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Legacy mining metal exposures contribute to circulating oxidized low density lipoprotein and serum inflammatory potential in a native community

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LEGACY MINING METAL EXPOSURES CONTRIBUTE TO CIRCULATING OXIDIZED LOW DENSITY LIPOPROTEIN AND SERUM INFLAMMATORY POTENTIAL IN A NATIVE COMMUNITY

by

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DEDICATION

I dedicate this dissertation to my daughter, Lily. You are only 4 years old right now, but you have been the sunshine in my heart and the laughter in my soul. Thank you for having to tolerate all of the times when I had to focus more on school than on you (thankfully that was mostly only the last few weeks). I know that you did not like it, not one little bit. But I hope that by watching your mommy go to school that someday you will pursue big goals and big dreams, and I promise to help you in every way that I humanly can. I love you, Lily, more than anything in the entire universe, always and forever. “Doop-ee doop-ee doo.”

I also dedicate this dissertation to my parents, Diane and Dr. Gary Harmon. I thank you from the bottom of my heart for your love and for supporting me during graduate school. I am so thankful that Lily got to spend many moments of her early childhood with you these past few years. Thank you forever for affording me the opportunity to continue mountain biking and to simply go to the mountains, for these things are as much a part of my existence as the air I breathe.

I equally dedicate this dissertation to my wonderful mentor Dr. Matt Campen for allowing me this opportunity, while also supporting me as a parent along the way.

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Legacy Mining Metal Exposures Contribute to Circulating Oxidized LDL and Serum Inflammatory Potential in a Native Community

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ABSTRACT

Numerous abandoned uranium mines (AUM) within the Navajo Nation contribute uranium (U), arsenic (As) and other metals to the soil, air and groundwater that continue to pose potential health risks to Navajo residents. The prevalence of inflammatory-related cardiovascular disease (CVD) and type 2 diabetes (T2D) has increased among the Navajo community in recent decades. Environmental exposure to metal contaminants may alter the circulation in ways that are associated with the inflammation-driven process of atherosclerotic plaque formation. Thus, we hypothesize that exposures to AUM metal contaminants are associated with pro-atherogenic changes in the circulation as evidenced by increased low-density lipoprotein (LDL) oxidation and serum inflammatory potential.

To assess the relative contribution of mining metals exposures on community health, we assessed oxLDL and its relationship to CVD and T2D biomarkers in the Navajo, and then these data were linked to mining contaminant exposure metrics (water intake and urine metals). As proof-of-concept and to examine a possible mechanism, acellular assays investigated if As and U could directly oxidize purified human LDL cholesterol. Lastly, since serum represents the balance of inflammation, i.e. inflammatory potential, transcriptional responses of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and chemokine (C-C motif) ligand (CCL2) were measured from primary endothelial cells treated with participant serum. These responses were linked to mining contaminant exposures (water intake and AUM proximity).

Regression modeling showed that estimated annual intake of As from drinking water is a significant predictor of oxLDL in this population, despite oxLDL levels being similar to levels in other populations without evidence of CVD. We found that As, but not U, oxidized the protein but not the lipid components of LDL cholesterol. Linear regression modeling showed that AUM proximity solely and strongly predicted serum inflammatory potential as measured by endothelial cell transcription of VCAM-1, ICAM-1 and CCL2.

Taken together, our data suggest that exposure to U mining waste in a population with several CVD risk factors influences pro-atherogenic changes in the circulation, and that other exposure routes, metals, and mixtures should be investigated. Our data may aid in modifying clinical treatment decisions and exposure reduction strategies.

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ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
AIC	Akaike information criterion
ANL	Argonne National Laboratory
ANOVA	analysis of variance
ApoB	apolipoprotein B
ApoE	apolipoprotein E
As	Arsenic
AUM	abandoned uranium mine
BMI	body mass index
BP	blood pressure
CAD	coronary artery disease
CCL2	chemokine (C-C motif) ligand 2
CDC	Centers for Disease Control
CHD	coronary heart disease
CRP	c-reactive protein
Cu	copper
$CuSO_4$	copper sulfate
CV	cardiovascular
CVD	cardiovascular disease
DBP	diastolic blood pressure
DiNEH	Dine Network for Environmental Health
DMA	dimethylarsinic acid
ELISA	enzyme linked immunosorbent assay
EPA	Environmental Protection Agency
ESRD	end-stage renal disease
HbA1c	glycated hemglobin
hCAEC	human coronary artery endothelial cell
HDL	high-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICAM-1	intercellular adhesion molecule 1
ICP-MS	inductively coupled plasma mass spectrometry
IL6	interleukin 6
IQR	interquartile range
LDL	low-density lipoprotein
LXR	liver x receptor
MDA	malondialdehyde
MMA	monomethylarsonic acid
mRNA	messenger ribonucleic acid
NaAsO2	sodium arsenite
NFκB	nuclear kappa-factor-light-chain enhancer of activated of B cells

NHANES	National Health and Nutrition Survey
Ni	nickel
OxLDL	oxidized low-density lipoprotein
PBS	phosphate buffered saline
ppb	parts per billion
ppm	parts per million
qPCR	quantitative real-time polymerase chain reaction
R^2	correlation of determination
ROS	reactive oxygen species
SBP	systolic blood pressure
SHS	Strong Heart Study
T2D	type 2 diabetes
TBARS	thiobarbituric acid reactive substances
TC	total cholesterol
TG	triglycerides
U	uranium
UA	uranyl acetate
US	United States
V	vanadium
VCAM-1	vascular cellular adhesion molecule 1
VLDL	very low-density lipoprotein
WHO	World Health Organization

I. Chapter 1

INTRODUCTION

Communities of the Navajo Nation reside in a geographical area rich in uranium ore. Natural deposits and historical mining activities have left a legacy of abandoned uranium mines (AUM) and exposure risks from the remaining metal contaminants. The Navajo have expressed ongoing concerns about how their health may be affected by these contaminants. The high prevalence of cardiovascular related health conditions, including type 2 diabetes (T2D) and obesity, present in this vulnerable population may be exacerbated by mining contaminants. Chronic, unalleviated exposures to contaminants such as those present in mining wastes may result in changes in the circulation that interact with the endothelium leading to permanent remodeling and worsening of disease. To assess the potential impact of these contaminants on the cardiovascular health of exposed individuals, we examined traditional (interleukin-6, IL6; C-reactive protein, CRP) and novel oxidized low-density lipoprotein (oxLDL) plasma cardiovascular disease (CVD) biomarkers as well as serum inflammatory potential and linked these cardiovascular (CV) indicators to measures of exposure including water intake and household proximity to AUM.

Epidemiology of Cardiovascular Disease

Cardiovascular disease is the leading cause of death globally, and according to the World Health Organization (WHO 2015), approximately 17.5 million deaths in 2012 were due to CVDs, nearly one-third of all deaths worldwide. In the United

States, CVD has been the leading cause of death for the last 80 years and is a major cause of disability with estimated costs in the U.S. exceeding $270 billion, which is expected to triple by 2030 (Heidenreich et al. 2011). Nearly 7.4 million of all worldwide CVD-related deaths were due to coronary artery disease (CAD) (WHO 2015). Atherosclerosis is the principle cause of CAD. Risk factors include hyperlipidemia, hypertension, obesity, diabetes, insulin resistance as well as family history, age, poor nutrition, physical inactivity, and smoking. The pathogenesis of atherosclerosis involves an imbalance in lipid metabolism and a perpetual immune response leading to chronic inflammation of the arterial wall (Weber and Noels 2011).

Cholesterol metabolism

Although a minimal amount of cholesterol comes from the diet, the liver forms most cholesterol. Lipids absorbed from the intestine are packaged into chylomicrons (large, triglyceride-rich particles), which undergo lipolysis to form chylomicron remnants and are taken up by the liver. The liver secretes very low-density lipoproteins (VLDLs) (triglyceride-rich lipoproteins); this is the first step of the endogenous lipoprotein pathway. VLDL are converted via intermediate-density lipoproteins to low-density lipoprotein (LDL) or taken up by the liver. High circulating levels of LDL are a risk factor for atherosclerosis. Normally, LDL are removed from the circulation by the liver and other tissue cells via LDL-receptor-mediated endocytosis. Apolipoprotein B-100 (ApoB 100) is the major

apolipoprotein carrier of chylomicrons, VLDL, intermediate density lipoprotein and LDL (Dieplinger and Dieplinger 2012).

High density lipoprotein (HDL) cholesterol plays an important role in the reverse transport of cholesterol from peripheral tissues to the liver. HDL is synthesized by the liver and intestines, or from macrophages via cellular cholesterol efflux, and then transported to the liver, i.e. reverse cholesterol transport, which is important for removal of peripheral cholesterol. ApoA-I is the protein carrier for HDL and helps mediate cholesterol efflux from macrophages via binding to ATP Binding Cassette Transporter A1 (ABCA1) or scavenger receptor BI on macrophages and takes up free cholesterol. During inflammation, cholesterol efflux is reduced via inhibition of the liver x receptor (LXR) and retinoid x receptor causing levels of circulating cholesterol to increase (Dieplinger and Dieplinger 2012).

Atherosclerosis and Inflammation

Atherosclerosis, the main cause of CAD, is the slow, chronic inflammatory process by which lipids accumulate in the vascular intima leading to lesion formation. The eventual rupture of the atherosclerotic lesion and resulting thrombosis, and is the main cause of myocardial infarction and stroke. The endothelium is the first cell type to encounter and respond to changes in the circulation. Endothelial response to challenges (e.g. toxins) in the circulation is largely driven by inflammatory cytokines (Ross e al. 1999). The initial response

is an increase in the permeability of the arterial wall.. This disruption of the endothelial barrier and formation of gaps between endothelial cells depend on cytoskeletal changes (contractile elements including microfilaments and myosin) (Kumar et al. 2009). Next, the endothelium is activated through receptor-mediated gene transcription of inflammatory cytokines (IL6, interleukin-8), chemokines including chemokine (C-C motif) ligand (CCL2), adhesion molecules including E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which serve to recruit immune cells (monocytes, T-cells) to the site of injury, and tissue factor (involved in coagulation and fibrin formation). The result is transformation of the endothelium from an anti-thrombotic to a pro-thrombotic phenotype (Ross 1999; Hunt and Jurd 1998).

Monocytes extravasate into the vascular intima following a chemokine gradient (chemotaxis) through the basement membrane and associating with the extracellular matrix via integrin binding (Ross 1999). Monocytes differentiate into macrophages once they move into tissue. Macrophages secrete cytokines, reactive oxygen species, nitric oxide, proteases, and growth factors that promote fibrosis and angiogenesis. Macrophages engulf LDL, especially oxidized LDL, eventually forming foam cells. Foam cells in turn secrete inflammatory cytokines, which trigger smooth muscle cell proliferation, lipid accumulation and plaque formation, and eventual rupture of the fibrous cap and thrombosis (Weber and

Noels 2011). Eventual outcomes of this process are ischemia, myocardial infarction and stroke.

Pathological changes and endothelial dysfunction may result if the activating stimulus is unalleviated (Ross 1999). Homeostatic mechanisms of the vasculature will eventually be unable to compensate, resulting in plaque accumulation, vascular remodeling and clinical disease (Ross 1999), which may be exacerbated when other independent risk factors (e.g. diabetes, hypertension etc.) (Gutiérrez et al 2013) or environmental insults are present (e.g. air pollution, metal contaminants) (Brook et al. 2010; Prozialeck et al. 2008), such as might occur with chronic exposure to metals from mining waste.

Oxidized LDL

The LDL molecule is a complex arrangement of lipid components around a single ApoB 100 molecule, a very large amphipathic protein (Segrest et al. 2001) which contains 25 cysteine residues as part of its primary structure (Yang et al 1994). The lipid components consist of an external phospholipid monolayer and free cholesterol and a lipid core that contains cholesterol esters and triglycerides (TG).

The oxidation of LDL is known to be involved in the initiation of atherosclerosis. LDL is retained in the intima by binding of ApoB to the extracellular matrix (Lusis et al. 2000). Oxidation of LDL may occur in the vessel wall, especially in

atherosclerotic plaques; while circulating in the plasma; or even intracellularly (Satoh and Tokunaga 2002), but the exact mechanisms and sites of oxidation are not well understood (Itabe et al. 2011; Yoshida and Kisugi 2010). LDL may be modified by enzymes (myeloperoxidase, lipoxygenase), reactive oxygen species (ROS), reactive nitrogen species and metal ions (Yoshida and Kisugi 2010).

LDL can be minimally- or fully- oxidized, and modifications to LDL can be diverse leading to differing effects. Minimally-modified LDL is still recognizable by the LDL receptor (LDL-R). Fully-oxidized LDL is not recognized by LDL-R (Yoshida and Kisugi 2010), but rather is recognized by scavenger receptors on endothelial cells (Ox-LDL receptor 1, scavenger receptor A) and macrophages (scavenger receptors A and B, and cluster of differentiation-36 receptor) (Zani et al. 2015), which may be a result of evolutionary immune responses to “non-self” antigens. The oxidation-specific epitopes such as those present on oxLDL are considered danger-associated molecular patterns that are recognized by pattern recognition receptors, such as cluster of differentiation 36 and toll-like receptor-4, resulting in pro-inflammatory gene expression (nuclear kappa-factor-light-chain enhancer of activated of B cells; NFκB and activator protein-1), antigen presentation, and lipid uptake (Miller et al. 2011). OxLDL induces endothelial cell and macrophage activation and triggers matrix metalloproteinases, surface expression of adhesion molecules such as ICAM-1 and VCAM-1, and chemokines including CCL2 (Weber and Noels 2011).

CVD Biomarkers

Because CVD develops over a long periods of time (decades), but culminates in life-threatening events such as myocardial infarction or stroke, the derivation of personal risk factors for a CVD-related event have been a valuable prognostic tool for physicians. While several non-modifiable risk factors are included in risk calculations (age, gender, familial history), numerous biomarkers (CRP, IL6) have also been examined as a potential means of improving the accuracy of event predicting a CVD event.

Circulating oxLDL, a novel CVD biomarker, are associated with subclinical CVD and metabolic syndrome (Holvoet et al. 2007) and are predictive of future cardiovascular events (Holvoet et al 2004, Johnston et al. 2006, Meisinger et al. 2005). OxLDL is a major player in the development of atherosclerotic lesions and, has not been assessed in any Native American population to date.

Epidemiology of CVD Native Americans

Native Americans generally are known to have a high prevalence of type 2 diabetes, metabolic syndrome, obesity and hypertension, which are all major risk factors for CVD (Howard et al 1999, Hoy et al 1995, Sugarman et al 1990, Welty et al 1995). CAD, hypertension and diabetes rates in Native Americans exceed those of the general U.S. population (Centers for Disease Control, CDC, 2011, 2014; Galloway 2002; Howard et al. 1999). Findings from the Navajo Health and

Nutrition Survey, conducted in the 1990s, also concluded that the Navajo population has a high prevalence of overweight, diabetes, and hypertension (Percy et al. 1997, White et al. 1997, Will et al.1997).

In the 1980s, the Strong Heart Study (SHS) began assessing cardiovascular disease and its risk factors in 13 Native populations from Arizona, North and South Dakota, and Oklahoma since (Lee et al.1990). The Pima Indians of Arizona have a high prevalence of T2D, insulin resistance and obesity, but a lower disposition for atherosclerosis relative to the native communities studied in Oklahoma and North and South Dakota (Howard et al. 1995), and the incidence of fatal coronary artery (heart) disease was less than half that found in the Framingham population after controlling for age, sex, and diabetes, which has been suggested to be due in part to lower LDL levels (Nelson et al. 1990; Weyer et al. 2002). Many Native communities, including Navajo (Coulehan et al.1986), have historically had lower incidences of myocardial infarction, ischemic heart disease, and LDL levels (Becker et al. 1988; Sievers et al. 1968) in comparison to the general population despite a high prevalence of diabetes, obesity and hypertension, but more recently CAD has been on the rise (Klain et al. 1988; Mendlein et al.1997; Sugarman et al. 1992), especially in those with diabetes (Howard et al. 1999; Xu et al. 2012).

Inflammation plays a significant role in the pathogenesis CV-related conditions including atherosclerosis, CAD, obesity, diabetes, and hypertension. CRP is a

well-established inflammatory marker that is elevated in CVD, T2D, and obesity. Only a handful of studies have looked at inflammation in Native American communities. The SHS found that CRP was predictive of CVD in these populations, especially in women but not in those with type 2 diabetes (Best et al. 2005). In Pima Indians, CRP and ICAM-1 were increased with increasing adiposity and ICAM-1 and E-selectin were associated with insulin resistance (Weyer et al. 2002). IL6 is a cytokine that is increased in inflammation and causes the release of CRP. A polymorphism in the IL6 promoter is associated with type 2 diabetes in Caucasians and Pima Indians (Vozarova et al. 2003), although in another longitudinal study, circulating markers of inflammation (CRP, IL6) and endothelial function (ICAM1, VCAM1) were not associated with the development of diabetes in Pima Indians (Krakoff et al. 2003). Little information exists on CVD-related inflammatory or endothelial markers in Native populations, especially Navajo.

Uranium Mining on the Navajo Nation

The Navajo Nation is located on the Colorado Plateau, an area rich in the mineral carnotite, a significant source of U. Carnotite (K2(UO2)2(VO4)23H2O), consists of approximately 53% U, 11% vanadium (V) and trace amounts of radium. (Dias de Cunha et al. 2014) Early Navajo and Ute Indians are said to have used the red and yellow ores as body paint. Soon after the discovery of radium by Marie and Pierre Curie in 1898, radium began to be mined from this region. (US Environmental Protection Agency, EPA, 2008, 2013). This was followed by V

mining in the 1920s, first for its uses in steel and then in the armament industry during WWII. With the arrival of the Atomic age, U was extensively mined starting in the mid-1940s. The U mined from the northern area of this region was used for the atomic bomb.

Exposure sources

Nearly four million tons of high grade U ore was extracted in the Grants U District on and near the Navajo Nation during mining operations from 1944 to 1986 under lease agreements with the Navajo Nation, leaving behind a legacy of over 500 AUMs, four U processing mills, 1,100 mine features (e.g. portals, prospects, rim strips, pits, vertical shafts or waste piles), and hundreds of contaminated wells (U.S. Environmental Protection Agency, US EPA, 2008, 2013). In the mid-1990's the Navajo Nation AUM regions became a part of the EPA's Superfund Program. The Diné Network for Environmental Health (DiNEH) Project, using a culturally appropriate community-based participatory approach, originally began addressing community concerns about the high rate of kidney disease in this population and was later expanded to address broader health risks including CVD in this exposed population. The DiNEH Project is a partnership of the University of New Mexico Community Environmental Health Program, Southwest Research and Information Center, Crownpoint Service Unit, University of Texas-Houston Medical Center and communities from 20 Chapters of the Eastern Navajo Agency.

Exposure pathways

Much of the metals contamination of water, soil, air and local biota is known to occur from mining and milling sites, rather than from naturally present U deposits (Mkandawire et al. 2013). U mine wastes consist of toxic metals, metalloids, and remains of U, as well as ground rock particles, non-extracted radionuclides, and leach and mill chemicals. Metals such as Arsenic (As), lead, cadmium, and mercury are discharged into water and air pathways via the leaching process and through blowing dust (Mkandawire et al. 2013). Transport of metals through the environment is strongly influenced by their chemical form. More soluble forms can move down through soil into underlying groundwater. (Argonne National Laboratories (ANL) 2005). Water is a major exposure pathway for metals and metalloids, as well as an important transport pathway for environmental mobilization and redistribution of contaminants (Csavina et al. 2012). Up to 30% of the population on the Navajo Nation does not have access to a public water system (US EPA 2008).

Metals and metalloids typically adhere to soils of finer particle size near mining operations can be suspended in air as atmospheric particulates (Csavina et al. 2012). These movements through air can contaminate soils, water and biota in distant regions over long periods of time. Climate change may increase exposure risks due to increased evaporation of contaminated water and increased wind erosion uncovering previously buried sites (US Department of Agriculture 2008). Some metals including U are absorbed much more readily if inhaled than ingested (ANL 2005). Residents living near a petrochemical

complex in Taiwan had urine levels of As and V that correlated with airborne particulate matter suggesting a distance-to-source gradient (Yuan et al. 2015).

Traditional agricultural practices (livestock and crops) and use of native plants in ceremonial practices may be potential sources of ingested contaminants, although there has been a significant decline in agriculture in recent decades (Diné Policy Institute. 2014). Uranium tends to adhere to soil particles, with soil concentrations typically higher than in water. Nickel (Ni) also adheres to soil and is not usually a major contaminant in groundwater (ANL 2005). Uranium can bio-concentrate in certain agricultural crops and in some land animals (Mkandawire et al. 2013). Many plant species can take up As whereby it enters the food chain (Zhao et al. 2010). Arsenic does not seem to bio-accumulate in animals. Children are highly susceptible to ingestion of contaminated soils due to "hand-to-mouth" behaviors typical of their age. Urine concentrations of metals in children have been found to correlate with soil concentrations near metal smelters (Hwang et al. 1997).

Metabolism of Metals

Metals exposures occur mainly through ingestion, but can also occur via inhalation and dermal routes. Metabolism of metals is an attempt by the body to reduce toxicity, although, as in the case of As, this sometimes can serve to produce more toxic species (Jomova and Valko 2011). The speciation or oxidation state and solubility are also important factors in toxicity (Crans et al.

