models to lead to the generation of antibodies that react with nuclear antigens [27]. This suggests that a convergence of factors; iHg, MeHg, and specific immune challenge, such as malaria infection increases the probability of autoimmunity.

Other than exposures to additional environmental contaminants, selenium (Se), and fatty acids consumed alongside MeHg in fish may account for some of the uncertainty in the associations between Hg-contaminated fish consumption and autoimmunity. A study of Hg miners in China who had correlated elevated Hg and Se found increased selenoproteins and glutathione peroxidase (GSH-Px) [30], which may mitigate the adverse effects of Hg exposure contributing to the development of

autoimmunity. The principle source of Se is through dietary animal protein [31], and some authors state that Se, like MeHg, biomagnifies within predatory fish [32]. Others suggest that Se accumulates at the base of the food chain and that significnt concentrations of Se may be ingested through plants grown in a Se-enriched environment [33]. A follow-up study in Amazonian Brazil found an inverse relationship between blood Hg and blood Se but no overall relationship between fish consumption and Se even though fish consumption was high [34]. Although the primary source of Se intake is unclear, Se and Hg are correlated in both the environment and the human body, and there is evidence that they have opposing mechanisms of action.

n-3 Poly-unsaturated fatty acids (n-3 PUFA) present in fish may also counteract the negative effects of Hg on the immune system. n-3 PUFAs are known to have the ability to regulate transcription factor activation and pro-inflammatory signaling pathways, and may modulate pathways involved in autoimmune disorders [35,36]. This potentially explains why the Long Island study [25], the only one to measure n-3 PUFAs in participants, found correlations between n-3 PUFAs and detection of ANA only at lower titer concentrations.

Finally, it is worth mentioning that common markers of autoimmunity such as ANA and ANoA are generally observed at low frequency at the conservative titers (1:80 or more dilute) used in the human studies cited here, and many cytokine measurements lie below the limit of detection. This, and limited population sample size, poses additional obstacles to reaching a firm conclusion about the role of MeHg fish in the development of autoimmune biomarkers.

Conclusions and Future Avenues of Study

The findings of the few human studies incorporating MeHg exposures through fish consumption do not provide a conclusive answer as to whether or not these exposures significantly contribute to autoimmune development. In our study with the CRST [23], which exhibits elevated levels of certain autoimmune diseases, the main question from community members was, "Is it safe to eat local fish?" Reframed, the question is, "Does MeHg from fish consumption exacerbate the development autoimmunity?" Current studies do not provide a clear consensus. It appears that high total Hg body burden is necessary in order to observe significant changes in autoimmune biomarkers. A combination of both iHg and MeHg exposures may be required to exacerbate autoimmune development, since the various forms of Hg affect the immune system differently. Because Amazonian Brazil populations evidenced both relatively high total Hg and increased likelihood of exposure to malaria, it is possible that development of Hg-driven autoimmunity in humans depends upon a convergence of factors: iHg, MeHg, and specific immune challenge, such as malaria infection. It is likely that nutritional elements in fish, including Se and n-3 PUFAs, attenuate the immune effects of Hg exposures. The limited evidence in human populations about the role of fish MeHg in autoimmunity concurs with the current public health consensus to retain or increase fish consumption, especially of species with lower MeHg, for nutritional benefits while decreasing other exposures to Hg.

To elucidate the question of whether or not MeHg through fish consumption contributes significantly to alterations in autoimmune markers in humans, a larger, more robust set of human studies is needed. Autoimmune biomarkers could be measured in

populations exposed to MeHg through fish consumption, beginning with the many communities world-wide in which Hg biomonitoring in fish tissue and/or human biological samples has already been done [39–48]. Estimated MeHg exposure, calculated from accurate species-specific tissue MeHg concentrations, should be modeled as a predictor alongside measures of iHg exposure with autoimmune biomarkers as the outcomes. This would help disentangle fish consumption's role in autoimmunity from that of other Hg exposures in order to inform public health recommendations.

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III. CHAPTER 3

Mercury, autoimmunity and environmental factors on Cheyenne River Sioux Tribal Lands

Published (Autoimmune Diseases)

Ong J, Erdei E, Rubin RL, Miller C, Ducheneaux C, O'Leary M, et al. Mercury, autoimmunity, and environmental factors on Cheyenne River Sioux Tribal lands. Autoimmune Dis 2014;2014. doi:10.1155/2014/325461.

ABSTRACT

Mercury (Hg), shown to induce autoimmune disease in rodents, is a ubiquitous toxicant throughout Cheyenne River Sioux Tribe (CRST) lands. CRST members may be exposed to Hg through fish consumption (FC), an important component of native culture that may supplement household subsistence. Our goals were to ascertain whether total blood Hg levels (THg) reflect Hg exposure through FC and smoking, and determine whether THg is associated with the presence of anti-nuclear antibodies (ANA) and specific auto-antibodies (sAuAb). We recruited 75 participants who regularly consume fish from CRST waters. Hg exposure through FC and smoking were assessed via questionnaires. Whole blood samples were collected from participants, and THg was measured using ICP-MS. ANA and sAuAb in serum were modeled using demographic and exposure information as predictors. Female gender, age, and FC were significant predictors of THg and sAuAb; self-reported smoking was not. 31% of participants tested positive for ANA≥2+. Although ANA was not significantly associated with Hg, the interactions of gender with Hg and proximity to arsenic deposits were statistically significant ($p<0.05$). FC resulted in a detectable body burden of Hg, but THg alone did not correlate with the presence of ANA or sAuAb in this population.

INTRODUCTION

For more than a century, mining from greater than 900 mines in the Black Hills, including gold mines in which Hg was used for amalgamation purposes, has released contaminants into watersheds draining onto CRST lands [1]. Additionally, approximately one ton of airborne Hg is emitted per year from coal power plants in Montana, Wyoming, North Dakota and South Dakota [2], and carried downwind to CRST lands where precipitation and dust wash this mercury out of the air into water and soil. Thus, Hg is virtually ubiquitous throughout the CRST reservation. Studies over the last decade conducted by the tribe, United States Environmental Protection Agency (USEPA) and University of Colorado [3] have documented high mercury concentrations in mid-flow water samples and sediment [4], invertebrates [5] and fish [5–7]. As a result of the widespread presence of Hg in the environment, fish consumption warnings have been posted along the Cheyenne River since 1974, yet no comprehensive health studies have ever been conducted in the CRST population to assess the health effects of consuming fish from tribal waters. In spite of posted warnings, CRST members still consume locally-caught fish for complex reasons. Fishing and fish consumption are not only important in Lakota culture, but high rates of poverty (~50%) [8,9] and unemployment (88%) [10] on the CRST reservation increase the community's likelihood of using fish to supplement household subsistence. Therefore, the safety of eating mercury-contaminated fish caught on tribal lands was a prime concern for CRST members. To address the CRST's environmental health concerns, a research partnership, *Environmental Justice on Cheyenne River*, was established in 2003 among the CRST Department of Environment and Natural Resources (DENR), the Black Hills Center for

American Indian Health, and the University of New Mexico Community Environmental Health Program (UNM CEHP). Through community forums and discussions with tribal leaders, the partnership identified a major concern that a perceived increase in autoimmune disease (AD) prevalence in the CRST population might be related to Hg exposures through fish consumption, as well as a widespread frustration that actual health studies had not occurred in spite of Hg warnings posted for nearly 40 years. Although de-identified numbers of autoimmune cases were obtained from Indian Health Service (IHS) data sources, interpretation of the prevalence is difficult in identification of an appropriate denominator, and determination of an appropriate comparison figure for Native American populations. Data on antinuclear antibody (ANA) prevalence in Native populations has not been evaluated. Prevalence of ANA in other US populations was recently derived from National Health and Nutrition Survey data (NHANES) [11,12], but values for Native American populations could not be extracted due to no representation in that sample. Reference values for specific AD in tribal populations relative to the US total population are also not readily accessible.

Since tribal populations are comparatively more homogenous than other studied US populations, it may be tempting to ascribe any elevations in AD in the CRST merely to genetics. However, while genetic susceptibility has long been acknowledged as an important causative factor in the development of AD, and evidence [13,14] exists that genetic composition may predispose CRST members to AD, it is estimated that genetic factors only account for one third of disease risk, and that gene-environmental interactions play a vital role in the onset of autoimmunity [15]. The growing role of environmental factors, including aluminum metal compounds and thimerisol in vaccines,

as adjuvants to the pathogenesis of autoimmunity has been studied extensively [16]. In addition, studies [17,18] indicate that Hg toxicity and autoimmunity may be synergistically enhanced by various infectious and non-infectious triggers. It is reasonable that chronic stimulation of the immune system by environmental Hg may act through similar mechanisms. To address the community's concerns and begin to address existing gaps in knowledge about the effects of chronic low-level environmental exposures to metals, we sought to systematically examine the relationships among fish consumption, THg and basic immune system markers in the CRST population in this study.

Existing knowledge about the effects of metals on the immune system comes mainly from the use of rodent models. In these models, relatively high doses of inorganic mercury administered to genetically-susceptible mouse strains lead to the development of lupus-like autoimmune syndrome, which includes increased circulating antibodies to nuclear targets (antinuclear autoantibodies, ANA) [16,17]. Further, exposure to inorganic *or* organic mercury exacerbates and accelerates the development of lupus-like disease in susceptible mouse strains [18–21]. Rodent models of mercury-induced autoimmunity [22–24], as well as their consistency with sex differences in autoimmune disease incidence observed in humans, suggest it is biologically plausible that Hg and other metals contribute to autoimmune pathogenesis in humans. Yet, with the exception of a few epidemiologic studies investigating the role of mercury amalgam fillings in multiple sclerosis [25,26], and studies of ANA and cytokines in mercury-exposed Amazonian Brazil populations [27–30], too few [31,32] have investigated the potential role of chronic environmental metal exposures as risk factors in the development of AD in

humans. While relationships between metal exposure and immune dysfunction have been demonstrated in animals, limited data exist in humans. Since Hg has long been linked to development of AD-like symptoms in animal models [17], we hypothesized that increased mercury exposure, primarily through fish consumption, would be associated with higher levels of circulating autoantibodies in the CRST population. In order to test this hypothesis and respond to community concerns, we modeled ANA and specific autoantibody concentrations in blood collected from CRST community members using THg, fish consumption, smoking, age, gender and proximity to high-concentration arsenic sediment deposits as predictors.

METHODS

Human Subjects

The protocol and study design was approved by Executive Committee of the Cheyenne River Sioux Tribe Tribal Council (Tribal Resolution #: E-302-08-CR and extended under E-343-2009-CR) and by the University of New Mexico Health Sciences Center Human Research Protection Office (HRPO#: 08-486). As de-identified serum samples were sent to the Scripps Research Institute Department of Molecular and Experimental Medicine, the Scripps Research Institute's Institutional Review Board provided approval for an analysis of serum ANA and specific autoantibodies.

Participants were recruited by using community-based communication tools and procedures previously developed by this team and applied in the *Environmental Justice on Cheyenne River* study. Outreach, enrollment and sampling were conducted in conjunction with local collaborators, notably Missouri Breaks Industries Research, Inc.

(MBIRI), who were crucial contributors in several previous federally funded research projects among Cheyenne River Tribal communities, and collaborating staff from the CRST DENR. The recruitment was targeted toward fishermen and their family members, who were known to local collaborators as regular consumers of fish caught from the Cheyenne River and its tributaries.

Written informed consent was obtained from a total of 75 adults living on the CRST Tribal Lands during the peak of fishing season. The study population includes members from multiple communities including Eagle Butte, Cherry Creek, Dupree, Timber Lake, Red Scaffold, Bridger, Takini and Howes (Figure 1). At each location, enrollment was conducted and biological samples were collected in community centers. These communities, of which some are in close proximity to rivers, lakes and ponds on CRST lands and others are not, include both commercial centers and rural areas, as well as members whose primary source of food is store-bought versus acquired from the local environment (subsistence lifestyle), and therefore reflects a wide range of potential exposures to Hg through fish consumption. Smoking status was also of concern as an alternate contributor to THg based on previously-reported increases in smoking on the CRST reservation [33], and the contribution to THg from cigarette smoking [34,35]. MBIRI team interviewers collected demographic (e.g. age, gender), health condition, fishing and smoking habit information through personal interviews conducted in English using a Centers for Disease Control (CDC)-developed fish consumption survey and our own short smoking exposure questionnaire. When participants needed information or clarification spoken in their native language, the community-certified nurse interviewers provided the answers.

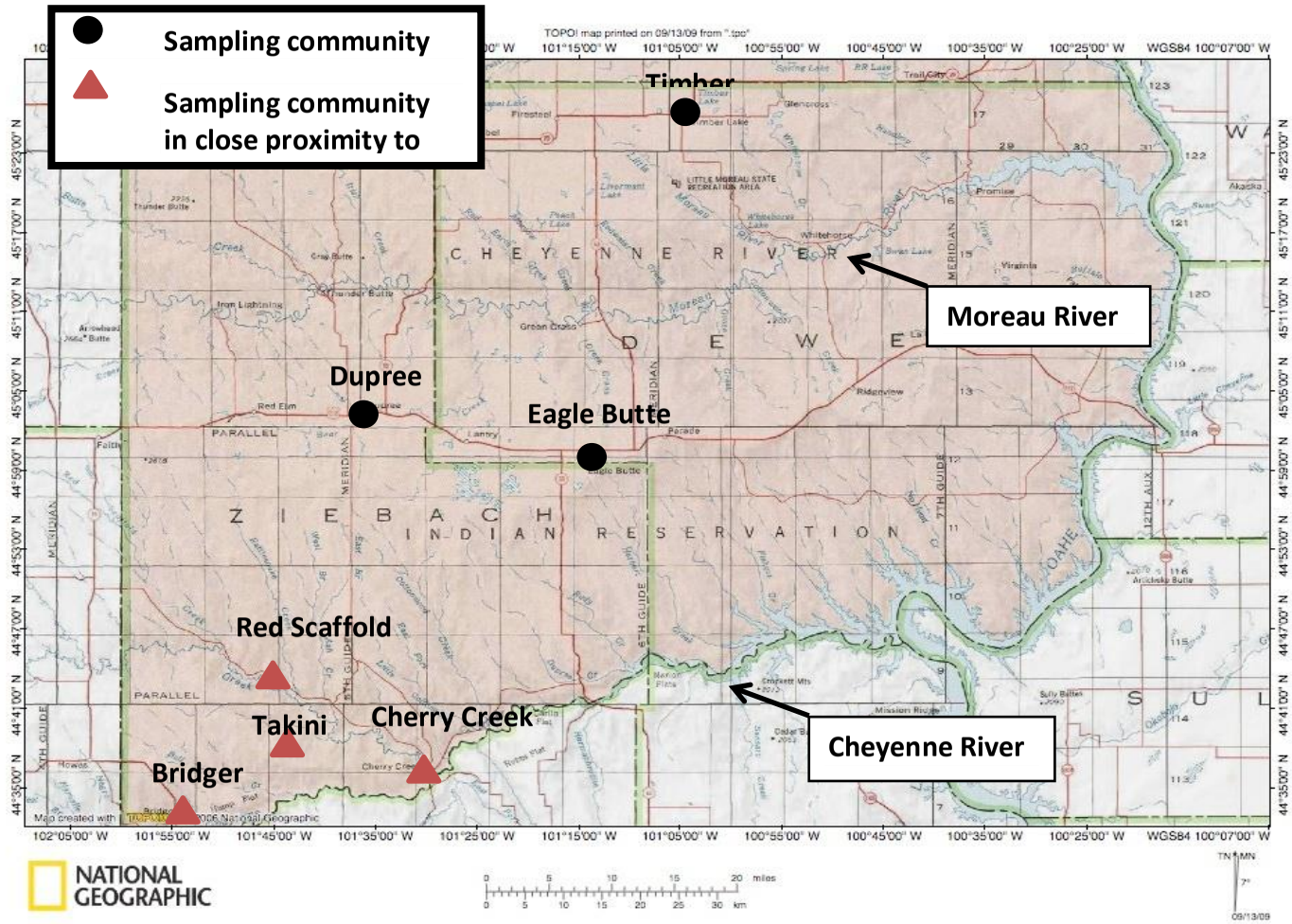


Figure 3.1. Map of Cheyenne River Sioux Tribal lands and sampling communities

Surveys

Fish Consumption

A CDC questionnaire, as well as local collaborators' knowledge of CRST community members' fishing habits, were used to assign a categorical rating of 1, 2 or 3 to each participant's fish consumption, with 1 designating minimal to no fish consumption, and 3 corresponding to high fish consumption. For reference to the local environment and consumption patterns, the safe amount of fish intake per month was previously recommended by our *Environmental Justice on Cheyenne River* study using DENR Hg measurements from local fish and USEPA guidelines [36]. One monthly-recommended serving was defined as one northern pike, two bass or perch, three walleye or four catfish. A rating of 1 denotes consumption of <1 serving of fish per month; 2 denotes 1-2 servings/month; and 3 denotes >2 servings/month.

Smoking

To account for smoking as both a potential source of Hg and contributor to immune system effects, participant smoking data were collected via questionnaire. The questionnaire was based on coauthor PNH's previous work [33] on smoking among tribal members, and was given to all participants in order to obtain self-reported information regarding smoking exposures. The questionnaire encompassed both direct as well as second-hand exposure to cigarette smoke. There were seven questions total; smoking score was coded as low (1) when fewer than two questions were answered affirmatively; medium (2) when 3-4 questions were answered affirmatively; and high (3) when greater

than five questions were answered affirmatively. A participant was considered an "active smoker" if he/she answered "yes" to the included question, "Do you smoke currently?"

Arsenic Proximity

During the analytic phase of this study, elevated sedimentary arsenic deposits were discovered in high land-use areas in close proximity to several of the sampling-site communities in this study (Figure 3.2). Ongoing collaborations among DENR, Dr. Lewis, and USEPA Region 8 are surveying residents and characterizing exposure pathways, frequencies and duration. However, as these deposits were identified subsequent to consent for this study, no arsenic biomonitoring data were obtained from the population in the original design, nor were exposure activities involving these sedimentary deposits identified. Due to studies in humans and animals indicating that arsenic suppresses autoimmunity [37,38], while mercury may either suppress or increase autoimmune response [28,32], a surrogate of participant arsenic exposure was also incorporated into models to address potentially-competing exposures. A binary surrogate for arsenic exposure was derived; the designations of "near" or "far" proximity to known quantified environmental arsenic deposits by USEPA were given according to self-reported participant residence data. The designation of "near" was given to participants who live in the communities of Cherry Creek, Takini, Bridger and Red Scaffold (Figure 3.1). Surveys of residents have identified potential exposure pathways which include common land-use practices such as fishing; herb, fruit and firewood gathering; inhalation of wood combustion products during sweat lodge and ceremonial practices; and roping/other horseback riding activities along the Cheyenne River near the identified alluvial arsenic deposits (Figure 3.2) (personal communication C. Ducheneaux

and J. Lewis). Participants residing in the Eagle Butte, Dupree and Timber Lake (Figure 3.1) communities more distal to the arsenic deposits were given a designation of "far" for arsenic proximity in this pilot assessment. This binary variable was incorporated to determine if further studies on the relationship of these exposures to AD were warranted.

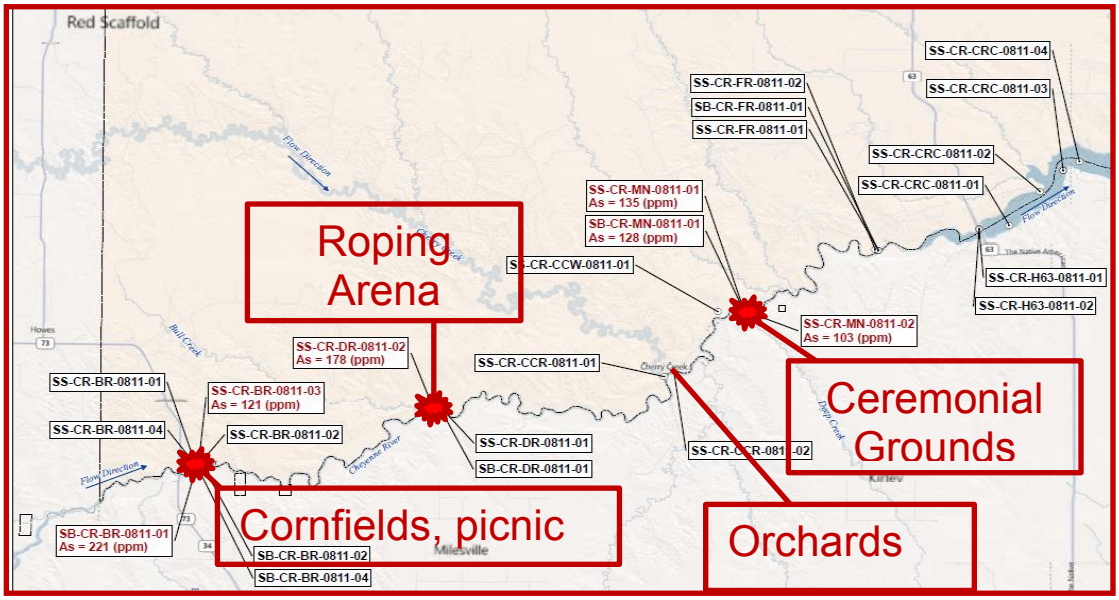


Figure 3.2. Map of arsenic sampling conducted by USEPA and CRST DENR. Concentrations of arsenic and exposure relevant sites are marked (personal communication C. Ducheneau

Biological Sample Collection

Blood and Serum Samples

Venous blood samples were collected by venipuncture at community centers or during home visits by a trained and certified phlebotomist or registered nurse. One red top (9 ml) for serum collection and one purple top (7 ml) Vacutainer tube were collected for biomonitoring from each participant. After clotting, serum samples were spun at 2,500 rpm for 10 minutes and separated into cryovials and placed into a -80ºC freezer. At a later

time point, sera were shipped to the UNM HSC laboratory and subsequently to the Scripps Research Institute.

