Arsenic association with circulating oxidized low-density lipoprotein in a Native American community

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Abstract

More than 500 abandoned uranium (U) mines within the Navajo Nation contribute U, arsenic (As) and other metals to groundwater, soil and potentially air through airborne transport. The adverse cardiovascular health effects attributed to cumulative exposure to these metals remains uncertain. The aim of this study was to examine whether environmental exposure to these metals may promote or exacerbate the oxidation of low-density lipoprotein (LDL) cholesterol in this Native American population. The correlation of cardiovascular biomarkers (oxidized LDL (oxLDL) and C-reactive protein (CRP)) from a Navajo cohort ($n = 252$) with mean annual As and U intakes from water and urine metals was estimated using linear regression. Proof-of-concept assays were performed to investigate whether As and U directly oxidize human LDL. Mean annual As intake from water was positively and significantly associated with oxLDL, but not CRP in this study population, while U intake estimates were negatively associated with oxLDL. In an acellular system, As, but not U, directly oxidized the apolipoprotein B-100 component of purified human LDL. Neither metal promoted lipid peroxidation of the LDL particle. Both the population and lab results are consistent with the hypothesis that As promotes oxidation of LDL, a crucial step in vascular inflammation and chronic vascular disease. Conversely, for outcomes related to U, negative associations were observed between U intake and oxLDL, and U only minimally altered human LDL in direct exposure experiments. Only urine U was correlated with CRP, whereas no other metals in water or urine were apparently reliable predictors of this inflammatory marker.

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Introduction

Navajo Nation community members in the south-western United States (US), specifically in New Mexico, Utah, and Arizona, display significant risks for environmental exposure and potential adverse health effects from metals, including arsenic (As) and uranium (U), both from natural deposits and legacy of U mining (deLemos et al. 2009; Hund et al. 2015; Lewis et al 2017) which was extensive from the 1940s through the 1980s (Brugge and Goble 2002; US Environmental Protection Agency n.d.). Chronic exposure to several metals is associated with cardiovascular disease (CVD) (Lind, Olsén, and Lind 2012; Prozialeck et al. 2008; Shim et al. 2017; 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). Recently Harmon et al. (2017) found that proximity to abandoned mine sites may contribute to circulating inflammatory potential, defined as an inflammatory transcriptional response in cultured endothelial cells treated with serum from subjects in this community. While diet and lifestyle are important contributors to increasing rates of CVD, the contribution from exposure to metals in mining waste is unknown.

As often coexists with U deposits and is present in legacy U mining wastes (Blake et al. 2015; Katsoyiannis et al. 2007), contributing to contamination of soil, air, and water. Approximately 20% of unregulated water sources throughout the Navajo Nation exceed current US Environmental Protection Agency (EPA) maximal contaminant levels for U (US Army Corps of Engineers 2000). Recently Hoover et al. (2017) noted that little improvement has occurred, with estimates of 15% exceedances for As and 13% exceedances for U in tested water sources. Navajo Nation EPA estimated that more than 30% of the Navajo population lack access to public water supplies and concerns exist regarding As and U exposure through consumption of unregulated water (Navajo Access Workgroup 2010).

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; Tseng et al. 2003; Wang et al. 2002; Yang 2006). Accumulating epidemiological and mechanistic evidence suggests that the cardiovascular system is sensitive to environmentally relevant levels of As exposure (Howard et al. 1999; James et al. 2015; Lemaire et al. 2011; Moon et al. 2017, 2013; Wu, Molinaro, and Chen 2014). In contrast, little is known regarding cardiovascular-related effects of chronic environmental exposure to U. Limited evidence suggests a potential relationship between estimates of U exposure and circulatory systemic disease (Guseva Canu et al. 2012) and increased blood pressure (Kurttio et al. 2006). In addition, exposure to U mine waste on Navajo was linked to an enhanced likelihood of hypertension (Hund et al. 2015). Maximal containment levels for U were developed for outcomes related to cancer and kidney diseases, but not CVD, while maximal containment level for As takes cardiovascular outcomes into consideration (Hoover et al. 2017).