2013). The most common physiological sources tested for metals exposure include blood, urine, hair, and nails, as these are readily obtainable. The focus for this project is on As and U because they are the major water contaminants in the Navajo Nation, but other metals, such as Ni, V and copper (Cu), as well as metals mixtures, may contribute to health effects.

Uranium

Uranium exists in different oxidation states and in other compounds, and solubility is an important factor in exposure. Uranium is mostly ingested and is excreted before it can enter the bloodstream. Based on a biokinetic model and bone-ash samples from Health Canada, the absorption fraction of U from the gastrointestinal tract was shown to be approximately 2% in adults, which is mostly distributed in the bone and kidneys. (Chen 2011, ANL 2005). The total body burden of U in humans has been estimated to be 40 µg, with nearly 10% of this being present in the blood (ANL 2005; Igarashi et al. 1987). U in blood is about 40% plasma bound and the remainder is found as the soluble uranyl ion. (Briner et al. 2006). In the circulation, U is associated primarily with erythrocytes (Fisenne and Perry 1985). Uranium forms a complex with albumin in equilibrium with another complex formed with carbonate (Moss 1985). Uranyl compounds have a high affinity for phosphate, carboxyl and hydroxyl groups, and therefore can easily and stably bind with proteins and nucleotides. Inhalation of U can result in a small proportion being deposited in the deep lung where it may remain for several years and may also enter the bloodstream if the U is in a soluble form

(ANL 2005). Inhaled lead and U dust both have pulmonary half-lives of about 4–5 years. (Briner et al. 2006)

Arsenic

As exists in organic and inorganic forms, as well as in different valence or oxidation states, most commonly trivalent (III) and pentavalent (V). Inorganic As is soluble and most commonly found in drinking water. About 95% of ingested As is absorbed and about 40%-60% of Inhaled As is absorbed (Solenkova et al. 2014). Inorganic As is readily taken up by cells (Cohen et al. 2006; Hughes et al. 2011). As in the trivalent oxidation state (III) are considered to be the most toxic. As(III) is primarily metabolized by the liver via progressive methylation which may involve glutathione into a number of different species including monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) (and other less common but more toxic thioAsal) (Chen et al. 2013, Raml et al. 2007; Stea 2014). Most excreted As in the urine is in the form of DMA, some as MMA, and approximately a quarter is excreted as inorganic (Stea 2014; Vahter 2000). Drinking water levels of As correlate well with nail and urine As concentrations (Chen et al. 2013, Karim et all. 2013, WHO 2011). As has an affinity for thiol-containing molecules (Hughes et al. 2011; Schmidt et al. 2009) and is known to bind plasma proteins including the antioxidants glutathione and thioredoxin. In rats exposed to As (III) in food for 42 days, DMA(V) was the predominant form of As found in both plasma and bound to erythrocytes at doses as low as 1 parts per million (ppm), whereas As (III) was found in plasma only at the highest

exposure dose (100 ppm) (Chen et al. 2013). Rodents are more sensitive to As thus making translations to humans is uncertain.

Nickel

Other metal contaminants found in drinking water and presumably in soils and air on the Navajo Nation include Ni, V, and Cu. Most Ni is absorbed through the gut. About 5% of the absorbed Ni moves from the intestines into the bloodstream, and approximately 20-35% of inhaled Ni is absorbed through the lungs. Most of the Ni that reaches the blood is rapidly excreted in urine. Much of the remaining Ni is evenly distributed throughout the body. The biological half-life of Ni is more than 3 years. (ANL 2005)

Vanadium

Vanadium also exists in many oxidation states making it a particularly complex metal in the human body (Mukherjee et al. 2004). The toxicity of V generally increases as the valence state increases, with the eyes and lung be most affected (Barceloux 1999). Diet is the main source of V, and most ingested V is not absorbed; V that is absorbed is rapidly transported by proteins to various tissues. Most V in the blood is bound to albumin and transferrin. Vanadium has been found to inhibit many enzymes. Vanadium may be incorporated into many various organs and tissues including bone, liver, kidney, brain, muscles and heart (Mukherjee et al. 2004). Little to no toxicity has been shown after long-term

supplementation of V compounds. Vanadium compounds may affect glucose (Srivastava and Mehdi 2005) and lipid metabolism (Zhang et al. 2014)

Copper

Copper, which is primarily obtained from the diet, rapidly appears in the bloodstream and is taken up mainly by the liver (Gaetke and Chow 2003). Women generally have higher levels of Cu than men (Milne 1998). The majority of Cu is bound to ceruloplasmin, the most abundant of many Cu -binding proteins, while much of the remainder is contained within several enzymes including superoxide dismutase and cytochrome c oxidase (Milne 1998). Copper is an essential nutrient and a common cofactor that is generally maintained at normal levels due to strong homeostatic control mechanisms (Gaetke and Chow 2003; Milne 1998). Deficiency is more common than toxicity, and low or high levels of Cu can be detrimental, including to the vasculature (Ford 2000; Schuschke et al. 1997). The main mechanism by which damage occurs is thought to be through Cu-induced oxidative stress; Cu is also known to oxidatively modify LDL cholesterol (Gaetke and Chow 2003).

Metals exposures and CVD

The Southwestern U.S. has disproportionately higher levels of As in drinking water where many Native Americans reside. The communities of the Navajo Nation, located in the four-corners region of the southwestern United States, have exposure risks from a legacy of AUMs (USEPA 2008, 2013; deLemos 2009). While lifestyle, socioeconomic challenges and genetic factors play a

significant role in the development of CV-heath conditions. Remaining U deposits along with other co-occurring metals including As, lead, V, Ni, and Cu may contribute to CV health effects. Environmental exposures to toxic metals have been associated with CVD and diabetes.

Epidemiology: Arsenic and CVD

Numerous epidemiological studies have linked groundwater contamination to adverse CV health effects. High As levels in drinking water are associated with increased the risk of coronary heart disease, peripheral artery disease, stroke, and carotid atherosclerosis in studies worldwide. In the 1990s, Bangladesh was the site of large-scale As exposure in which thousands of residents were exposed via drinking water. Many other regions have significant As exposures, which have manifested as peripheral artery disease (also known as Blackfoot disease) implicated the vasculature as a target of As toxicity (Tseng 2002). A follow-up study in Taiwan assessed the interaction between As exposure and urinary As speciation on the risk of peripheral artery disease (Tseng et al. 2005). This study found that aging was associated with a decreased ability to methylate inorganic As and that women were better able to methylate As than men. In addition, peripheral artery disease risk increased with a higher cumulative As exposure and a lower capacity to methylate As (Tseng et al. 2005). Another study in Bangladesh found a dose-response relationship between exposure to As in well water assessed at baseline and mortality from

ischemic heart disease and other heart disease, with hazard ratios increasing with greater quartiles of As drinking water concentration (Chen Y et al. 2011).

The majority of studies, both epidemiological and mechanistic, have focused on high As concentrations, and more studies are needed to assess the relationship between low to moderate As exposure and CVD. Meliker et al (2007) examined mortality in a U.S. population with <100 µg/L water As concentrations and found increased mortality rates for all cardio- and cerebrovascular diseases, diabetes and kidney diseases. Another study conducted in Spain found higher mortality rates for cardiovascular, coronary, and cerebrovascular disease in a population with drinking water concentrations >10 µg/L versus those with concentrations <1 µg/L (Medrano et al. 2007).

Low to moderate As exposure may be a cardiovascular risk factor with no apparent threshold (Schmidt et al. 2014). EPA maximum contaminant level of 10 parts per billion (ppb) in drinking water was established based on risk of developing kidney cancer, but the cardiovascular system may be sensitive to lower concentrations. Most study designs have been ecological and there is a need to examine As exposure at the individual level. (Moon et al. 2012; Navas-Acien et al. 2005)

In populations from the SHS, urine levels that indicated low to moderate inorganic As exposure, were prospectively associated CVD and CVD mortality, and these associations remained after adjustment for socio-demographic factors,

lipids, and smoking. The findings were attenuated with further adjustment for hypertension, diabetes, and measures of kidney disease, which the authors suggest may variables in the causal pathway (Moon et al. 2013). Several studies from the As-endemic regions of Taiwan and Bangladesh have found links between As exposure and vascular inflammation and endothelial dysfunction. In the Health Effects of As Longitudinal Study, urinary As and well-water As were positively associated with plasma levels of soluble VCAM-1 (sVCAM-1). They also found that associations between As exposure and increased levels of plasminogen activator inhibitor-1 and soluble VCAM-1 were stronger among people with higher body mass index (BMI) (Wu 2012). Another study in Bangladesh found that well-water As was positively associated with both ICAM-1 and VCAM-1 and with changes in these two markers over time (Chen et al. 2007). More recently, Karim et al. (2013) found that OxLDL, HDL, and CRP showed dose-response relationships with As exposure. Ox-LDL/HDL ratios were also increased with the increasing concentrations of As in the water, nails and hair.

Microarray data of peripheral blood mononuclear cell from 24 individuals exposed to intermediate- to high-levels of As in Taiwan suggest that a number of pro-inflammatory genes are upregulated, including IL6, CCL2, and interleukin-1β. Multivariate analysis of 64 exposed individuals from the same study showed that the association of plasma CCL2 levels with blood As remained significant after adjustment for other CVD risk factors (Wu et al. 2003), but other results have

been inconsistent which may indicate possible influences due to ethnic and/or environmental conditions (Salgado-Bustamante et al. 2010).

Toxicology: Arsenic and CVD

Several mechanistic studies have found links between As exposure and manifestations of CVD. In apolipoprotien E deficient ($ApoE^{-/-}$) mice that were exposed to 20 or 100 µg/mL sodium arsenite ($NaAsO_2$) in drinking water for 24 weeks, lesions of the aorta were increased significantly compared with non-treated control mice (Simeonova et al. 2003). They also found an accumulation of As in the vessel wall without changes in serum cholesterol. Mice also ate cocoa butter in their diet for 2 weeks, which resulted in higher serum cholesterol levels and only slight increases in lesion size in both control and As-exposed $ApoE^{-/-}$ mice. In the same study, As treatment of endothelial cells induced the expression of inflammatory mediators, including interleukin-8 (Simeonova et al. 2003). When Srivastava et al. (2009) exposed $ApoE^{-/-}$ mice to a high level (49 ppm) of As for 24 weeks, they found increased lesion formation by 2- to 3.6-fold in the aortic valve, the aortic arch and the abdominal aorta. Lesions also showed an increase in macrophage accumulation as well as pro-inflammatory molecules, such as CCL2, IL6, protein-hydroxynonenal and protein-malondialdehyde (MDA)-adducts, in As-exposed mice. Plasma concentrations of CCL2, IL6 and MDA were also significantly elevated in As-exposed mice. Lemaire et al. (2011) did similar studies in $ApoE^{-/-}$ mice with lower As concentrations (200 ppb) and again found increased atherosclerotic lesions compared to controls. When they tried

high and low concentrations, interestingly, the lower exposure concentration (200 ppb) was more atherogenic than the higher concentration (1000 ppb). As-enhanced lesions were also associated with increased proinflammatory cytokines and decreased LXR target gene expression, which is involved in cholesterol regulation (Lemaire et al. 2011).

Although still not well understood, several modes of action of As toxicity have been proposed including: interactions with sulfhydryl groups on proteins and enzymes, alteration of cellular signal transduction, arsenate substitution of phosphate (since both share physiochemical properties), substitution for zinc in zinc finger binding domains, and genotoxic effects (Flora 2011; Hughes et al. 2011). Other indirect processes via the formation of ROS (Barchowsky et al. 1996) could also contribute to the pathogenic mechanism for the CV effects of As. (Stea et al. 2014) Modification of cysteines, especially dithiols, on a number of molecules by As may affect redox status. Some studies have suggested that NFκB, a major transcription factor that regulates inflammatory processes, can be induced in arsenite-exposed macrophages and endothelial cells through oxidative stress. (Barchowsky et al. 1996; Hossain et al. 2013). Inflammatory cytokines may be induced in the liver due to the metabolism of As or hepatic injury.

Epidemiology: Uranium and CVD

Few epidemiology studies have looked at chronic, low-dose exposure to U, especially in CVD. A dose-response relationship has been identified between U exposure has been associated with both diastolic and systolic blood pressures (DBP, SBP, respectively); cumulative U intake has been associated with increased urinary excretion of glucose but these effects may be secondary to kidney toxicity (Kurttio et al. 2006). Most of U's effects are nephrotoxic. Kidney disease can be a comorbidity of CVD and T2D. U has also been associated with neurological and reproductive health effects (Brugge and Buchner 2011).

Toxicology: Uranium and Inflammation/Oxidative Stress

Few animal or cell studies have looked at the CVD associated with U exposure. The following animal studies with U looked at processes related to CVD: inflammation and oxidative stress. One study examined if chronic ingestion of depleted U (40 mg/L) for 3, 6, or 9 months would lead to inflammatory changes in the intestine and found varied responses. Messenger RNA was increased for interleukin-1β and interleukin-10 at 6 months, and decreased for CCL-2, and this was associated with decreased macrophage density. Neutrophils were increased number was observed at 3 (31.7) and 9 months (33). They also observed decreased endothelial NO synthase messenger RNA at 6 months. Another study examined the free radical scavenging activity of a carbohydrate polymer (a potential drug candidate for treatment of depleted U toxicity) on oxidative stress induced by depleted U (uranyl acetate, UA) in isolated rat hepatocytes. Rats treated with 50μM but there were no other details provided.

They found increased ROS formation in isolated hepatocytes, as well as glutathione depletion, mitochondrial membrane potential collapse and lysosomal membrane rupture before hepatocyte lysis occurred, which were prevented by the carbohydrate polymer (Pourahmad et al, 2010). Briner et al (2005) looked at short- and long-term behavioral effects and lipid oxidation in brains from rats exposed to UA exposure (0, 75, or 150 mg/L in drinking water, equivalent to 0.18 mM and 0.35 mM, respectively) for either 2 weeks or 6 months. Behavioral differences were seen in male rats after short-term exposure and in female rats after long-term exposure. Increased brain lipid oxidation was seen for the highest dose group for both genders and they correlated significantly with the behavioral measures suggesting that UA can cross the blood brain barrier in these rats.

Other Metals Associated with CVD

Cadmium, mercury, lead and Cu are also suspected to be clinically and mechanistically associated with various measures of CVD (Jomova and Valko 2011; Solenkova 2014). Cadmium exposure has been associated with atherosclerosis development in the hypercholesterolemic rabbit (Subramanyam et al. 1992), increased carotid intima-media thickness in young healthy women (Messner et al., 2009), and with peripheral artery disease as measured by ankle brachial index, in participants from the SHS (Tellez-Plaza 2013). In a prospective 4-year follow-up study in a population-based sample of men from Finland, baseline hair mercury content was the strongest predictor of increased mean intima-media thickness as determined by ultrasonographic assessment, after

adjusting for other atherosclerotic risk factors (Salonen et al. 2000). Numerous studies have shown an association between lead exposure and hypertension and several clinical cardiovascular outcomes and mortality (Navas-Acien et al. 2007). Copper, a redox active metal, is also associated with CVD, atherosclerosis, CVD mortality, and LDL oxidation (Jomova and Valko 2011). Many effects of Cu can be attributed to the induction of oxidative stress either by the formation of ROS via a Fenton-like reaction and by decreasing glutathione levels. Copper is known to oxidize LDL in vitro (as well as HDL), and can interact with another CVD risk factor, homocysteine, to oxidize LDL (Burkitt 2001; Jamova and Valko 2011).

Role of Metals in Lipid Oxidation

LDL may be oxidized by reactive oxygen or nitrogen species, metal ions, and enzymes secreted by immune cells such as myeloperoxidase (Yoshida et al. and Kisugi 2010). Copper sulfate ($CuSO_4$) is commonly used to induce oxidation of LDL (Burkitt 2001). The oxidation of LDL by Cu *in vitro* may occur differently from the process occurring *in vivo*, but metal ions, including Cu as well as iron, have been shown to be required for cell-mediated LDL oxidation. *In vitro*, it is believed that Cu binds to histidine residues within the ApoB-100 protein of the LDL particle where it may cause lipid oxidation through a variety of reactions, which may or may not involve a reducing agent (Burkitt 2001; Wagner and Heinecke 1997). Several studies have found associations between metals exposures and carotid atherosclerosis (Lind et al. 2012) and lipid oxidation (Ani

et al. 2007; Elis et al. 2001; Kapiotis et al. 2002). However, it is unknown if As or U can lead to LDL/lipid oxidation.

Metals exposures and changes in serum composition

Increased levels of reactive oxidants and decreased antioxidant capacity have been found in plasma from humans exposed to As in drinking water Wu et al. 2001). Many metals can inactivate or alter proteins due to an affinity for sulfhydryl groups, which is significant since proteins in the plasma may account for up to 50% of the anti-oxidative capacity of plasma. In addition, inflammation can increase binding of modified-LDL to endothelium and smooth muscle (Ross 1999).

We have previously shown that inflammatory potential, as measured by endothelial cell responses to serum derived from humans exposed to diesel exhaust, was exhibited by increases in ICAM-1 and VCAM-1 messenger ribonucleic acid (mRNA) (Channell et al. 2012). It has also been shown that when human umbilical vein endothelial cells and/or THP-1 monocytes were exposed to metal-based nanomaterials, there were increased gene expression levels of heme-oxygenase-1 and interleukin-8, ICAM-1, and VCAM-1, as well as increased adhesion of THP-1 monocytic cells onto endothelial cells (Danielsen et al. 2015). It is plausible that exposure to metal-mining contaminants could lead to changes in the circulation in Navajo residents, including increases in LDL oxidation and serum inflammatory potential.

Rationale for Research

Numerous AUMs within the Navajo Nation have left behind a mixture of metals including U and As in water, soil, and likely air. The Navajo are an underserved population that in recent decades have seen high prevalence rates of obesity and overweight, hypertension, and diabetes, which historically have been lower in Native Americans than in the general population. Rates of coronary artery disease and metabolic syndrome are also on the rise. We hypothesize that endothelial activation and inflammation in the circulatory system, which are involved in the pathophysiology of these CV-related conditions, are exacerbated by exposures to metals from legacy mining.

The circulation represents the balance of inflammation, i.e. inflammatory potential, to which the endothelium responds by expressing adhesion molecules and chemokines, which recruit macrophages to sites of inflammation. Scavenger receptors on immune and endothelial cells recognize and internalize oxidatively-modified LDL leading to a progressive inflammatory response central to atherosclerotic plaque formation. Circulating oxidized LDL is an emerging CV biomarker. Metals exposures are associated with lipid oxidation (Ani et al. 2007; Elis et al. 2001; Kapiotis et al. 2002). It is unknown if As and U are able to directly oxidize LDL. Metals exposures may also influence inflammatory changes in the circulation. The circulation contains the balance of pro- and anti-inflammatory mediators, i.e. inflammatory potential, which we have assessed using a novel method in our lab (Cung et al. 2015, Channell et al. 2012), but this

novel method has not been applied at the community level. Because the major cause of non-accidental mortality in Navajo, as well as worldwide, is due to CVD, even a slight increase in risk, such as due to exposure to toxic metals, may indicate a large quantity of excess mortality (Moon et al. 2013).

Central Hypothesis: Exposures to AUM contaminants are associated with pro-atherogenic changes in the circulation.

AIM 1: Characterize oxLDL and its relationship to CVD and T2D risk factors in a subset of the DiNEH participants.

Rationale: OxLDL is a novel CVD biomarker that is predictive of acute MI and subclinical atherosclerosis (Meisinger et al. 2005; Wallenfeldt et al. 2004) and is associated with other CV-related outcomes including metabolic syndrome/diabetes (Holvoet et al. 2004; Hussein et al. 2007). However, oxLDL and its relationship to CVD and type 2 diabetes risk factors have never been characterized in the Navajo population. To assess this, we obtained survey health information and laboratory assessments from a subset of volunteers from the DiNEH study and measured plasma oxLDL by an enzyme linked immunosorbent assay (ELISA). Pearson correlations and linear regression were used to evaluate associations between oxLDL and CVD and type 2 diabetes risk factors.

AIM 2: Determine the association between drinking water intake of U and As and oxLDL in a subset of the DiNEH participants, and determine the extent to which these metals directly oxidize LDL.

Rationale: Navajo have expressed the need for epidemiological studies investigating health concerns potentially due to U mining contamination (USEPA 2008). As and U are the major metal contaminates of drinking water on the Navajo Nation. Chronic As is associated with CVD, but few studies have assessed the association of U with CVD. This population has an increasing prevalence of CV-related health conditions. OxLDL is a novel biomarker of CVD and CVD risk. Metals exposures have been associated with oxLDL as a biomarker (Karim et al. 2013), carotid atherosclerosis (Lind et al 2012) and lipid oxidation (Ani et al. 2007). We tested the hypothesis that As and U intake from drinking water would be associated with oxLDL in the DiNEH subset, and that these metals would directly oxidize LDL in an acellular system, as proof-of-concept. We developed linear regression models to evaluate the relationship between participant oxLDL levels and drinking water intakes of As and U and potential covariates. To test if As and U could oxidized LDL, we designed an acellular assay in which purified human LDL was incubated with varying concentrations of the same metals and measured oxLDL by ELISA and lipid peroxidation by the thiobarbituric acid reactive substances (TBARS) assay.