Experimental use of collected biological samples

Biomonitoring

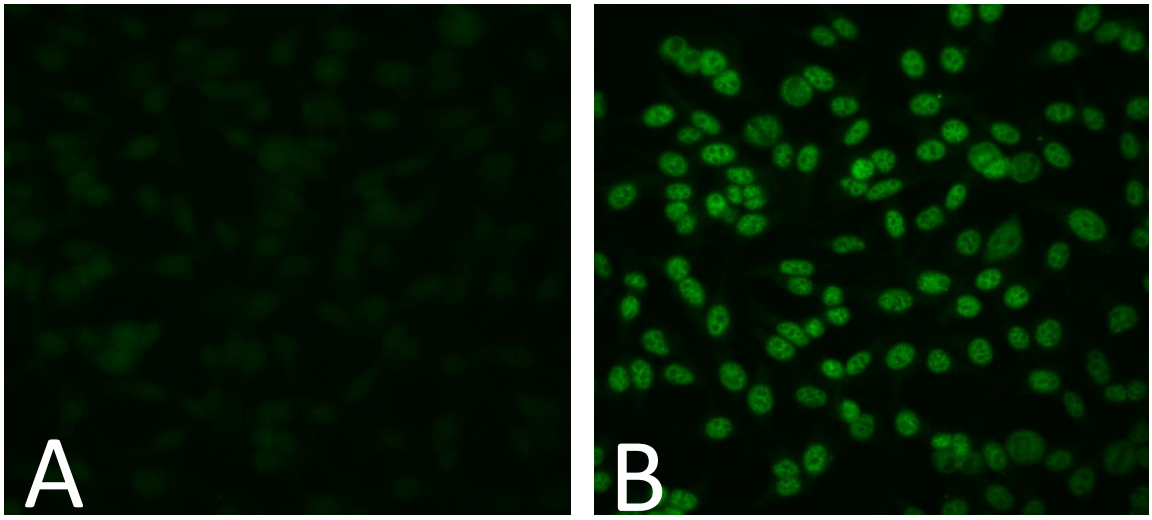
The EDTA-containing whole blood samples were transported to the CDC ONDIEH/NCEH Environmental Health Laboratory where inductively coupled plasma mass spectrometry (ICP-MS) was used to determine THg concentrations. The limit of detection was 0.32 µg/L.

Detection of Autoantibodies

Antinuclear antibodies (ANA)

The presence of ANA was determined by indirect immunofluorescence (IIF) microscopy using HEp-2 cells as substrate (MBL-BION, Des Plaines, IL) and Alexa Fluor 488 Goat Anti-Human IgG (H+L) (Life technologies, NY, USA) as detecting reagent. Sera were diluted 1:100 in serum diluent, and detecting reagent 1:200 with anti-Ig diluent as previously described [39]. Slides were viewed by a single observer (KMP) blinded to participant identity on a BH2-RFCA fluorescence microscope (Olympus, Lake Success, NY). Intensity of fluorescence was graded on a scale of 0–4+. A reading of ≥2+ was considered significant and further used in our statistical modeling. This cutoff value reflects a stricter value based on literature [40,41]. Example immunofluorescence images for ANA determination can be found in Figure 3.3 for negative (0) and ANA ≥2+ readings.

Figure 3.3. Examples of ANA determination by immunofluorescence. Human sera were incubated with HEp-2 cells followed by fluorescent anti-human IgG. The sample on the left (A) is ANA negative while the sample on the right (B) was considered 2+ ANA positive, showing fine speckled nuclear staining sparing the nucleolus.



Specific autoantibodies(sAuAb)

Commercially available kits (INOVA Diagnostics, San Diego, CA) were used as described by the manufacturer to detect and quantify serum autoantibodies to the following antigens: chromatin, Sm, RNP, SSA, SSA-52, SSB, Scl-70, RNA Pol III, CENP-A/B, Ribo-P, Jo-1, M2 EP (MIT3) and primary biliary cirrhosis (PBC) Screen, a panel of antigens (M2 EP, gp210 and sp100 IgG/IgA). Assay-specific positive controls were used to convert optical density values to units in order to determine whether the result of assays for a Sm, RNP, SSA, SSA-52, SSB, Scl-70, RNA Pol III, Ribo-P and Jo-1 were negative/equivocal (<20 units), weakly positive (20-39 units), moderately positive (40-80 units), or strongly positive (>80 units). The tests for M2 EP and the PBC screen were interpreted as being equivocal from 20.1-24.9 units and positive for >25 units. Centromere-A/B (CENP-A/B) has negative/equivocal results for <20 units, weak positive for 20-30 units, and a strong positive for >30 units. Chromatin has a negative/equivocal

reading <20 units, moderate positive between 20-60 units, and a strong positive >60 units.

Additional assays to chromatin, denatured DNA (single-stranded, dDNA), native DNA (nDNA) and histones were quantified by enzyme-linked immunosorbent assays (ELISA) as previously described in [42,43]. Briefly, Immulon 2HB microtiter plates (Dynex Laboratories, Inc., Alexandria, VA) were coated with antigen at 2.5 μg/ml concentrations. For the anti-chromatin assays, in-house-prepared H1-stripped chromatin was used as the solid-phase antigen. S1-nuclease (Invitrogen)-treated DNA (Calbiochem) was used in the anti-native DNA assay, and DNA was heated for 10 min and then quickly cooled for preparation of the dDNA antigen. Plates were pre-coated with poly(lys-phe) (Sigma) prior to addition of DNA. Total histone was from Worthington. Serum samples were diluted 1:200, and incubated on the plate for 2 hours at room temperature with gentle shaking. Each sample was run in duplicate. The bound antibodies were detected with peroxidase-conjugated anti-human IgG (Southern Biotech, AL) and 2,2′ azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (MP Bioproducts) as the secondary substrate. Optical densities (OD) beyond the range of direct measurement at 1 h in the ELISA were extrapolated from OD at earlier time-points as described [44]. Positive and negative control sera were always included in each assay, and values determined in different assays were normalized by multiplying by the ratio of the reactivity of the positive control sera tested in each assay.

Statistical Analysis

Total blood mercury results lower than the limit of detection (LOD=0.32 µg/L) were analyzed with the value of $LOD/\sqrt{2}$, because fewer than 50% of participants had a biomonitoring value <LOD. Total blood mercury results are presented as median values with the interquartile range, since the median is a better indicator of the true population value for the distribution of the collected data. The mean and 95% confidence interval for THg are also presented for ease of comparison with published NHANES population data. When comparing groups (e.g. male vs female) in relation to THg and ANA status, Fisher's exact test was used.

To characterize the complex exposures on CRST lands and their relationships to immune system responses and autoantibody production, several statistical models incorporating biomonitoring data, fish consumption score, smoking exposure score, distance to arsenic contamination and immune system markers were developed. The approaches included multiple linear, logistic and Poisson regression models to evaluate relative contributions of environmental exposures to circulating autoantibodies. They also included accepted risk factors such as age and gender. Multiple linear regression was used to model THg in relation to environmental exposure and risk factors while logistic regression was used to model ANA ≥2+ in relation to predictors. Poisson regression was used to model the numbers of specific autoantibodies (both determined via INOVA and additional assays) with environmental exposure and risk factors. Poisson models accommodate count information with non-normal distribution, thereby enhancing the analytical capacity to understand the exposure factors' underlying contribution to risk. Full models were fitted using all demographic, biomonitoring and exposure data as

predictors. Reduced models were selected using the Akaike information criterion (AIC), which is a measure of the relative quality of a statistical model. The openly-available statistical software R [45] and the stepAIC function from the package MASS [46] were used to complete this AIC model selection where,

$$AIC = 2k - 2\ln(L)$$

And $k = 2$ and L is the likelihood of each model. The AIC selection criterion minimizes the distance between the predicted values of the model and the true values while also favoring models with fewer parameters.

Specific approach to analyze specific autoantibody results from INOVA assays

Since positivity for individual specific autoantibodies was expected to be lower in frequency, we pooled all participants who tested positive for any specific autoantibodies to examine an overall prevalence of specific autoantibodies and the contributing exposure factors. By summing the number of specific autoantibodies for which each participant tested positive, and then conducting Poisson regression on the count variable generated, biomonitoring data and exposure data were incorporated as well. This count variable also makes biological sense because it follows established clinical AD diagnosis criteria; individuals present with different specific autoantibodies.

Approach to analyze specific autoantibody results

Poisson regression was used to model several combinations of autoantibodies that would otherwise be rarely detected. The combinations modeled were selected in order to examine different possible scenarios of positive autoantibody response. Individuals with

detectable autoantibody response were classified into groups according to the following scenarios based on literature [42–44]:

a) Presence of any autoantibody response (nDNA, dDNA, histone, chromatin)

b) Detectable levels of potentially environmentally-related autoantibodies (dDNA and histone)

c) Disease-associated autoantibodies (nDNA and chromatin).

Results of these models are summarized in several tables presented in the next section.

Because anti-chromatin autoantibodies were detected using both INOVA and in-house assays, we evaluated the reproducibility of this antigen. We applied a non-parametric correlation (Spearman r-value) and used a z-score.

Reporting of significant results.

While our primary results will follow a more standard reporting cut-off of $p<0.05$, we will report those with probabilities up to 0.1 to guard against Type 2 error and to ensure comprehensive consideration of predictors in designing follow-up investigations. This decision is warranted given 1) the importance of the results to the communities, 2) the lack of prior studies in this area and in this population, and 3) the lack of biomonitoring data at this time on other potential environmental exposures including arsenic resulting in imprecise measures for that variable.

RESULTS

Mercury Exposure and Population Characteristics

Population characteristics, including gender, smoking score, fish consumption score, community size, and proximity to identified high-concentration sedimentary arsenic deposits are summarized in Table 3.1. Total blood mercury concentrations (THg) ranged from below the limit of detection (LOD, 0.32 µg Hg/L) to 4.14 µg Hg/L, with a median lower than the LOD (Figure 3.4). For most population characteristic categories, the median THg was below the LOD, with the exception of males (0.37 µg Hg/L) and participants with "medium" or "high" fish consumption scores (0.35 and 0.54 µg Hg/L, respectively.

Total blood mercury in the CRST depended on gender, age and fish consumption but not smoking. The reduced multiple linear regression modeling results for THg as a response with demographic and exposure information as predictors are summarized in Table 3.2. Male gender and older age were significant predictors of THg ($p=0.0084$ and 0.022, respectively); fish consumption approached significance as a predictor for THg ($p=0.053$).

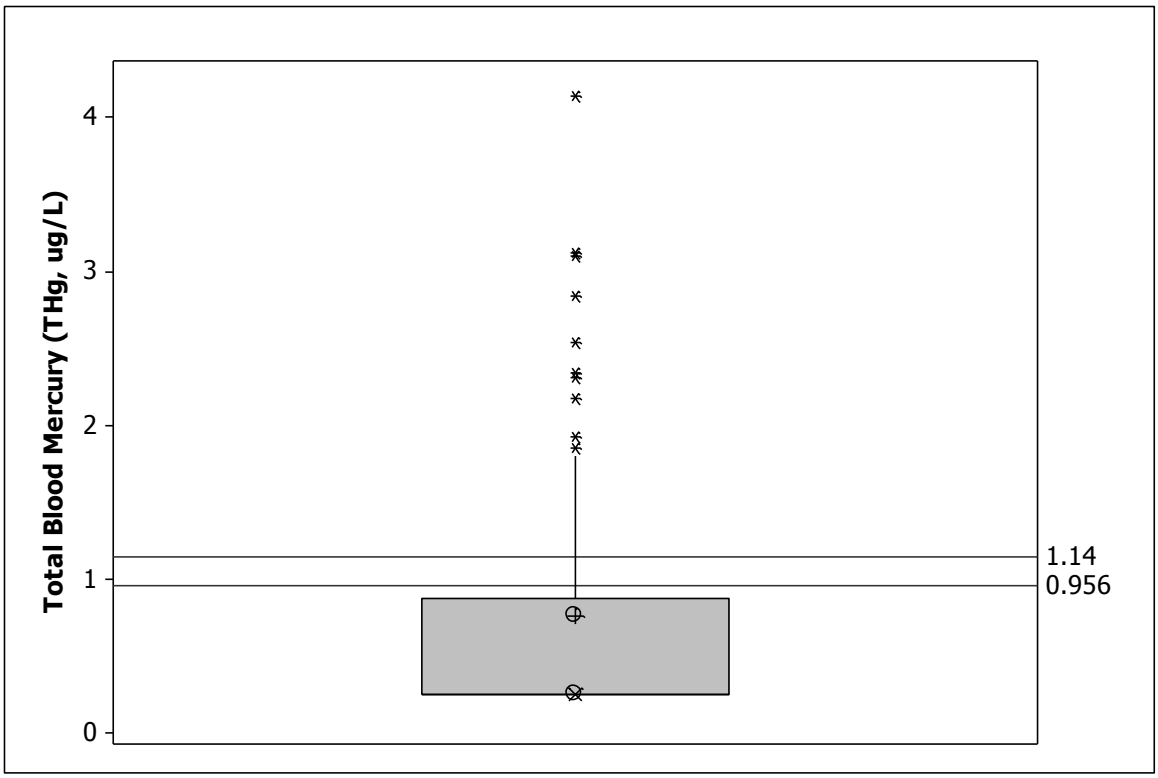


Figure 3.4. Scatterplot of total blood mercury (THg) for sample population with median denoted by an encircled "X," and mean denoted by an encircled cross. The reference lines at 0.956 µg/L and 1.14 µg/L indicate the 95% CI for THg in the US population from NHANES [41].

Table 3.1. Biomonitoring and ANA 2+ results linked with study participant characteristics

Population characteristic	***N***	**Participants with THg >LOD***	**Hg biomarker median (inter-quartile range)**	**Hg biomarker mean (95% CI)**	**ANA reading ≥2+ (*n*, %)**
All participants	75	36 (36%)	<LOD (<LOD-0.87)	0.75 (0.55-0.95)	23(31%)
Gender					
Male	38	23 (61%)	0.37 (<LOD-1.81)	1.01 (0.67-1.37)	2(5%)
Female	37	13 (35%)	<LOD (<LOD-0.56)	0.48 (0.32-0.63)	9(24%)
Smoking Score					
1 (Low)	32	17 (53%)	0.37 (<LOD-0.74)	0.67 (0.41-0.92)	8(25%)
2 (Medium)	18	8 (44%)	<LOD (<LOD-1.24)	0.82 (0.36-1.28)	3(17%)
3 (High)	25	11 (44%)	<LOD (<LOD-1.21)	0.81 (0.39-1.23)	7(28%)
Active Smoker					
Yes	42	19 (45%)	<LOD (<LOD-0.96)	0.77 (0.49-1.05)	4(10%)
No	31	16 (52%)	0.37 (<LOD-0.87)	0.76 (0.40-1.06)	7(23%)
Fish Score					
1 (Low)	41	17 (59%)	<LOD (<LOD-0.60)	0.59 (0.36-0.82)	7(17%)
2 (Medium)	18	10 (56%)	0.35 (<LOD-1.53)	0.90 (0.38-1.43)	2(11%)
3 (High)	16	9 (56%)	0.54 (<LOD-1.89)	0.99 (0.51-1.48)	2(13%)
Arsenic Proximity					
Yes	23	7 (30%)	<LOD (<LOD-0.52)	0.45 (0.25-0.64)	7(30%)
No	52	29 (56%)	0.37 (<LOD-1.27)	0.89 (0.62-1.16)	4(8%)
*LOD = 0.32 µg/L					

Table 3.2. Reduced model (multiple linear regression) for total blood mercury

	Estimate	***p*-value**	**Std. Error**
Intercept	0.412	0.46	0.56
Gender*	-0.545	*0.0084*	0.2
Age	0.0181	*0.022*	0.0077
Smoking Score	-0.0127	0.91	0.12
Fish Score	0.246	0.053	0.13
Arsenic Proximity	-0.526	*0.020*	0.22
p-values less than or equal to 0.05 are italicized. *Male gender was used as the reference, so the estimate describes the effect of being a female.			

Prevalence of ANA in the CRST population

Antinuclear antibodies (ANA) were analyzed in serum samples from all participants. Data are presented in Table 3.1 and representative images of negative and ANA ≥2+ readings are shown in Figure 3.3. Approximately thirty-one percent of participants had an ANA reading ≥2+. For readings ≥2+, ANA prevalence was significantly higher in women than men (24% vs 5%; p=0.025). ANA prevalence was also larger in community members living in proximity to high-concentration sedimentary arsenic deposits (30% vs 8%; p=0.028).

Table 3.3. Point estimates and 95% confidence intervals (CI) for coefficients and odds ratios (OR) for fitting logistic regression models for ANA ≥2+

	OR	**95% CI**	**p-value**
(Intercept)	0.011	N/A	0.93
Age	1.1	0.96-1.17	0.081
Gender	2.4	0.046-645.5	0.37
THG	0.4	0.045-1.75	0.89
Fish Score	2.9	0.56-20.70	0.092
Gender:THG	13.8	0.97-487.8	*0.026*
Gender:Fish Score	0.1	0.0013-1.12	0.97
Gender:Arsenic Proximity	27.1	0.68-2101	*0.040*
Arsenic Proximity	0.3	0.015-4.26	0.82
p-values less than or equal to 0.05 are italicized.			

Gender and fish consumption were significant predictors of ANA ≥2+, and gender modifies the effects of environmental exposures with respect to ANA. The logistic regression model information is shown in Table 3.3. Age and fish consumption are borderline predictors ($p<0.10$) of ANA ≥2+ ($p = 0.081$ and $p = 0.092$, respectively), with age and fish consumption positively associated with the probability of ANA ≥2+ level of circulating ANA. Gender, THg and proximity to arsenic, by themselves, do not strongly correlate with the probability of ANA ≥2+; however, the *interactions* of gender with THg and arsenic proximity are significant, and their odds ratios are greater than one (OR=13.83, p=0.026 and OR=27.71, p=0.04, respectively).

Specific Autoantibodies in the CRST Population

Of the panel of several specific autoantibodies tested for in the collected sera, autoantibodies to SSA, SSA/52, CENP-A/B, M2 EP and the antigens in the primary biliary cirrhosis (PBC) panel were noteworthy. These results are summarized in Figure 3.5 and Table 3.4. Fifteen percent of participants tested positive for autoantibodies to M2 EP while 24% were positive for autoantibodies to the PBC panel. The number of specific autoantibodies detectable by INOVA kit increased with female gender and fish consumption score. Information for the reduced Poisson model for the number of detectable specific autoantibodies using INOVA assays can be found in Table 3.5a. The number of specific autoantibodies detectable from INOVA assays was associated significantly with female gender ($p=0.0064$). The model indicated that the mean number of specific autoantibodies detectable in serum is increased by a factor of 6.5 in female versus male community members. Age and fish consumption had significant ($p=0.012$ and $p=0.0073$, respectively), but smaller effects on the number of specific autoantibodies in the collected serum samples. In particular, the mean number of specific autoantibodies detectable by INOVA assay were 2.6 times greater in participants with a high (3) versus a low (1) fish consumption score. The number of participants positive for autoantibodies to native DNA, histone and chromatin using in-house assays was small in our study. No significant associations were found between any demographic or exposure predictors, including smoking, and various combinations of in-house autoantibodies except in the case of dDNA and histone. The values for the reduced model can be seen in Table 3.5b. Fish score was a significant predictor ($p=0.035$) of the number of subjects with elevated

anti-dDNA and anti-histone autoantibodies detectable using in-house assays. An increase of one fish score category predicts a 2.5 factor increase in the number of dDNA and histone autoantibodies. Smoking was a borderline predictor (p=0.065) with a 0.4 factor of decrease in the number of dDNA and histone autoantibodies for an increase of one smoking category.

Anti-chromatin positivity and reproducibility between INOVA and additional assays was confirmed in all positive serum samples (5/75); there was 100% agreement in detection using INOVA and in-house assays. The mean number of specific autoantibodies detectable in serum is increased by a factor of 6.5 in female versus male community members. Age and fish consumption had significant (p=0.012 and p=0.0073, respectively), but smaller effects on the number of specific autoantibodies in the collected serum samples. In particular, the mean number of specific autoantibodies detectable by INOVA assay were 2.6 times greater in participants with a high (3) versus a low (1) fish consumption score. The number of participants positive for autoantibodies to native DNA, histone and chromatin using in-house assays was small in our study. No significant associations were found between any demographic or exposure predictors, including smoking, and various combinations of in-house autoantibodies except in the case of dDNA and histone. The values for the reduced model can be seen in Table 3.5b. Fish score was a significant predictor (p=0.035) of the number of subjects with elevated anti-dDNA and anti-histone autoantibodies detectable using in-house assays.