Oxidized low-density lipoprotein (oxLDL) is an emerging biomarker that is mechanistically associated with CVD (Gómez et al. 2014; Holvoet et al. 1998; Trpkovic et al. 2014) and predictive of cardiovascular outcomes including acute coronary artery disease (Meisinger et

al. 2005) and myocardial infarction (Johnston et al. 2006). CRP is a well-established biomarker of inflammation associated with increased CVD risk (Danesh et al. 2004). Karim et al. (2013) reported that As exposure in Bangladesh residents was associated with oxLDL and CRP levels, but Moon et al. (2017) found no marked association between urine As and plasma CRP. Several metals are known to contribute indirectly to oxidation of LDL and lipids (Chisolm and Steinberg 2000; Jomova and Valko 2011). However, it is unknown whether As and U directly oxidize LDL cholesterol.

Few studies examined the relationship between chronic low-level As or U exposure and CVD in at-risk communities such as those on the Navajo Nation where CVD and diabetes are significant public health concerns (Moon et al. 2017, 2013). The aim of this investigation was to assess a potential association between these metals and plasma levels of oxLDL and CRP in several Navajo communities located in the southwestern United States with varying levels of As and U exposures. It was postulated that environmental exposure to As and U may be correlated with elevated plasma oxLDL and CRP concentrations. In addition, proof-of-concept acellular assays were performed 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 Diné Network for Environmental Health (DiNEH) Project using an interviewer-administered survey. More detailed descriptions of the survey and population may be found elsewhere (deLemos et al. 2009; Harmon et al. 2016, 2017; Hund et al. 2015). Surveys were completed by 1,304 Navajo Nation adult members between 2005 and 2010. Data for the current study represent a subset of these participants ($n = 252$) who volunteered to provide non-fasting blood and urine samples between 2010 and 2011 at which time height and weight were measured to determine body mass index (BMI). These 252 participants were therefore not a randomly selected group. 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 178 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 representative water sources (101 unregulated, 23 regulated) from 2003–2010 following US EPA Water Quality Standards Handbook, 2007 (https://www.epa.gov/wqs-tech/water-quality-standards-handbook). In US EPA region, nine labs conducted water quality analyses for unregulated sources using US EPA analysis method 200.8 (Inductively Coupled Plasma Mass Spectroscopy, ICP-MS). A small subset of samples (approximately 8%) was analyzed at the Carlsbad Environmental Monitoring and Research Center or at Stanford University. Navajo

Nation EPA provided public water quality data. The compiled water data included 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).

Only unregulated sources with water quality information were included in the analyses and are representative of commonly used sources. A comprehensive description of As and U in unregulated water sources on the Navajo Nation may be found elsewhere (Hoover et al. 2017). Public, regulated water system sources, store purchased and bottled water without As or U measurements were presumed to be in compliance with Safe Drinking Water Act regulations for As ($< 10 \ \mu g/L$) and U ($< 30 \ \mu g/L$) (US Environmental Protection Agency 2015).

Annual As and U intakes from water were estimated from self-reported volume of water consumed and metal concentration for each water source used. Participants indicated how frequently they visited the source, the typical volume of hauled water and % used for cooking or drinking. This information was used to calculate the individual annual intake of As or U (mg/year) from drinking water using the associated As or U concentration for each separate water source.

Once the cumulative annual intake of water As and U from water was determined for each participant, a binary variable was employed to indicate low versus high metal consumption, based upon a threshold consumption of 1.4 mg/year in the distributions of both metals. This threshold represented a natural division in data distribution for both metals. In addition, subjects who utilized only regulated drinking water sources, were assigned to the low consumption groups for both As and U. Those considered to be in the high intake group were a small fraction of the 252 people in the cohort (10% for As and 15% for U).

Urine metals concentrations

Urine metals were determined by ICP-MS. Five-point standard curves were generated for each metal. Total As was measured without regard to specific species. Urinary concentrations of vanadium (V 51), nickel (Ni 60), As (AsO 91), and U (U 238) were available for 188 participants. Urinary copper (Cu) was available for a subset of participants ($n = 94$). Approximately 50% of urine U levels were below the level of detection (0.1 $\mu g/L$), therefore available U data were examined as a binary variable reflecting detect or non-detect as the threshold. The limit of detection (LOD) for urinary As was 0.2 $\mu g/L$.

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 (ApoB 100) 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 lab (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 Tinaquant assay (Roche Diagnostics, Indianapolis, IN).