AIM 3: To determine the contribution of AUM exposures to community-level serum inflammatory potential

Rationale:

Residents living in close proximity to AUMs have a near doubling of risk for hypertension as compared to those living further away (Hund et al. 2015). Chronic exposures to a mixture of mining contaminants may occur through multiple pathways (drinking water, inhalation). Increased free radical production and inflammatory factors may be induced by the presence of toxic metals in plasma or atherosclerotic lesions (Shi et al. 2004). Inflammatory changes in the circulation that reflect chronic environmental exposure may be useful in assessing health effects of mining waste exposures, such as that experienced by Navajo Nation communities. We hypothesized that exposure to mining waste contaminants, in terms of drinking water intake and AUM proximity, would be associated with serum inflammatory potential of naïve cultured endothelial cells treated with serum from the Navajo volunteers. To test this hypothesis, we applied our novel inflammatory potential assay using serum obtained from DiNEH volunteers. We then developed linear regression models to then test if AUM exposures could predict these inflammatory responses.

II. CHAPTER 2

Associations of Circulating Oxidized LDL and Conventional Biomarkers of Cardiovascular Disease in a Cross-sectional Study of the Navajo Population

In press (PLOS ONE)

ABSTRACT

The prevalence rates of CVD and T2D have increased among the Navajo Native American community in recent decades. OxLDL is a novel CVD biomarker that that has never been assessed in the Navajo population. We examined the relationship of oxLDL to conventional CVD and T2D risk factors and biomarkers in a cross-sectional population of Navajo participants. This cross-sectional study included 252 participants from 20 Navajo communities from the DiNEH Project. Plasma samples were tested for oxLDL levels by a sandwich enzyme-linked immunosorbent assay. Univariate and multivariate analyses were used to determine the relationship of oxLDL and oxidized- to non-oxidized lipoprotein ratios to glycated hemoglobin (HbA1c), CRP, IL6 and demographic and health variables. Type 2 diabetes, hypertension and obesity are very prevalent in this Navajo population. HbA1c, CRP, body mass index (BMI), high-density lipoprotein, and TG were at levels that may increase risk for CVD and T2D. Median oxLDL level was 47 (36.8-57) U/L. Correlational analysis showed that although oxLDL alone was not associated with HbA1c, oxLDL/HDL, oxLDL/LDL and CRP were significantly associated with HbA1c and glucose. OxLDL, oxLDL/HDL and oxLDL/LDL were significantly associated with CRP. Multivariate analysis showed that TG were a common and strong predictor of oxLDL, oxLDL/HDL and oxLDL/LDL. OxLDL was trended with HbA1c and glucose but did not reach significance, however, HbA1c was an independent predictor of OxLDL/HDL. CRP trended with oxLDL/HDL and was a weak predictor of oxLDL/LDL. This Navajo subset appears to have oxLDL levels comparable to

subjects without evidence of CVD reported in other studies. The high prevalence of T2D, hypertension and obesity along with abnormal levels of other biomarkers including HbA1c indicate that the Navajo population has a worsening CVD risk profile.

Introduction

Cardiovascular diseases and T2D were rarely reported in the Navajo population until the 1930s (Salsbury 1937). In recent decades, the prevalence of cardiovascular related health conditions such as T2D, overweight and hypertension has increased (CDC 2014; Percy et al. 1997; Mendlein et al. 1997; Will et al. 1997; Hoy et al. 1995; Howard et al. 1999), with CVD currently being the leading cause of non-accidental death among Navajos (Percy et al. 1997; Hoy et al. 1995), as well as in other Native American populations [Howard et al 1999; Rhoades et al. 2005). It has been well-established that diabetes increases the risk for CVD, and Navajo are now developing T2D at a rate four times higher than the United States average (Will et al. 1997). In 1997, the last published comprehensive look at Navajo-specific health status, the Navajo Health and Nutrition Survey, reported that nearly 40% of Navajos over the age of 45 had T2D (Will et al. 1997). According to a recent Center for Disease Control report, the incidence of newly diagnosed T2D among American Indian/Alaskan native youth aged 10-19 is higher than any other racial/ethnic group in the United States (U.S.) (CDC 2014). T2D, along with obesity and hypertension, have become major public health concerns in a population that has become increasingly at risk for CVD.

Clinically, numerous circulating biomarkers including CRP and IL6 have been useful in predicting CVD outcomes and assessing risk (Ridker et al. 2009; Pepys et al. 2003). CRP has long been established as a marker of chronic systemic inflammation and is regulated by IL6, a pro-inflammatory cytokine and important

inducer and regulator of chronic inflammation (Yudkin et al. 2000; Gabay et al. 2006). Novel biomarkers for CVD are emerging including oxidized LDL (oxLDL) cholesterol. OxLDL is increased in subclinical atherosclerosis (Wallenfeldt et al. 2004) and is often a stronger predictor of acute CAD than standard lipid measures or other conventional risk factors (Meisinger et al. 2005). OxLDL levels are reportedly able to distinguish patients with CAD from healthy cohorts (Huang et al. 2008), and serve as a predictor of future myocardial infarction in patients with unstable CAD (Johnston et al. 2006). Oxidized LDL is also associated with T2D (Njajou et al. 2009). More research is needed to establish oxLDL as a clinically useful biomarker.

The relationship between oxLDL and CVD risk factors and biomarkers in the Navajo community is unknown. Given the high prevalence of T2D in this population, our purpose was to characterize CVD biomarkers in a cross-sectional Navajo population and to evaluate the association of oxLDL with HbA1C, widely accepted as a major biomarker of glycemic control, ultimately to better understand how these metrics currently trend in an understudied ethnic group.

METHODS

Study Population and Survey Methods

The geographic study area for the study population was located in the northwestern region of New Mexico, in the political division of the Navajo Nation known as the Eastern Agency. Demographic and health data were obtained from the interviewer-administered Water and Land Use, Environmental and Health Survey, designed by the DiNEH Project (de Lemos et al 2009; 2007)

which implemented a community-based participatory approach to enroll 1,304 participants from 20 Chapters of the Navajo Nation between 2005 and 2010. The DiNEH Project was initiated to evaluate kidney disease in the Navajo due to potential exposures to U from numerous abandoned U mines., The study was later expanded to evaluate the overall health of this population, including cardiovascular, immunological and neoplastic diseases. Height and weight were measured at the time of survey administration to determine BMI. From these DiNEH participants, we had a recruitment goal for a follow-up blood collection of 450 participants; we had a 40-90% recruitment success rate on any given day from 2010-2011 for a final total of 252 Navajo participants. Non-fasting plasma samples were collected along with clinical assessments conducted by a Navajo Area Indian Health Service mobile unit at multiple community locations. All serum biomarker data were derived from this subset. Blood pressure was measured by automatic cuff. All participants provided written informed consent, and the study was approved by both the University of New Mexico Human Research Review Committee and the Navajo Nation Human Research Review Board.

Biomarker Measurements

Plasma samples were tested for oxLDL by a sandwich enzyme-linked immunosorbent assay, according to manufacturer's instructions (Mercodia, Uppsala, Sweden) (n = 252). The full volume of intended blood samples was not available for all participants so subsequent analyses were prioritized resulting in the N noted below for each of the markers reported herein. IL6 was determined by MILLIPLEX ultrasensitive human magnetic bead set (Millipore Inc; Billerica,

MA) and multiplexing technology to obtain serum cytokine concentration measures (in pg/ml) (n = 236). Magnetic bead detection was carried out on a MAGPIX machine and multiplexing platform capable of performing quantitative analysis of low concentration of protein markers (Luminex Corporation, Austin, TX). Analytical values were determined by xPONENT 4.2 Software (Luminex Corporation, Austin, TX) by standards provided in the assay kit for each cytokine. All remaining biochemical analyses were performed by a reference laboratory (LabCorp, Phoenix, AZ), reported to Indian Health Services and compiled in a clinical database (RedCAP). HbA1c (n = 249) was determined by the Roche Tina-quant assay. CRP (n = 249) was assessed quantitatively by latex immunoturbidimetry. There were 217 samples available for assessing total serum cholesterol, HDL, and TG, which were measured by conventional clinical analytical methods; LDL was measured directly (n = 211).

Statistical Analysis

Descriptive summary statistics were reported as median and interquartile range (IQR) for continuous variables, unless otherwise indicated. Non-Gaussian distributions were normalized using a logarithmic transformation. Pearson correlations and multivariate analysis were performed to assess the relationship of oxLDL to other CVD and T2D risk factors and biomarkers. A value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using R version 2.12.1 (The R Foundation for Statistical Computing, 2010, 64-bit) and GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). All multivariate models were adjusted for age, gender, BMI, total cholesterol (TC),

TG, diastolic and systolic blood pressures, non-fasting glucose, HbA1c, CRP, and IL6. The model for oxLDL/HDL was also adjusted for LDL cholesterol, and the oxLDL/LDL model was adjusted for HDL cholesterol. Model selection was performed based on Akaike Information Criterion (AIC) or Bayesian Information Criterion.

RESULTS

Clinical Characteristics and Prevalence of Health Conditions in the Navajo Population

Table 2.1 shows the characteristics of the Navajo population subset (n = 252) relative to the original DiNEH study mean age of 55.3 ±14.3 years versus 51.5 ±17.4 years, respectively; both were similar in gender proportion, BMI and self-reported health conditions. Diabetes and hypertension were the predominant conditions self-reported by both the subset and original DiNEH population, at 26.2% (subset) and 25.1% (DiNEH) for diabetes and 38.1% (subset) and 35.9% (DiNEH) for hypertension; heart disease, stroke and myocardial infarction were less prevalent in both groups.

The prevalence of obesity (BMI ≥30 kg/m²) in the Navajo subset was 47.6%, which was higher than the prevalence in the original DiNEH study population (41.2%) (Table 2.1). Over one-third of both study populations were overweight (BMI 25.0-29.9 kg/m²). Furthermore, the majority of participants in both the subset (84.7%) and original DiNEH population (76.2%) had BMI values

considered either “overweight” or “obese.” Only 23% of original DiNEH participants and 14.5% of the subset were within a normal BMI range (18.5-24.9 kg/m²).

Median systolic blood pressure in the Navajo subset was pre-hypertensive; for 68% of this study population, systolic pressures were at or above pre-hypertensive levels (>120 mmHg) according to American Heart Association guidelines (Chobanian et al. 2003) (Table 2.1). Median diastolic blood pressure was normal although almost half (47%) of the subset had diastolic pressures that were measured at or above pre-hypertensive levels (>80 mmHg). Blood pressure measurements were not available for the original DiNEH population. Thirty-five percent of the subset reported taking hypertension medication.

Levels of Biomarkers Associated with CVD and T2D

Plasma biomarker levels for the Navajo subset are shown in Table 2.1. The median oxLDL level in the Navajo subset was 46.9 (36.8-57) U/L. The median IL6 level was 5.9 (1.8-12.5) pg/ml. Currently, reference ranges for oxLDL and IL6 as biomarkers have not been established. The median CRP level was 2.1 (0.9-4.8) mg/L (average risk), however, 38.5% had levels greater than 3.0 mg/L, which is considered high risk for heart disease (Pearson et al. 2003). Circulating lipid levels are shown in Table 2.1. Based on American Heart Association guidelines (National Cholesterol Education Program 2002), median TC in this

Table 2.1 Characteristics and clinical parameters of the Navajo subset and original DiNEH participants.

Variable	DiNEH Subset	n	DiNEH Participants* (n = 1304)
Age, years	55.3 ± 14.3	252	51.5 ± 17.4
Female, %	57.5	252	56.4
Self-reported health conditions		252	
Type 2 Diabetes, %	26.2		25.1
Hypertension, %	38.1		35.9
Heart Disease, %	6		5.4
Myocardial Infarction, %	4.4		3.1
Stroke, %	5.2		3.5
Body mass index (BMI), kg/m²	29.7 (26.8–33.6)	252	28.3 (25.1-32.6)
Underweight (BMI ≤ 18.4), %	0.8		0.7
Normal (BMI = 18.5-24.9), %	14.5		23
Overweight (BMI = 25.0-29.9), %	37.1		35
Obese (BMI ≥ 30), %	47.6		41.2
Total Cholesterol, mg/dL	182 (162–204)	217	
LDL, mg/dL	105 (90–123)	211	
HDL, mg/dL	45 (38–54)	217	
TG, mg/dL	183 (127–251)	217	
Systolic BP (mmHg)	129.5 (117-143)	248	
Diastolic BP (mmHg)	78 (71–86)	248	
CRP, mg/L	2.1 (0.9–4.8)	249	
CRP > 3.0 mg/L, %	38.5		
oxLDL, U/L	46.9 (36.8–57)	252	
IL6, pg/ml	5.9 (1.8-12.5)	236	
Glucose (non-fasting), mg/dL	91 (78–121)	249	
HbA1c, %	6.2 (5.8–7.1)	249	
% Normal (≤ 5.6%)	15.7		
% Pre-diabetes (5.7-6.4%)	45.8		
% Diabetes (≥ 6.5%)	38.6		

*Biomarker levels and blood pressure were not available for the DiNEH participants. Data are presented as median (IQR) or %. LDL, low-density lipoprotein; HDL, high-density lipoprotein; BP, blood pressure; HbA1c, glycated hemoglobin; CRP, C-reactive protein; oxLDL, oxidized low-density lipoprotein; IL6, interleukin 6.

subset was “desirable,” and LDL cholesterol levels were near optimal. However, HDL cholesterol was bordering on low, and TG were high; both are risk factors for heart disease. No data were available for the use of lipid-lowering medications in this subset.

As mentioned, a diagnosis of T2D was self-reported by 26.2% of the Navajo subset (Table 2.1), however, our data indicate that 84% of HbA1c levels in the Navajo subset as a whole were pre-diabetic (5.7-6.4%) or higher (Table 2.1, Figure 2.1), as classified by the American Diabetes Association (2012). Indeed, the median HbA1c in the Navajo subset was pre-diabetic at 6.2% (5.8-7.1%). Furthermore, as shown in Figure 2.1, of those who denied a diagnosis of diabetes at the time of survey administration, 59% had pre-diabetic levels of HbA1c with a mean of 6.3% ± 1.3. HbA1c levels greater than 6.5% are indicative of diabetes. For participants self-reporting a diagnosis of diabetes, 89% were in the diabetic range with a mean HbA1c of 8.9% ± 2.5. As a whole, more than a third (39%) of the subset had diabetic levels of HbA1c, and nearly half (46%) were in the pre-diabetic range (Table 2.1). Only 16% were in the normal HbA1c range (<5.6%). The median non-fasting glucose of this Navajo subset was 91 (78-121) mg/dl (Table 2.1), which is comparable to a normal fasting glucose level. Twenty-five percent of the subset reported taking oral medication and/or insulin for diabetes.

Oxidized LDL Interactions

Table 2.2 Univariate relationships between novel CVD biomarkers with conventional biomarkers and self-reported health conditions

Variable	oxLDL		oxLDL/HDL		oxLDL/LDL		CRP		IL6		HbA1C	
	r	p	r	p	r	p	r	p	r	p	r	p
Age (years)	-0.07	0.268	**-0.135**	**0.046**	-0.065	0.35	**-0.208**	**0.001**	0.018	0.773	0.104	0.1
Gender	0.02	0.756	-0.111	0.104	-0.001	0.986	0.064	0.315	-0.005	0.934	**0.146**	**0.021**
BMI (kg/m^2)	0.004	0.947	0.058	0.392	0.036	0.602	**0.235**	**<0.0001**	0.115	0.067	**0.157**	**0.013**
Hypertension*	-0.03	0.638	0.034	0.616	0.109	0.116	-0.026	0.679	0.064	0.314	**0.294**	**<0.0001**
Heart attack*	-0.014	0.822	0.026	0.701	**0.141**	**0.04**	-0.006	0.92	0.029	0.645	0.086	0.177
Diabetes*	**-0.127**	**0.044**	-0.035	0.603	0.06	0.385	0.013	0.836	0.096	0.13	**0.596**	**<0.0001**
TC (mg/dl)	**-0.409**	**<0.0001**	**-0.744**	**<0.0001**	-0.434	0.492	-0.186	0.666	**0.056**	**0.032**	-0.143	0.679
TG (mg/dl)	**0.592**	**<0.0001**	**0.363**	**<0.0001**	**-0.377**	**<0.0001**	0.005	0.274	**-0.125**	**0.024**	**0.031**	**0.019**
HDL (mg/dl)	**0.095**	**<0.0001**	**0.118**	**<0.0001**	**0.006**	**<0.0001**	**-0.008**	**0.006**	-0.003	0.412	**-0.003**	**0.037**
LDL (mg/dl)	**0.165**	**<0.0001**	**0.209**	**<0.0001**	**0.067**	**<0.0001**	0.16	0.943	-0.028	0.071	0.023	0.652
SBP (mmHg)	0.614	0.137	0.389	0.085	-0.048	0.929	0.03	0.904	-0.145	0.964	0.028	0.963
DBP (mmHg)	**0.564**	**0.009**	**0.7**	**0.002**	0.457	0.34	**0.075**	**0.012**	-0.153	0.658	0.16	0.715
log oxLDL (U/L)	…	…	**0.829**	**<0.0001**	**0.523**	**<0.0001**	…	…	…	…	…	…
log CRP (mg/L)	**0.147**	**0.021**	**0.167**	**0.014**	**0.147**	**0.034**	…	…	…	…	…	…
log IL6 (pg/ml)	**-0.163**	**0.009**	-0.121	0.076	-0.076	0.272	**0.249**	**<0.0001**	…	…	…	…
log HbA1C (%)	0.118	0.062	**0.172**	**0.012**	**0.138**	**0.047**	**0.196**	**0.002**	0.084	0.189	…	…
Glucose (mg/dl)	**0.163**	**0.01**	**0.203**	**0.003**	**0.186**	**0.007**	**0.209**	**0.001**	0.027	0.667	**0.826**	**<0.0001**

*Participant self-reported health conditions; heart disease and stroke did not correlate with any biomarkers. Pearson correlation coefficients (r). P < 0.05 was considered statistically significant (bold). Non-Gaussian distributions were log transformed. CVD, cardiovascular disease; oxLDL, oxidized low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HbA1c, glycated hemoglobin; CRP, C-reactive protein; IL6, interleukin 6, SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index

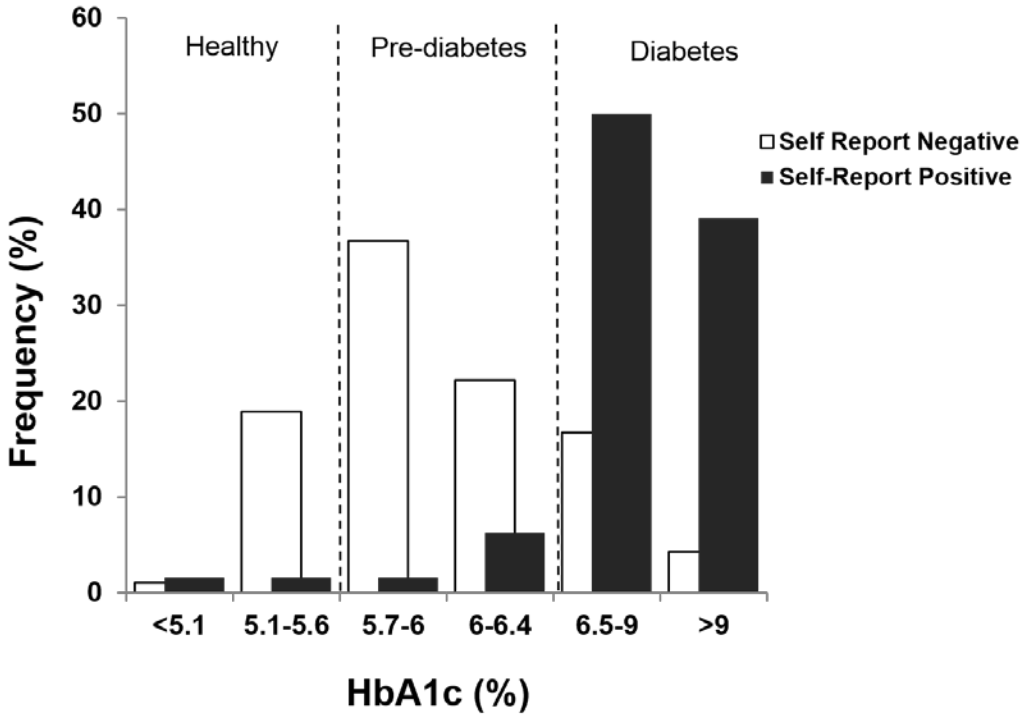

Figure 2.1 Frequency histogram of self-report diabetes versus actual HbA1c values. Type 2 diabetes (positive) or no type 2 diabetes (negative). Dashed lines correspond to HbA1c category cutoffs. Normal: HbA1c ≤ 5.6%; Pre-diabetes: HbA1c = 5.7-6.4%; Diabetes ≥ 6.5%

Table 2.2 shows the Pearson correlations of oxLDL and oxidized- to non-oxidized lipoprotein ratios (oxLDL/HDL and oxLDL/LDL) to more conventional risk factors and biomarkers of CVD and T2D in this Navajo subset of volunteers. OxLDL trended positively with HbA1c but did not reach significance. OxLDL was moderately correlated with non-fasting glucose but negatively correlated with self-report of diabetes. When oxLDL levels were compared between normal, pre-diabetic, and diabetic levels of HbA1c, no differences were found; the same was true for the oxidized-to non-oxidized lipoprotein ratios (data not shown). Both oxLDL/HDL and oxLDL/LDL were moderately correlated with HbA1c and non-fasting glucose. CRP, but not IL6, correlated moderately with HbA1c and non-fasting glucose. HbA1c was strongly correlated with non-fasting glucose and self-report of diabetes. OxLDL and oxLDL/HDL were strongly correlated with DBP, and CRP was weakly correlated with DBP, but HbA1c was the only biomarker associated with a self-report of high blood pressure. OxLDL/LDL was significantly correlated with self-report of heart attack. OxLDL, oxLDL/HDL, and oxLDL/LDL correlated significantly with each other and with all conventional lipid biomarkers, TC, TG, HDL and LDL (with the exception that oxLDL/LDL did not correlate with TC). HbA1c was weakly correlated with TG and negatively correlated with HDL. CRP was also negatively correlated with HDL. IL6 was weakly correlated with TC and negatively correlated with TG. OxLDL, oxLDL/HDL and oxLDL/LDL were moderately correlated with CRP. OxLDL was negatively correlated with IL6. CRP was moderately correlated with IL6.