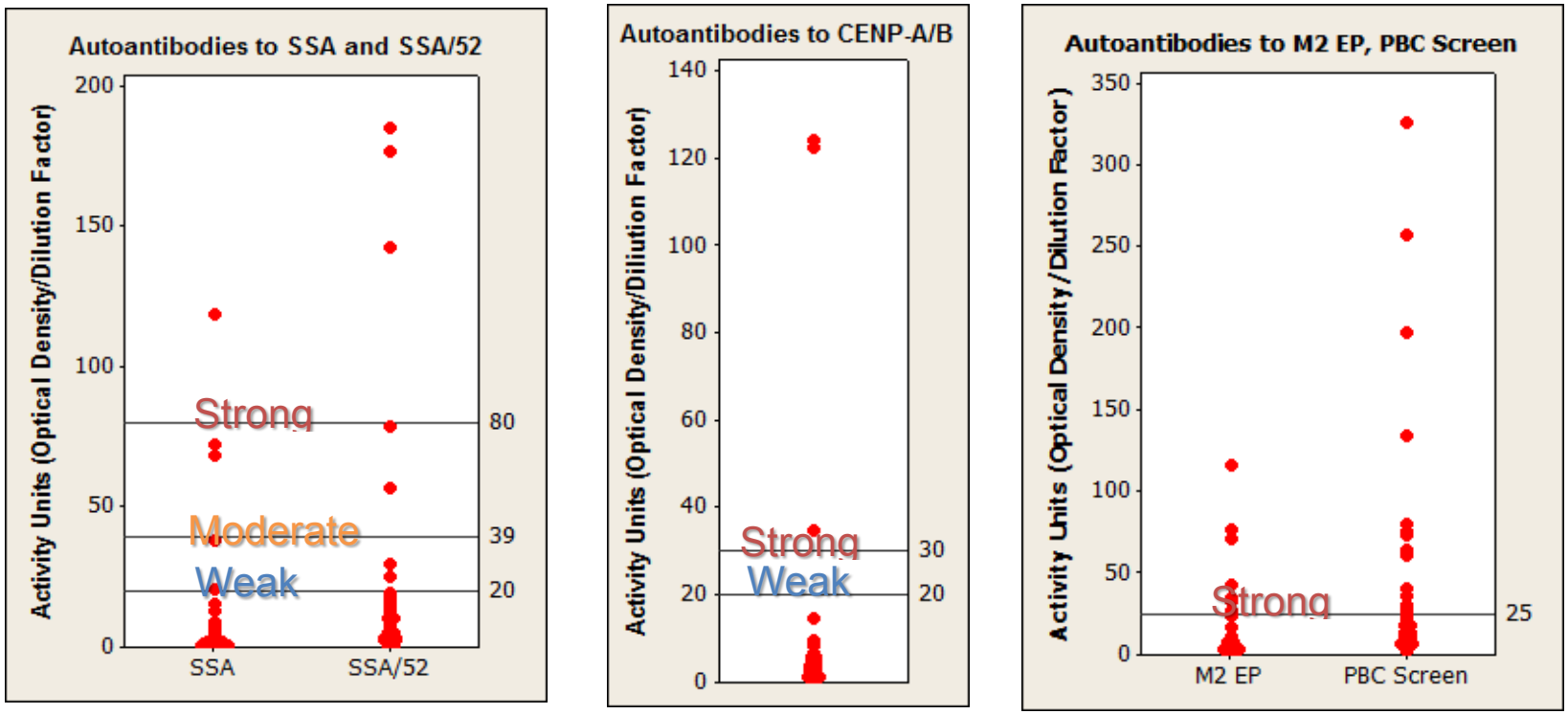


Figure 3.5. Dot plots of the activity units of participants for specific autoantibodies with clinical labels for clinical cutoffs. Note: In clinical practice there is no "moderate" positive reading for CENP-A/B and no "weak" or "moderate" positive readings for M2 EP and the PBC screen.

Table 3.4. Results of selected specific autoantibody results from the CRST population sample

n = 75 Autoantibody	Negative	Moderate Positive	Strong Positive	Total Positive
SSA	72 (96%)	2 (3%)	1(1.3%)	3 (4%)
SSA-52	70 (93%)	2 (3%)	3 (4%)	5 (7%)
CENP-A/B	72 (96%)	0 (0%)	3 (4%)	3 (4%)
M2 EP	64 (85%)			11 (15%)
PBC Panel	57 (76%)			18 (24%)

Table 3.5a. Model (Poisson regression) for the number of detected specific autoantibodies using INOVA assays

	Factor of Change	**95% CI**	**p-value**
(Intercept)	0.0	0.00-0.18	0.0012
Gender*	6.5	1.69-24.78	*0.0064*
Age	1.0	1.01-1.04	*0.012*
Fish Score	1.6	1.13-2.16	*0.0073*
Smoking Score	1.9	0.60-5.77	0.29
Gender:Smoking Score	0.5	0.25-1.01	0.053
p-values less than or equal to 0.05 are italicized.			

Table 3.5b. Model (Poisson regression) for number of detected specific denatured DNA and histone autoantibodies from in-house assays

	Factor of Change	**95% CI**	**p-value**
(Intercept)	0.2	0.004-9.831	0.406
Age	1	0.919-1.055	0.658
Smoking	0.4	0.127-1.064	0.065
Fish Score	2.5	1.067-5.980	*0.035*
Arsenic Proximity	0	0.000-3.752	0.117
Age:Arsenic Proximity	1.1	0.984-1.244	0.092
p-values less than or equal to 0.05 are italicized.			

An increase of one fish score category predicts a 2.5 factor increase in the number of dDNA and histone autoantibodies. Smoking was a borderline predictor (p=0.065) with a 0.4 factor of decrease in the number of dDNA and histone autoantibodies for an increase of one smoking category.

Anti-chromatin positivity and reproducibility between INOVA and additional assays was confirmed in all positive serum samples (5/75); there was 100% agreement in detection using INOVA and in-house assays.

DISCUSSION

We assumed that, due to consumption of locally-caught fish, community members would have elevated levels of total blood mercury (THg). We hypothesized that THg would correspond to an increased level of autoantibodies, as has been shown in animal models

[17]. Contrary to expectations, although Hg deposition in fish tissue had been documented in CRST sources by DENR, the detected THg levels in participants were low with a median THg <LOD, despite sampling during the middle of fishing season when fish consumption was considered to be maximal. The median THg for all participants (<LOD) was lower than the published results of the NHANES survey [47]. NHANES reported a mean THg of 0.944 µg/L for those 12 years and older [47]. Native American populations were not stratified in that study; the data were compiled under "other" ethnicity. The low levels of blood mercury among the CRST members found in our study confirmed the THg levels reported in a 2008 collaborative study between our team and CDC [48,49]. Potential reasons for the observed low THg include variation due to race/ethnicity and possible physiological and metabolic changes among CRST community members, or possible alterations in deposition and clearance with repeated exposure in this population.

While gender, age and fish consumption showed an impact on THg levels in the CRST population, smoking did not (Table 3.2). This finding is puzzling since 56% of the participants reported current smoking, yet only 45% of that group had THg above the detection level. The trends in our data parallel those seen in the NHANES survey [50]. Males have greater mean THg versus females, and THg increases with age. Males may consume larger quantities of locally-caught fish, or engage in activities that increase dust and particulate exposures to mercury (e.g. agricultural work, horse-tending). The age-dependent increase in THg found in this study, as well as in Wolkin et al. [48] and the NHANES survey [50], is likely due to the accumulation of metals in the body over time.

Thirty-one percent of participants had an ANA reading ≥2+. ANA production could be associated with chronic toxicant exposure, which introduces self-antigens to antigen presenting cells, resulting in the breakdown of T-cell tolerance. While no single predictor was significantly associated ($p<0.05$) with ANA ≥2+, fish score was a borderline predictor ($p=0.092$). A larger proportion of ANA-positive participants was female, which concurs with the literature and clinical findings about autoimmune diseases [24], and may support a possible role for female hormones in AD and immune dysregulation. Although THg and proximity to high-concentration arsenic deposits, by themselves, did not correlate with the probability of ANA ≥2+, the *interactions* of female gender with THg and female gender with arsenic proximity are significant ($p=0.026$ and $p=0.040$, respectively) and the odds ratios were large (OR=13.8 and 27.1, respectively). Gender differences may reflect alterations in the molecular mechanisms by which gender-specific detoxification occurs within the human body.

Another interesting finding is that current smokers were less likely to have ANA≥2+ results (Table 3.1). Additionally, the specific autoantibody model estimates for smoking were negative with ORs less than one (Table 3.5a and 3.5b), suggesting a protective effect of smoking. The fact that fewer autoantibodies were detected in this subgroup of smokers sheds lights on probable molecular mechanisms by which smoking induces immunosuppressive effects.

There were significant associations between predictor exposure variables and the presence of autoantibodies to dDNA and histone (Table 3.5b). This is potentially similar to previously-observed instances of xenobiotic-induced antibody responses such as drug-

induced lupus [42]. Autoantibody production to dDNA and histone may also be linked to epigenetic changes triggered by environmental stimuli.

The CRST population exhibited strong positivity for M2 EP autoantibodies and autoantibodies detectable with the PBC screen, both of which are associated with liver diseases. It is possible that the medical problem of high rates of idiopathic liver cirrhosis in Sioux communities (personal communication J. Henderson) may have environmental etiology. Similar findings were reported among Alaskan Natives [51]. As with ANA≥2+ models, fish score was a significant predictor of *specific* autoantibodies using both detection methods ($p<0.01$ for INOVA kit detection and $p<0.05$ for in-house ELISA).

As to the specific mechanisms responsible for mercury and metal/metalloid-induced autoimmune responses in the CRST population, several mechanisms should be considered. In susceptible individuals, environmental metals may behave as adjuvants that prolong or enhance antigen-specific immune response through various mechanisms such as molecular mimicry [52], polyclonal activation of B cells [53], bystander activation [54] and epitope spreading [55]. Additionally, chronic exposures to environmental metals, including Hg and arsenic, are well-known to induce oxidative stress. As has been characterized with thimerisol [56], this oxidative stress could lead to sensitization of inositol 1,4,5-triphospate (IP3) receptors, resulting in enhanced intracellular calcium release and subsequently the dysregulation of immune cells and autoimmunity. Another possibility includes the role of chronic gut exposures to ingested dietary nanoparticles of soil and minerals, which induce inflammasome production and the breakdown of immune tolerance via enhanced gastrointestinal antigen presentation. Since fish consumption was an important predictor of antibody production in this study,

dietary exposure may be one potential pathway through which molecular markers of autoimmunity are generated, especially among Native community members who are more likely to inhale and ingest large quantities of dust and metals due to their rural location, cultural practices, and subsistence and agricultural activities.

We hypothesize that fish consumption reflects multiple exposures, including *co*-exposures to mercury, arsenic and other environmental toxicants, such as pesticides, pharmaceuticals and infectious agents. In animal and cell studies, Hg toxicity and autoimmunity is synergistically enhanced by co-exposure to additional xenobiotics. These ideas will be explored in future studies, and additional activities that increase inadvertent exposure to toxicants will also be examined. Future studies will include a larger sample size, participant AD medical record history, and biomonitoring for arsenic and smoking exposures in order to address this study's limitations. We also acknowledge that technical issues with indirect immunofluorescence assays (IIFA) for the detection of ANA limit the comparability of these data to other population information and previous publications. However, IIFA is the gold-standard technique for ANA detection [57], and we attempted to minimize variability by using only one evaluator of staining (KMP, coauthor).

In this study, compelling evidence that the CRST population exhibited elevated levels of both ANA and specific autoantibodies was found. The observed results highlighted environmental toxicants that may contribute to autoantibody production in this population and also underscored the need to characterize the CRST communities' lifestyles and behaviors to better understand how complex exposures contribute to autoimmune health effects. There is a large knowledge gap concerning environmental

influences on the development of AD, and it is imperative that they be addressed within the context of environmental health disparities issues, particularly in tribal communities. Information will empower CRST community members and leaders by aiding them in making informed decisions about health, health services, the environment, and the preservation of their culture, in which for example fishing plays and should play vital role.

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IV. CHAPTER 4

Chronic Community Exposure to Environmental Metal Mixtures is Associated with Selected Cytokines in the Navajo Birth Cohort Study (NBCS)

Work in progress

ABSTRACT

The Navajo Birth Cohort Study (NBCS) was established to address community health concerns about chronic exposure to metals from abandoned uranium mines and waste sites. Many tribal populations are characterized by health disparities including infection, kidney function, diabetes, cancer, and autoimmune disease (AD), which are all mediated by the immune system. In particular, community members were concerned about perceived elevated prevalence of AD. Based on past and ongoing work with Navajo Nation and other tribes, we hypothesize that chronic low-level environmental exposure to metal mixtures from mine waste is associated with immune system differences. In this study, we used population samples (N = 120) to analyze and model metals/metal exposure profiles with serum cytokine expression. Samples of whole blood and urine were collected from NBCS participants and analyzed by Centers for Disease Control and Prevention (CDC) laboratories for a panel of 35 metals. We used Meso Scale Discovery (MSD) multiplexing technology to measure six circulating cytokines (IFNα, IFNγ, IL-4, IL-7, IL-17, IL-29) known to be related to autoimmunity. To begin to better understand exposure patterns and the effects of mixed metals, we analyzed metal and cytokine data using Spearman's correlation coefficients, univariable and multivariable linear regression, and profile regression. From these, we arrived at a reduced list of metals (As and As species, Mn, Hg, Pb, U, Se, Cs) that appear to influence cytokine levels. Profile regression also identified distinct exposure profile subsets ("exposure clusters" (EC)) associated with differences in levels of two cytokines (IFNγ and IL-7), most notably differences in participants with high concentrations of arsenic species in comparison to the lower metal EC reference group. Our data demonstrate that there are differences in

cytokines based on single metal, as well as metal profile, exposures. It is important to understand the relationships between chronic mixed metal exposures and immune alterations to better understand the potential health effects related to exposure. This would ultimately enable more effective health risk assessment and interventions, as well as aid in environmental clean-up and exposure reduction.

BACKGROUND AND INTRODUCTION

There are more than 160,000 abandoned hard rock mines in the western United States (US), and over 500,000 mine waste sites (Figure 4.1) [1]. As a result, 40% of watersheds in the western US are contaminated by mine waste and related metals [2]. Mining waste sites are often located on or contiguous to the watersheds of tribal lands, and mobilized wastes may migrate through the environment. Due to proximity and traditional or cultural practices, Native American community members are more likely to be in contact with mines/mine waste sites, or metal mixtures that have migrated from these sites.

This is true of Navajo Nation (NN), which is located in the Four Corners Region of the Southwestern US with a land area equivalent to the state of West Virginia (Figure 4.2). NN is the largest Native American reservation in the US, covering parts of Arizona, New Mexico, and Utah. Although active mining and milling on NN ended in 1986, the legacy from the atomic bomb and Cold War Era production includes 521 abandoned uranium (U) mines and >1100 of the 10,400 U waste sites identified in the western US (Figure 4.2). In addition to U, the wastes associated with these sites contain multiple metals and metalloids including, but not limited to, those cited by the World Health Organization (WHO) as of public health concern: arsenic (As), copper (Cu), lead (Pb), cadmium (Cd), and mercury (Hg) [3]. Navajo Nation community members may be chronically exposed to these metal mixture wastes through multiple pathways: consumption of local water and crops, contact with contaminated soil and dust from mine features, and inhalation of metals released from combustion for home heating. Drinking water is of primary concern, because 8-10% of unregulated water sources serving the

>30% of Navajos without access to public water systems (PWS) exceeded the U maximum contaminant level (MCL) while nearly 15% had elevated As [4]. Additionally, major PWS's on Navajo Nation are known to have been repeatedly out of compliance with one or more water standards for metals [5]. Traditionally Navajos have consumed locally-grown crops, locally-grazed cattle, and locally-foraged tea, all potential exposure pathways due to metal contaminant uptake from the soil. Combustion of local wood and coal for home heating and cooking, as well as emissions from the coal-fired Four Corners Generating Station power plant must also be considered. Combustion of coal, in particular, is a well-documented source of exposure to arsenic and mercury [6]. Numerous publications document arsenic- and mercury-associated immune alterations, including our mini-review of environmental mercury as a potential factor in the development of autoimmunity [7] and our manuscript examining associations between arsenic, mercury, and autoantibodies [8].

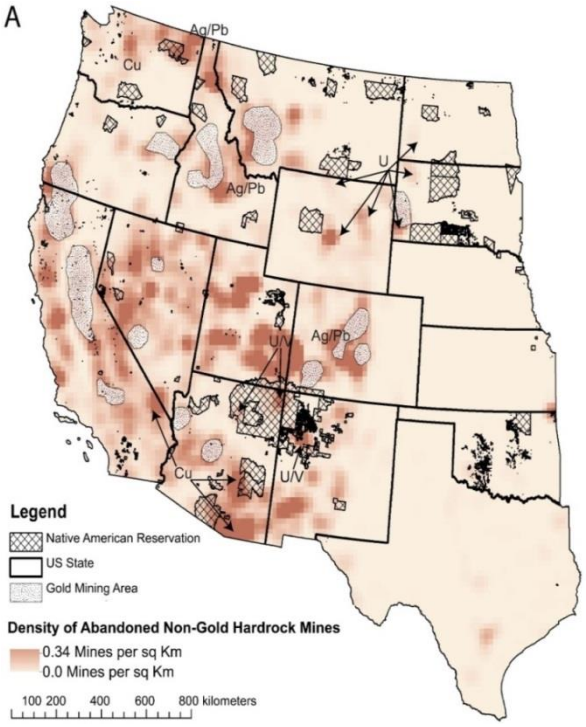

Figure 4.1. Map of the western United States showing the locations of Native American reservations and the density of non-gold hard rock mines

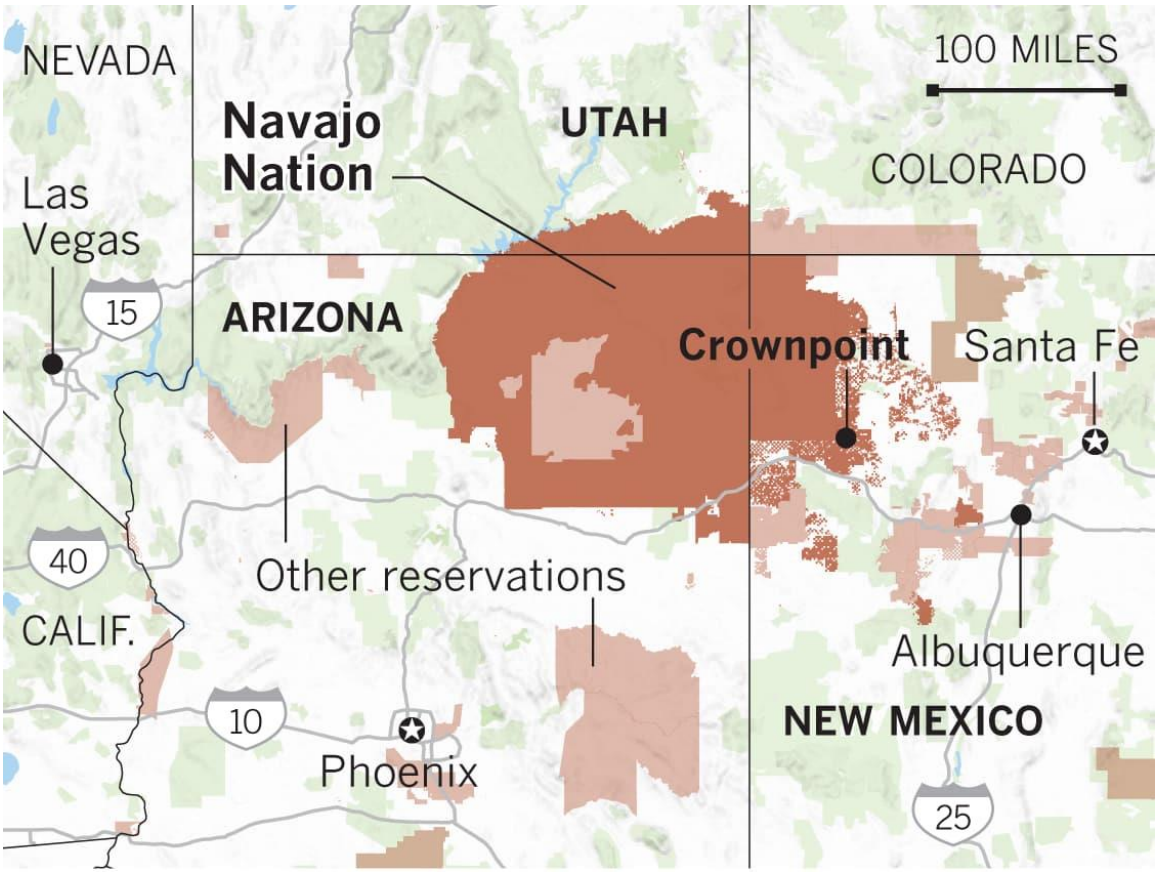

Figure 4.2. US Census Bureau map of Four Corners Region with Navajo Nation marked in dark brown.

Although Navajo communities have long been concerned that environmental exposure to mine waste contributes to poor health outcomes among tribe members, no comprehensive characterization of metal body burden of this population had been conducted prior to the Navajo Birth Cohort Study (NBCS). Tribal populations are not well-represented in the National Health and Nutrition Examination Survey (NHANES); tribal populations are aggregated with various racial/ethnic groups into the "Other" group which only comprises 5.3% of the most recent NHANES study population [9].

Epidemiologic studies have linked chronic low-dose exposures to U or As through drinking water to adverse health effects including kidney damage, various cancers, cardiovascular diseases and hypertension [10–19]. The associations between this list of adverse health effects and U or As exposures parallel the list of health concerns on Navajo Nation: cancer, autoimmunity, kidney disease, diabetes and hypertension. Furthermore, IHS clinicians report increased frequency and severity of infections and unusually high numbers of severe combined immunodeficiency (SCID) cases and lupus (personal communication), and studies implicate immune complex deposition in Native American/American Indian (NA/AI) populations to end-stage renal disease [20,21]. It is plausible that the adverse health outcomes observed in Navajo Nation community members are mediated or modulated by the immune system. Other than U and As, Navajo Nation community members are exposed to additional environmental metals, which have been shown to be both immunosuppressive [22] and immunostimulatory [23,24]. Our previously published work with the Cheyenne River Sioux Tribe (CRST) [8] and literature review [7] indicate an association between environmental metal exposure and autoimmune markers. Early results from the NBCS

reveal that increased maternal urinary As, Hg, and U are significant predictors of changes in newborn cytokine production (in progress).