OxLDL/HDL and CRP were negatively correlated with age. HbA1c and CRP were moderately correlated BMI. HbA1c was the only biomarker correlated with gender.

Multivariate analysis

Multivariate analysis confirmed that oxLDL was independently and significantly associated with TC, LDL and TG but not CRP or IL6. OxLDL was marginally associated with age, HbA1c and glucose, but these associations did not reach significance (Table 2.3). TG were a very strong predictor of oxLDL/HDL. OxLDL/HDL was independently and positively associated with HbA1c but negatively associated with glucose. OxLDL/HDL was weakly and negatively associated with age and TC and positively associated with LDL. OxLDL/HDL trended positively with CRP but did not reach significance. OxLDL/LDL was independently associated with TG and weakly associated with CRP. OxLDL/LDL was negatively associated with TC and BMI (Table 2.3).

DISCUSSION

Our goal was to measure oxLDL and examine its relationship to health characteristics and T2D in a cross-sectional subset of the Navajo Nation, as oxLDL has never been assessed in this population. The median oxLDL level in this subset was 47 U/L (IQR: 36.8-57) and ranged from 12.7-138.2 U/L. The

Table 2.3 Coefficient estimates of final models for oxLDL, oxLDL/HDL and oxLDL/LDL

	OxLDL			OxLDL/HDL			OxLDL/LDL		
Variable	**Estimate**	**Error**	***P***	**Estimate**	**Error**	***P***	**Estimate**	**Error**	***P***
Age	-0.112	0.061	0.067	-0.005	0.002	0.009			
Gender	-0.71	1.59	0.656	0.107	0.052	0.041			
BMI	-0.23	0.147	0.12				-0.003	0.001	0.046
SBP	0.052	0.051	0.303						
DBP	-0.061	0.082	0.456						
TC	0.168	0.064	0.01	-0.006	0.001	<0.0001	-0.001	0.0002	<0.0001
LDL	0.14	0.057	0.015	0.01	0.001	<0.0001			
HDL	-0.317	0.086	0.0003						
TG*	6.254	2.963	0.036	0.851	0.059	<0.0001	0.169	0.016	<0.0001
HbA1c	0.636	0.361	0.08	0.062	0.023	0.0094			
Glucose*†	2.75	1.437	0.057	-0.203	0.098	0.039			
CRP*	0.454	0.669	0.498	0.042	0.022	0.057	0.014	0.006	0.028
IL6*	-1.013	0.809	0.212						
n	191			204			206		
Adjusted R^2	0.649			0.638			0.351		
AIC	1426.084			161.012			-336.174		
BIC	1471.616			194.193			-316.206		

P < 0.05 was considered statistically significant. *Non-Gaussian distributions were log transformed. †Non-fasting. OxLDL, oxidized low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, TG; HbA1c, glycated hemoglobin; CRP, C-reactive protein; IL6, interleukin

mean oxLDL level (48.5 ± 17.0 U/L) was similar to mean oxLDL levels measured in subjects without evidence of atherosclerosis reported in other studies (Supplementary Table 2.1; (Meisinger et al. 2005; Gomez et al. 2014; Mascarenhas-Melo et al. 2013; Pawlak et al. 2013; Girona et al. 2008) that also used the same murine monoclonal antibody 4E6 based ELISA (Holvoet et al. 1998). However, oxLDL levels varied widely in these studies and consistent associations related to CAD or diabetes disease status are difficult to discern.

Increased levels of HbA1c, a biomarker for T2D, have been associated with CAD, overall CVD, and all-cause mortality (Khaw et al. 2014; Liu et al. 2011). For the present Navajo subset, the median HbA1c level was in the pre-diabetic range with the majority of participants having pre-diabetic (46%) or diabetic (39%) levels of HbA1c. Other studies have shown an association between oxLDL and HbA1c (Njajou et al. 2009), but in the present study univariate analysis showed that the oxLDL/HDL and oxLDL/LDL ratios correlated significantly with HbA1c, while oxLDL itself correlated only weakly with HbA1c ($p=0.062$). We examined the ratios of oxLDL/HDL and oxLDL/LDL as previous reports suggest that these metrics may be more informative than oxLDL alone (Huang et al. 2012; Johnston et al. 2006; Lankin et al. 2011). In a similar study of T2DM, oxLDL/HDL and oxLDL/LDL, but not oxLDL alone, were associated with atherosclerosis, even when adjusting for BMI, gender, age and hypolipidemic treatment (Girona et al. 2008). LDL derived from diabetics is more susceptible to oxidation, and this susceptibility is associated with HbA1c levels (Matsuda et al. 2013; Hussein et al. 2007). The present Navajo subset also

showed a similar lipid profile as seen in metabolic syndrome/diabetes, wherein LDL is normal, but HDL is low and TG are high (Howard et al. 2000; Mendlein et al. 1997; Nesto et al. 2005; Schumacher et al. 2008). Thus, although LDL may be lower, it may also be more susceptible to oxidation further increasing CVD risk in this population.

T2D (26%) and hypertension (38%) in the Navajo subset and original DiNEH population (25% and 36%, respectively) have increased compared to previous reports (Mendlein et al. 1997; Will et al. 1997) and are notably greater than the U.S. prevalence (CDC 2014; Go et al. 2014). Overweight and obesity continue to be very prevalent (at least 76%) in the Navajo population. In the Navajo subset, BMI was moderately correlated with CRP and HbA1c but not oxLDL or oxidized- to non-oxidized lipoprotein ratios. OxLDL was correlated with both CRP and IL6, suggesting a potential role for the contribution of overall inflammation to the health status of this population. CRP was also correlated with oxLDL/HDL and oxLDL/LDL. The Navajo subset exhibited moderate CRP levels (2.1 mg/dl; IQR: 0.9-4.8), but over 38% had levels greater than 3.0 mg/dl consistent with increasing risk for CVD. Mechanistic studies have demonstrated that CRP and oxLDL may be interacting in concert to promote atherosclerosis (Singh et al. 2008; Li et al. 2015). Despite low self-report of heart disease, heart attack and stroke, these data suggest that CVD- and T2D-related conditions may become more prevalent in this middle-aged Navajo population.

It is not surprising that standard lipid measures are predictive overall of oxLDL and oxLDL ratios with HDL and LDL. TG in particular were positively associated

with all three response-variables, which is consistent with other studies that have assessed lipid oxidation in diabetes (Verges et al. 2009; Gowri et al. 1999; Makimattila et al. 1999). HbA1c seems to be somewhat predictive of oxLDL/HDL and perhaps oxLDL but not oxLDL/LDL. Non-fasting glucose was negatively associated with oxLDL/HDL but slightly positively associated with oxLDL possibly reflecting more of a post-prandial response. It has been suggested that LDL oxidation is associated with dyslipidemia as diabetes progresses (Kopprasch et al. 2002). Although not measured in this study, small dense LDL particles, HDL abnormalities and diminished serum anti-oxidative capacity in diabetics are strongly associated with metabolic syndrome and diabetes (Verges et al. 2009; Gowri et al. 1999; Makimattila et al. 1999; Brizzi et al. 2003). Inflammation is a component of both atherogenesis and the development of diabetes. CRP was somewhat predictive of the oxLDL ratios with HDL and LDL but not of oxLDL itself. Age was negatively associated with oxLDL and oxLDL/HDL suggesting that these variables decrease in middle age in the Navajo population. OxLDL/HDL appears to be higher in women.

Study Limitations

An important limitation of our study was that some plasma obtained for biomarker assessments (2010-2011) was collected at the end of the survey administration (2005-2010). Thus, subjects may not have initially reported health conditions for which they were later diagnosed. Although review of medical records was attempted, complete records were not available for all volunteers so not all self-reported health conditions could be validated. Navajo also engage in traditional

medical practices, which would not be included in clinical medical records. However, for the self-reported measures of BMI (data not shown) and diabetes where sufficient clinical data were available at the time of blood collection, concordance with self-report was >80% with errors. Thus, given that the time between survey and clinical assessment could range as long as 5 years, observed discrepancies were consistent with a clinical finding of disease subsequent to the survey. Although bias may present due to self-selection, the Navajo subset was similar to the larger DiNEH study population based on age, gender, BMI and self-reported disease conditions. Non-fasting blood samples may impact some of the biomarker levels, however, non-fasting lipid levels may better predict risk of cardiovascular events (Langsted et al. 2011). Participant use of diabetic and hypertensive medications was not included in our initial analysis.

The cross-sectional nature of this study does not allow for the determination of causality. The results of this study cannot be generalized to other populations. Finally, this population is known to have significant environmental exposure risks to metals from the legacy of U mining. However, the study population reported herein spans the full range of those exposures, including a significant proportion of unexposed participants (Hund et al. 2015), improving the generalizability of our results.

Conclusions

The Navajo population faces a dramatic increase in overall CVD risk profile. This population is greatly in need of better control of diabetes, hypertension and

obesity to prevent future CVD-related health complications. Concentrations of the CVD marker oxLDL in the Navajo population trend with indices of metabolic syndrome and T2D, but were consistent with several other published cohorts. The relationship between oxLDL and absolute CVD risk in individuals with pre-diabetes and diabetes from this underserved ethnic group remains unclear. Ongoing and future analyses are exploring the potential role of chronic environmental exposures and other possible socioeconomic and lifestyle risk factors in contributing to cardiometabolic disease.

SUPPLEMENTAL INFORMATION

Table 2.1S Comparison of Navajo subset mean oxLDL levels, age, BMI, and HbA1c and/or diabetes status with other studies using monoclonal antibody 4E6 to measure circulating oxLDL

Population studied	oxLDL (U/L)	Age (yrs)	BMI (kg/m^2)	HbA1c (%)	Diabetes (%)	*N*	Country	Reference
Navajo population	*48.5*	*55*	*30*	*7*	*38.6*	*252*	*U.S.*	
General population							Spain	Gómez et al. 2014
No CAD event	53.2	49.5	27	n/a	11.4	2690		
CAD event	65.1	60	29.1	n/a	26	103		
No subclinical atherosclerosis	51.8	45.4	26.9	n/a	7.9	1121		
Subclinical atherosclerosis	57.8	58	28.3	n/a	16.7	306		
Patients w/o CVD							Portugal	Mascarenhas-Melo et al. 2013
Normal HDL	45.7	57.6	27	6	n/a	51		
Low HDL	39.2	57.9	28.8	6.3	n/a	22		
Patients w/ risk factors for CVD								
Normal HDL	35.7	62	29.2	8.1	n/a	119		
Low HDL	40.1	60	29.9	9.4	n/a	50		
Subjects w/o ESRD or CVD	38.3	53.2	26	n/a	none	20	Poland	Pawlak et al. 2013
ESRD on peritoneal dialysis	27.3	52.8	25.5	n/a	25	52		
ESRD on hemodialysis	30.8	57.7	25	n/a	14.8	54		
Type 2 diabetes							Spain	Girona et al. 2008
No atherosclerosis	67.4	60.8	31	7	n/a	93		
Atherosclerosis	70.4	64.9	29	7	n/a	73		
Patients w/o CHD event	93	61.3	27.7	n/a	none	258	Multi-national; men only	Meisinger et al. 2005
Patients w/ CHD event	110	61.1	28.6	n/a	none	88		

*Some subjects were taking lipid-lowering medications. oxLDL, oxidized LDL; BMI, body mass index; HbA1c, glycated hemoglobin; LDL, low-density lipoprotein; CAD, coronary artery disease; HDL, high-density lipoprotein; CVD, cardiovascular disease; ESRD, end-stage renal disease; CHD, coronary heart disease. All values are means or percent as indicated, reported by the corresponding study

III. CHAPTER 3

Arsenic Contribution to Circulating Oxidized LDL in a Native Community

In preparation for submission to Environmental Health Perspectives

ABSTRACT

More than 500 abandoned U mines within the Navajo Nation contribute U, As and other metals to groundwater, soil and potentially air through Aeolian transport. The cardiovascular health effects of cumulative exposure to these metals remain uncertain. We tested the hypothesis that environmental exposure to these metals may promote or exacerbate the oxidation of oxLDL cholesterol in a Native American population. CV biomarker data (oxLDL and CRP) from a Navajo cohort (N=252) were linked to mean annual As and U intakes from water and urine metals concentrations using linear regression. Proof-of-concept assays were performed to investigate whether As and U could directly oxidize human LDL. Mean annual As intake from water was a robust predictor of oxLDL, but not CRP in this Navajo group, while U intake estimates were negatively associated with oxLDL. In an acellular system, As, but not U, directly oxidized the ApoB 100 component of purified human LDL. As in combination with U may additively oxidize LDL. Neither metal promoted lipid peroxidation of the LDL particle. In summary, these findings suggest As intake from drinking water may influence oxidative modifications of LDL in the Navajo population where ~20% of unregulated water sources are known to contain As exceeding the US EPA maximum contaminant level.

INTRODUCTION

Navajo Nation community members in the southwestern United States have significant risks for environmental exposure to metals, including As and U, both from natural deposits and the legacy of U mining (deLemos JL et al. 2009; Hund et al. 2015), which was extensive from the 1940s until the 1980s (Brugge and Goble 2002; USEPA 2008). Chronic exposure to several toxic metals (e.g. As, lead, chromium) is associated with cardiovascular disease (CVD) (Lind et al. 2012; Prozialeck et al. 2008; Solenkova et al. 2014). In recent decades, the Navajo population has experienced an increased prevalence of metabolic syndrome (Schumacher et al. 2008) and coronary heart disease (National Cholesterol Education Program 2002). While diet and lifestyle are important contributors to this change, the contribution from exposure to metals in mining waste is unknown.

As often co-exists with U deposits and is present in legacy U mining wastes (Blake et al. 2015; Katsoyiannis et al. 2007), contributing to contamination of soil and water. Approximately 15-20% of unregulated water sources throughout the Navajo Nation produce exceed current U.S. Environmental Protection Agency (USEPA) maximum contaminant levels (U.S. Army Corps of Engineers 2000). Navajo Nation EPA has estimated that more than 30% of the Navajo population lack access to public water supplies and concerns exist about As and U exposure through consumption of unregulated water.

Chronic As exposure is associated with ischemic heart disease, hypertension, atherosclerosis, diabetes and cardiovascular mortality (Medrano et al. 2010; Navas-Acien et al. 2006; Rahman et al. 1999; Wang et al. 2002; Tseng et al. 2003). Accumulating epidemiological and mechanistic evidence confirms that the cardiovascular system is sensitive to low-to-moderate levels of As exposure (James et al. 2015; Moon et al. 2013; Lemaire et al. 2011). In contrast, little is known about CV-related effects of chronic low-level exposure to U. Limited evidence suggests a potential relationship between U exposure and circulatory system disease (Guseva Canu et al. 2012) and increased systolic and diastolic blood pressures (Kurttio et al. 2006). In addition, exposure to U waste on Navajo has been linked to an increased likelihood of hypertension (Hund et al. 2015).

OxLDL is an emerging biomarker that is mechanistically associated with CVD (Gomez et al. 2014; Holvoet et al. 1998; Trpkovic et al. 2014) and predictive of cardiovascular outcomes such as acute coronary artery disease (Meisinger et al. 2005) and myocardial infarction (Johnston et al. 2006). CRP is a well-established conventional biomarker of inflammatory status associated with increased CVD risk (Danesh et al. 2004). Recently, oxLDL and CRP levels were reported to be associated with As exposures in Bangladesh (Karim et al. 2013). Several essential and non-essential metals are known to contribute indirectly to oxidation of LDL and lipids (Chisolm and Steinberg 2000; Jamova and Valko 2011). It is unknown if As and U can directly oxidize LDL cholesterol.

Few studies have examined the relationship between chronic low-level As or U exposure and CVD in an at-risk community such as the Navajo Nation where CVD and diabetes are significant public health concerns (Moon et al. 2013). We assessed the potential impact of these metals on plasma levels of oxLDL and CRP in several Navajo communities located in the southwestern United States with varied levels of As and U exposures. We hypothesized that environmental exposure to As and U is associated with increased plasma oxLDL and CRP. We also performed proof-of-concept acellular assays to investigate whether As and U directly oxidize LDL cholesterol.

METHODS

Community-Derived Biomarkers and Exposure Assessments

Cohort Recruitment and Demographic Information

Demographic, water use and clinical data were obtained in partnership with the DiNEH Project (deLemos et al. 2009; Hund et al., 2015) using their Water and Land Use, Environmental and Health Survey. This interviewer-administered survey was completed by 1,304 Navajo Nation members between 2005 and 2010. A volunteer subset of this initial cohort of Navajo participants (n = 252) provided non-fasting blood and urine samples between 2010-2011 at which time height and weight were also measured to determine BMI. All data for the current study were derived from this volunteer subset. All participants provided informed

consent, with oversight and approval from the UNM Human Research Review Committee and the Navajo Nation Human Research Review Board.

Water Analysis and Human Exposure Assessments

Study participants reported obtaining water from a total of 180 distinct sources, including 122 unregulated sources such as wells, springs or livestock watering stations; 42 public water supply sources; and 14 sources that could not be classified. In order to evaluate metal exposure via drinking water, DiNEH staff collected water samples for 124 water sources (101 uregulated, 23 regulated) from 2003 to 2010 following USEPA protocol #2007. USEPA Region 9 laboratories conducted water quality analyses for unregulated sources using USEPA analysis method 200.8 (Inductively Coupled Plasma Mass Spectroscopy, ICP-MS). (A small subset of samples was analyzed by the Carlsbad Environmental Monitoring and Research Center or at Stanford University.) Navajo Nation EPA provided public water quality data. The compiled information resulted in As measurements for 113 sources (15 public water sources and 98 unregulated sources) and U measurements for 108 sources (16 public water sources and 92 unregulated sources).

Water quality information was unavailable for the remaining 167 unregulated sources identified by participants so these sources were excluded from subsequent analyses. For public water system sources without chemical data, (As, n=52 and U, n=48) the source was assumed to be in compliance with Safe Drinking Water Act regulations for As (< 10 µg/L) and U (< 30 µg/L) (USEPA

2013). Multiple imputation was used to generate a set of U and As concentration estimates from a uniform distribution within the bound of 0 and 2 µg/L. This process was also used to estimate As and U concentrations in store purchased and bottled water. Although the 2 µg/L value is well below the US EPA maximum contaminant level for both As and U, the effect of this choice would be to bias exposure estimates towards the lower end while creating numeric estimates of consumption.

Annual consumption of As and U was estimated based on participant self-reported water use and the metal concentration for each water source. Participants indicated how frequently they visited the source ($F_{i,j}$) (where *i* indicates the participant and *j* indicates the water source), the typical volume of hauled water ($V_{i,j}$) and the percentage used for cooking or drinking ($P_{i,j}$). This information was used to calculate the annual volume of water each individual consumed from each water source ($W_{i,j}$):

$$W_{i,j} = F_{i,j} \times V_{i,j} \times P_{i,j} \qquad (1)$$

An individual annual intake of As or U (mg/year) from drinking water was then calculated using the associated As or U concentration where *C(As)* indicates the As concentration and *C(U)* indicates the U concentration:

$$AsIntake_i = \sum_{j \in S(i)} (W_{i,j} \times C(As)_{i,j}) \qquad (2)$$

$$UIntake_i = \sum_{j \in S(i)} (W_{i,j} \times C(U)_{i,j}) \qquad (3)$$

Once the cumulative annual intake of water As and U from water was determined

for each participant, the continuous variable was translated to a binary variable: low or high metal consumption at the threshold of 1.4 mg/ year, because, for subjects who took all their drinking water from regulated sources, the estimated intake was always less than 1.4, for both As and U.

Urine Metals Concentrations

Urinary concentrations of V (V 51), Ni (Ni 60), As (AsO 91), and U (U 238) were available for 188 participants. Urinary Cu (Cu 63) was available for a subset of participants (n=94). Urine metals were determined by ICP-MS. Detection limits for U were high and resulted in many non-detects, therefore, available U data were examined as a binary variable reflecting detect or non-detect as the threshold.

Serum Biomarker Analysis

Plasma samples were tested for oxLDL by a sandwich ELISA that uses a monoclonal antibody specific for an epitope (monoclonal antibody 4E6) of the apolipoprotein B100 portion of the LDL particle, in accordance with manufacturer's instructions (Mercodia, Uppsala, Sweden) (N = 252). All other biochemical analyses were performed by a Navajo Area Indian Health Service reference laboratory (LabCorp, Phoenix, AZ). CRP was assessed quantitatively by latex immunoturbidimetry (LabCorp, Phoenix, AZ) (n= 249). Glycated hemoglobin (HbA1c) (n = 249) was determined by the Roche Tina-quant assay.

<u>Acellular Assays of Metal-induced LDL Oxidation</u>

Materials

Acellular studies utilized $CuSO_4$ and $NaAsO_2$ (Sigma-Aldrich Co., St. Louis, MO, USA); UA (Electron Microscopy Sciences, Hatfield, PA, USA); and highly purified human LDL (Lee Biosolutions, St. Louis, MS, USA).

Determination of Protein and Lipid Oxidation of LDL

Direct apoprotein B oxidation of the metal-treated LDL samples was determined by ELISA (as above). Lipid peroxidation in the LDL-metal samples was determined using a TBARS assay kit, which normalized total lipid peroxides to a malondialdehyde standard (Cayman Chemical Company, Ann Arbor, MI, USA) per manufacturer's instructions.

Treatment of LDL by Metals

Direct metal-induced oxidation of human LDL cholesterol was determined by performing individual dose responses with $CuSO_4$, $NaAsO_2$, and UA with purified human LDL. $CuSO_4$ was used as a positive control since it is frequently used experimentally to oxidize LDL (Burkitt 2001). A concentration of 100 mg/dl human LDL was incubated with CuSO4 (20, 50, 150 and 450 μM for the oxLDL ELISA; 15, 25, 45 and 75 μM for the TBARS assay), NaAsO2 (0.02, 0.07, 0.2 and 0.7 μM for the oxLDL ELISA; and 0.2, 0.7, 2 and 7 μM for TBARS assay), UA (0.3, 3, 30 and 300 nM). LDL was co-incubated with combinations of NaAsO2 and UA (0.02 μM NaAsO2 and 0.3 nM UA, 0.02 μM NaAsO2 and 3 nM UA, or 0.07 μM NaAsO2 and 0.3 nM UA). CuSO4 and NaAsO2 were dissolved in phosphate buffered saline (PBS) to achieve desired concentrations. UA was

first dissolved in water at a concentration of 100 μM and then diluted to 300 nM in HEPES to obtain desired concentrations. For the experiments that tested the combined effect of NaAsO2 and UA, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was used to dissolve both metals. After treatment, metal-treated purified human LDL samples were placed in a chamber with nitrogen flow for 10 minutes to reduce the contribution of atmospheric oxygen to LDL oxidation. Samples were then sealed and incubated at 37°C for 1-5 hours. EDTA (4 mM) was immediately added to the final samples at each time point to chelate the reaction. Samples were immediately frozen at -80°C until further analysis. Lower concentrations of metals tested were physiologically relevant to normal and/or background levels of each metal in plasma (Ivanenko et al. 2013; ATSDR 2007; McMillin et al. 2009; Byrne and Benedik, 1991).

Statistical Analyses

We constructed four linear regression models each for oxLDL and *log* CRP. All models (1-4, Tables 3 and 4) used demographic (age, gender) and physiological variables (BMI, HbA1c), and estimated annual water intakes of U and As as predictors. Models 3 and 4 also included all urinary metals as predictors. Models 2 and 4 included 2nd order interactions with age and gender. The majority (> 80%) of HbA1c levels in the participants were in pre-diabetic or diabetic categories; therefore a binary variable was used (HbA1c ≤6.4% or >6.4%). Estimated annual intakes of As and U from drinking water also used the binary variables as previously described (Water As_{bi}, Water U_{bi}). Urinary metals were skewed and thus log transformed except in the case

of urine U, a binary variable was used as previously described. Reduced models were derived by model selection using the AIC with stepwise selection allowed in both directions; models were successively revised so as to reduce AIC. A value of $p < 0.05$ was considered statistically significant. Statistical analyses were performed using R version 2.12.1 (The R Foundation for Statistical Computing, 2010, 64-bit).

The time and dose-response relationships of purified human LDL and metal treatments were analyzed by two-way analysis of variance (ANOVA) followed by the Dunnett multiple comparison test using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Community-Level Association between Mining Metal Exposure and LDL Oxidation

This Navajo subset is known to have a high prevalence of diabetes, hypertension and overweight/obesity (Hund et al. 2015; Harmon et al, In submission). Table 3.1 shows clinical characteristics and biomarker levels by gender. Both genders were similar in age and had similar TC, LDL cholesterol and non-fasting glucose levels. Women were generally heavier, had higher HDL levels, lower TG, and lower blood pressures than men. Both genders had similar median HbA1c levels, but more women fell into pre-diabetic and diabetic categories of HbA1c

Table 3.1 Navajo demographic and clinical characteristics by gender

Variable	Female (n = 145)	Male (n = 107)
Age (years)	55.5 (±14)	55.1 (±14.7)
Body mass index, kg/m²	31.2 (27.7-34.9)	28.2 (26.1-31.5)
Total Cholesterol, mg/dL	182.5 (163.3-201.8)	182 (159.5-204.5)
Low Density Lipoprotein (LDL), mg/dL	105 (90.3-123.8)	104 (88-122)
High Density Lipoprotein, mg/dL	47.5 (39-57)	42 (34-49.5)
TG, mg/dL	177 (127.5-247.3)	191 (125-261.5)
Systolic Blood Pressure (mmHg)	126 (114-142)	132 (121.5-145.5)
Diastolic Blood Pressure (mmHg)	76 (70-85)	81 (73-88.5)
Glucose (non-fasting), mg/dL	90 (77-122.8)	92 (79-120)
Median HbA1c, %	6.3 (5.9-7.3)	6 (5.6-6.9)
% Normal (≤ 5.6%)	7.7	26.4
% Pre-diabetes (5.7-6.4%)	50.3	39.6
% Diabetes (≥ 6.5%)	42	34
Oxidized LDL, U/L	45.7 (36.7-57)	48.7 (36.7-57.2)
C-Reactive Protein (CRP), mg/L	2.5 (0.9-5.2)	1.8 (0.9-4.2)
CRP > 3.0 mg/L, %	44	32

Data are presented as median (IQR) or %. HbA1c, glycated hemoglobin

classification. Women had lower levels of oxLDL and higher CRP.

Median urine As [4.21 (2.25-6.78) µg/L] was below National Health and Nutrition Survey (NHANES) 50th percentile [6.09 (5.22-7.12) µg/L] (CDC 2015; Table 3.2). However, 14.6% of participants had urine U concentrations that exceeded NHANES 95th percentile for all races, age, and gender by 4.2 fold. Median values and IQR for other measured urine metals were: Cu, 12.45 (8.15-17.02) µg/L; Ni, 13.08 (6.67-25.24) µg/L; and V, 1.05 (0.61-1.56) µg/L; NHANES values for these urine metals are not presently available.

Overall median estimated mean annual water intakes for As and U were similar, 0.49 mg/year (IQR 0-1.09) and 0.46 mg/year (IQR 0-1.13), respectively. As and U concentrations in drinking water ranged from 0-120 µg/L and 0-110 µg/L, respectively. Water As intake was not correlated with urine As, and similarly water U intake did not correlate with urine U (data not shown).

Reduced multivariate linear regression models for oxLDL are shown in Table 3.2. Models 1 and 2 had very low adjusted correlation of determination (R^2), however, in both models estimated annual intake of water As (Water As_{bi}) was significantly associated with oxLDL. In Model 2, water U_{bi} was also significantly but negatively associated with oxLDL ($p = 0.033$). In Model 3, urine metals were included (no interactions) and the adjusted R^2 improved (0.106). Water As_{bi} remained a positive predictor, and urine As was a marginally ($p = 0.045$) negative

Table 3.2 Reduced regression models showing coefficient estimates for oxLDL

Coefficient	Estimate	Standard Error	*P* value	*N*
Model 1				*249*
Water As_{bi}	10.98	3.788	0.004	
Adjusted R^2 = 0.029, p = 0.004				
Model 2				*249*
Age, years	-0.052	0.081	0.526	
Female	-1.12	2.288	0.625	
Water As_{bi}	10.05	4.232	0.018	
Water U_{bi}	-31.66	14.73	0.033	
Adjusted R^2 = 0.037, p = 0.02				
Model 3				*94*
Water As_{bi}	15.9	7.086	0.027	
Water U_{bi}	-11.66	5.305	0.031	
Urine As	-3.889	1.916	0.045	
Urine U_{bi}	11.68	4.392	0.009	
Adjusted R^2 = 0.106, p = 0.007				
Model 4				*94*
Age, years	-1.568	0.823	0.06	
Female	-54.19	24.07	0.027	
Water As_{bi}	16.09	6.978	0.024	
Water U_{bi}	-35.28	17.89	0.052	
Urine As	-6.023	2.143	0.006	
Urine U_{bi}	8.833	4.281	0.042	
Urine Cu	-25.14	13.84	0.073	
Female x Urine Cu	17.42	7.427	0.021	
Adjusted R^2 = 0.203, p = 0.002				

P < 0.05 was considered statistically significant

Model 1: OxLDL modeled with physiological [age, gender, body mass index (BMI), glycated hemoglobin (HbA1c)], and binary ($_{bi}$) water intake variables [As (As), U (U)]
Model 2: Model 1, and 2nd order interactions with age and gender
Model 3: Model 1, and urine metals (As, U_{bi}, Copper (Cu), Nickel, Vanadium)
Model 4: Model 3, and 2nd order interactions with age and gender

predictor of oxLDL; water U_{bi} remained a negative predictor and urine U was a positive predictor ($p = 0.009$) of oxLDL. Model 4 seemed to best fit the data, but the number of observations was reduced (n = 94; also for Model 3) due to the limited data for urine Cu. In Model 4, adjusted R^2 was higher (0.203) indicating that including urine metals in the model is important. There was a significant interaction for gender and urine Cu ($p = 0.021$) suggesting that women in this Navajo with higher urine Cu have higher levels of oxLDL. Gender was a negative significant predictor indicating that women have lower oxLDL, consistent with what is shown in Table 3.1. Water As_{bi} again remained a positive predictor ($p = 0.024$), but water U_{bi} was no longer significant ($p = 0.052$). Urine As was again a significant negative predictor of oxLDL, and urine U remained a positive predictor. BMI, HbA1c, and urine Ni and V did not appear as predictors in any model.

Reduced multivariate linear regression models for log CRP are shown in Table 3.3. Models 1 and 2 had low adjusted R^2 values (0.09). Age was a negative predictor and BMI was a positive predictor in Models 1, 2 and 3, and in Model 2 the interaction of gender and BMI was marginally negatively correlated with *log* CRP ($p = 0.49$). Adjusted R^2 was improved in Model 3 (0.157) and further improved in Model 4 (0.246), indicating that including urine metals is important.

Table 3.3 Reduced regression models showing coefficient estimates for CRP

Coefficient	Estimate	Standard Error	*P value*	*N*
Model 1				*247*
Age, years	-0.015	0.005	*0.002*	
BMI, kg/m²	0.038	0.014	*0.007*	
Adjusted R^2 = 0.085, p = <0.0001				
Model 2				*247*
Age, years	-0.016	0.005	*0.001*	
Female	1.625	0.852	*0.058*	
BMI, kg/m²	0.055	0.017	*0.002*	
Female x BMI	-0.059	0.03	*0.049*	
Adjusted R^2 = 0.092, p = <0.0001				
Model 3				*94*
Age, years	-0.019	0.007	*0.011*	
Female	-0.327	0.205	*0.115*	
BMI, kg/m²	0.049	0.022	*0.026*	
Adjusted R^2 = 0.157, p = 0.001				
Model 4				*94*
Age, years	0.004	0.012	*0.706*	
Female	-0.943	1.348	*0.486*	
BMI, kg/m²	0.051	0.021	*0.017*	
Urine U_{bi}	-0.788	0.38	*0.042*	
HbA1c $_{bi}$	2.62	0.954	*0.007*	
Age x HbA1c $_{bi}$	-0.04	0.017	*0.018*	
Female x Urine U_{bi}	1.296	0.589	*0.031*	
Adjusted R^2 = 0.246, p = 0.0003				

P < 0.05 was considered statistically significant

Model 1: *log* CRP modeled with physiological [age, gender, body mass index (BMI), glycated hemoglobin (HbA1c)], and binary ($_{bi}$) water intake variables [As (As), U (U)]
Model 2: Model 1, and 2nd order interactions with age and gender
Model 3: Model 1, and urine metals (As, U_{bi}, Copper, Nickel, Vanadium)
Model 4: Model 3, and 2nd order interactions with age and gender

Model 4 seemed to best fit the data, but the number of observations was reduced (n = 94; also for Model 3) due to the limited data for urine Cu. In Model 4, there was a significant interaction with gender and urine U_{bi} (*p = 0.031*) suggesting that women with higher urine U_{bi} have higher levels of CRP. Urine U_{bi} was also a significant independent predictor of CRP but the association was negative (*p = 0.042*). BMI continued to be a positive predictor of CRP, but age was removed. HbA1c appeared in the model and was positively associated with CRP (*p = 0.007*). Water As_{bi} and U_{bi} and urine As, Cu, Ni or V did not emerge as predictors in any of the models of CRP.

Acellular Studies of LDL oxidation

Protein oxidation of LDL cholesterol by As and U

Copper sulfate is a known oxidizer of LDL, and the $CuSO_4$-treated LDL showed a time and dose dependent response as expected, for $CuSO_4$ concentrations greater than 50 μM as compared to untreated LDL (Figure 3.1A). Sodium arsenite appeared to directly oxidize LDL earlier at lower concentrations and later at higher concentrations, but the level of oxLDL was similar at each time-point (Figure 3.1B). Uranyl acetate (UA) only appeared to oxidize LDL at 3 hours, but inconsistently at only the lowest (0.3 nM) and highest (300 nM) concentrations (Figure 3.1C). Interestingly, in combined treatments, oxLDL levels were increased at all time-points when both $NaAsO_2$ and UA were present in the reaction mixture (Figure 3.1D). Only the lowest combined

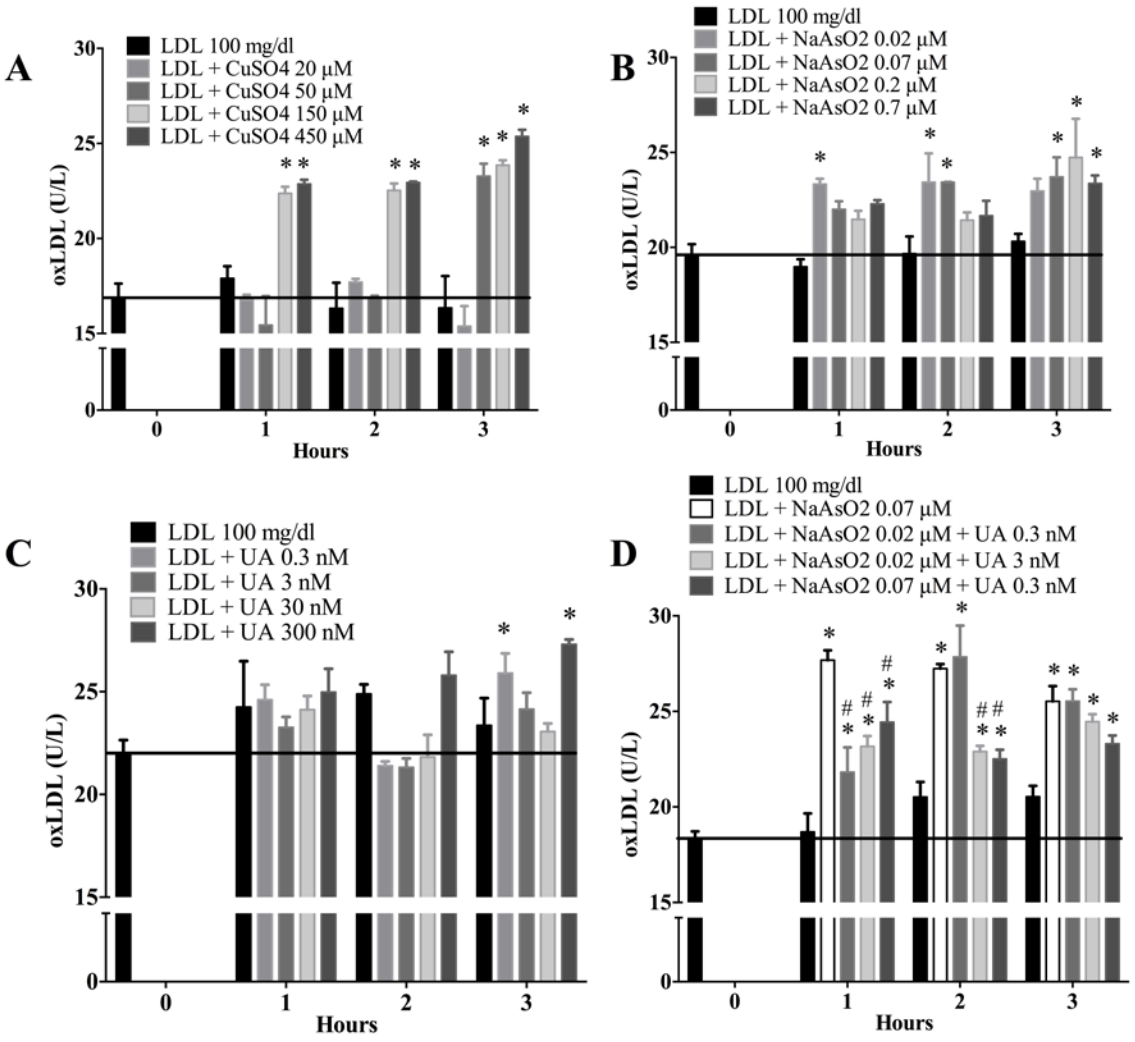

Figure 3.1 Sodium arsenite directly oxidizes the apolipoprotein B component of LDL cholesterol. (A) $CuSO_4$ was used as a positive control. Effects of different concentrations of (B) Sodium arsenite ($NaAsO_2$); (C) Uranyl acetate (UA), or (D) the combination of $NaAsO_2$ and UA on the apoprotein B (ApoB) of human purified LDL as measured by the 4E6 ELISA antibody. *significant difference from LDL untreated. $P < 0.05$ was considered statistically significant.

concentrations of $NaAsO_2$ (0.02 μM) and UA (0.3 nM) increased LDL oxidation to the same level as LDL treated with $NaAsO_2$ alone (0.07 μM), however, LDL treated with the same concentration of $NaAsO_2$ (0.07 μM) plus UA (0.3 nM) was significantly lower than LDL treated with $NaAsO_2$ alone at 1 and 2 hours but was similar at 3 hours. LDL treated with 3 nM of UA alone was not significantly increased but when the same concentration was combined with $NaAsO_2$, LDL oxidation was increased at all time-points. These data suggest that $NaAsO_2$ directly oxidizes LDL, and that when combined, $NaAsO_2$ and UA may additively contribute to ApoB oxidation as measured by ELISA, especially at later time-points in this experimental system.

Lipid peroxidation of LDL cholesterol by As and U

CuSO4 induced lipid peroxidation (MDA equivalents) of human LDL as expected, with clear concentration- and time-dependent trends observed (Figure 3.2A). The level of lipid peroxides was not affected by $NaAsO_2$, UA, or the combination of both metals (Figure 3.2 B-D) especially in comparison to $CuSO_4$-treated LDL, used repeatedly as a positive control (Figure 3.2A). These data suggest that neither $NaAsO_2$ nor UA promote lipid oxidation of LDL cholesterol under these conditions.

Overall, these findings are coherent with our regression modeling data in that As may promote increased LDL oxidation, however, it appears that As more likely may contribute directly to the oxidation of the protein (Apo B)

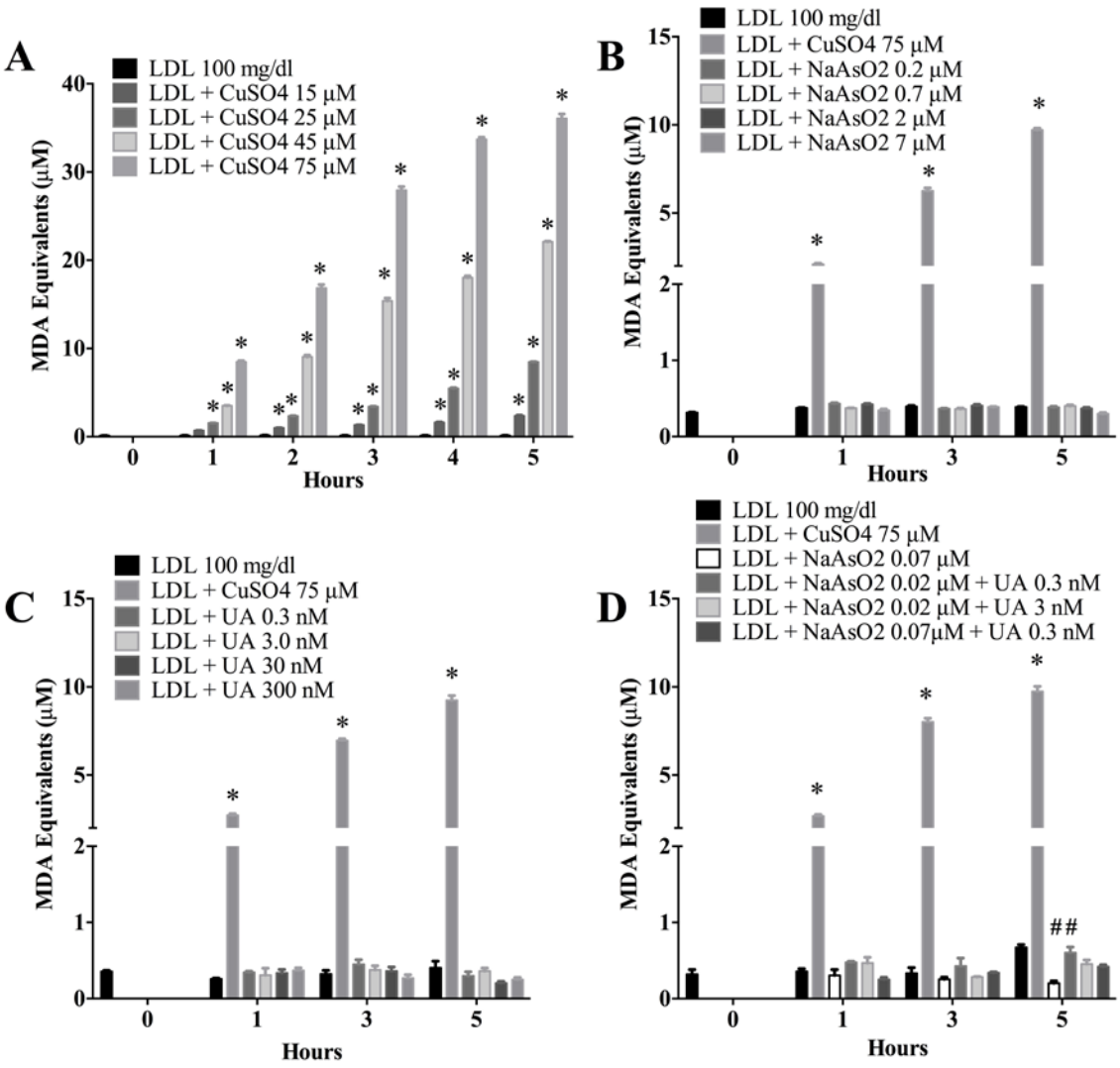

Figure 3.2 Sodium arsenite and uranyl acetate do not increase lipid peroxidation of LDL TBARS assay with malondialdehyde (MDA) equivalents. (A) $CuSO_4$ was used as a positive control. Effects of different concentrations of (B) $NaAsO_2$, (C) UA, or (D) the combination of $NaAsO_2$ and UA on lipid peroxidation of human purified LDL. *significant difference from LDL untreated; # significant difference from LDL treated with $NaAsO_2$ at 0.07 µM. $P < 0.05$ was considered statistically significant.

component and not the lipid components of the LDL particle. U seems to be associated with oxLDL but the relationship is unclear.