Although U and As exposures are linked to alterations in cellular and humoral immune responses in animals [25,26], little is understood about the impact of these metals on immune system alterations associated with populations chronically exposed to mixed metal mine waste [27–29]. Few previous studies have been conducted to examine the effects on the immune system of dual exposure to U and As, much less chronic exposure to the broad suite of metals contained in mixed mine wastes. Though studies have been conducted to examine the immune system effects of occupational metal exposure [30–32], human epidemiological studies of chronic environmental metal and immune system biomarkers are lacking. Circulating cytokines, measured from comparatively easily-to-collect serum samples, are possible biomarkers to help assess immune alterations and evaluate potential health risks due to chronic mixed metal exposure.

We measured participant metal biomonitoring and circulating cytokine levels, and then examined the relationships among metals and cytokines using multiple statistical methods. We hypothesized that chronic low-level environmental exposure to metal mixtures results in measurable metals in Navajo Nation community members, which is associated with cytokine concentration differences.

METHODS

Inclusion Criteria

The study site of Navajo Nation is roughly the size of West Virginia and is located in the Four Corners region of the southwestern United States (US) (Figure 4.2). The NBCS was initiated to address Navajo community members' concerns about the potential health effects of chronic environmental exposure to uranium mine wastes. In 2013 the NBCS began recruiting pregnant women between 14 and 45 years of age who had lived on Navajo Nation for at least 5 years. To be included in the study, women had to be willing to deliver at a participating hospital, and have their child followed up for one year postnatally.

Survey Information

At enrollment, a survey of socioeconomic, demographic, lifestyle, and reproductive history information was administered by trained community health environmental research staff. The responses were entered into the research database RedCap in accordance with all privacy and security requirements of both University of New Mexico and Navajo Nation institutional review boards.

Biological Sample Collection

Trained hospital staff collected and prepared biospecimen samples. Samples intended for metal biomonitoring analysis were collected using pre-screened metal-free cups, transfer pipettes, and Nalgene cryo-vials provided by the Centers for Disease Control (CDC)

National Center for Environmental Health (NCEH) Division of Laboratory Sciences (DLS). At enrollment and 36 weeks gestation prenatal visits, participants provided 40-50 mL of urine in a sterile collection cup. Laboratory staff aliquoted 1.8 mL urine samples into separate Nalgene cryo-vials for multi-element metal, total arsenic (As), speciated As, creatinine, and mercury (Hg)/iodine (I) analysis. Hospital laboratory staff also collected peripheral blood via venipuncture and then allowed the blood to clot at room temperature for 30 to 40 minutes. Once clotted, laboratory staff centrifuged the blood tube at 2,400 revolutions per minute for 15 minutes to separate the serum. 1.8 mL aliquots of serum were transferred to 2.0 mL Nalgene cryo-vial. After processing urine and serum samples, hospital staff placed all cryo-vials in a −80°C freezer for storage. Samples were transferred from participating facilities on Navajo Nation on dry-ice to freezer storage facilities at UNM. Chain of Custody forms were completed, reviewed, and validated at each stage of collection, storage and analysis. Samples are stored, analyzed, and disposed of in accordance with participant wishes as indicated on consent forms. Samples not consumed during analysis will be returned to participants after the close of the study analysis period if they desire, in accordance with cultural practices.

Metal Biomonitoring

Samples were examined for quality control purposes by UNM laboratory staff; then urine vials and one serum vial were mailed to CDC. UNM staff shipped samples on dry ice to CDC DLS for analysis. Urine concentrations of antimony, arsenic (total), barium, beryllium, cadmium, cobalt, cesium, iodine, lead, manganese, mercury, molybdenum, platinum, strontium, tin, thallium, tungsten, and uranium, were measured using

inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) [33–37]. Blood concentrations of cadmium, manganese, total mercury, and lead, and serum concentrations of zinc, selenium, and copper were also measured using ICP-DRC-MS [38,39]. The limit of detection for these elements in urine, blood or serum ranged from 0.002 to 24.48 ng/mL, depending on the analyte and 134 biological media.

Serum Cytokines

Cytokine measurements were performed using the Meso Scale Discovery multiplex four- or ten- spot 95-well electrochemiluminescence detection platform. Serum samples were diluted 1:2 in appropriate assay buffer and incubated for two hours. The plates were washed with PBS-Tween and corresponding Sulfo-Tag secondary reagents were added. They were then incubated for an additional two hours. Plates were read using the MESO QuickPlex SQ 120 microplate reader and data was analyzed using the Discovery Workbench 4.0 software.

Statistical analysis

Summary statistics

Urine and serum metal measurements below the limit of detection (LOD) were replaced by the LOD divided by two. Summary statistics including median (interquartile range) and mean (standard deviation) for continuous variables and frequency (%) for categorical variables were used to described the demographics, environmental characteristics, urine metals, serum metals, and cytokine concentrations of NBCS participants.

Cytokine selection

Six cytokines were selection measured and included in this study: interferon alpha (IFNα), interferon gamma (IFNγ), interleukin-4 (IL-4), interleukin-7 (IL-7), interleukin-17 (IL-17), and interleukin-29 (IL-29). These six cytokines were chosen for early analysis from NBCS population data because they have been linked to autoimmunity [40]. Navajo community members, as well as clinicians who serve the community, have expressed concerns about the possibility of environmental metal exposure exacerbation of autoimmune diseases development, and our previous work also suggests an increase in early autoimmune markers related to metals [8,41].

Correlations

Spearman (non-parametric) correlation coefficients were calculated between metal concentrations in biological samples, and between metal concentrations in biological samples and cytokine levels. Spearman correlations were classified as significant at a 5% level.

Linear regression: Univariable and multivariable modeling

For univariable and multivariable modeling, metal biomonitoring and cytokine concentrations were log-transformed to reduce skewness and approximate normal distributions for the analysis. In order to reflect literature about metal exposure and public health effects [3], as well as address community concerns about mixed metal mine wastes exposure, metal predictors in the univariable and multivariable models were limited to uranium (urine), manganese (blood and urine), mercury (blood), total arsenic

(urine), arsenite (As(III) urine), dimethylarsinic acid (DMA urine), and monomethylarsonic acid (MMA urine).

Since there was no statistically significant difference between cytokine values between sociodemographic groups by education level, marital status, and annual income, these were not included in the univariable and multivariable modeling. Gestational age and fetal sex have been shown to influence both body metal concentrations and cytokines, so these were included in the univariable and multivariable modeling [42,43] . Multivariable regression models with backwards selection based on the AIC criteria were performed. When discussing the univariable and multivariable results, p=0.10 was the cutoff for predictor significance. Since this is the first assessment of these associations between exposures and cytokine production in this population, and because the concerns about exposure and health are of high importance to the population, 0.10 was used to increase likelihood of identifying potential contributors important in future investigations.

Profile regression

Bayesian Profile Regression (BPR) uses a Dirichlet process mixture model to identify subgroups of observations with similar patterns in the levels of each exposure covariate. BPR was selected for this analysis due to no prior information about the optimal number of clusters and to the need to understand what metals might be driving clustering. BPR also allows for missing values in the exposure covariates, thus allowing the analysis to proceed with the full range of observations. This method has been used previously for environmental and epidemiology research [44–48].

BPR uses a Dirichlet process (DP) as the prior for the mixing distribution, defined as $P \sim \mathrm{DP}(P_{\theta 0}, \alpha)$ where α is a scale parameter that affects the shape of the Dirichlet distribution and $P_{\Theta 0}$ is the base probability distribution [49]. BPR is a data driven clustering algorithm because it implements a Dirichlet process mixture model (DPMM) that allows for an infinite possible number of clusters and then applies a dissimilarity matrix to determine the optimal number of subgroups [50]. Additionally, BPR sets the DPMM in a Bayesian framework with Markov chain Monte Carlo (MCMC) estimation to propagate uncertainty in cluster assignment. Uncertainty in cluster membership is ascertained with Bayesian model averaging. Additional information about BPR can be found in other recent publications [49,51,52]

In a full BPR analysis the exposure predictors are fit jointly with the response variable such that the predictor and response relationship is cluster specific. The relationship between biomonitoring (exposure predictors) and each of the cytokines IFNγ, IL-4, IL-7, IFNα, IL-29 (response variables) was modeled for this analysis. Cytokines were modeled as a continuous variable with normally distributed errors. We added trimester of sample collection and fetal sex as fixed effect confounders. Continuous covariates were mean-centered to facilitate MCMC convergence and to help facilitate interpretation of cluster effect posterior distributions provided by the full BPR model output.

For each cluster of metal biomonitoring, henceforth denoted as "exposure cluster" (EC), the posterior distributions were derived from the MCMC iterations of the cytokine values. We focus our Bayesian inference on the difference in specific cytokine concentration for each EC compared with the specific cytokine concentration of the

lowest exposure EC (defined as the EC that resulted in posterior distributions with the highest proportion of observations in the lowest quartile across all metals). Because the participants in this study represented the full continuum of exposures on Navajo Nation, this method allows us to take advantage metals exposures profiles occurring in the population, allowing comparisons between the low exposure group and other groups with other exposure profiles.

Exposure variables included concentrations of 27 metals/metal species measured in blood, serum, or urine. Some urinary metals, such as beryllium and platinum, were excluded from analysis because more than 90% of individuals had concentrations less than the limit of detection (LOD). Measured biomonitoring concentrations were converted to quartiles for clustering. Due to left-censoring, total blood mercury, inorganic blood mercury, and urine manganese were transformed into binary variables that represent non-detectable and detectable concentrations. Additionally, urine mercury was converted into tertiles because approximately one-third of the observations were less than the LOD. All observations less than the LOD were assigned to the first quantile. Trimester at sample collection and fetal sex were included in the BPR model as confounding variables. A sensitivity analysis was conducted where clusters were fit without an outcome. The same model parameters were used for the sensitivity analysis and the fully adjusted BPR model - a burn-in of 20,000 iterations and 200,000 MCMC sweeps. All BPR analyses were implemented in R (3.6.0) using the PReMiuM package (3.2.2) with default priors, and MCMC output was checked for convergence using trace plots of betas for the fixed effects [49]. For more details on BPR, see [48–50,52].

RESULTS

Participant Demographics

Demographic and socio-economic information are summarized for the subset of NBCS maternal study participants included in this study (Table 4.1). Mothers enrolled during all three trimesters of pregnancy. At study enrollment the mean maternal age was 28.1 years. Pre-pregnancy body mass index (BMI) was classified as "underweight or normal" for 28.4% of participants, with the remaining participants split between the "overweight" and "obese" classifications. Forty-one percent of participants have earned a high school diploma, but 60% report an annual household income of less than $20,000. Most participants (78.4%) were married or living with a partner. Slightly more participants delivered male versus female children. No significant differences in these variables were observed between the subset of individuals selected for this analysis and the NBCS cohort as a whole.

Table 4.1. Summary of sociodemographic characteristics of study cohort (N=133)

Variable	Category	Result
Maternal age (years)	Mean (SD)	28.10
Education level	No high school diploma (%)	78 (59.1)
	High school diploma (%)	54 (40.9)
Household income	<$20,000/year (%)	64 (59.8)
	>$20,000/year (%)	42 (40.2)
Marital status	Married or living with a partner (%)	105 (78.4)
	Not married or living with a partner (%)	29 (21.6)
Pre-pregnancy BMI	Underweight or normal (%)	42 (28.4)
	Overweight (%)	53 (35.8)
	Obese (%)	53 (35.8)
Trimester at sample collection	1st (%)	24 (16.0)
	2nd (%)	60 (40.0)
	3rd (%)	66 (44.0)
Sex of child	Male (%)	80 (52.6)
	Female (%)	72 (47.4)
NOTE: No difference in variables was observed between the subset of individuals selected for this study analysis and the NBCS as a whole.		

Table 4.2. Summary statistics for metal concentrations of biomonitoring included in linear regression modeling

Abbr	Metal/Metabolite - Matrix	Units	Number	Mean (SD)	Median (IQR)	NHANES 50th%tile (95th%tile)
BMN	Manganese - Blood	µg/dL	140	19.96 (6.7)	18.7 (15 - 24.18)	9.2 (16.1)
UDMA	Dimethylarsinic acid - Urine	µg/L	140	5.17 (3.26)	4.32 (3.09 - 6.34)	2.95 (12)
UMN	Manganese - Urine	µg/L	142	0.32 (0.28)	0.23 (0.14 - 0.39)	0.13 (0.28)
UTAS	Total arsenic - Urine	µg/L	141	7.23 (6.16)	5.81 (4.18 - 8.11)	5.74 (49.9)
UUR	Uranium - Urine	µg/L	143	0.04 (0.15)	0.02 (0.01 - 0.03)	0.005 (0.031)
UAS3	Arsenite (As(III)) - Urine	µg/L	140	0.54 (0.41)	0.42 (0.23 - 0.71)	0.12 (1.11)
UAS5	Arsenate (As(V)) - Urine	µg/L	140	1.09 (1.3)	0.63 (0.38 - 1.21)	0.79 (0.79)
UMMA	Monomethylarsonic acid - Urine	µg/L	140	0.54 (0.45)	0.4 (0.27 - 0.67)	0.28 (1.45)
THG	Total mercury - Blood	µg/dL	140	0.41 (0.26)	0.33 (0.2 - 0.51)	0.74 (4.66)

Body Metal Concentrations

Biomonitoring results for the subset of metals included in linear regression modeling are summarized in Table 4.2. This subset of metals was selected in order to reduce the dimensionality of predictor variables. Uranium, manganese, mercury, and arsenic/arsenic species were chosen because these are metals with documented immune system effects. A complete table with summary statistics for participant biomonitoring and the 27 metals measured is available in Table 4.S1.

Joint Distribution of Metals

Although a subset of metals was used in the univariable and multivariable analyses, the full set of metals, except for those metals for which >90% of samples were below the limit of detection, was included in determining cluster profiles with Bayesian Profile Regression (BPR). Therefore, the full correlation matrix for metals used in BPR is included here. Spearman's ρ values ranged from -0.32 to 0.79 (Figure 4.3). Moderate to moderately-strong positive Spearman's correlation coefficients were observed for the majority of urine metals including manganese, barium, strontium, molybdenum, tungsten, cesium, thallium, cobalt, lead, antimony, arsenic, tin, cadmium, and uranium. Strong positive correlations were observed between total arsenic and the arsenic species As(III), As(V), MMA, and DMA. Serum zinc was negatively correlated with most metals in blood or urine with the exceptions of cadmium (urine), selenium (serum), cesium (urine), MMA (urine), and lead (blood). The presence of multiple correlated, and several highly-correlated metals, indicates that our data are appropriate for analysis using BPR.

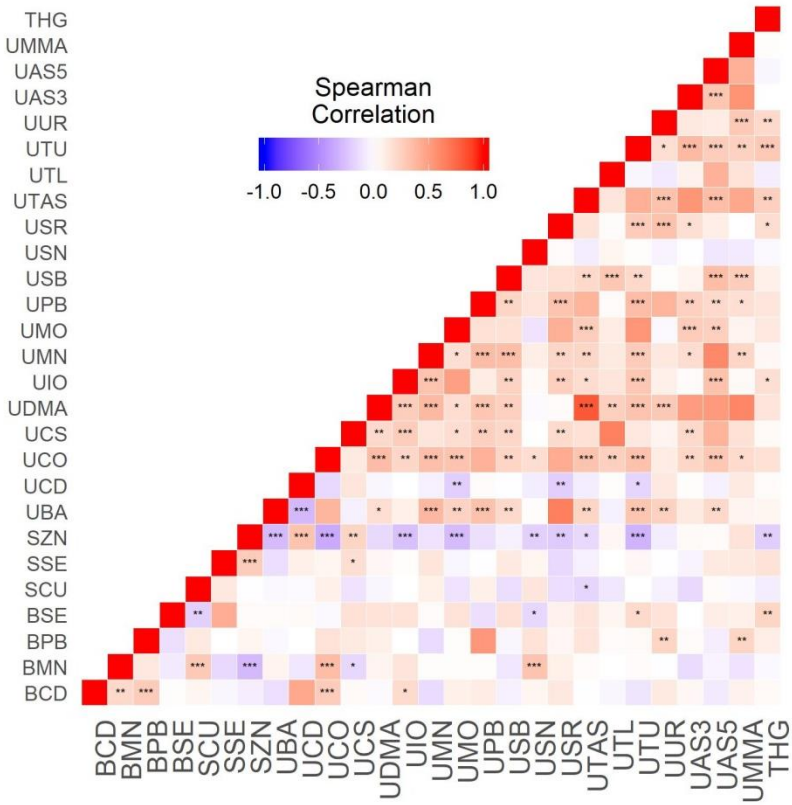

Figure 4.3. Spearman's correlations between metal biomonitoring results. Correlation coefficient is designated by color with blues designating negative correlations and reds designating positive correlations. Asterisks denote significant correlations at the $p<0.05$ (*), 0.01 (**), and 0.001(***).

Cytokine Results

Summary statistics for study participants are summarized in Table 4.S2. There were no significant differences in mean or median cytokine concentrations across sociodemographic or other confounder groups except for IFNγ. First trimester IFNγ was higher than second and third trimester IFNγ concentrations.

Spearman's Correlation Coefficients

The significant Spearman's correlation coefficients between metal biomonitoring and cytokine are summarized in Figure 4.4 and Table 4.3. There are no significant correlations between IL-4 or IL-7 and any metals. The significant correlation coefficients between IFNα , IFNγ, and IL-29 range in magnitude from 0.15 to 0.27. Aside from the correlations between IL-29 and selenium (blood), and IL-17 and As(III) and cesium (urine), the significant correlations between cytokines and biomonitoring are positive.

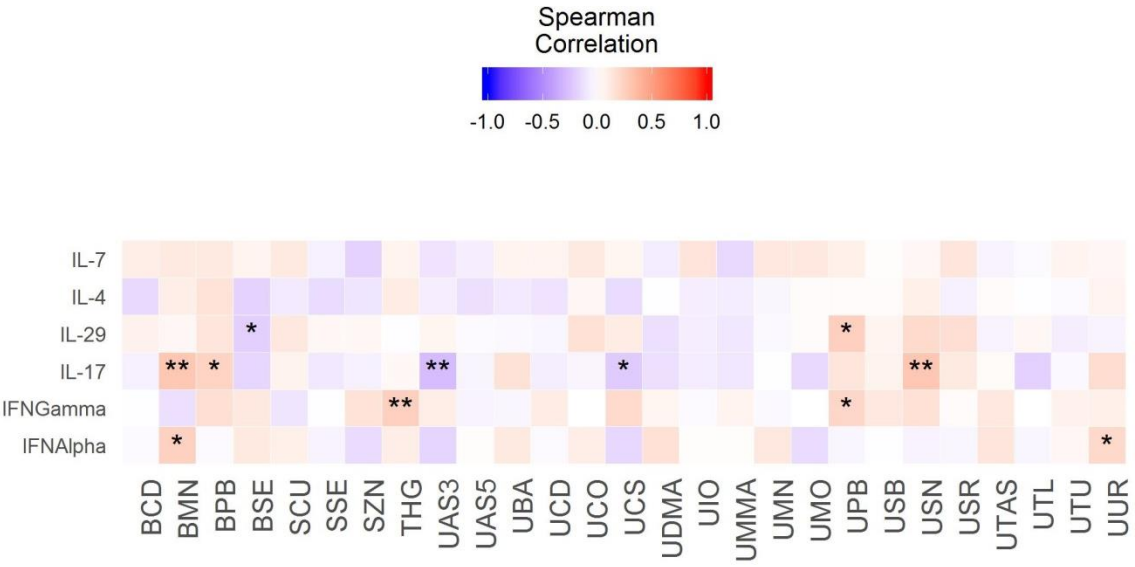

Figure 4.4. Spearman's correlations between metal biomonitoring and cytokines. Correlation coefficient is designated by color with blues designating negative correlations and reds designating positive correlations. Asterisks denote significant correlations at the $p<0.05$ (*) and 0.01 (**).

Table 4.3. Summary of significant ($p<0.05$) Spearman's correlations between cytokines and biomonitoring metals

Cytokine	Metal	Coefficient	p-value
IFNα	BMN	0.24	0.016
	UUR	0.19	0.048
IFNγ	UPB	0.21	0.030
	THG	0.25	0.010
IL-17	BMN	0.29	0.005
	BPB	0.23	0.031
	AS3	-0.28	0.007
	UCS	-0.22	0.035
	USN	0.29	0.005
IL-29	BSE	-0.20	0.048
	UPB	0.25	0.010

Linear Regression Modeling

When discussing the univariable and multivariable results, p=0.10 is used as the cutoff for predictor significance. A summary of significant univariable relationships between log-transformed cytokines and metal biomonitoring is contained in Table 4.4. A summary of significant predictors after model selection is summarized in Table 4.4. Arsenic species recur frequently as associated with cytokines in both the univariable analysis, and final multivariable model after variable selection. Arsenic species are positively associated with cytokines with the exception of As(III), which is negatively associated with IL-4, IL-17, and IL-29. Urine uranium is positively associated with IL-17.

Table 4.4. Summary of univariable modeling of cytokines and exposure variables (UUR, BMN, UMN, THG,UTAS, As3, DMA, and MMA) for variables with p<0.10.

Cytokine	n	Significant metal in univariable model	Estimate	Std Error	p-value
IFNα	165	log(UTAS)	0.58	0.27	0.035
		log(DMA)	0.9	0.25	0.001
		log(MMA)	0.43	0.19	0.027
INFγ	133	log(THG)	0.23	0.12	0.070
IL-4	133	log(As3)	-0.16	0.081	0.057
IL-7	133	log(UMN)	0.077	0.044	0.085
IL-17	98	log(UUR)	0.22	0.09	0.016
		log(BMN)	0.47	0.19	0.015
		log(As3)	-0.22	0.089	0.016
IL-29	137	log(BMN)	0.81	0.46	0.079
		log(As3)	-0.34	0.19	0.070
		log(DMA)	-0.72	0.27	0.010
		log(MMA)	-0.5	0.2	0.015

Table 4.5. Summary of final multivariable models of the relationship between exposure variables (UUR, BMN, UMN, THG,UTAS, As3, DMA, and MMA) and cytokines after variable selection

Cytokine	n	Metal predictors retained in multivariable model	Estimate	Std Error	p-value
IFNα	165	log(DMA)	0.9	0.25	0.00049
INFγ	133	log(THG)	0.26	0.13	0.044
IL-4	133	log(As3)	-0.16	0.081	0.058
IL-7	133	log(UMN)	0.088	0.048	0.066
		Log(MMA)	-0.078	0.051	0.13
IL-17	137	log(UUR)	0.19	0.093	0.052
		log(BMN)	0.38	0.19	0.052
		log(As3)	-0.32	0.11	0.0038
IL-29	133	log(BMN)	0.9	0.46	0.051
	137	log(DMA)	-0.67	0.28	0.017

BPR Clustering

BPR analysis identified two to four exposure profile groups depending on which cytokine was modeled as the outcome. BPR clustering analysis showed a significant difference between cytokine concentrations across EC groups for INFγ and IL-7, but no significant difference related to EC for IFNα, IL-4, IL-17, and IL-29 (Table 4.6). Therefore, only INFγ and IL-7 will be discussed below in relation to EC differences.

Table 4.6. Summary of BPR clustering by cytokine. Significance was determined as $p<0.05$.

Cytokine	Number of BPR Clusters	ANOVA p-value	Significant difference in cytokine level between cluster groups?
IFNα	3	0.92	No
INFγ	4	*0.005*	Yes
IL-4	3	0.175	No
IL-7	3	*0.002*	Yes
IL-17	2	0.392	No
IL-29	3	0.36	No

BPR assesses the clustering patterns of metal predictor variables along with the specified cytokine outcome. The latent selection weights (ρ) generated for each metal capture the probability that a metal plays a role in clustering patterns, and it is informative for variable selection. For INFγ and IL-7, results will be presented by identifying the primary metals that drive participant placement into separate ECs. The metals discussed in following sections are those that with $\rho>0.70$. Heat map visualizations of the ECs generated for each cytokine using BPR will be presented, along with descriptive text in order to identify the relative magnitudes of metals observed across exposure groups. The observed empirical mean of the cytokine outcome for each of the ECs will be presented, as well as the relative shift in the posterior distribution in

comparison with the low exposure group, highlighting the clusters where significant shifts in posterior distributions are found.

INFγ Metal Exposure Clusters (EC)

The fully adjusted BPR model for INFγ indicated an optimal clustering of four subgroups containing 36, 39, 19, and 26 individuals, respectively. Of the four identified subgroups, EC2 represents individuals with relatively low biomonitoring for all metals that drive clustering. The median concentrations of metals in EC2 are in the first or second quartile (Figure 4.5). EC3 represents an intermediate degree of exposure to metal mixtures, with median concentrations for most of the metals in the second quantile, and the rest in the third quartile. The remaining two subgroups (EC1 and EC4) are characterized by comparatively high exposures to several metals. EC1 is characterized by median fourth quantile concentrations in total arsenic, arsenic species, and urine lead, while EC4 has median fourth quantile urine barium, molybdenum, and tungsten. EC1 has a significantly higher empirical mean than the other EC subgroups, as well as the highest adjusted posterior mean (Table 4.7). The probability that the posterior distribution of expected IFNγ for EC1 is greater than EC2, the lowest exposure group, is 92.3% (Table 4.7).

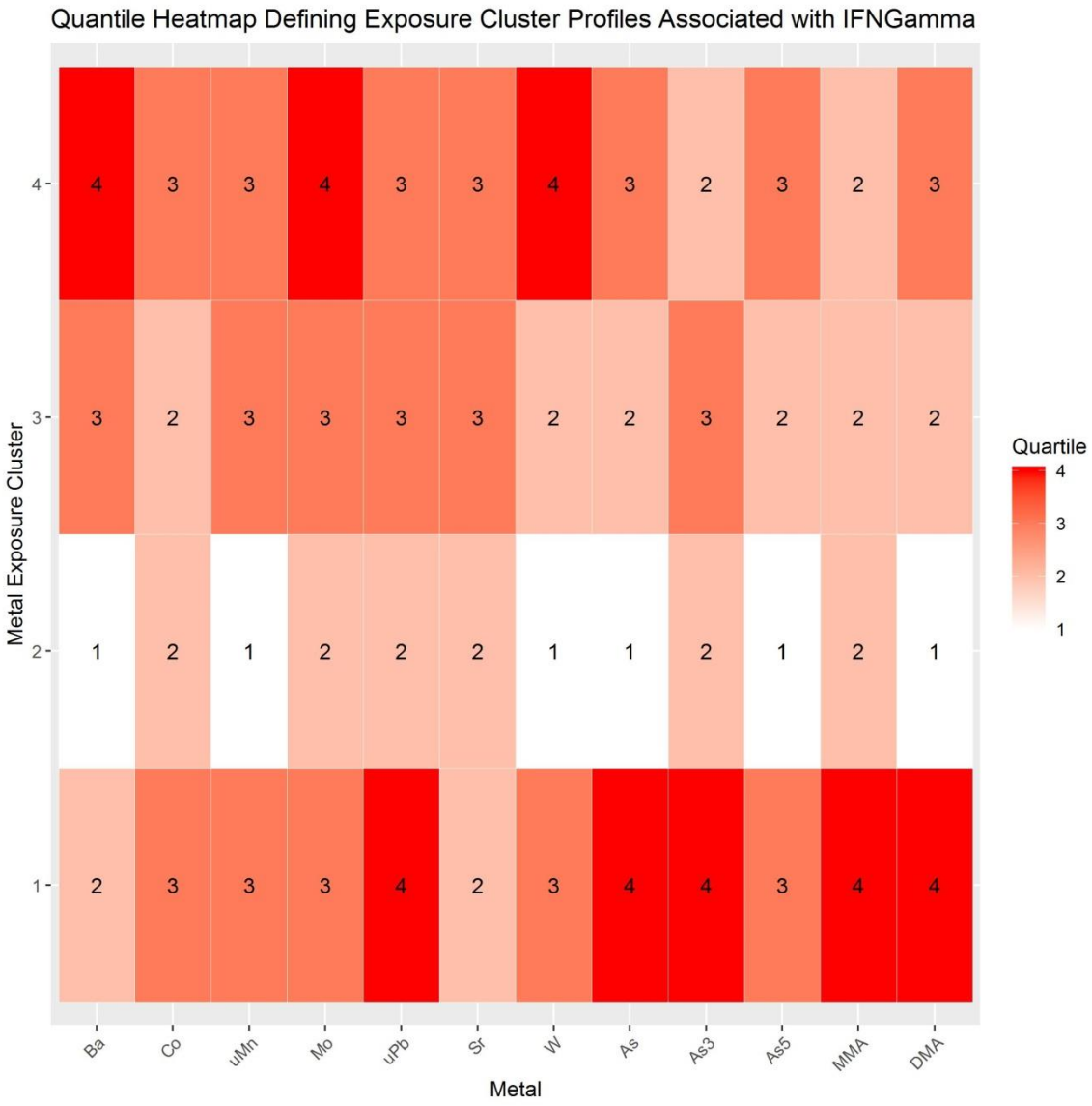

Figure 4.5. Quantile heat map displaying median metal biomonitoring concentration for each metal retained in BPR analysis by exposure cluster where IFNγ is the outcome. Each column represents a metal while each row represents a cluster profile (EC1 through EC4). The numeric values represent quantile score, i.e. the quantile in which the mean of biomonitoring concentrations for that metal for individuals in the cluster falls (1 is the lowest and 4 is the highest).

Table 4.7. Summary of empirical concentrations of IFNγ for each cluster, adjusted posterior means of IFNγ by exposure profile cluster, and the estimated difference compared with lowest exposure profile cluster (n = 120)

	n	**Empirical Mean (95% CI)**	**Adujusted Posterior Mean IFNγ (95% Credible Intervals)**	**Probability IFNγ_i > IFNγ_2**
Overall	120	9.99 (7.16, 12.82)		
Cluster				
EC1[1]	36	17.48 (8.87, 26.08)	11.614 (8.095-15.588)	0.923
EC2	39	6.73 (4.29, 9.17)	7.955 (5.062-10.891)	Ref group
EC3	19	9.12 (5.48, 12.75)	8.643 (5.64-11.735)	0.658
EC4	26	5.16 (3.911, 6.41)	8.379 (5.039-11.692)	0.575
1 - Significant difference between empirical mean and other clusters (p=0.005)				

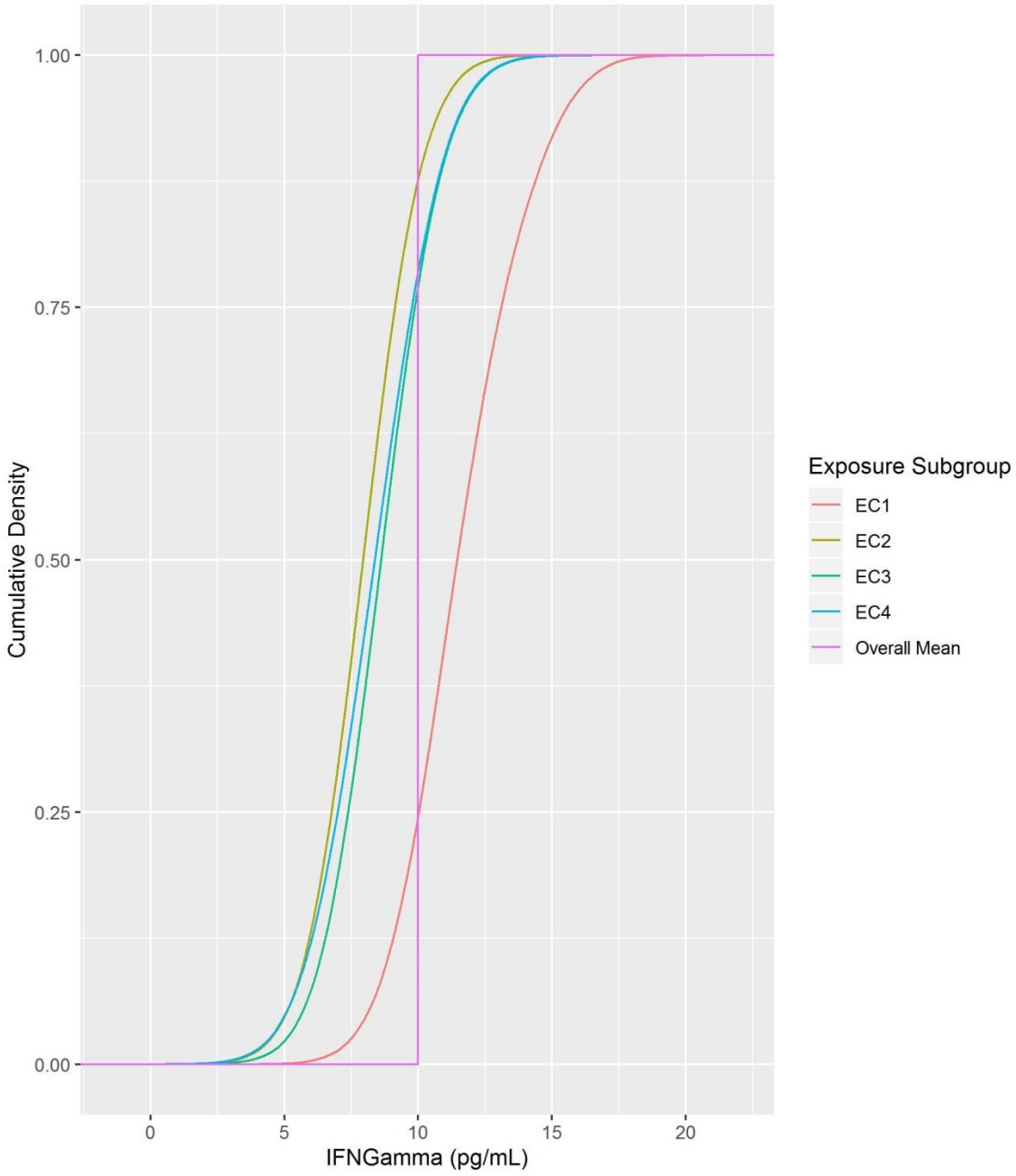

Figure 4.6. Cumulative probability density plots of cluster-specific posterior adjusted IFNγ distribution. Baseline IFNγ was determined using an average across all clusters at each iteration (mean=10.17 pg/mL)

IL-7 Metal Exposure Clusters (EC)

The fully adjusted BPR model for IL-7 indicates an optimal clustering of three subgroups sized 57, 39 and 24 individuals, respectively. Of the three identified subgroups, EC2 represents individuals with low biomonitoring. The median concentrations of metals in EC2 are in the first or second quantile (Figure 4.7) indicating relative low overall exposures. Median concentrations for most of the metals in EC3 are somewhat higher — in the second or third quantiles. The remaining subgroup, EC1, is characterized by comparatively high exposures, with all metals supporting the clustering having a mean in the third or fourth quantile. Notably, As(III), As(V), DMA, and MMA have mean fourth quantile concentrations. The cumulative density plots for both EC1 and EC3 are shifted left in comparison with EC2, the low exposure group (Figure 4.7). Though EC1 has the highest median quantile metal exposures, only EC3 has a significantly lower empirical mean for IL-7 compared across ECs, as well as the lowest adjusted posterior mean (Table 4.8). The probability that the posterior distribution of expected IL-7 for EC3 is lower than that for EC2 is 93.0% (Table 4.8).

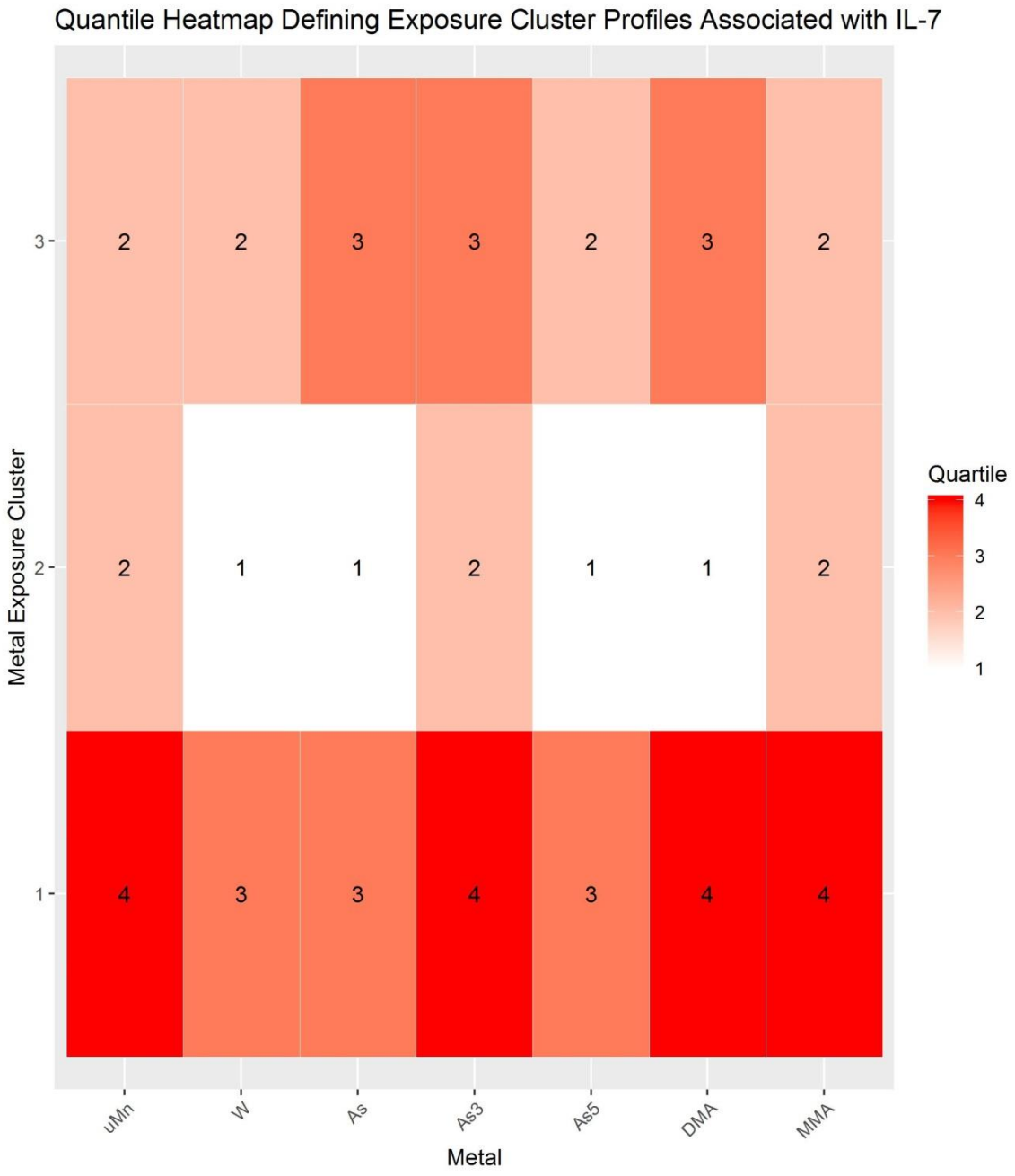

Figure 4.7. Quantile heat map displaying median metal biomonitoring concentration for each metal retained in BPR analysis by exposure cluster where IL-7 is the outcome. Each column represents a metal while each row represents a cluster profile (EC1 through EC3). The numeric values represent quantile score, i.e. the quantile in which the mean of biomonitoring concentrations for that metal for individuals in the cluster falls (1 is the lowest and 3 is the highest).

Table 4.8. Summary of empirical concentrations of IL-7 and adjusted posterior means of IL-7 by exposure profile cluster, and the estimated difference compared with lowest exposure profile cluster (n = 120)

	n	**Empirical Mean (95% CI)**	**Adjusted Posterior Mean IL7 (95% Credible Intervals)**	**Probability IL7_i > IL7_2**
Overall	120	15.63 (14.521, 16.748)		
Cluster				
EC1	57	15.70 (14.176, 17.232)	14.91 (13.589-16.228)	0.779
EC2	39	17.66 (15.40, 19.91)	15.996 (14.243-17.719)	Ref group
EC3[a]	24	12.18 (10.47, 13.90)	13.59 (11.663-15.491)	0.930
a - Significant difference between empirical mean and other clusters (p=0.002)				

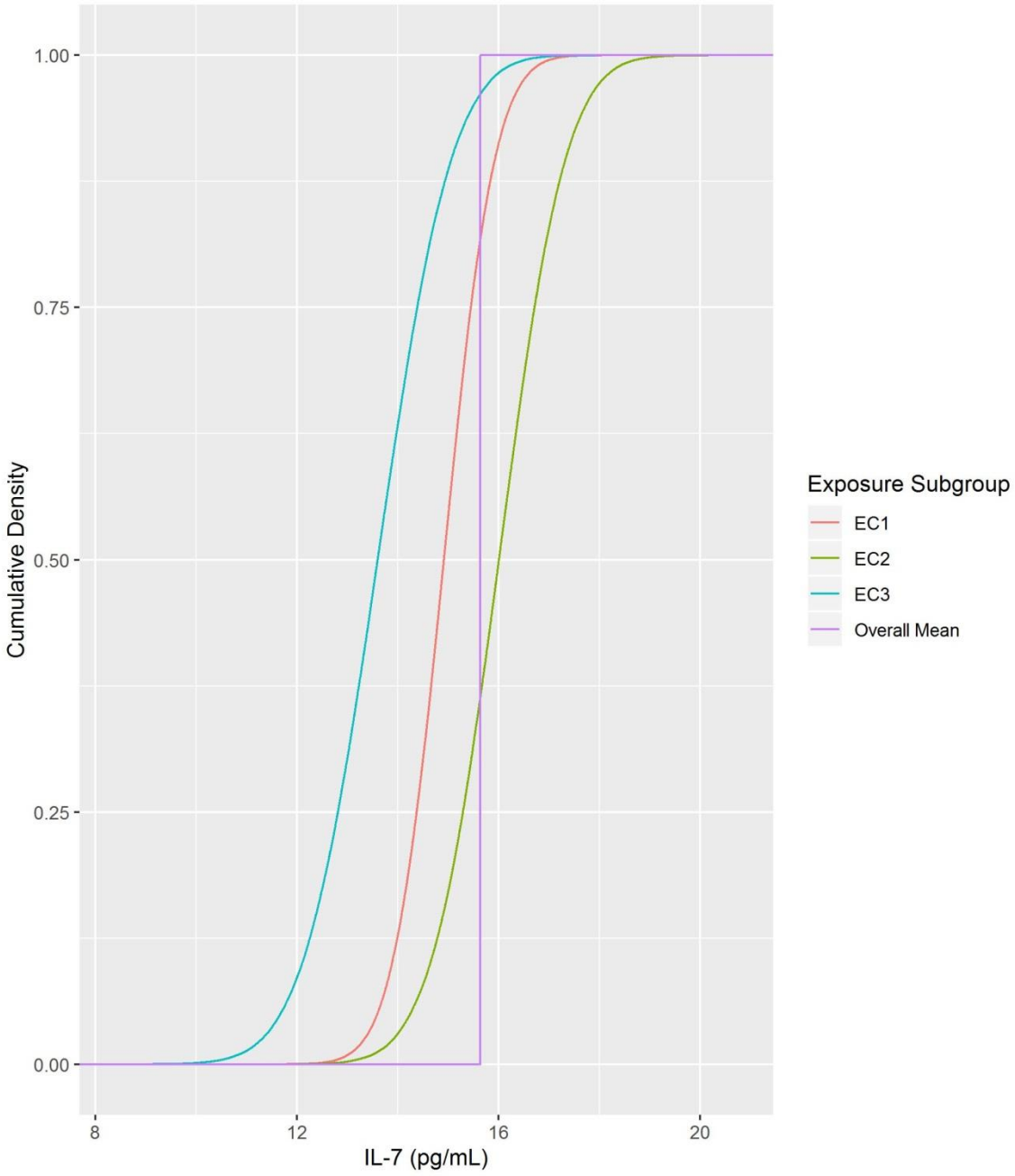

Figure 4.3. Cumulative probability density plots of cluster-specific posterior adjusted IL-7 distribution. Baseline IL-7 was determined using an average across all clusters at each iteration (mean=15.59 pg/mL).