DISCUSSION

We investigated whether CVD biomarkers, oxLDL and CRP, were associated with annual estimated drinking water intake of As and U, as representative mining metal contaminants. To establish proof-of-concept, we also assessed if these metals could directly oxidize LDL. Both data sets are consistent with the hypothesis that As may promote oxidation of LDL, a crucial step in vascular inflammation and chronic vascular disease. Outcomes related to U were also consistent, in that negative associations were observed between U intake from water and oxLDL, and U only
minimally altered human LDL in direct exposure experiments. Urine As and U were also associated with oxLDL, albeit in opposite directions from each other and from the water intakes of these metals. Only urine U seemed to have an association with CRP whereas no other metals in water or urine were predictors of this inflammatory marker.

Numerous epidemiological studies have shown that As contributes to CVD development, but limited evidence is available for As effects on the intermediate biochemical changes associated with atherogenesis. Recently, chronic As exposure from drinking water has been associated with biomarkers of inflammation and endothelial cell activation including oxLDL, the ratio of oxLDL with HDL, CRP, ICAM-1 (Karim et al 2013) and VCAM-1 (Wu et al. 2012) in

Bangladesh residents. Another recent study in Bangladesh showed that As exposure was associated with increased plasma CRP, especially in participants with greater plasma glutathione redox potential (Peters et al. 2015).

OxLDL contains heterogeneous lipid and protein components that vary in degree and type of oxidation that can induce a variety of pro-atherogenic effects (Reis et al. 2015). Our data suggest that As, in direct contact with LDL particles, can modify the ApoB 100 component in a manner that makes it recognizable by the 4E6 antibody. While As has been shown to promote chronic vascular remodeling, there is conflicting evidence whether As increases lipid peroxide levels in ApoE$^{-/-}$ mice (Simenova et al. 2003; Srivastava et al. 2009). In the present study, As had minimal acute effects on lipid peroxidation of purified human LDL. Minimally-oxidized LDL is sufficient to promote vascular inflammation and pathological remodeling, and is typically characterized by ApoB oxidation with lower levels of TBARS (Itabe et al. 2003). The direct interaction of As with ApoB may, however, be sufficient to drive the minimally-oxidized form of oxLDL and thereby play a role in systemic vascular disease.

It remains unknown how metals such as As and U may influence oxidative modifications of LDL. As is redox inactive but has an affinity for thiol-containing molecules (Jomova and Valko 2011). The ApoB-100 protein component of LDL contains cysteine residues (Segrest et al. 2001) that could potentially be modified by As. As is also known to bind hemoglobin on erythrocytes as well as other

proteins in the plasma, including thioredoxin and glutathione (Hughes et al. 2011; Schmidt et al. 2009), which may influence the bioavailability and pharmacokinetics of As as well as influence the behaviors of the bound-proteins.

Little is known about the effects of chronic low-level exposure to U, particularly in terms of CVD prevalence and pathogenesis. Exposure to depleted U is associated with increased lipid oxidation in rat brains (Briner and Murray 2005) and the limited studies on endothelial cells toxicity are negative (Dobson et al. 2006). However, our results suggest that in an isolated acellular system, low concentrations of U do not directly modify either the protein or lipid components of LDL, and U seemed to reduce the oxidizability of the ApoB 100 protein by As. Furthermore, measures of U in drinking water associated negatively with oxLDL and not at all with CRP levels in the Navajo population. An important consideration for mine wastes is the mixture of metal contaminants, not simply the residual ores of interest, which can be most toxic to exposed populations. Thus, while the vast majority of abandoned mine sites in this region were a result of U extraction, numerous other metals such as As, V, Ni, and Cu may be left behind creating a legacy environmental health risk.

While our findings indicate the potential for direct effects between metal contaminants and LDL oxidation, the contribution of indirect effects should not be dismissed. Increased levels of reactive oxidants and decreased antioxidant capacity have been found in plasma from humans exposed to As in drinking

water (Wu et al. 2001). Changes in redox status or increased free radical production (Lankin et al. 2014) and inflammatory factors may be induced by the presence of toxic metals in plasma or atherosclerotic lesions (Shi et al. 2004).

Limitations

The cross-sectional nature of our analysis does not allow for temporal relations between exposure and CVD outcomes to be established, however, this is the first epidemiologic study to demonstrate that As promotes LDL oxidation in an exposed population in the U.S. Selection bias is a possibility in this volunteer population; however, no obvious biases in exposure risks have been found (deLemos et al. 2009; Hund et al. 2015). Participants represented the full range of exposures including a significant proportion of unexposed volunteers, improving the generalizability of our results (Hund et al. 2015). Blood samples were non-fasting, and medication use was not included in this initial analysis, which could influence LDL modifications. Because of the long storage times, we were unable to assess TBARS in the banked serum from the DiNEH study as a parallel to the acellular assays.

Epidemiological study designs prevent conclusions of causality and provide limited mechanistic information. In order to establish biological plausibility, we performed proof-of-concept acellular assays, which were chosen to represent direct oxidation of LDL by As and U in the circulation thereby contributing to the atherosclerotic process, but this could also represent what may be occurring

within developing lesions. It is thought that most modifications to LDL probably occur *in situ* within the atherosclerotic lesion (Itabe et al. 2011), and more research is needed to assess whether As and/or U can accumulate in vulnerable vascular lesions.

Several metals, including As, have been detected in atherosclerotic lesions and cardiovascular tissue (Simeonova et al. 2003; Stadler et al. 2004). Importantly, our acellular data match what was observed in the human serum, in terms of ApoB modifications. Lastly, although the buffers used in these experiments (PBS and HEPES) are stable and non-reactive, they do not share the same milieu of factors that may contribute to the atherosclerotic process or affect metal-LDL interactions, such as anti-inflammatory factors and metal-binding or carrier proteins, respectively.

Conclusions

These findings suggest that As intake from contaminated drinking water may influence oxidative modifications of low-density lipoproteins in the Navajo population. Direct oxidation by metals may be an overlooked contributing mechanism to explain chronic CVD health outcomes, although future research will need to explore such relationships in more physiologically-relevant experimental systems. For the Navajo community, new mining sites are planned (e.g. near Grants, New Mexico), and there remains a dearth of literature exploring the potential health effects of unremediated wastes and tailing from

mining operations. Continued research into mining waste-related community health outcomes, and pathophysiological basis thereof, is essential to ensuring that mineral resources can be extracted with minimal negative impacts on ecological and public health.

IV. CHAPTER 4

Abandoned U Mine Proximity Predicts Endothelial Inflammatory Potential of Serum from Chronically Exposed Navajo Communities

In preparation for submission

ABSTRACT

Members of the Navajo Nation reside at varying distances from hundreds of AUMs, which contribute U, As and other metals to the soil, water and air. These contaminants continue to pose potential health risks to these communities where the prevalence of inflammatory-related health conditions including coronary artery disease, metabolic syndrome and hypertension have increased in recent decades. Endothelial cells, essential players in the development of coronary plaques, respond to cumulative circulating pro- and anti-inflammatory mediators in a manner that reveals the balance of inflammation, i.e. inflammatory potential, which we define as the changes in transcriptional responses of inflammatory markers from primary endothelial cells treated *in vitro* with serum derived from exposed populations. Our goal was to determine the contribution of AUM waste exposures to community-level serum inflammatory potential. Serum was obtained from a subset of Navajo volunteers (n = 145). Transcriptional responses of VCAM-1, ICAM-1, and CCL2 were measured from Navajo serum-treated primary endothelial cells and linked to AUM proximity and mean annual As and U intakes from water using linear regression. Linear regression modeling showed that household AUM proximity strongly predicted CCL2, VCAM-1 and ICAM-1 mRNA (p = <0.0001 for each response) from endothelial cells treated with serum from exposed Navajo participants, whereas annual water intakes of As and U did not. In summary, this is the first application of this novel measure of cardiovascular health in a community population. Our data reveal a broader

effect of mining waste exposure on overall inflammation, and routes of exposure beyond drinking water should be investigated.

INTRODUCTION

One of the richest U ore deposits in the United States, located in the Four Corners region of the Southwest, was actively mined on the Navajo Nation between the 1940s and 1980s (McLemore 2010). Today, there are more than 500 AUMs and 4 mill sites that continue to pose a risk to these communities (deLemos et al. 2009). U, As, Ni, V and Cu are among the major contaminants still found in the soil, air and ground water to which this population may be chronically exposed (Blake et al. 2015). The impact of such exposures on cardiovascular disease remains poorly researched.

American Indians, including Navajo, are known to have a high prevalence of inflammatory conditions including cardiovascular disease, hypertension, diabetes, and obesity (Nava et al. 2015). As such, there is concern that this population may be vulnerable to added cardiovascular stressors, such as environmental contaminant exposure. Recent studies have found a small link between As in the drinking water and the levels of oxidized LDL cholesterol (Harmon et al. 2015). However, assessment of single circulating factors (e.g., cytokines, etc.) may fail to capture the overall inflammatory and pathological influence that all circulating components convey to the endothelial wall. Endothelial cells respond to cumulative circulating pro- and anti-inflammatory mediators in a manner that reveals the balance of inflammation, *i.e.*, inflammatory potential, which we define as the transcriptional responses of inflammatory markers in primary endothelial cells treated *in vitro* with serum

derived from human subjects (Smoliga et al. 2013). Endothelial cell expression of adhesion molecules and chemokines (e.g., CCL2 or monocyte chemoattractant protein-1) is a principal activity in the pathogenesis of chronic atherosclerotic vascular disease (Aird 2008; Aukrust et al. 2008).

In humans, serum inflammatory potential, as determined by stimulation of responses in human primary coronary artery endothelial cells (hCAECs), has been linked directly to cardiovascular disease (Cung et al. 2015) as well as to inhalation exposures to diesel emissions (Channell et al. 2012; Schisler et al. 2015). Furthermore, it has been reported that polyphenols (namely resveratrol), as a nutraceutical treatment, led to reduced serum inflammatory potential in healthy subjects, as compared to placebo (Agarwal et al. 2013). Such studies are coherent with animal toxicological research showing that the inhalation of various toxicants, including gases and particles, leads to inflammatory and anti-dilatory alterations in the serum composition (Aragon et al. 2015; Paffett et al. 2015; Robertson et al. 2013).

In the present study, we assessed the inflammatory potential of serum from a population of Navajo community members. We hypothesized that serum inflammatory potential would be adversely influenced by exposure to AUMs in the Southeastern corner of the Navajo Nation. While drinking water was also considered as an exposure route, linear regression models showed that simple proximity to AUM had a strong and independent influence in the overall serum

inflammatory potential. This is the first application of this novel and unbiased metric of cardiovascular health in a community population.

METHODS

Study Population

Demographic, water use and clinical data were obtained in partnership with the Diné Network for Environmental Health (DiNEH) Project (deLemos et al. 2009) using the Water and Land Use, Environmental and Health Survey. Surveys were administered in interview fashion to a cohort of 1,304 participants from 20 Chapters of the Navajo Nation between 2005 and 2010. A volunteer subset of this population (n = 145) provided non-fasting serum samples between 2010-2011 at which time height and weight were also measured to determine BMI; all data for the current study was derived from this volunteer subset. All participants provided informed consent, with oversight and approval from the UNM Human Research Review Committee and the Navajo Nation Human Research Review Board.

Geospatial data

The locations of the homes of the survey participants were determined by using a hand-held Global Positioning System (GPS) instrument. The mean duration of residence in a current home was 32.1 years. The locations and surface areas (in m^2) of the abandoned U mines and mills within the study area were obtained from the U.S. Army Corps of Engineers documentation compiled for the USEPA (USEPA, 2007a).

Exposure metrics

Mean annual water intakes of As and U were estimated as previously described in Chapter 3 (p. 67-69).

AUM proximity was calculated as the log of the sum of the inverse distances of participant reported household geographical location to all AUM features (portals, prospects, rim strips, pits, vertical shafts or waste piles) in the study area, weighted by the surface area of each feature. The formula is noted below: $part_i$ denotes the geographical position of participant *i*. m_j denotes the geographical position of abandoned mining site *j*. *N* is the number of abandoned mining sites used in this formula. $area_j$ is the area of mining site *j* in square meters. The formula for AUM for participant *i* is then

$$AUM_i = \sqrt{\sum_{j=1}^{N} \frac{wj}{dist(part_i i, m_j)}}$$

where the weights $\{w_j\}$ are defined by

$$w_j = \hat{w}_j \Big/ \sum_{k=1}^{N} \hat{w}_k \,, \hat{w}_j = \sqrt{area_j}.$$

Inflammatory Potential Assay and Transcriptional Responses

The inflammatory potential assay (Figure 4.1) was conducted as previously described (Agarwal et al. 2013; Cung et al. 2015). Briefly, hCAEC (Lonza; Allendale, NJ) were grown to confluence in complete media on a series of 24-

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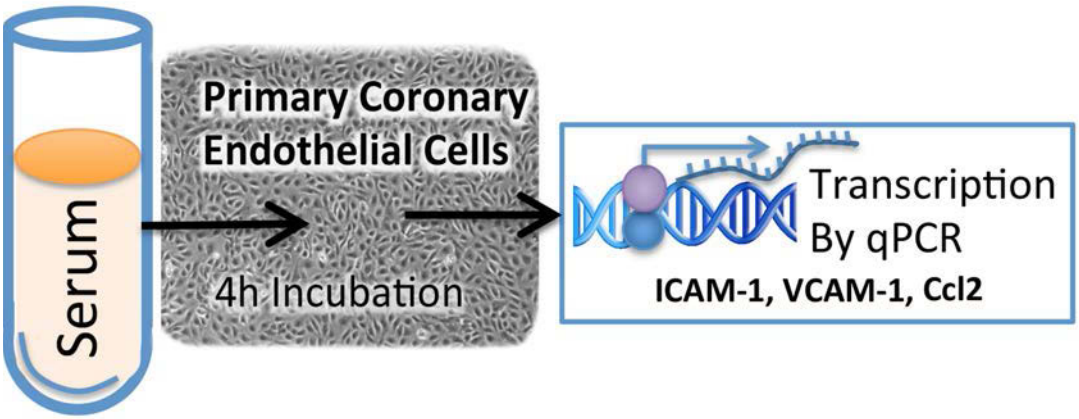


Figure 4.1 Schematic of serum inflammatory potential assay. Primary human coronary artery endothelial cells were incubated with complete serum from study participants for 4h, after which endothelial cell responses were assessed by RT-qPCR for ICAM-1, VCAM-1, and CCL2

well plates. For 24 hours prior to serum treatment, the cells were serum-starved with Basal Media (Lonza). Confluent hCAECs were then incubated with 10% serum (vol:vol) obtained from DiNEH volunteers (n=145). The samples were incubated for 4 hours at 37°C. Human CAECs were washed with PBS, lysed, and cell lysates were immediately collected for RNA purification. Total RNA was isolated from samples using RNeasy Mini Prep Kits (Qiagen), and RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kits (Applied

Biosystems) prior to quantitative real-time PCR (qPCR) assessment of endothelial adhesion markers. Amplification of target message was performed in TaqMan Universal Master Mix according to manufacturer's recommended conditions with TaqMan gene expression assays for intercellular adhesion molecule 1 (ICAM-1; Hs00164932_m1), vascular cell adhesion molecule 1 (VCAM-1; Hs01003372_m1), and chemokine (C-C motif) ligand (CCL2; Hs00234140_m1), with TATA box–binding protein (TBP; Hs00427620_m1) as the endogenous reference gene (Applied Biosystems 7900HT). Relative gene expression was analyzed by the $2^{-\Delta\Delta}C_T$ method using a relative amount of mRNA for each sample normalized to TATA box–binding protein (Livak and Schmittgen 2001).

Statistical Analysis

Descriptive summary statistics were reported as median and IQR for continuous variables, unless otherwise indicated. Non-Gaussian distributions were normalized using a logarithmic transformation. Distributions for untransformed

CCL2, ICAM-1 and VCAM-1 mRNA are shown in Fig 4.1S. Pearson correlations were performed to assess the relationship of CCL2, ICAM-1 and VCAM-1 mRNA to other CVD risk factors and biomarkers. Linear regression models were used to link the endothelial mRNA transcriptional responses (ICAM-1, VCAM-1, and CCL2) from the inflammatory potential assays as response variables to the exposure metrics including AUM proximity and annual As (As) and U (U) intakes from water and potential confounders age, gender, BMI and HbA1c. Estimated annual intake of As and U from drinking water sources were binary variables (as described in Chapter 3, p. 69). Reduced models were derived by model selection using the AIC. A value of $p < 0.05$ was considered statistically significant for models and their component variables. Statistical analyses were performed using R version 2.12.1 (The R Foundation for Statistical Computing, 2010, 64-bit) and GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Demographic and clinical characteristics of the DiNEH subset are shown in Table 4.1. The mean age was 56 years (S.D. 14.8), and females represented 38% of this Navajo subset. Median BMI was 30 kg/m^2 with more than a third of this subset (34.5%) being overweight and 50% were obese. In the original DiNEH study of 1304 participants, 41.2 % were obese (Harmon et al., 2015 in press) but a similar percentage was overweight. Median TC and LDL were within recommended guidelines based on American Heart Association guidelines (National Cholesterol Education Program Expert Panel on Detection and Treatment of High Blood Cholesterol in 2002). HDL cholesterol was somewhat

Table 4.1 Characteristics of DiNEH participants

Variable		n
Age, years	56.1 (SD 14.8)	145
Female, %	38	145
Body mass index (BMI), kg/m²	30 (26.8-34.0)	142
Normal (BMI = 18.5-24.9), %	14.8	
Overweight (BMI = 25.0-29.9), %	34.5	
Obese (BMI ≥ 30), %	50	
Total Cholesterol, mg/dL	185 (164-204)	127
Low Density Lipoprotein, mg/dL	105 (91.5-121.5)	127
High Density Lipoprotein, mg/dL	45 (38-55)	127
TG, mg/dL	184 (130-263)	125
Systolic Blood Pressure (mmHg)	129 (116-144)	143
Diastolic Blood Pressure (mmHg)	78 (72-87)	143
Glycated hemoglobin (HbA1c), %	6.2 (5.8-7.6)	143
% Normal (≤ 5.6%)	16	
% Pre-diabetes (5.7-6.4%)	42.7	
% Diabetes (≥ 6.5%)	41.3	
Estimated mean annual intake from water		
As (mg/year)	0.83 (0-1.15)	144
U (mg/year)	0.82 (0-1.14)	144

Data are presented as median (IQR) or %, except where noted. Abandoned U Mine (AUM)

low, and TG were elevated. Median systolic blood pressure was pre-hypertensive, but median diastolic blood pressure was normal (Chobanian et al. 2003). Median glycated hemoglobin (HbA1c) was in the pre-diabetic range as defined by the American Diabetes Association (American Diabetes 2012). Over 80% of this subset was pre-diabetic or diabetic based on HbA1c. Estimated Navajo subset. Median BMI was 30 kg/m^2 with more than a third of this subset (34.5%) being overweight and 50% were obese. In the original DiNEH study of 1304 participants, 41.2 % were obese (Harmon et al. 2015 *in press*) but a similar percentage was overweight. Median TC and LDL were within recommended guidelines based on American Heart Association guidelines mean annual As and U intakes from water for this subset were both about 0.8 mg/year.