DISCUSSION

The primary aim of this study was to probe the relationship between metal exposures and the cytokines associated with autoimmunity in chronically exposed Navajo Nation community members. This study builds upon of previous work with the Cheyenne River Sioux Tribe (CRST) [8] and Navajo Nation [41]. These studies linked environmental metal co-exposures in Native American/American Indian (NA/AI) participants to arsenic and mercury [8], and arsenic and uranium [41], to early autoimmune markers. To further investigate the relationship between complex metal mixtures and autoimmunity, six cytokines associated with autoimmunity (IFNα, IFNγ, IL-4, IL-7, IL-17, and IL-29) were analyzed and modeled with participant biomonitoring information for 27 metals/metal metabolites. Common methods such as summary statistics, correlation, and linear regression (univariable and multivariable) were employed, as well as Bayesian Profile Regression (BPR). Each method of statistical analysis provides slightly different, but important, related information about our dataset. A summary of the cytokine-metal relationships that we observed in this study, along with literature that provides insight into the potential mechanisms of action follows below.

With the exception of IFNγ, there was no significant difference in mean cytokine concentrations related to trimester. This is contrary to studies documenting differences in circulating cytokine levels in both mice [53,54] and humans [55,56]. Over the course of human pregnancy, IFNγ [55] and IL-4 [56] are expected to increase, while IL-17 [42] is expected to decrease, by the third trimester. We only observed an increase in IFNγ in the third trimester in comparison to samples collected early in pregnancy, but no significant difference by trimester for IL-4 and IL-17. In this case, no difference in circulating

cytokines by trimester may actually be an indicator of an alteration in immune processes, since the mother's circulating cytokine profiles are not changing throughout pregnancy as anticipated. Supporting this idea is the fact that the cytokine levels of mothers who have preeclampsia are different (closer to first trimester means) than mothers who do not present with preeclampsia [42].

We observe significant Spearman's correlations with cytokine expression levels and manganese, uranium, lead, mercury, tin, molybdenum, and selenium (Table 4.2). Noticeably, arsenic and arsenic species are absent (except As(III) for IL-17) from the table of significant Spearman's correlations for the cytokines measured, yet arsenic (total urine) and arsenic species (As(III), MMA, DMA) appear as significant predictors in several univariable linear regression models. In the univariable models (Table 4.4), arsenic and arsenic species are significantly associated with IFNα, IL-4, IL-17, and IL-29. IL-17 levels are also associated with blood manganese (BMN) and urine uranium (UUR). In the univariable modeling, IFNγ is only significantly associated with total blood mercury (THG), and IL-7 is only associated with urine manganese (UMN).

Metal predictors retained after variable selection in the multivariable models (Table 4.5) are subsets of the metals that were found to have significant associations in the univariable models with the exception of IL-7. MMA is retained in the multivariable model for IL-7 (though it was not significant in the univariable analysis). DMA is the only metal predictor retained in the reduced multivariable model, while DMA and BMN are the two predictors retained in the multivariable model for IL-29.

The complexity of both cytokine responses and combined metal exposures, particularly in a human population, makes straightforward interpretation of the analyses

difficult. For example, human studies that measure the effect of the combined administration of arsenic and IFNα as a treatment for adult T-cell leukemia (ATL) show a shift from a T(reg)/Th2 phenotype before treatment toward a Th1 phenotype after treatment [57]. This immune phenotype shift in patients includes a decrease in IL-4 and increase in IFNγ [57]. Not only does this parallel our finding in terms of arsenic effects on specific cytokines that we measured, but it also illustrates the way in which even a single metal and single cytokine, administered together, can have broad-ranging effects, including immune phenotype shift and concomitant alterations in multiple cytokines. On the other hand, several studies report perturbations of cytokine responses associated with single metal exposures, and even general observation of differences in cytokine levels dependent upon metal exposures supports our hypothesis that chronic environmental metal exposures play a role in altered immune responses. The following section summarizes literature relevant to the associations that we found between cytokines and metals.

A possible mechanism for our observed positive correlation between IFNα and BMN is through manganese superoxide dismutase (MnSOD). The role of MnSOD in the induction of the IFNα antiviral state has been described in cells [58,59] and rats [60]. Additionally, low-level ionizing radiation from terrestrial uranium deposits has been documented to cause mutations in the hIFNα-2b gene and decreased downstream protein [61], but a later study was not able to detect these changes in a different uranium-exposed group [62]. It is unclear why UUR is significantly positively correlated with IFNα in our study, but possible explanations include uranium appearing as a surrogate in analysis, or affecting IFNα additively or synergistically with other metals. Regardless of the

direction of change in IFNα, those studies, as well as this one, suggest a possible contribution of uranium to immune system alterations that are associated with autoimmune markers. Work by our team revealed increased autoantibodies in Navajo Nation residents living in close proximity to uranium mines [41], and systemic lupus erythematosus (SLE) has been associated with uranium exposure in the Fernald Community Cohort residing near a uranium processing plant [63].

Treatment of cells with As(III) significantly inhibited IL-4 secretion by splenocytes from young mice [64], but not human T cells [65]. IFNγ was significantly correlated with urine lead (UPB) and total blood mercury. Environmental and occupational exposure to lead [66] and mercury [67–70] have been shown repeatedly to increase proinflammatory markers including IFNγ. While the immune system effects of arsenic [71], lead [72] , and mercury [73] have been studied in detail, information about the immune system effects of uranium is sparse [61,74–77], as are studies of the effects of metals on IL-7, IL-17, and IL-29. Research regarding the immune system and manganese focuses primarily how manganese as a micronutrient provides protection against infection and help to maintain immune system balance [78].

Examining the correlation, univariable, and multivariable analysis together, it can be seen that arsenic and arsenic species, mercury, uranium, and manganese recur as significant predictors of the cytokine levels assessed in this study. Overall, our correlation and linear regression results concur with available literature about relationships between single metals and cytokines, which increases confidence in methodology and validity of the study. The fact that multiple modeling methods

associate these metals consistently with cytokines strengthens the case that metal exposures play a role in levels of cytokines associated with autoimmunity.

Complementary to correlation and linear regression analysis, we also used Bayesian Profile Regression (BPR). Multiple metal exposures may have several exposure pathways and exposure routes. BPR allows us to see which metal exposures are frequently observed together, and then examine the effects of these metal exposure profiles, as opposed to single metals, on cytokines. In this work, BPR was able to classify our participants into subgroups with distinct joint-patterns of biomonitoring metal measurements and identify subgroup associations with IFNγ and IL-7. Although univariable and multivariable analyses showed significant influences of individual metals on concentrations of IFNα, IL-4, IL-17, and IL-29, there was no significant difference across EC groups for the BPR analysis of these cytokines. This indicates that individuals' levels of IFNα, IL-4, IL-17, and IL-29 do not strongly influence cluster membership. Rather the level of these cytokines is more likely to be influenced predominantly by the significant metal predictors in the linear regression modeling, and less so by the overall metal exposure patterns identified through BPR's clustering process.

In contrast, using blood, serum, and urine biomonitoring measurements of 27 metals, distinct patterns of exposure were observed and significantly associated with IFNγ and IL-7. In both the model for IFNγ and the one for IL-7, BPR identified one subgroup (EC2) with low exposures for most measured metals ($N_{IFN\gamma}=N_{IL\text{-}7}=39$, 32.5% of cohort) and another subgroup (EC1) with high exposures for nearly all metals ($N_{IFN\gamma}=36$, 30.0% of cohort; $N_{IL\text{-}7}=57$,47.5% of cohort). The profiles of metals defining the clusters

differed for the two outcome cytokines, but some commonalities were observed. Notably, EC1 has the highest mean quantile for total arsenic, As(III), MMA, and DMA, again supporting the conclusion that arsenic species are significant in perturbation of cytokine responses. EC1 and EC2 clusters account for more than half of participants, suggesting that these subgroups represent the most common exposure patterns in our population. EC2, the low exposure cluster has been used as the reference group, since it is impossible to have a completely unexposed group in this case. Both the BPR subgroups for IFNγ and IL-7 included an intermediate cluster (EC3) with slight elevation in some metals ($N_{IFN\gamma}$=19, 15.8% of cohort; $N_{IL\text{-}7}$=24, 20.0% of cohort). An additional subgroup (EC4) is observed for IFNγ, which also shows high exposures; in comparison to EC1, this cluster is characterized by fourth quantile urine barium and urine tungsten. This suggests two patterns of metal exposure for study participants in the highest exposure clusters. A possible source of EC4's barium and tungsten biomonitoring is occupational exposure in machining or electronics industries [79]. Despite total arsenic measurements close to the US national average, EC1's levels of As(III) and As(V), as well as DMA and MMA, suggest environmental exposure to inorganic rather than, or in addition to, dietary exposure, the most common source of arsenic exposure nationally [9]. It is worth remarking that the primary dietary sources of arsenic observed nationally, seafood and rice, are rarely consumed in our study population, increasing the likelihood arsenic levels in participants originate from the local environment.

Although two high metal ECs emerged from BPR for IFNγ, only EC1 corresponded to a significant difference in IFNγ, as shown both by the empirical mean and adjusted posterior mean as compared with reference subgroup EC2 (Table 4.6). This

suggests that arsenic exposure, along with overall elevated metal exposures, is associated with increased IFNγ. Published work in this same cohort documents an association between arsenic and oxidative stress markers. Evidence of arsenic exposure-associated increases in both IFNγ and oxidative stress markers suggest that arsenic may be contributing to inflammation in this cohort, which is considered to be a contributor to chronic disease states, including autoimmunity.

Interpretation of BPR for IL-7 is less clear because there is no monotonic "dose-response." EC2, the low exposure reference cluster has the highest empirical and adjusted posterior means for IL-7. EC1, the high exposure subgroup, characterized by fourth quantile inorganic arsenic, DMA, and MMA has intermediate levels of IL-7. EC3 has significantly lower empirical and adjusted posterior means for IL-7 than the other two subgroups. It is possible that arsenic may have an opposing effect on IL-7 compared with the other metals with which it is strongly correlated like manganese, leading to its association with a median IL-7 concentration between that of the reference group and the middle metal exposure group (EC3). Animal studies indicate that IL-7 deficiency in mice impairs the development of both T and B cells [80] and that IL-7 may play a significant role in numerous T cell-driven chronic inflammatory autoimmune diseases [81]. In particular, it has been shown that very low doses of MMA were capable of suppressing IL-7 signaling [82].

While our correlations and linear regression modeling are informative, BPR helps to address concerns that arise when using these types of analysis with a large set of interrelated covariates. Conventional multivariable regression struggles to estimate combined effects for a large number of exposures, particularly when, as in our case, the

number of parameters is large in comparison with the number of observations. Due to the highly-correlated nature of groups of metal exposure, conventional regression likely over-estimates the effect of a single metal on cytokines while BPR provides information on combined exposure. However, the significant effects observed in the univariable and multivariable results, especially in the association of uranium as a predictor of IL-17, indicate the importance of investigating these complex relationships with multiple analytic approaches. Uranium, though expected to be associated with other metal exposures, did not appear a significant driver in cluster membership, despite its significant role in predicting IL-17. This finding, and other significant univariable and multivariable results could be indicative of specific, targeted effects of these metals alone that may influence overall patterns, while other perturbations are more likely to occur as a result of exposures to combinations of metals in mixtures.

Limitations

Caveats to this study include small sample size and limitations to the information obtainable from single time point biological sample collection. In a human study with a large number of predictors, a larger sample size would reduce variance and increase statistical power. Additionally, multiple biological sample collection time points would yield data that were more representative of participants' chronic environmental exposures and typical immune system state. Urine samples were collected once (spot urines) rather than over a 24-hour period, introducing variance due to differences in collection time and half-lives of metal metabolism. It is also possible that participants were undergoing acute immune responses, such as infection or allergies, at the time blood samples were collected, which could impact the cytokines measured through increases in population

variance. It is worth noting that it is difficult to interpret levels of circulating cytokines. While circulating cytokine measurements are practical for human studies, because they can be measured from serum, it is complicated to link cytokine concentrations to specific, localized immune responses.

Though BPR allows us to examine how the groups of metals and their concentration profiles can jointly influence cytokine production, it cannot, by itself, indicate the relative contributions of each metal in an exposure cluster. Furthermore, it is possible that metals will be missed using BPR modeling not because they do not have a significant effect on cytokines, but because they are minimized or dropped from the model due to low correlation with other metal exposures.

Future work

Future work should address the aforementioned limitations. Total enrollment for the Navajo Birth Cohort Study (NBCS) included 781 women. These findings are persuasive evidence to analyze additional serum samples for cytokine levels in order to determine whether exposure subgroup proportions and their trends with cytokines remain the same as those observed in this study. Modeling the relationship of metal exposure subgroups with the six single cytokines in this study is a first step.

Our results indicate that individual metals, as well as patterns of mixture exposure, influence cytokines in a non-random manner. Both individual metal influences, and mixture influences, will likely be necessary to interpret the impact of metal exposure on the immune system. Yet, the complexity of cytokine interactions makes it difficult to predict specific immune system responses linked to specific changes in exposures or cytokine perturbation. Cytokines are one piece of the puzzle, and the net

effect of the identified perturbations will require integration of data such as these with other immune responses such as lymphocyte profiles and phenotypic analyses.

In order to address concerns about whether or not single, circulating cytokines provide meaningful information about the immune system as a whole, one step would be to conduct multivariate analysis using biologically-relevant subgroups of cytokine and lymphocyte measures as a joint outcome. For example, TH1-associated IFNγ, IL-2, TNFβ, and CD4/CD8 ratio of T cells could be used as the outcome for BPR rather than IFNγ alone. Similar groupings with selected markers for TH2 and Treg responses could also be modeled as outcomes. Though the immune system is complex with dozens of interrelated cytokines and cell types, recall that one of the major strengths of BPR is dealing with highly-correlated inputs. BPR could be used to cluster immune markers without an outcome, potentially revealing distinct immune "phenotypes" of the study population. Then metal EC membership counts, as well as summary statistics of metal biomonitoring, for each "phenotype" could be examined to determine if particular metals or metal combinations seem to alter steady-state immune function in participants.

To assess relative contributions of specific metals to the change in cytokine within an EC, a statistical method that allows for fitting multiple correlated exposures jointly into the same model, while also evaluating each parameter's relative importance should be used. Bayesian kernel machine regression (BKMR) is a recently-developed approach for estimating the health effects of mixtures that could be applied to our data. BKMR has previously been applied to environmental exposure studies on the impact of metal mixtures [83], pesticides [47], and insecticides [84] on child neurodevelopment.

Future work should include further examination of total arsenic and arsenic species in order to better characterize Navajo Nation community members' arsenic exposures, find arsenic exposure sources, identify potential alterations in arsenic metabolism, and implement the findings for both personal and larger-scale environmental health risk reduction. BKMR could elucidate questions about which specific forms of arsenic measured in biomonitoring appear to be driving immune effects. Modeling the ratios of arsenic species measured in biomonitoring, particularly of the ratio of the excretion metabolites MMA to DMA, would likely be informative.

The relationships among micronutrients, metals, and immune system markers must also be probed in future studies. Urine manganese is identified as highly correlated with other metals ($\rho_{Mn}>0.9$), which may be indicative that the source of manganese is environmentally-related. Whether manganese exposure is through environmental exposure or diet, it has the potential to be biologically active, affecting cellular processes, including those in the immune system. Though measured, zinc, selenium, and copper do not appears in the reduced set of metals shown in the BPR subgroups generated for IFNγ and IL-7 because they are not highly correlated with the other metals ($\rho_{Zn}=\rho_{Se}=\rho_{Cu}=0.000$) and the cytokine outcome. The fact that these micronutrients are not included in BPR clusters does not necessarily mean that they do not have an association with cytokines or play a role in immune alteration. They merely do not move in unified manner with other metal biomonitoring values. This is plausible if the metals that strongly drive clustering and IFNγ and IL-7 are from an environmental exposure, versus diet or supplementation, which are common sources of micronutrient intake. Since evidence exists that zinc in particular may ameliorate cellular damage caused by

arsenic [85], additional analysis should include statistical modeling of the interactions between zinc and arsenic species.

Conclusions

Using data from the Navajo Birth Cohort Study (NBCS), the aim of this manuscript was to probe the relationship between metal exposures and autoimmune-associated cytokines in chronically exposed Navajo Nation community members by using both conventional statistical analysis and Bayesian Profile Regression (BPR). Significant associations between both single metals and cytokines, and between distinct patterns of metal exposure and cytokines, were observed. Highly-correlated arsenic species in biomonitoring appear to be the strongest drivers of observed subgroup differences in cytokine levels. Taken as a whole, our results suggest that chronic community-level exposure to mixed metals on Navajo Nation plays a role in altering immune response. In our approach, we are treating circulating cytokines as potential biomarker indicators of immune status and function. The fact that we observe statistically significant changes in circulating cytokines associated with metal biomonitoring concentrations and profiles, despite caveats to these measurements, strengthens the case that environmental metal exposure contributes to immune system disruption. Further investigation is needed elucidate which immune system mechanisms are most impacted by chronic environmental metal mixture exposures, as well as the interaction of metal micronutrients with environmental metals. The findings from human studies such as this one must also inform translational work in both directions: basic science experiments such as in vitro and animal studies, as well as health and regulatory policy.

SUPPLEMENTAL INFORMATION

Table 4.S1. Complete table of summary statistics for metal concentrations of participant biological samples (biomonitoring)

Abbr	Metal/Metabolite - Matrix	Units	Number	Mean (SD)	Median (IQR)
BCD	Cadmium - Blood	µg/dL	140	0.34 (0.17)	0.3 (0.23 - 0.4)
BMN	Manganese - Blood	µg/dL	140	19.96 (6.7)	18.7 (15 - 24.18)
BPB	Lead - Blood	µg/dL	140	0.43 (0.48)	0.33 (0.25 - 0.44)
BSE	Selenium - Blood	µg/dL	140	173.15 (15.95)	171.44 (163.2 - 180.05)
SCU	Copper - Serum	µg/L	145	225.76 (49.49)	222.2 (192.7 - 255.8)
SSE	Selenium - Serum	µg/L	145	107.47 (11.34)	108.7 (100 - 115.5)
SZN	Zinc - Serum	µg/dL	145	63.02 (16.73)	61 (50.2 - 74)
UBA	Barium - Urine	µg/L	142	5.03 (6.22)	3.02 (1.56 - 6.05)
UCD	Cadmium - Urine	µg/L	143	0.24 (0.18)	0.21 (0.13 - 0.31)
UCO	Cobalt - Urine	µg/L	142	1.15 (1.12)	0.91 (0.6 - 1.45)
UCS	Cesium - Urine	µg/L	143	5.25 (2.18)	4.68 (3.89 - 6.23)
UDMA	Dimethylarsinic acid - Urine	µg/L	140	5.17 (3.26)	4.32 (3.09 - 6.34)
UIO	Iodine - Urine	µg/L	142	240.94 (373)	120.34 (79.67 - 230.93)
UMN	Manganese - Urine	µg/L	142	0.32 (0.28)	0.23 (0.14 - 0.39)
UMO	Molybdenum - Urine	µg/L	143	60.64 (33.01)	52.39 (37.86 - 79.02)
UPB	Lead - Urine	µg/L	143	0.4 (0.67)	0.29 (0.2 - 0.39)
USB	Antimony - Urine	µg/L	143	0.09 (0.08)	0.07 (0.05 - 0.11)
USN	Tin - Urine	µg/L	142	3.05 (4.29)	1.46 (0.84 - 3.24)
USR	Strontium - Urine	µg/L	142	229.96 (163.5)	183.7 (118.86 - 312.6)
UTAS	Total arsenic - Urine	µg/L	141	7.23 (6.16)	5.81 (4.18 - 8.11)
UTL	Thallium - Urine	µg/L	143	0.16 (0.08)	0.14 (0.11 - 0.2)
UTU	Tungsten - Urine	µg/L	143	0.2 (0.27)	0.11 (0.08 - 0.22)
UUR	Uranium - Urine	µg/L	143	0.04 (0.15)	0.02 (0.01 - 0.03)
UAS3	Arsenite (As(III)) - Urine	µg/L	140	0.54 (0.41)	0.42 (0.23 - 0.71)
UAS5	Arsenate (As(V)) - Urine	µg/L	140	1.09 (1.3)	0.63 (0.38 - 1.21)
UMMA	Monomethylarsonic acid - Urine	µg/L	140	0.54 (0.45)	0.4 (0.27 - 0.67)
THG	Total mercury - Blood	µg/dL	140	0.41 (0.26)	0.33 (0.2 - 0.51)

Table 4.S2. Summary statistics for cytokine measurements (ng/pL)

Cytokine	Number	Mean (SD)	Median (IQR)
IFNα	137	0.5393 (1.0802)	0.2806 (0.034 - 0.6984)
IFNγ	120	9.9917 (15.6406)	5.6695 (3.8768 - 8.366)
Il-4	120	0.0155 (0.0616)	0.0076 (0.0062 - 0.0086)
IL-7	120	15.6344 (6.1617)	15.5703 (11.4095 - 18.325)
IL-17	98	0.8065 (0.5220)	0.6615 (0.4020 - 1.063)
IL-29	137	0.076 (0.1885)	0.0056 (0.0024 - 0.0854)

Table 4.S3 - BPR latent selection weights (ρ) for IFNγ, which indicate probability of contributing to the clustering structure of the dataset

Media	Metal	Code	Latent Selection Weight
Blood	Cadmium	BCD	0.000
Blood	Manganese	BMN	0.000
Blood	Lead	BPB	0.000
Blood	Selenium	BSE	0.000
Serum	Copper	SCU	0.000
Serum	Selenium	SSE	0.000
Serum	Zinc	SZN	0.000
Urine	Barium	UBA	0.793263
Urine	Cadmium	UCD	0.000
Urine	Cobalt	UCO	0.700469
Urine	Cesium	UCS	0.000
Urine	Dimethylarsinic acid	UDMA	0.983313
Urine	Iodine	UIO	0.000
Urine	Manganese	UMN	0.935426
Urine	Molybdenum	UMO	0.60731
Urine	Lead	UPB	0.613644
Urine	Antimony	USB	0.0280104
Urine	Tin	USN	0.000
Urine	Strontium	USR	0.856381
Urine	Arsenic, total	UTAS	0.963852
Urine	Thallium	UTL	0.000
Urine	Tunsten	UTU	0.830282
Urine	Uranium	UUR	0.000
Urine	Arsenite	UAS3	0.942623
Urine	Arsenate	UAS5	0.916414
Urine	Monomethylarsonic acid	UMMA	0.910149
Blood	Mercury, total	THG	0.000

Table 4.S4 - BPR latent selection weights (ρ) for IL-7, which indicate probability of contributing to the clustering structure of the dataset

Media	Metal	Code	Latent Selection Weight
Blood	Cadmium	BCD	0.000
Blood	Manganese	BMN	0.000
Blood	Lead	BPB	0.000
Blood	Selenium	BSE	0.000
Serum	Copper	SCU	0.000
Serum	Selenium	SSE	0.000
Serum	Zinc	SZN	0.000
Urine	Barium	UBA	0.012046
Urine	Cadmium	UCD	0.000
Urine	Cobalt	UCO	0.457691
Urine	Cesium	UCS	0.04600835
Urine	Dimethylarsinic acid	UDMA	0.983174
Urine	Iodine	UIO	0.000
Urine	Manganese	UMN	0.903875
Urine	Molybdenum	UMO	0.1528665
Urine	Lead	UPB	0.270264
Urine	Antimony	USB	0.01573105
Urine	Tin	USN	0.000
Urine	Strontium	USR	0.000
Urine	Arsenic, total	UTAS	0.971196
Urine	Thallium	UTL	0.000
Urine	Tunsten	UTU	0.560736
Urine	Uranium	UUR	0.000
Urine	Arsenite	UAS3	0.9355855
Urine	Arsenate	UAS5	0.944144
Urine	Monomethylarsonic acid	UMMA	0.881082
Blood	Mercury, total	THG	0.000

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V. CHAPTER 5

Conclusions and Future Directions

The work in this dissertation contributes to understanding disparities in Native American health in several ways. First, informed by historical, cultural, and scientific knowledge, this work was able to identify possible associations between metal exposures and autoantibodies, as well as potential upstream pathways (e.g. fishing/fish consumption, drinking water) that could be responsible for the observed immune effects. This research has gone beyond epidemiological recording of apparent relationships between exposure and autoimmune disease disparities in Native American/American Indian (NA/AI) communities. This work takes the first steps to reveal plausible mechanisms by which mixed-metal exposures contribute to common pathways that can lead to immune dysregulation and possible downstream adverse health impacts, which includes autoimmunity and other chronic diseases with increased prevalence in tribal populations. The identification of specific cytokines linked to inflammation and autoimmunity, and the metals and metal exposure profiles associated with them, can provide early indicators of health risk. Progressing from observations of associations between metals and autoantibodies to potential mechanisms of metal-related immune dysregulation provides a stronger basis for early recognition of disease risk and for developing health interventions and risk reduction strategies to decrease exposure and toxicity.

Work in this dissertation has started to fill gaps in knowledge about the effects of single metals on the immune system (e.g. uranium), metal co-exposures (e.g. mercury + arsenic), and mixtures (metal exposure clusters) on the immune system. The study with

the Cheyenne River Sioux Tribe in Chapter 3 underscores the additive and/or synergistic role arsenic may play alongside mercury in antibody levels. Prior to our work, only mercury was suspected in its role in autoimmunity rather than arsenic, or mercury and arsenic together. The work with Navajo Nation in Chapter 4 demonstrates the way in which metal exposure profiles, in addition to single metals, may associate with immune markers. Further, this shows the importance of using multiple methods of statistical analysis when examining environmental health data not only to confirm findings, but also to reduce the likelihood that important information about health-relevant exposures is missed. Our statistical work provides a scaffold upon which other researchers may build to analyze and interpret other multifactorial datasets in order to answer complex environmental health questions in a statistically rigorous way. On the other hand, nother important outcome of our work was to reduce and prioritize a long list of metal toxicants by examining metal biomonitoring levels, as well as their associations with immune markers. The 10-12 metals that were identified in Chapter 4 as significantly influencing specific cytokine production will help to prioritize future population studies and basic mechanistic research.

This dissertation work has progressed from initial identification of a relationship between exposures to multiple metals and autoimmunity, to an understanding of what is known about one of the observed metal relationships with autoimmune disease, and then back to an initial attempt to understand metals' plausible underlying contributions to immune dysfunction leading to autoimmune responses. In these initial investigations, single measurements, such as single autoantibodies or cytokines, were modeled as outcomes. Our work suggests a link between chronic low-level community exposures to

metal mixtures and immune alteration. While autoimmunity as a primary outcome has been the focus of this dissertation, the immune system potentially mediates health effects ranging from cognitive development and obesity, to autoimmunity and cancer. Thus, the immune system provides an opportunity for early detection of health risk via immune biomarkers, as well as a potential point for intervention. Because nearly all populations are exposed to some degree of mixtures of metals, whether rural or urban, mining-impacted or not, it is vital that research concerning mixed metal exposure and the immune system continues, both in the laboratory and population studies.

Future avenues of research to elucidate the associations among mixed metals and adverse health outcomes, and immune-related mechanisms of action, have been discussed in the previous chapters. Broadly, they fall into the following categories:

- Continuing and further population study work
- Additional statistical analysis methods to understand/interpret mixtures
- Basic mechanistic research, including animal models, cell, and cell-free systems

Ideally, longitudinal data of biomonitoring and immune system markers in our population samples would increase the understanding of exposure patterns, reducing variability inherent in spot urines. This would in turn increase confidence in observed associations, if any, among chronic environmental metal exposure, immune system biomarkers, and health outcomes. Additionally, closer examination of available participant information, especially medical record and exposure survey information could help to reduce uncertainty in these results. The ongoing work in the Navajo Birth Cohort Study (NBCS) can also specifically improve our understanding of patterns and critical timing of environmental metal exposure through annual measurements of metals via

biomonitoring and matched outcome assessments, and help us understand the relationship between the timing of exposures relative to development of the immune system, and potential downstream impacts. Additionally, we can extend the findings from the NBCS by continuing to examine relationships between metals exposures and profiles of exposures with a variety of other outcomes such as cognitive development and obesity.

While several approaches to statistical analyses have been investigated in this dataset, additional statistical methods can further refine our understanding and help to tease out complex effects of the multiple input and outcome variables, especially the application of techniques that are equipped to handle large sets of interrelated variables *and* interrelated outcomes. As seen in our work, community members living in proximity to hardrock mines/wastes have a range of metal biomonitoring values for dozens of metals. Individuals can be subset by biomonitoring patterns into exposure "clusters" or profiles. While it is rare in human studies to see "clean" exposure to individual toxicants, individual toxicants can play unique roles in modifying observed effects of clustered metals as well. Therefore, in population work, it may be important to examine the health impacts of combined overall mixed metal exposures through cluster analyses, as well as to distinguish the relative importance of single metals in driving outcome changes. This type of effect was seen in the Chapter 4 clustering of metal exposure patterns on IFNγ where inclusion of individual metals not selected in the analysis as major components of the cluster actually were integral in distinguishing between observed clusters. Likewise, uranium was a strong predictor of IL-17, a cytokine that has been reported to have an important role in autoimmunity, and likely contributing to some of our overall population outcomes. Uranium, as a ubiquitous environmental contaminant on NAVAJO NATION,

does not cluster directly with the other groupings of metals significantly influencing the responses of IFNγ or IL-7. Yet uranium does indeed seem to individually contribute to perturbation of immune pathways.

Future work should model multiple, interrelated biomarkers as outcomes, such as groups of cytokines, immune cell populations, and/or autoantibodies that, taken together, characterize immune system status or classes of response. For example, TH1-associated IFNγ, IL-2, TNFβ, and CD4/CD8 ratio of T cells could be used as the outcome rather than IFNγ alone. This approach for using related immune system measurements as the outcome mirrors the way in which clinicians already diagnose autoimmune diseases, particularly diseases with complex and variable physical symptoms and test results, and extends it to research. In bidirectional pollination, statistical analysis of this type would be more readily translatable back to clinical practice by helping to identify appropriate groups of biomarkers for early detection, assessment, and intervention of immune dysregulation, especially in patients with concerns over metals exposures.

For several metals (e.g. uranium, tungsten, barium) and cytokines (IL-7, IL-17, IL-29) that we studied, the scientific literature is sparse. Basic science experiments in cell systems and animal models are needed to help fill gaps in knowledge about chronic low level single, and combined, metal exposure and the immune system, especially to demonstrate plausibility of relationships identified in human studies and to probe those observations to develop mechanistic understanding and potentially identify mitigation approaches based on that knowledge.

Overarching Conclusions and Future Work

While pursuing my scientific research goals, personal development dissertation goals emerged throughout the process. My broad personal development goals for my dissertation work included utilizing, integrating and channeling the broad skillset and knowledge from my background in nuclear engineering and health physics with my interests in community environmental health issues. I wanted to cultivate skills to channel the diversity of perspectives from both grass-roots and scientific communities into a focused approach that addresses environmental health problems in a culturally-appropriate, community-relevant, and scientifically-rigorous manner. By incorporating multiple aspects, I have pursued an interdisciplinary, translational and holistic approach to community environmental health issues. The multi-faceted nature and dual-population work of my dissertation cultivated experience with team science, in which knowledge and benefits are not only translated between bench to community, but cross-discipline and cross-community.

This dissertation aims to explore a thin cross-section, rather than a segment of a single layer, of the enormous question, "How does environmental exposure to mixed metals contribute to human health outcomes?" Rather than confining study to one of the following: environmental metal measurements, human metal body burden, or health outcomes, this work aimed to integrate across all three of these layers and examine the relationships among them. Broadly, this dissertation endeavors to present examples of synthesized approaches useful for probing complex, multidimensional environmental health questions, approaches which are applicable to other interdisciplinary work.

The beginning of my dissertation journey started out with the primary intent of focusing on environmental epidemiology to answer the two primary questions, "Are

metals found in measurable levels in community members living in proximity to mine waste sites?" and "Do metal biomonitoring levels in community members associate with measured immune system biomarkers?" My interest in potential immune system alterations stemmed, in part, from the desire to find a common link among environmental exposures and multiple adverse health outcomes and disparities observed on Cheyenne River Sioux Tribe (CRST) lands and Navajo Nation. Encountering the complexity of characterizing both the immune system and chronic environmental metal exposure in a human population rapidly led to the realization that statistical analysis would be challenging. In this way, my dissertation work grew, and also pivoted in a sense, to interrogate the ways in which to analyze a research question with both multidimensional inputs and outputs, and perhaps more importantly, how to interpret the analysis in a meaningful way to begin to answer a complex environmental health research question.

My interest in investigating the immune system as a possible common link between environmental exposure and multiple health outcomes in a human population through multiple statistical methods, in two populations, with all of the inherent messiness, is a testament to my desire to answer questions and solve problems across systems. My dissertation has been valuable in exposing me to the full spectrum of research: benchwork, sample collection, survey administration, and statistical analysis. While this knowledge will undoubtedly be applicable in my future endeavors, arguably the most important lessons from my graduate work are non-quantifiable and range beyond academic scientific learning. These are the lessons I have learned from CRST and Navajo community members about conceptualizing the world relationally, embracing

multiple ways of knowing, and allowing the totality of one's knowledge and experience to harmoniously inform action, broadly and in science.

With this framework, it is clear that, aside from the research that must be done to address gaps in scientific knowledge concerning chronic mixed metal exposure and health impacts, particularly in Native American/American Indian (NA/AI) communities, future work must extend to systemic change in multiple sectors: healthcare, local and federal government, and education. The term "translational" for many scientists often does not extend to this scope, but the only way to effect substantive, ongoing, sustainable change for communities dealing with environmental injustice is through improvement in these areas. Not only is engagement with healthcare and regulatory agencies required, but increased inclusion and representation of community members within these organizations is necessary in order to pursue interdisciplinary, culturally-appropriate, holistic solutions to environmental health problems. As I can attest from having the privilege of working alongside CRST and Navajo Nation community members, grassroots activism and personal stories are powerful. Combining bottom-up grassroots activism with top-down involvement from the multiple involved agencies is the only way to apply the sustained pressure required to address complex environmental health issues impacting indigenous people and lands, and halt propagation of harm to future generations.

Harnessing both grassroots and top-down organizational power will require intergenerational and multicultural collaboration. At meetings and community events, Navajo elders often express their desire to fight for environmental restoration and environmental health research for their children and grandchildren. Many Navajo elders

have, and continue to, share their stories with researchers, government officials and the public, in spite of an oftentimes high literal and emotional cost to themselves. Elders' experience; wisdom; and deep commitment to stewardship of land, culture, and community needs to be combined with the energy (even if partially-derived from anger), skills, and viewpoints of the younger generation to solve environmental health problems and sustain tribes for another ten generations.

Oftentimes the topic of the communication difficulties among community members, healthcare workers, scientists, and policymakers arises. While there is undoubtedly difficulty in translating English into Navajo and vice versa, I argue that overarching "translation" difficulties lie in cross-cultural communication, where "culture" in this case is broadly defined as assumed reference points that may vary depending on language, age, lived experience, and worldview. The need for "translators," who simultaneously embody multiple cultures, experiences, and ways of knowing, underscores the need to aggressively prepare, and encourage, younger generations to work in science, healthcare, regulatory, and government careers in order to shift understanding and priorities. As I have heard from the indigenous community members and trainees numerous times, this process needs to start with high-quality, culturally appropriate K-12 education, and freedom from worrying about basic needs.

This is not at all to say that all responsibility lies on adversely impacted communities, but to point out that a long-term plan to shift decision-making to community members is necessary. As I have observed firsthand, despite potential logistical difficulties, misunderstandings, and time requirements, creating space and opportunities for community members, researchers, clinicians, and policymakers to

interact and experience each other's everyday reality is beneficial and necessary. While I expected academic and agency collaborators to find the experience of visiting tribal lands to be eye-opening, I did not anticipate community members' reaction after they presented at a scientific conference with us. Specifically, community members arrived at an appreciation of the ways in which researchers doing community work juggle multiple priorities.

By nature, community environmental health research is complex and therefore requires dynamic, diverse teams that work synergistically together. The experiences during my dissertation work highlight the way in which seemingly heterogeneous groups must work together to develop a common set of experiences and language for effective communication. However, my experiences have also warned me of the need to be cognizant of power dynamics and assimilationist expectations, particularly in community research. Though everyone involved may be invested towards a common goal, existing underlying power dynamics often require community members to bend farther in terms of code switching and/or make them reluctant to contribute or ask for clarification.

Future personal work includes increasing capacity to act as a liaison and buffer among individuals, groups, and organizations involved in community environmental health work. This entails continuing to improve my abilities to think relationally, function multiculturally, and communicate science and values effectively to varied audiences. It also requires ongoing self-education in history, politics, and economics, particularly the ways in which they intersect with environmental health issues on tribal lands. I want to help communicate across the gaps, whether those gaps are cross-disciplinary, cross-agency, or cross-cultural, and also help to train and support others who

want to, or must, fulfill a similar role. In order to function in those liminal spaces, I seek to increase my resilience and stamina, because community environmental health research is, by nature messy, nonlinear, and long-term. It is my hope that this dissertation is one step on that journey, both scientifically and personally.

VI. APPENDIX

Active smoking, secondhand smoke exposure and serum cotinine levels among Cheyenne River Sioux communities in context of a Tribal Public Health Policy

In press (Tobacco Control)

O'Donald ER, Miller CP, O'Leary R, Ong J, Pacheco B, Foos K, et al. Active smoking, secondhand smoke exposure and serum cotinine levels among Cheyenne River Sioux communities in context of a Tribal Public Health Policy. Tob Control 2019. doi:10.1136/tobaccocontrol-2019-055056.

ABSTRACT

Tribal communities face disproportionately high active smoking and environmental tobacco smoke (ETS) exposures. The Cheyenne River Sioux Tribe is among the first Tribal Nations actively controlling tobacco exposures in public. We described tobacco use, ETS prevalence and identified predictors of serum cotinine (SC) concentrations among Tribal members enrolled into an environmental health study in which we had an opportunity to explore effects of the new tobacco policy. Self-reported survey and SC concentrations were used in generalized mixed linear models and quantile regression to explore changes and risk factors of SC levels. Among 225 adults, extreme rates of combustion tobacco smoking were detected, and 58% reported current ETS exposure. Among smokers, 16% were dual users consuming smokeless tobacco product. Significant differences in SC median values were found among participants with and without current ETS exposure. Substantial concentration drop was observed in the intermediate SC group (3-15 ng/ml) and in high SC group (>15 ng/ml) across the years. Current smokers had 6 times higher chance to be in the high SC group compared to non-smokers. Participants enrolled in 2014 had 13 times higher chance to be included in the high SC group than participants enrolled in 2016. Significant predictors of SC levels were sampling year, current smoking, and smokeless tobacco use. Gender and age had homogenous effects on smoking. The “Smoke-Free Clean Air Act" was implemented shortly before the 2015 sampling and already shown some positive changes in ETS exposures among CRST Tribal members.

INTRODUCTION

A declining trend in adult cigarette smoking has been demonstrated in the US across diverse ethnic groups [1-3], 21% of US adults reported smoking in 2005, which plummeted in 2014 to 17% with a further decrease to 15.5% in 2016. South Dakotans reported higher rates of smoking, compared with national rates [4-6]. However, no such positive trends in decreasing tobacco use can be observed among American Indian and Alaska Native (AI/AN) adults. Among them, 32% were current smokers in 2005, and 29% still reported active smoking in 2014, which stayed almost unchanged or even increased in 2016 (32%). Previous research demonstrated that even within Tribal communities, differences in cigarette smoking prevalence [7] and early initiation of smoking [8] were found. Further research confirmed that Northern Plain Indians had higher smoking prevalence than Southwestern Tribes [9].

Cigarette smoking is shown to have many adverse health effects that exacerbate known respiratory and cardiovascular problems [10]. Besides exposure to numerous organic, carcinogenic compounds, mainstream cigarette smoke also contributes to mercury, cadmium and arsenic exposures [11]. Community-based participatory collaboration with the Cheyenne River Sioux Tribe (CRST) has aimed to identify various mine waste metal exposures and their potential health effects on the CRST lands for more than nine years. The focus of the study centered on fishing and fish consumption as culturally significant activities; self-reported smoking and environmental tobacco smoke exposures were also assessed using a short smoking survey.

The Cheyenne River Sioux Tribe is one of the few Native communities – including White Earth Nation and Red Cliff Band of Lake Superior Chippewa Indians – recognizing tobacco smoking as an indoor air pollution problem and a serious public health threat to their communities [12, 13]. Tribal Clean Air Ordinance 77 was introduced on CRST in May 2015 controlling the high level of nicotine exposure in public places. This paper is intended to study self-reported smoking exposures and their association with serum cotinine levels among Tribal members in the time period before and after the Clean Air Ordinance 77 was implemented on CRST.

METHODS

Population and Sample Collection

The study enrolled 225 adult participants who provided informed consent in during the summers of 2014-2016. All participants were CRST Tribal members living in the Tribal Land in South Dakota. We recruited anglers/fishermen and study participants who reported outdoor activities bringing them to contact with the Cheyenne River. Each participant was interviewed using fishing, land-use, and smoking surveys and also provided a blood sample for laboratory serum cotinine analysis. The smoking survey is available in the Supplemental Materials for this paper. Participants' age, gender, and community location were also recorded. Geographical areas of enrollment were defined as zones based on the presence of predominant environmental toxicants of the Tribal Land. This study received UNM HSC HRRC approval (HRPO# 08-486) and Tribal Executive Resolution (E-135-2014-CR) supporting this academic collaboration,

community-based outreach, recruitment, enrollment and all proposed, and approved research activities on the CRST Sovereign Nation Land and among its population.