Figure 4.2A shows a heat map of the study area and area-weighted proximity to abandoned U mine and mill sites. AUM proximity distribution is shown in Figure 4.2B. Median AUM proximity was 0.207 (IQR 0.179-0.224). Univariate analysis (Table 4.2) showed that the weighted (based on surface area) proximity to AUM was significantly correlated with all endothelial transcriptional inflammatory markers (Fig 4.2S). CCL2, ICAM-1 and VCAM-1 mRNA were positively and significantly correlated with each other, which is consistent with their roles as inflammatory response mediators. Age, gender, BMI, HbA1c, lipids, and systolic or diastolic blood pressures were not correlated with any of the transcriptional responses. Final reduced regression models are shown in Table 4.3. Of all the variables used as potential predictors, the one strong independent predictor of

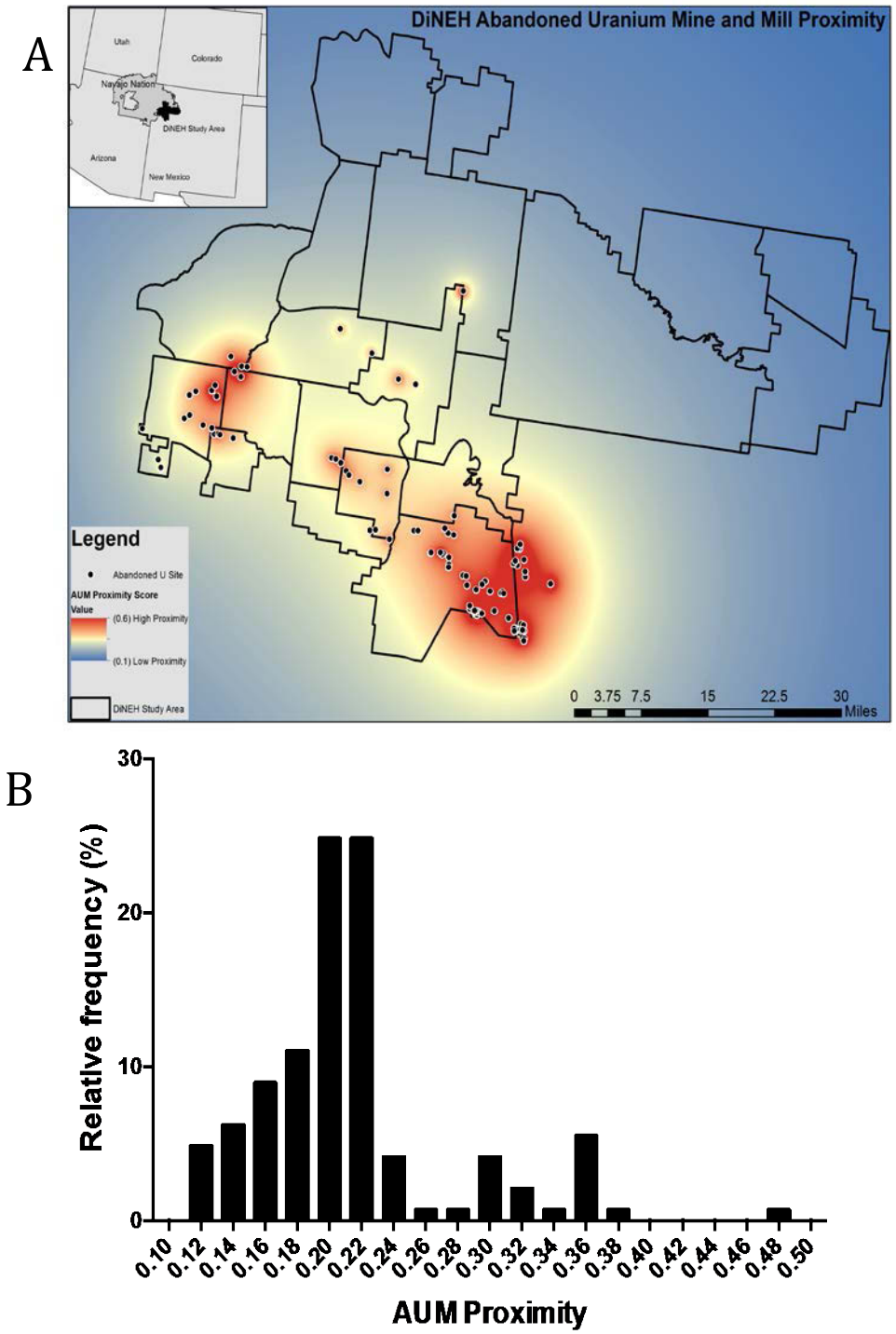


Figure 4.2 Study area heat map and frequency distribution of AUMs A) Study area and heat map of area-weighted proximity to abandoned U mine and mill sites. The Southeastern region of the Navajo Nation sits within the Northwestern portion of New Mexico and contains approximately 100 mine sites. **B)** Frequency histogram showing distribution of Navajo participants' household weighted proximities to abandoned U mines (AUM), with higher numbers indicating more proximal residences

Table 4.2 Pearson correlations between mRNA transcriptional responses and demographic and clinical risk factors and exposure metrics

Variable	VCAM1		ICAM1		CCL2	
	r	*p*	r	*p*	r	*p*
Age	-0.047	*0.572*	-0.037	*0.657*	0.007	*0.93*
Gender	0.072	0.39	0.066	0.429	0.02	0.813
BMI	-0.07	*0.409*	-0.082	*0.333*	-0.102	*0.229*
Total Cholesterol (mg/dL)	0.096	*0.285*	0.125	*0.161*	0.034	*0.703*
LDL (mg/dL) Direct	0.098	*0.277*	0.091	*0.311*	-0.027	*0.766*
HDL Cholesterol (mg/dL)	0.135	*0.13*	0.162	*0.068*	-0.0001	*0.999*
TG (mg/dL)	-0.013	*0.888*	0.033	*0.715*	0.135	*0.131*
SBP (mmHg)	-0.026	*0.756*	-0.071	*0.397*	-0.02	*0.813*
DBP (mmHg)	0.001	*0.989*	-0.005	*0.952*	-0.009	*0.917*
HbA1C (%)	-0.019	*0.825*	0.003	*0.968*	0.065	*0.441*
IL-6 (pg/ml)	-0.108	*0.213*	-0.117	*0.176*	-0.134	*0.122*
CRP (mg/L)	-0.099	*0.239*	-0.182	***0.029***	-0.103	*0.221*
Water U*	0.087	*0.298*	0.127	*0.129*	0.157	*0.059*
Water As*	0.084	*0.317*	0.075	*0.37*	0.111	*0.185*
AUM Proximity	0.226	***0.006***	0.378	***<0.0001***	0.471	***<0.0001***

Pearson correlation coefficients (r). P < 0.05 was considered statistically significant (bold). *continuous variable. Non-Gaussian distributions were log transformed. LDL, low-density lipoprotein; HDL, high-density lipoprotein; HbA1c, glycated hemoglobin; CRP, C-reactive protein; IL6, interleukin 6, SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; U, U; As, As; AUM, abandoned U mine

Table 4.3 Reduced regression models showing AUM proximity is the sole predictor of endothelial mRNA responses to serum from Navajo participants

Endothelial Response Variable	Coefficient Estimate of AUM Proximity	Standard Error	*p-value*	Adjusted R^2	*p-value of model*
VCAM-1	5.582	1.304	*3.41E-05*	0.107	*< 0.0001*
ICAM-1	5.335	0.964	*1.45E-07*	0.171	*< 0.0001*
CCL2	6.954	1.142	*9.99E-09*	0.2	*< 0.0001*

Reduced models are shown. Age, gender, body mass index, glycated hemoglobin, and water intake variables as potential predictors dropped out of models after model selection. Chemokine (C-C motif) ligand 2 (CCL2); intercellular adhesion molecule 1 (ICAM-1); vascular cell adhesion molecule 1 (VCAM-1); Abandoned U Mine (AUM).

endothelial mRNA transcriptional responses was AUM proximity. The associations were strong and positive for all responses ($p < 0.0001$). The highest R^2 (adjusted) was 0.20 for CCL2, followed by 0.17 for ICAM-1 and 0.11 for VCAM-1. Age, gender, BMI, HbA1c and estimated annual water intakes of As and U dropped out of the model.

DISCUSSION

Proximity of residence to AUMs has a strong influence on the degree of inflammatory potential in the circulation of Navajo community participants in the present study. Although the power of the final models was not large (adjusted R^2 values for the final models were 0.107 for VCAM-1, 0.171 for ICAM-1, and 0.200 for CCL2), considering the known influence of genetic and lifestyle factors on CV health, and the high prevalence of obesity and diabetes in the Navajo, these numbers indicate that AUM proximity has significant predictive power for these endothelial transcriptional responses to serum. In previous work with patients who had previously experienced a major cardiac event (infarction or angina), we saw comparable influences of risk factors on serum inflammatory potential (Cung et al. 2015). While a head-to-head comparison across these studies is not valid because of the differences in the demographics, the correlation coefficients (R) for proximity to AUM and endothelial inflammatory responses were comparable and even greater than the reported influences of inflammatory markers such as CRP, circulating lipids, and incidence of diabetes. Thus, the degree of association between proximity to AUMs and circulating inflammatory potential is

striking.

In our previous assessments of individual cardiovascular disease markers in this Navajo population, we found only a modest influence of the drinking water levels of As on oxidized LDL, with no association between exposure and CRP (Chapter 3, p. 76-78) or IL6 (data not shown). However, the data for As in the water was limited for the vast majority of this study population and effects were driven by less than 10% with the highest As levels. The present data reveals a broader effect of mining waste exposure on what we believe to be a more sensitive and reliable metric of overall inflammation than single cytokines or acute phase protein measures. Proximity to AUMs, and not our estimates of oral As or U intake, associates strongly with the serum-stimulated responses of ICAM-1, VCAM-1 and CCL2 mRNA. This relationship suggests that alternate exposure pathways or indirect health impacts are induced by the presence of the AUMs. Potentially windblown dusts from unremediated surface mines may present a hazard of metal-rich particulate matter, which is a known driver of cardiovascular toxicity (Bell et al. 2009; Lippmann et al. 2006; Niu et al. 2013). Windblown events and Aeolian transport of such contaminated dusts have been increasing and climate change models predict this trend to continue in the foreseeable future (Munson et al. 2011; Stovern et al. 2014).

We assume that changes in circulatory inflammatory potential are, in the short term, temporary and reversible (Agarwal et al. 2013). Factors that contribute to life-long risk of cardiovascular disease, such as smoking, poor diet, sedentary

lifestyle, often are reversible in the short term (Estruch et al. 2006; Lessiani et al. 2015; Xu et al. 2013), but chronic unrelieved adverse behaviors and exposures will ultimately lead to vascular inflammation and irreversible remodeling. On the assumption that our findings reflect a causal relationship between exposure and circulating inflammatory potential, relocation of residence or remediation of such sites could therefore allow for reversal of this cardiovascular stressor. Any vascular plaque development up to that intervention, however, would not likely reverse. Importantly, this is a population with a high degree of residential stability, with the average participant having a residence time of greater than 30 years.

Limitations of this novel assay and, by extension, the study conclusions relate primarily to the lack of quantitative reference data due to the responses of different batches and passages of endothelial cells being difficult to compare across studies. Unlike measurement of circulating proteins, which have a clear unit of concentration, the present data are derived from a single origin of endothelial cells and normalized within the study population. While quality control protocols are in place to ensure that Ct values are within acceptable ranges for all studies, it would not be feasible to conduct head-to-head comparisons of the present data with previously published data, although within-study trends are certainly comparable in a broader sense. Additionally, as this assay remains novel, pure risk estimates of cardiovascular disease are not available. Unlike CRP protein or IL6, which have been characterized thoroughly in numerous large population studies (Framingham; NHANES; etc) (Bennet et al.

2003; Rutter et al. 2004; Wang et al. 2002), such extensive association to CVD outcomes in a large cohort has yet to be conducted for the present study. Study limitations related to the population have been previously addressed (Harmon et al., 2015, in press), but include a potential for self-selection and the lack of a clear unexposed population, although this study of 145 participants reflects a broad continuum of exposure and includes participants from regions of the Navajo Nation with no mining history. Because the Navajo population has unique genetic and cultural characteristics, comparisons with populations much farther from the Navajo Nation region (and AUMs) would be challenging if not impossible to properly match.

Finally, endothelium subjected to chronic exposures may not respond in kind with naïve endothelial cells in culture, however, this does not detract from the observation that detectable differences exist in endothelial responses to serum from unexposed versus exposed subjects. Serum represents not only the balance of inflammation but also adaptive responses (Williams and Iatropoulos 2002) by the endothelium to chronic exposures over time that often co-occurs with chronic disease progression. Endothelial activation serves as an important end point that shows potential for assessing CV health beyond traditional biomarkers and risk factors. More studies are needed to assess endothelial responses to serum from larger, chronically exposed populations with and without disease at different points in time. Additionally, future studies should also examine changes in endothelial expression and function that could occur with more chronic serum treatment times.

Despite some limitations, we have applied a novel and unbiased approach to assess endothelial activation by cumulative circulating inflammatory components using serum from community members overburdened by chronic CV health conditions with legacy mining exposures. This study supports the emerging idea that changes in serum composition caused by chronic toxic exposures, and transmitted by circulatory signals to the endothelium, can promote vascular inflammatory responses, although further study is needed to confirm any causal link between the exposures and health outcomes.

SUPPLEMENTAL INFORMATION

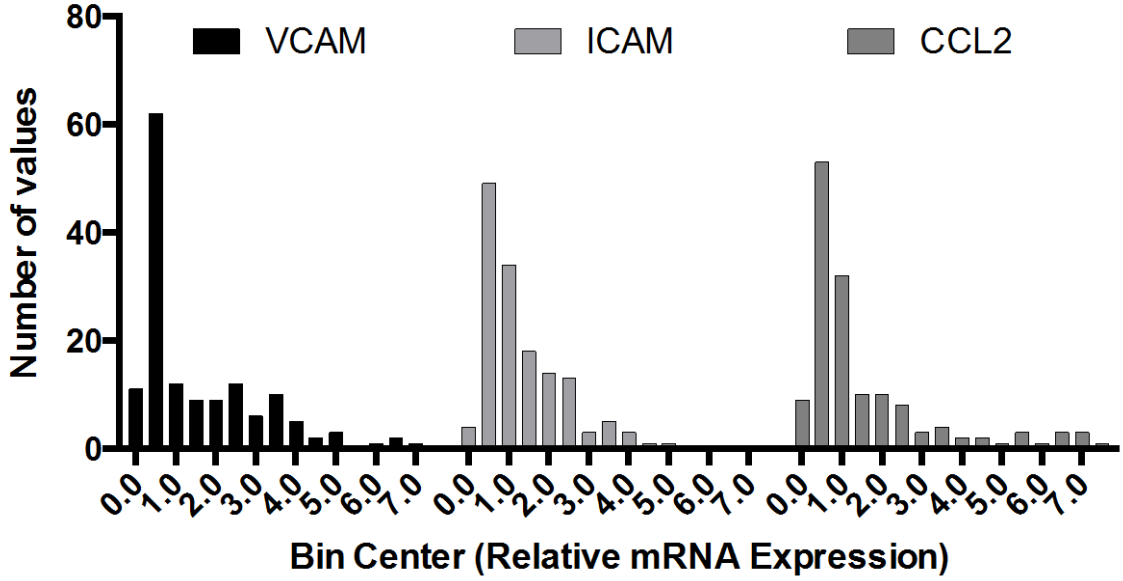
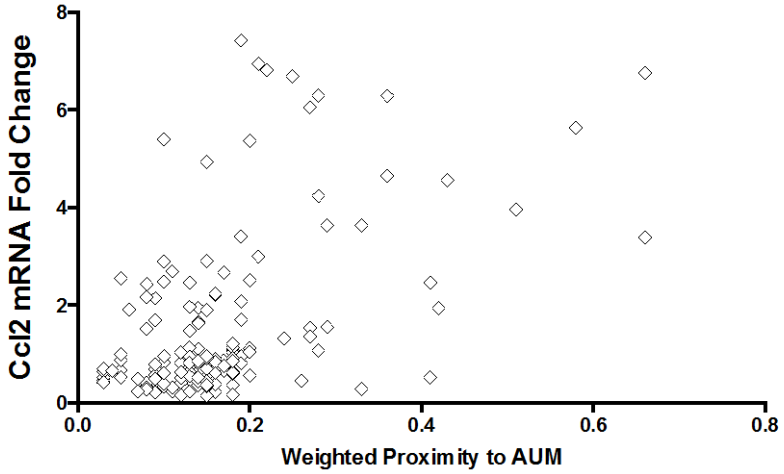
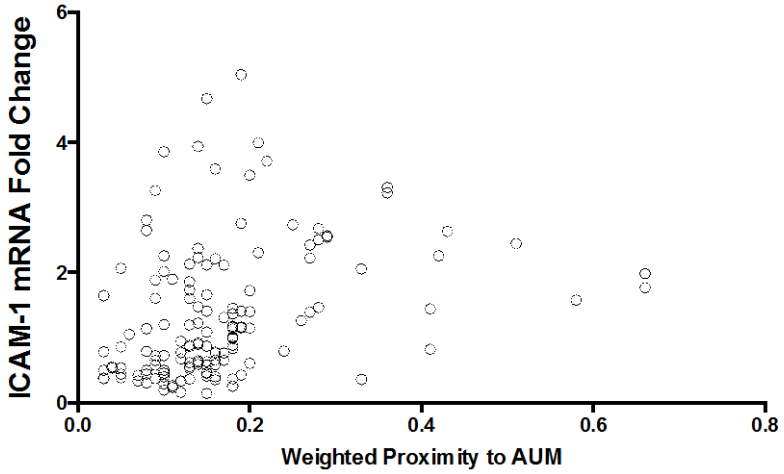


Figure 4.1S Distribution of relative endothelial mRNA expression responses to serum from Navajo participants





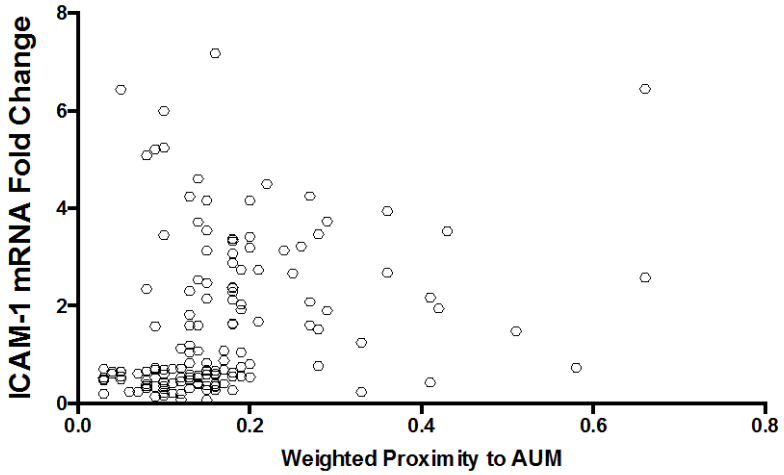


Figure 4.2S Plots of AUM proximity with fold change expression and endothelial responses to serum treatment

V. Chapter 5

Conclusions and Future Directions

I. *Demographic Associations with Oxidized LDL*

Conclusion

In the first study, our primary objective was to evaluate circulating levels of the novel CVD biomarker, oxLDL, from a cross-sectional population of the Navajo Nation (DiNEH Project), as oxLDL had never been assessed in a Native population. We also compared levels of oxLDL with other populations from studies that used the same antibody to measure oxLDL. We examined the relationship of oxLDL, and ratios of oxLDL to both HDL and LDL, to more conventional CVD risk factors and biomarkers. We were also able to provide updated information on the prevalence of CVD-related health conditions in this population.

Mean oxLDL levels in the DiNEH subset were similar to levels of oxLDL in subjects from previous studies with no detectable CAD. The DiNEH subset also appeared to have moderate dyslipidemia, evidenced by high TG and low HDL; however, LDL levels were near normal. OxLDL and oxLDL-ratios were correlated with CRP, but only the ratios were significantly correlated with HbA1c. Multivariate analysis showed that CRP and HbA1c were not predictive of oxLDL itself, but CRP was predictive of oxLDL/LDL and HbA1c was predictive of oxLDL/HDL. The relationship of oxLDL to other CVD-risk factors in this high-risk

group is unclear, therefore oxLDL may be a unique, independent biomarker, thus, relationships of CVD outcomes with oxLDL would be merited.

Obesity, hypertension, pre-diabetes, and diabetes (as measured by self-report survey data and BMI, blood pressure, and HbA1c, respectively), were prevalent in the Navajo population, consistent with earlier findings and likely more prevalent than previous studies have shown (Mendlein et al. 1997; Will et al. 1997). This population potentially also has a high prevalence of metabolic syndrome, based on criteria for this condition (Grundy et al. 2004; Schumacher et al. 2008), although this has not been directly measured. Navajo who had a diagnosis of diabetes at the time of survey appeared to have high (pre-diabetic and diabetic) HbA1c levels from subsequent blood draws up to 5 years later, suggesting that their diabetes is not being managed well (only 25% reported using insulin or other medication), further increasing the risk of CVD. It seems likely that the levels of oxLDL in this population will rise as the severity of CVD- and diabetes-related risk factors increase, especially without proper interventions. It is also known that populations with CVD-related conditions are more susceptible to environmental toxins.

Future Directions

The biggest gap in our study is that we have not done a long-term health study from which we can link oxLDL to CVD morbidity and mortality. This initial evaluation of oxLDL and the relationship to CVD risk factors applied relatively

facile statistical analyses due in part to the cross-sectional nature of the study (i.e. no longitudinal data or comparative population). It would be interesting to look more deeply at the data from this subset. For example, percentiles of various biomarkers (HbA1c or BMI) could be compared (t-tests, ANOVA) between genders and across age groups to discern if certain segments of the population are more at risk and potentially make comparisons with other native populations (e.g. Strong Heart Study, Navajo Health and Nutrition Survey). It also might be worth comparing some data from studies that have previously investigated health measures from Native populations that have accessible databases (Pima Indians, Strong Heart Study).

Additionally, there are other unexplored socioeconomic and lifestyle risk factors that may contribute to the cardiometabolic disease seen in this population that could be evaluated for potential interactions. Socioeconomic status, smoking, and dietary practices (for which some data is available for this subset) are commonly included in regression analysis as potential confounders and could have significant impact on health status. The majority of Navajo incomes are reportedly below the poverty line, opportunities for employment are minimal, and there is lack of access to health care and education, although this has improved in recent years (Diné Policy Institute 2014). Smoking tends to be uncommon except for ceremonial use, but alcohol use is prevalent despite being illegal on the Navajo Nation (Kunitz 2006), and has often been overlooked in previous Native population studies (Lu et al. 2003). Finally, the Navajo Nation is

considered a rural “food desert” due to lack of access by the population to adequate nutrition, which can drive disease and influence susceptibility to toxicants (Diné Policy Institute 2014). Understanding all of these risk factors and how they relate to each other will help in better assessing CVD risk in this population.

Many native populations, including Navajo, have historically had lower cholesterol levels as well as low a incidence/prevalence of CAD, myocardial infarction and stroke (Howard 1998). Diabetics also have lower serum antioxidant capacity and dysfunctional HDL (Gowri et al. 1999). Dyslipidemia with normal LDL is common in metabolic syndrome (Howard et al. 2000), and LDL derived from diabetics is more susceptible to oxidation (Vergès 2009). One explanation for the normal level of LDL but a high prevalence of CVD-related conditions and risk factors could be that the composition of the LDL particle itself is more pro-atherogenic, e.g. “small dense LDL”, which can pass more easily into the intima and be more easily oxidized (Vergès 2009). Thus, although LDL may be at a normal level, it may also be more susceptible to oxidation further increasing CVD risk in this population. It would therefore be of interest to determine LDL particle size in this population on a portion of the remaining serum (polyacrylamide gradient gel electrophoresis). Smaller oxLDL particles would theoretically associate more closely with ongoing inflammatory disease and/or increased risk of adverse CVD outcomes. It has been suggested that lipid management in metabolic disorders may need more aggressive treatment. In order to address

questions like these, the need exists for a larger longitudinal CVD outcomes study of the Navajo population which has not been included Framingham or similar studies thus far (D'Agostino et al. 2001).

II. *Relationship Between AUM Exposure Metrics and oxLDL*

Conclusion

Having studied the general demographic predictors of oxLDL in the Navajo participants, our next objectives were two-fold. First, we assessed if As and U exposure from drinking water (as binary variables described in Chapter 3, p. 69) would be associated with circulating inflammatory markers including oxLDL and CRP using linear regression modeling in a subset of the DiNEH study population. Second, we determined whether As (NaAsO2) and U (UA) could increase the oxidation of purified human LDL directly, including both the protein (ApoB 100) protein and lipid (represented by MDA) components of LDL.

Median estimated mean annual water intakes for As and U were similar, 0.49 mg/year (IQR 0-1.09) and 0.46 mg/year (IQR 0-1.13), respectively, although these have not been calculated per body weight in kg thus far which would not account for other routes of exposure. On the other hand, nearly 15% of subjects had urine U concentrations that exceeded NHANES 95th percentile. It is unknown if urine Cu, V, and Ni were similar to other populations, as Cu levels in urine are not available in NHANES and urine levels of V and Ni are mainly available for occupationally exposed populations. Kidney disease could affect excretion of As (urine levels; Zheng et al. 2015). In this subset, associations between biomarkers or renal function/pathology and urine metals have not been

evaluated. Also, we were unable to obtain different species of metals, particularly As, from urine samples.

The models differed substantially between oxLDL and CRP. All four reduced multivariate linear regression models for oxLDL (dependent variable) indicated that As intake (Water As_{bi}) was a significant independent predictor of oxLDL. It was important to include age and gender interactions and urine metals despite the reduced subject number due to fewer available Cu biomonitoring data). The only other urine metal influencing the model was Cu in an interaction with gender. Men and women may behave differently leading to different types of exposures and/or differences in ability to detoxify ingested metals. BMI, HbA1c, and urine Ni and V did not appear as predictors in any model.

For reduced multivariate linear regression models for CRP, it was again important to include age and gender interactions and urine metals as these models better fit the data and had higher adjusted R^2. The only metal to appear as a predictor in any model of CRP was urine U in an interaction with gender, suggesting that women with higher urine U_{bi} have higher levels of CRP. Urine U_{bi} alone was a negative predictor of CRP. BMI appeared as a predictor in each model, consistent with previous literature (Weyer et al. 2002), and suggests that subjects with higher BMI have an increased inflammatory status. Age was a negative predictor in all but the fourth model. HbA1c only appeared in the fourth model and was positively associated with CRP.

Diabetes is associated with CVD as well as As exposure (Kim et al. 2013), and the association of As with CVD mortality has been shown to be greater in diabetic participants (Solenkova 2014). We also assessed HbA1c as a response variable (data not shown) as well as a covariate of oxLDL, but we did not find any associations with exposure, or as an interaction with exposure, respectively. However, the majority of the study population was pre-diabetic or diabetic, with only a small number of participants having healthy HbA1c levels. It is unknown at present if other comorbidities could influence our results. Also, there is no time course or explicit control group, and epidemiological approaches are limited in that they do not allow for causation.

$NaAsO_2$ increased LDL oxidation as measured by ELISA, but this did not appear to be dose- or time-dependent in this experimental system. UA oxidized LDL only at latest time point (3 hours) but was inconsistent in that only the lowest and highest concentrations showed this effect. However, interestingly, when both metals were added, LDL was increased at all timepoints, though not to the same level as LDL treated with $NaAsO_2$ alone. As is known to bind thiol and sulfhydryl groups, and the ApoB protein contains 25 cysteine residues (Yang et al. 1994), but it is unknown if As can indeed bind these residues in the LDL molecule. Similarly, U is known to bind several plasma proteins in vitro, including ceruloplasmin, complement proteins, and even apolipoprotein A-I (a component of HDL; Vidaud et al., 2005), but it is unknown how U is interacting with LDL.

Additionally, we made assumptions that oxidation of LDL by As and U could occur in the circulation, thereby contributing to atherosclerosis development. It is unknown if circulating LDL could be directly oxidized by As in vivo. Additionally, although minimally modified-LDL and ox-LDL are detectable in the circulation, there is some controversy as to the pathologically-relevant location of LDL oxidation. It is generally thought that LDL first moves into the vascular intima and then is modified by ROS and reactive nitrogen species produced by activated macrophages (Itabe et al., 2011). Metal-ions (e.g. iron or Cu) can sometimes catalyze the oxidation process (Yoshida and Kisugi, 2010). Thus, assuming LDL can be modified by toxic metals/As *in vivo*, it is unknown if LDL oxidation could occur in the circulation or within developing plaques. Some evidence suggests that metals selectively accumulate in chronic vascular lesions (Hanć et al. 2011; Simeonova et al., 2003; Stadler et al. 2004), but it is unknown if As or U present in developing plaques can potentiate LDL oxidation.

In summary, our results show that water intake of As is associated with oxLDL but not CRP in a native population. Data from acellular experiments are in line with our regression modeling in that As, but not U, could oxidatively modify LDL cholesterol.

Future Directions

Population Studies

The complexity of human disease, i.e. “multi-morbidity” can make prediction of outcomes difficult and there are numerous potential confounding variables that were not included in the analysis. For example, the Navajo population is also known to have an increased risk of kidney disease associated with mining proximity (Hund et al. 2015), which may also intersect with cardiovascular disease prevalence and certainly influence circulating biomarkers. It would be helpful to assess kidney biomarkers (creatinine, BUN) and include them in our analyses. Along these lines, it would also be informative to assess liver enzymes in relation to biomarkers and exposure. As exposure in mice promotes inflammatory angiogenesis and vessel remodeling in the liver (Straub et al. 2007a, 2007b). As effects on liver vasculature may impair the liver’s ability to clear modified lipids and modified proteins (Mazumder et al. 2005). It is important to consider CVD co- and multi-morbidities, as populations with CVD-related conditions may be more susceptible to environmental toxins. Our study design did not allow for risk/odds ratios of clinical disease due in part to the limited size of the cohort and lack of long-term follow-up, but prevalence ratios could potentially be derived.

Other confounders, such as socioeconomic status, diet, smoking and alcohol use (already addressed in previous section) could also influence our exposure and outcomes analysis and we might consider using these potential covariates as well. However, covariates must be limited to what is relevant to the character of the population as well as to the number of observations. Furthermore, it is

doubtful that the range of income and access to health care in this population would have been sufficiently large to observe a strong influence on CVD biomarkers.

Alternative means of segregating the study population might also strengthen the conclusions. It would also be interesting to divide metals exposures, either from drinking water exposures or urine, into tertiles or quartiles to evaluate potential non-linear relationships with other response variables (oxLDL, oxLDL ratios, CRP). We were unable to directly compare co-exposure (e.g. low water As, low water U; high water As, high water U, etc) groups however due to low power and a lack of precise data in the low concentration exposure groups.

Finally, it would be informative to recall the DiNEH subset to re-interview and to obtain blood to follow-up on any long-term changes related to exposure (although the ongoing Navajo Birth Cohort Study may be addressing some of this). It would be a particular interest to assess changes in lipid profiles including oxLDL and perhaps employ an imaging technique (ultrasound to assess carotid intima-media thickness) to capture lesion development, both to understand the overall trend of CAD in this population as well as to assess in terms of exposure. It would also be important to know if exposures via drinking water are stable over time. Longitudinal study designs strengthen conclusions due to repeated measures analytical approaches and improved power, in addition to being able to observe temporal trends.

Mechanisms of Metal-Induced LDL Oxidation

$NaAsO_2$ increased oxidation of LDL in our experiments and there appeared to be additive effects when both $NaAsO_2$ and UA were present. Although $NaAsO_2$ could potentially reveal modifiable sites by UA, it seems more likely that the opposite is true, given that As is known to bind thiol and sulfhydryl groups, and the ApoB protein contains 25 cysteine residues (Yang et al.1994). U could be interacting with the apoB protein at sites other than what the antibody can detect or in a non-oxidative manner, potentially modifying the protein such that more/fewer oxidation sites are revealed that can be modified by As. A similar effect has been shown *in vitro* with aluminum and iron (Kapiotis et al. 2005). Some of the increased oxidation could be due to the presence of atmospheric oxygen remaining in the buffer, potentially leading to ROS formation, although we mitigated this effect by placing the samples under nitrogen flow and sealing samples before the incubation period.

Several alternative methods might be used to examine LDL oxidation by As (and U). Conjugated diene formation is a common method used to measure Cu-induced oxLDL. We also might want to determine if other oxidizing LDL protein modifications are occurring besides what the ELISA antibody can capture by using a protein-binding assay, such as one that can measure the number of thiol groups on proteins [5-5'-dithiobis-(2-nitrobenzoic acid)-thiol] (Sedlak and Lindsay 1968). We could also attempt to inhibit or block modifications with a thiol

compound, anti-oxLDL antibody, or an antioxidant (N-acetylcysteine) to narrow down how LDL is being modified and to rule out interactions with atmospheric oxygen in the buffers. We could also try methylated forms As (MMA, DMA) as these generally have more toxic effects (Aposhian et al. 2003). As an alternative to measuring MDA, we could use HPLC to evaluate protein adducts of 4-hydroxynonenal-amino acid (as opposed to free 4-hydroxynonenal); adducts may be more stable and offer a "footprint" of past oxidative stress. (Wakita et al. 2011)

Alternatively, the process of LDL oxidation may be an indirect effect of metals and, while our assays highlight the possibility that As may directly induce oxLDL, this is not proven *in vivo*. It has been shown that As can inhibit lipid metabolism by inhibiting the retinoid X receptor (Padovani et al. 2010), a nuclear receptor that heterodimerizes with the LXR. LXR is involved in the regulation of cholesterol transport and efflux and repression of inflammatory genes. LDL under normal conditions upregulates the ATP-binding cassette transporter (Liao et al 2002), which interacts with HDL during cholesterol efflux (Weber and Noels 2011). On the other hand, oxLDL has been shown to downregulate ABCA1 by inhibiting LXR (Zhu 2005). Recently, ABCA1 was found to promote As tolerance in human cells by reducing cellular As accumulation in ECV304 cells, which exhibit both endothelial and epithelial characteristics (Tan et al. 2014). Other studies have demonstrated that As or As-species can be readily taken up by endothelial cells (Hirano 2003, 2004). Taken together, these studies suggest

that As and oxLDL each independently lead to inhibition of reverse cholesterol transport and the accumulation of cholesterol in the vascular wall.

Our results suggest that LDL oxidation could occur directly by toxic metals in circulation, but this could also reflect what could be occurring within lesions, which was not addressed in our studies. It would be interesting to test if both As and lipids could accumulate and possibly co-localize in the vascular wall, which could lead to excessive cholesterol accumulation and direct oxidation of lipids by As. This might be one mechanism by which As contributes to chronic CVD.

Accumulation of metals in plaque lesions may enhance oxLDL formation *in situ*, and the circulation may be a reflection of this. Lind et al. (2012) found that circulating levels of Ni, aluminum and chromium were associated with atherosclerotic plaque sizes or intima-media thickness independently of cardiovascular risk factors, including lipids in an elderly population. Accumulation of As was found in cardiovascular tissue by atomic absorption analysis in ApoE$^{-/-}$ mice that received 20 or 100 µg/mL sodium As for 24 weeks, and this tissue accumulation was dose- and time-dependent (Simeonova et al. 2003). Iron has also been identified in *ex vivo* human arteries and carotid lesions versus healthy controls and also correlated with cholesterol levels (Stadler et al. 2004). We might approach this question by assessing oxLDL formation in blood and aorta from ApoE$^{-/-}$ mice (or other atherogenic models) exposed to low-to-moderate concentrations of U and/or As in drinking water. OxLDL levels within

atheromatous lesions would be measured using anti-oxLDL antibodies and levels of As and U in these lesions would be assessed by ICP-MS or Raman spectroscopy (Hanć et al. 2011), and then we would examine any correlations between these measures. Selective accumulation of metals in human vascular lesions would be difficult to assess, although postmortem studies or analysis of coronary arterial resections from patients undergoing coronary artery bypass grafts might allow for translational evidence of metal impacts.

III. *Cumulative Circulatory Inflammatory Potential and Mine Site Exposures*

Conclusion

Our objective was to examine whether indices of mine waste exposure could induce circulating inflammatory potential. To accomplish this, we assessed mRNA expression of inflammatory adhesion molecules and a chemokine in endothelial cells treated with serum from the DiNEH subset. We then used linear regression to assess if the mRNA transcriptional changes were correlated with exposure (metals intake, AUM). We found that AUM proximity was a strong, independent predictor of endothelial transcriptional responses to the serum, in terms of mRNA expression of CCL2, ICAM-1, and VCAM-1. All other covariates including water intake variables and CVD-risk factors (age, gender, BMI, and HbA1c) were not significant predictors of endothelial responses in the model.

Future Directions

Our results indicate that other routes of exposure should be considered besides that of water intake and likely could be airborne transport of contaminated dusts or uptake of metals into trees, which then might become a wintertime indoor exposure risk as firewood. In addition, these findings may reflect that it is an overall exposure to a mixture of metals may be responsible for the circulating inflammatory potential. Several approaches could be used to address these potential causes, for instance, mice could be exposed (via inhalation of instillation methods) to dust samples collected at various AUM proximities. Then the same inflammatory potential experiments could then be performed using serum from the mice (as well as several CVD and pulmonary endpoints). Furthermore, these techniques could be applied to a chronic time course to assess not only inflammatory markers, but also progression of cardiovascular pathology in specific mouse models. However, mice might not respond the same way as humans, therefore further epidemiological studies would be desirable that could somehow stratify different levels of exposure or obtain more serum to conduct a larger-scale, longitudinal study that included follow-up and access to medical records.

Serum represents the balance of pro- and anti-inflammatory circulating factors. In inflammatory CVD disease states and exposure scenarios, the serum is more atherogenic, and anti-inflammatory and athero-protective components would be reduced (Gowri et al 1999; Vergès 2009;). Most responses by the endothelium in this assay are assumed to be receptor-mediated leading to transcriptional

responses. Therefore, we have measured transcriptional levels of several pro-inflammatory molecules (ICAM-1, VCAM-1, CCL2, and interleukin-8) in this and other studies (Channell et al. 2012; Cung et al. 2015). In a slightly different study design, healthy subjects receiving grape seed extract (principally the polyphenol, resveratrol) for a month exhibited reduced inflammatory potential in their serum, compared to subjects taking placebo (Agarwal, Campen et al., 2013). This study suggests that the inflammatory potential of the serum is a sensitive metric of the net balance of pro- and anti-inflammatory factors, as healthy subjects revealed benefits of a nutraceutical (inflammatory cytokines were measured, but remained unchanged). Furthermore, this study asserts that the serum inflammatory potential is, in fact, modifiable.

In a similar vein, the effects of a more “ideal” serum inflammatory status could be obtained from trained humans (or rodents) involved in exercise studies, since exercise is known to reduce inflammation both acutely and over time (Gleeson et al. 2011; Neefkes-Zonneveld et al. 2015). In this case, we could perform the same experiments but expect reduced transcription of inflammatory mRNA and vice versa. Conducting intervention studies of exercise with the Navajo population affected by the AUM exposures may not directly explain the mechanism underlying the exposure-related insult to the cardiovascular system, but it would offer suggestions as to how lifestyle factors may help overcome environmental health risks.

The overall approach of using endothelial cells as a biomarker of circulating inflammatory state offers further opportunities to better understand the mechanisms and potential health outcomes. Some studies have suggested that NFκB, a major transcription factor that regulates inflammatory processes, can be induced in arsenite-exposed macrophages and endothelial cells through oxidative stress (Barchowsky et al. 1996; Hossain et al. 2013). Serum from humans exposed to diesel emissions was able to induce a profile of endothelial transcriptional changes consistent with NFκB activation (Schisler et al., 2015). To determine if NFκB is indeed mediating the responses seen in the present study, endothelial cells could be treated with exposed and unexposed serum (from humans or mice with appropriate cell type), and then NFκB activation (activated p50, p65 transcription factors, luciferase-linked promoter assay) could be measured. Alternatively, an NFκB inhibitor could be added to the serum-treatment (serum with and without the inhibitor, or exposed serum with and without the inhibitor), and then measure if transcriptional responses are inhibited. In a similar vein, numerous possibilities exist to mechanistically probe the role of other transcriptional or cell signaling pathways activated by serum components.

Changes in expression that occur following chemical exposure may also be adaptive responses (Dangleben 2013), and naïve endothelial cells may not respond in the same way as endothelial cells from chronically exposed humans. Therefore, we should also attempt to design studies that assess more chronic exposures to serum treatment. One way to do this might be to repeatedly

serum-treat endothelial cells over several days and then assess responses. It is also possible to collect and isolate endothelial cells from freshly obtained human blood for culture work, although this would be highly challenging in community studies. Indirect measures of endothelial function, such as brachial artery flow, vascular stiffness, or hyperemic responses might be more feasible in the field.

A direct link between exposure to As and other metals and endothelial dysfunction and inflammation in humans has not been thoroughly investigated (Stea et al. 2014). Our study has attempted to fill this gap. AUM proximity was the only predictor of inflammatory endothelial cell transcriptional responses to serum from exposed Navajo participants. These results have potentially opened a new avenue of exploring airborne sources of toxicity in this community. This novel inflammatory potential assay promises to be very useful and has many potential applications experimentally, and maybe eventually clinically. Chronic exposures to mining wastes may potentiate increasing inflammatory-related health conditions in this population.

SUMMARY AND CONCLUSIONS

In summary, the results from these studies suggest that there are potential CV effects from AUM-related exposures in a subset of the DiNEH survey participants, a highly underserved population with numerous CVD risk factors. We used several exposure metrics in our regression modeling including estimated annual water intakes, AUM proximity and urine metals, which

represent different exposure sources and pathways, and each had differing effects depending on the variable that was measured. Thus, certain exposure metrics may be more important at varying points along the continuum of atherogenesis. This work is also important because we included individual measures of exposure which is often lacking in many population toxicity studies.

Results from the inflammatory potential assay also suggest that there is a broader effect of mining waste on circulating inflammatory status than just drinking water exposure alone on a single circulating CV biomarker (oxLDL). Underlying health conditions could increase susceptibility to these exposures, and inflammation plays an important role in the pathogenesis of many health conditions that could be affected by these exposures. Thus, other routes of exposure, such as airborne transport and food consumption, as well as other metals and metal mixtures should be considered in future studies that include long-term CV outcomes.

As U and other metals continue to be mined throughout the world, with more mining operations in production due to an ever increasing demand (Organization for Economic Co-operation and Development 2014; Rogich and Matos 2008), many populations will continue to be exposed to mining waste from both current and historical operations. It is imperative to continue investigating the health effects of these exposures because the knowledge gained could guide

environmentally and socially responsible mining practices and clinical treatment decisions in affected communities.

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