Laboratory Analysis

Serum samples were obtained at enrollment from all participants by venipuncture and stored in -80^{o}C freezer until laboratory use. Competitive ELISA assay was carried out to measure serum cotinine concentrations (ng/ml) following manufacturer's instructions (Calbiotech Inc. El Cajon, CA). As the primary and stable metabolite of nicotine, serum cotinine level is used to measure nicotine absorption (within approximately 16 hours) and metabolism by the body [14, 15]. Cotinine is also used as exposure biomarker of active (>15 ng/ml) and secondhand smoking exposure (3-15 ng/ml) in population-based studies [9,16], although the cutoff value varied in the literature [17].

Data Collection and Statistical Analysis

Participants' current smoking and tobacco chewing were assessed based on answers to the survey questions (survey in Supplemental Materials). Former smoking and tobacco chewing activities were not estimated. Current secondhand, environmental tobacco smoke exposure (ETS) at the home, workplace, and during leisure time activities were also considered. In addition, childhood ETS was also included in modeling.

To consider the amount of smoking both at the personal and ETS-level, we inquired about the participants' own and others' tobacco consumption indoors. Survey information was coded to create binary tobacco exposure variables (yes/no) for current smokers and

chewers. A binary composite ETS (CoETS) (yes/no) variable (yes/no) was also used to capture smoking by others at home, in the workplace, or during leisure time activities.

Participants were separated into two age groups: <42 and ≥42 years old, based on the mean age (41.8 ± 13.4 years) of all study participants in order to examine age influence on serum cotinine concentrations (SC). SC concentrations (ng/ml) were used both as a continuous variable in modeling and dichotomized to create a binary outcome variable. Furthermore, SC groups were created using literature information on serum cotinine concentration values as thresholds [18,19]. The low SC group was formed, who were non-tobacco users without ETS (SC<3 ng/ml), and also a high SC group was used in modeling, who were mainly tobacco user participants (SC≥15 ng/ml). In addition, an intermediate SC group (3-15 ng/ml), was also examined in statistical analyses. To assess significant difference in median SC level among the groups, and the Wilcoxon (Mann-Whitney) rank-sum test and the Kruskal-Wallis test were employed (Table A1.) Chi-square test and the Fisher's exact test were also utilized for examining differences in proportions between the groups.

Binary outcome of SC was modeled using logistic regression modeling and the odds ratios (OR) and their 95% confidence intervals were provided (Table A2) for both low and high SC groups. An interaction between active smoking and tobacco chewing was also included as a predictor variable of dual use in final multivariable logistic models. The Akaike Information Criterion (AIC) of the final pseudo-model was applied to compare models and select the best fitting one.

In addition, quantile regression was also utilized to provide estimates that were more robust against outliers in the SC measurements especially because the distribution of SC concentrations was skewed with some very high concentrations detected among participants. Quantile regression model (QRM) plots were provided for visual examination. All statistical analyses were performed using SAS, v9.2 software (SAS Institute Inc, Cary, NC). All tests were two-sided and the p-value of <0.05 was considered for statistical significance. No Bonferroni correction was carried out, as predictor variables used in the models have documented influence on SC values and therefore were not randomly used in modeling.

RESULTS

Characteristics of the study subjects (gender, age group, fishing, and smoking status) are presented in Table A1. A larger proportion of participants lived in the community center area (Eagle Butte and surroundings). Mercury was the most prominent environmental contaminant affecting the community through multiple sources: persistent water contamination, fishing, and coal-burning power plants' particulate matter exposures. Fishing was linked to higher smoking exposures (Table A1), potentially through lifestyle factors.

Current smokers and tobacco chewers had significantly increased SC concentrations than non-smokers and non-chewers ($p<0.0001$ and $p=0.0457$, respectively). Furthermore, participants with CoETS had higher SC concentrations than others without ETS ($p=0.0357$).

There were significant decreases over the years in overall SC levels among all CRST participants (2014 -2016; $p=0.0062$), and also both among tobacco users ($p<0.0001$) and non-users ($p<0.0001$). However, SC levels of non-tobacco users with CoETS were not significantly different from SC levels of non-tobacco users without CoETS overall ($p=0.4783$). More current smokers reported also CoETS compared to non-smokers.

A substantial portion (43.9%) of Tribal tobacco chewers also smoked cigarettes. Similar to active smokers, the proportions of tobacco chewers and tobacco users did not significantly differ across the collections years (2014: 20%, 64%; 2015: 14.7%, 60%; 2016: 20%, 58.7%; $p=0.6205$, 0.7846, respectively, data not shown in Table A1). While we enrolled a new set of participants across the CRST lands in each sampling year, their age, gender, fishing and smoking status were not significantly different.

Table A1. Serum cotinine concentrations (ng/ml) presented by demographics and smoking status among CRST community members

Variable	**n (%)**	**Mean (±SD) Serum Cotinine** (ng/ml concentration)	**Median** (Range: Min, Max) **Serum Cotinine** (ng/ml concentration)	**p-value***
Total	225	89.5 (152.5)	12.9 (0.4; 859.1)	
Age group				
Younger (18-41 y/o)	118 (52.4)	79.7 (136.8)	12.3 (0.4; 650.9)	0.9779
Older (42-77 y/o)	107 (47.6)	100.3 (168.1)	13.1 (0.4; 859.1)	
Gender				
Male	109 (48.4)	90.4 (155.8)	14.8 (0.4; 761.3)	0.8603
Female	116 (51.6)	88.6 (149.9)	11.5 (0.4; 859.1)	
Presence of environmental exposures				
Community center area	98 (43.6)	80.5 (146.1)	11 (0.4; 761.3)	0.2704
Arsenic zone	46 (20.4)	84.1 (118.6)	17.6 (0.4; 506.3)	
Pesticide zone	56 (24.9)	115.5 (192.7)	12.2 (0.4; 859.1)	
Mercury zone	25 (11.1)	76.4 (130.8)	10.9 (0.4; 544.7)	
Current anglers/fishermen				
Yes	164 (72.9)	96 (162.5)	14 (0.4; 859.1)	0.3785
No	61 (27.1)	72.1 (120.9)	9.9 (0.4; 544.7)	
Current smoker				
Yes	114 (50.7)	137.5 (168.2)	79.6 (0.4; 859.1)	p<0.0001
No	111 (49.3)	40.2 (115.9)	8.8 (0.4; 761.3)	
Current tobacco chewer				
Yes	41 (18.2)	130.5 (205.4)	19.5 (0.4; 761.3)	0.0457
No	184 (81.8)	80.4 (137)	11.2 (0.4; 859.1)	
Current CoETS≠				
Yes	129 (57.6)	104.6 (161.1)	15.3 (0.4; 859.1)	0.0357
No	95 (42.4)	69.3 (139)	10.6 (0.4; 761.3)	

Table A1 (cont)

Tobacco user, by Collection Year				
Tobacco users in 2014	48 (21.3)	203.9 (175.9)	173.8 (0.4; 650.9)	p<0.0001[†1]
Tobacco users in 2015	45 (20.0)	173.9 (203.2)	106.7 (0.4; 859.1)	
Tobacco users in 2016	44 (19.6)	24.0 (45.1)	13.5 (8.8; 238.4)	
Non-users in 2014	27 (12.0)	36.4 (89.4)	3.5 (0.4; 356.9)	p<0.0001[†2]
Non-users in 2015	30 (13.3)	6.6 (26.1)	0.4 (0.4; 142.7)	
Non-users in 2016	31 (13.8)	9.3 (2.3)	9.0 (0.4; 17.4)	
Childhood ETS (at home)[≠]				
Yes	138 (61.6)	105.5 (167.9)	14.9 (0.4; 859.1)	0.0601
No	86 (38.4)	64.2 (121.4)	11.1 (0.4; 650.9)	
SC at 3 ng/ml threshold				
<3 ng/ml	50 (22.2)	0.7 (0.5)	0.5 (0.4; 2.7)	p<0.0001
≥3 ng/ml	175 (77.8)	114.9 (164.3)	21.4 (3.1; 859.1)	
SC at 15 ng/ml threshold				
<15 ng/ml	121 (53.8)	5.8 (4.9)	6.4 (0.4; 14.9)	p<0.0001
≥15 ng/ml	104 (46.2)	186.9 (180.9)	144.5 (15.0; 859.1)	
Smoking status: n (%)	**Mean Age (yrs, SD)[ǂ]**			
Non-smoker, non-chewer, no ETS: 53 (23.5)	41.9 (13.2)	15.7 (51.9)	3.5 (0.4; 356.9)	<.0001
Non-smoker, non-chewer, has ETS: 35 (15.5)	43.3 (15.7)	18.1 (55)	2.7 (0.4; 247.5)	
Chewer and non-smoker: 23 (10.2)	37.2 (8.7)	130.3 (213.1)	19.4 (0.4; 761.3)	
Smoker and chewer: 18 (8)	36.9 (7.2)	130.8 (201.2)	21.6 (0.6; 650.9)	
Smoker and non-chewer: 96 (42.6)	43.1 (14.2)	138.7 (162.5)	90.7 (0.4; 859.1)	

Figure A1 demonstrates changes in SC concentrations in all participants during the enrollment period (2014-2016). It is shown that both mean (dashed line) and median (solid line) SC concentrations decreased from 2014 to 2016. Statistical tests, however, did not indicate a significant trend that was different from zero.

Active smoking stayed overall high among participants (57.3% in 2014, 48% in 2015, and 46.7% in 2016; p=0.3773). In addition, a decreasing, however not statistically significant trend was detected in the frequency of CoETS in the respondents' environment over the years.

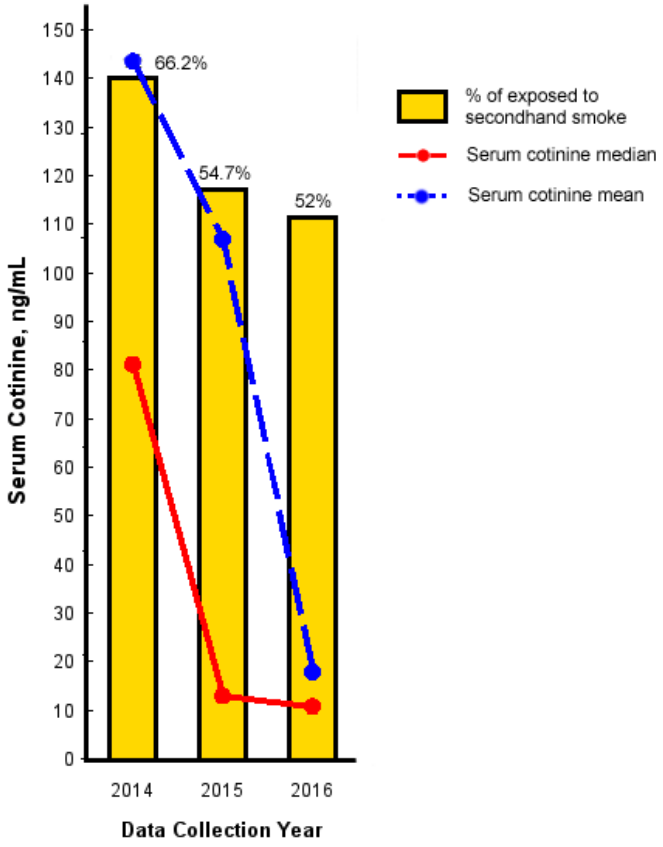

Figure A1. Serum cotinine levels and CoETS in all participants by the data collection year

Results of the multivariable logistic regression models are presented in Table A2 by the two groups of SC values, low and high. Results showed that both low (<3 ng/ml) and higher SC concentrations (≥15 ng/ml) were more likely to be found in 2014 and 2015 compared to the year of 2016 sampling. Based on these models, current smokers were predicted to have higher odds for increased SC levels (OR=16.6) and less likely to be in the low SC group. When interaction between active smoking and tobacco chewing was also considered, these odds ratios decreased except for the low SC group comparison in 2015 to 2016 and for the high SC group in comparison of 2014 to 2016 year's sampling.

Table A2. Logistic regression models predicting chances of CRST participants of having serum cotinine levels below and above the literature thresholds for smoking categories.

	Low SC group: OR (95% CI) for Serum Cotinine <3 ng/ml (compared to SC ≥3 ng/ml)		**High SC group: OR (95% CI) for Serum Cotinine ≥15 ng/ml (compared to SC <15 ng/ml)**	
Variable	No Interactions	With Interaction between Smoking and Chewing	No Interactions	With Interaction between Smoking and Chewing
Age (≥42 yrs)	1.01 (0.38 – 2.68)	1.06 (0.4 – 2.8)	1.54 (0.73 – 3.22)	1.6 (0.72 – 3.56)
Anglers/fishermen	1.26 (0.44 – 3.61)	1.3 (0.46 – 3.65)	0.88 (0.38 – 2.03)	0.78 (0.32 – 1.95)
Chewing tobacco	0.37 (0.09 – 1.55)	0.91 (0.26 – 3.15)	2.93 (1.09 – 7.92)	2.59 (1.01 – 6.59)
Data Collection (yrs)				
2014, compared to 2015	0.37 (0.12 – 1.16)	0.25 (0.08 – 0.8)	1.57 (0.62 – 3.99)	2.41 (0.85 – 6.84)
2014, compared to 2016	46.58 (4.62 – 469.51)	45.71 (5.18 – 403.06)	8.8 (3.36 – 23.09)	12.91 (4.5 – 37.07)
2015, compared to 2016	124.88 (11.44 – >999.999)	180.08 (18.08 – >999.999)	5.61 (2.12 – 14.84)	5.37 (1.95 – 14.77)
Female gender	1.44 (0.5 – 4.13)	1.23 (0.44 – 3.43)	0.78 (0.36 – 1.69)	0.83 (0.37 – 1.86)

Table A2. (cont)

Environmental Exposure Zones				
Community Center, compared to Arsenic EA	1.96 (0.38 – 10.23)	1.62 (0.33 – 7.99)	0.44 (0.16 – 1.24)	0.44 (0.15 – 1.32)
Community Center, compared to Pesticide EA	1.2 (0.37 – 3.85)	1.33 (0.43 – 4.16)	1.13 (0.45 – 2.87)	0.95 (0.35 – 2.58)
Community Center, compared to Mercury EA	1.1 (0.22 – 5.37)	1.06 (0.22 – 5.1)	0.87 (0.26 – 2.91)	0.9 (0.24 – 3.31)
Arsenic EA, compared to Pesticide EA	0.61 (0.1 – 3.66)	0.82 (0.15 – 4.59)	2.57 (0.79 – 8.34)	2.16 (0.62 – 7.49)
Arsenic EA, compared to Mercury EA	0.56 (0.07 – 4.65)	0.66 (0.08 – 5.17)	1.97 (0.48 – 8.1)	2.03 (0.44 – 9.26)
Pesticide EA, compared to Mercury EA	0.92 (0.18 – 4.7)	0.8 (0.15 – 4.1)	0.77 (0.21 – 2.77)	0.94 (0.23 – 3.83)
Childhood home ETS	0.44 (0.16 – 1.24)	0.44 (0.16 – 1.2)	1.4 (0.65 – 3.01)	1.12 (0.5 – 2.53)
CoETS	2.57 (0.86 – 7.71)	2.9 (0.98 – 8.57)	0.56 (0.25 – 1.26)	0.57 (0.24 – 1.34)
Current Smoker	0.03 (0.01 – 0.12)	0.11 (0.03 – 0.4)	16.63 (7.06 – 39.16)	6.06 (2.31 – 15.86)
Model Fitting Criteria:				
-2 Res Log Pseudo-Likelihood	1401.95	1399.05	1096.79	1138.24
Pseudo-AIC	1429.95	1429.05	1124.79	1168.24

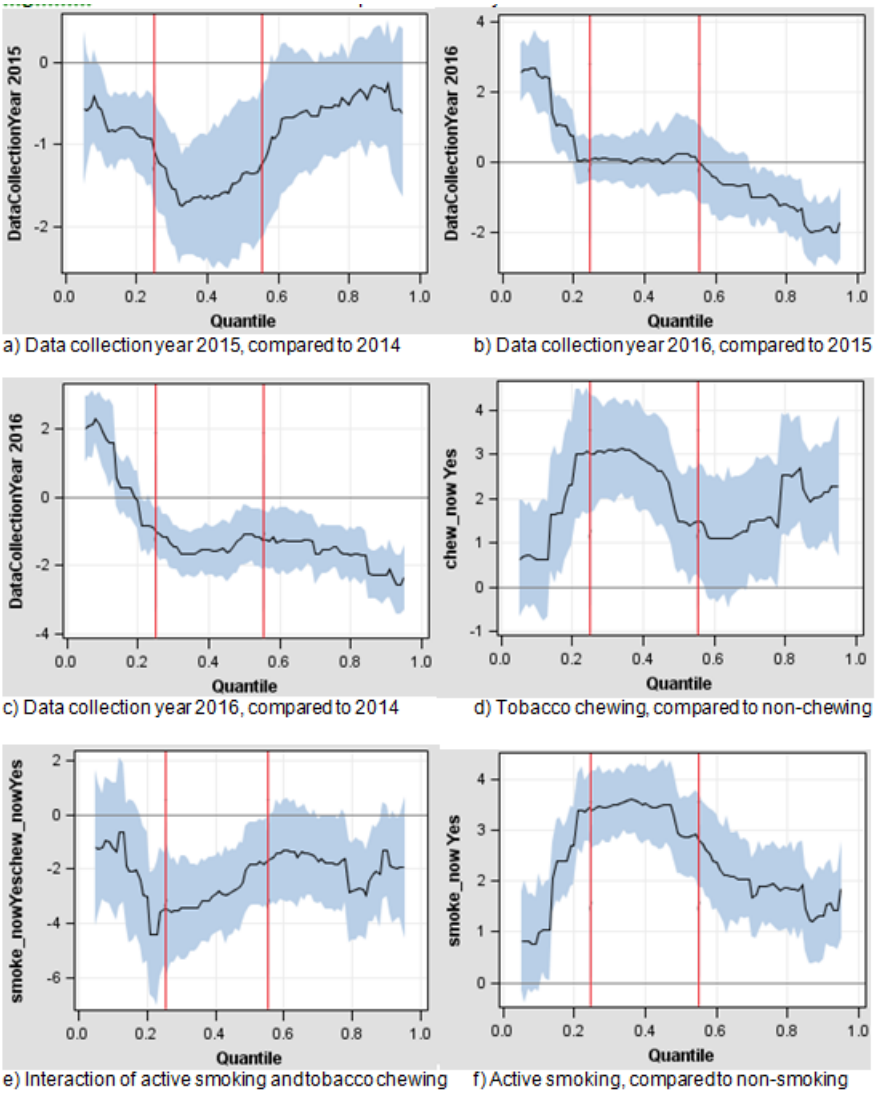

Figure A2. QRM: Selected estimated parameters by Quantile for Serum Cotinine natural log (with 95% CI)

When QRM was employed, similar results to logistic modeling were obtained, confirming that sampling years were critical contributors to increasing likelihood of low SC values in the study. Increased levels of SC concentrations were also predicted by self-reported smoking and chewing statuses in almost all quantiles of cotinine concentrations (see Figure A2). Low SC threshold of 3.08 ng/ml was associated with the 0.225-th SC

quantile while the higher SC threshold of secondhand exposure value, 15.02 ng/ml was associated with the 0.540-th SC quantile in the models documented in the graphs below.

CONCLUSIONS

Since using SC levels as biomarkers of exposure, we confirmed self-reported tobacco use. The reliable connection of survey information to confirmed tobacco exposures has continuously been an important discussion point in tobacco research [18-20, 23]. It is documented in several studies that self-reported surveys can significantly underestimate tobacco use.

However, CRST combustion tobacco user participants had significantly higher concentration of SC across all sampling years, compared to non-users. In addition, a decrease in SC mean and median levels were observed over the data collection years, which was most prominent in comparing 2014 to 2016. The year 2015 biospecimen sampling occurred shortly after the CRST Tribal Ordinance 77 was enacted by CRST Tribal Council under the "Smoke-Free Clean Air Act" on May 4, 2015. This decrease was also confirmed both for the intermediate and high SC groups even though did not reach statistical significance. In the low SC group, a decrease of SC levels was shown in 2015 (compared to 2014); and an increase in lower SC concentrations was found in 2016 compared to 2014. As no significant active smoking changes were reported in the community using the self-reported surveys, a detectable even though not statistically significant trend was seen over the collection years in CoETS. ETS exposure seems to be one important component of the detected extreme high SC concentrations observed in CRST combustion tobacco users.

Nevertheless, we also consider that possible genetic factors shown to be molecular drivers of high nicotine metabolizing capacity and the demonstrated prevalence of single nucleotide polymorphisms in *CYP2A6* gene among the same group of Great Plain Natives could also be part of the high SC associations [9,18]. The extremely high SC concentrations among Tribal members (highest concentration of 859.1 ng/ml) warrant further work as part of health disparity research. This work is also important in terms of engaging the community and urging the continuation of existing anti-smoking campaign efforts taken on by the Tribal health leadership and the CRST Canli Coalition.

Smoking survey data collected by self-report demonstrated consistency in CRST study participants and proven to serve the categorization of study participants' tobacco use statuses. However, in light of the importance of home ETS, inclusion of third-hand smoking exposure and confirmatory detailed environmental testing are suggested for future research on the CRST.

The reliability of serum cotinine testing in identifying individuals with active tobacco use was evaluated in this analysis. No known demographic variables such as age, gender, fishing status, or the participants' geographical location and their possible toxicant exposures altered the predictive association of sampling years and tobacco-related covariates on serum cotinine concentrations. That observation further promotes the usefulness of such accurate cotinine testing in community-based epidemiological studies. Smoking survey reliably categorized our study participants; furthermore, SC can be easily and successfully included in applications of various health outcome evaluations (e.g. immune system alterations, presence of chronic disease diagnosis).

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