Acellular assays of metal-induced LDL oxidation: materials

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

Determination of protein and lipid peroxidation of LDL

Direct ApoB 100 oxidation of the metal-treated LDL samples was determined by ELISA (as described earlier). Lipid peroxidation in the LDL-metal samples was determined using a thiobarbituric acid reactive substances (TBARS) assay kit, which normalized total lipid peroxides to a malondialdehyde (MDA) 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 of $CuSO_4$, $NaAsO_2$, and UA with purified human LDL. $CuSO_4$ was used as a positive control since it is frequently utilized 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 oxLDL ELISA; 15, 25, 45 and 75 μM for the TBARS assay), NaAsO2 (0.02, 0.07, 0.2 and 0.7 μM for oxLDL ELISA; and 0.2, 0.7, 2 and 7 μM for TBARS assay), and 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 4-(2-hydro-xyethyl)-1-piperazineethanesulfonic acid (HEPES) to obtain desired concentrations. For the experiments that tested the combined effect of NaAsO2 and UA, 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 min to reduce the contribution of atmospheric oxygen to LDL oxidation. Samples were then sealed and incubated at 37°C for 1–5 h. 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 or background levels of each metal in plasma (Agency for Toxic Substances and Disease Registry (ATSDR) 2007; Byrne and Benedik 1991; Ivanenko et al. 2013; McMillin, Travis, and Hunt 2009).

Statistical analyses

Summary statistics are reported as median and inter-quartile range (IQR) for continuous variables unless otherwise indicated. Logarithmic transformations were performed to normalize non-Gaussian distributions. Four linear regression models were constructed for each oxLDL and *log* CRP (Tables 2 and 3), all of which were derived from the full set of data and reduced by model selection using the Akaike Information Criterion (AIC) with stepwise selection allowed in both directions; models were successively revised so as to reduce AIC. All models include demographic (age, gender) and physiologic variables (BMI,

HbA1c), and estimated annual water intakes of U and As as predictors. Models 1 and 2 include data from 249 participants. Models 3 and 4 additionally include continuous concentrations of urinary copper (Cu), vanadium (V), and nickel (Ni), As (log transformed) and U (binary as described before) as predictors for a subset of 94 participants with available data for urinary Cu. Second order interactions are included Models 2 and 4, and significant interactions reported. The majority (> 80%) of HbA1c levels in the participants were in prediabetic or diabetic categories, therefore, a binary variable with HbA1c >6.4% as the threshold used in regression models. Estimated annual intakes of As and U from drinking water are also included as binary variables (Water As $_{bi}$, Water U $_{bi}$) as previously described. 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's multiple comparison test using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). The criterion for significance was set at $p < 0.05$.

Results

Community-level association between mining metal exposure and LDL oxidation

This Navajo subset is known to exhibit a high prevalence of diabetes, hypertension and overweight/obesity compared to the general US population (Harmon et al. 2016; Hund et al. 2015). Table 1 shows clinical characteristics and biomarker levels by gender. Both genders were similar in age and displayed similar total cholesterol (TC), LDL cholesterol and non-fasting glucose levels. Women generally exhibited higher BMI, high-density lipoprotein (HDL) levels, lower triglycerides (TG), and lower blood pressures than men. Both genders displayed similar median HbA1c levels, although more women fell into prediabetic and diabetic categories of HbA1c classification. Women showed lower levels of oxLDL and higher CRP levels.

The median 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. Water As intake was not correlated with urine As, and similarly water U intake did not correlate with urine U (data not shown).

Median urine As [4.21 (IQR 2.25–6.78) μg/L] was below National Health and Nutrition Survey (NHANES) 50th percentile for total As [7.9 (IQR 7.00–9.10) μg/L] (non-creatinine corrected, adults >20 years old) (Centers for Disease Control and Prevention (CDC) 2017). It is noteworthy that seafood intake may contribute substantially to heavy metal intake in the NHANES population, while the Navajo consume relatively little seafood (Jones et al. 2016; Navas-Acien et al. 2011). However, 14.6% of participants exhibited urine U concentrations that exceeded NHANES 95th percentile (survey years 2003–2004; 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.

Results of the linear regression models for oxLDL are shown in Table 2. In Models 1 and 2, estimated annual intake of water As (water As$_{bi}$) was significantly associated with oxLDL. In Model 2, water U$_{bi}$ was significantly, inversely associated with oxLDL. Including only

individuals with available urine Cu ($n = 94$) in Models 3 and 4, model fit improved to R^2 =0.11 (Model 3) and R^2 =0.203 (Model 4). In Model 3, which included urinary metals concentrations, water intake estimates for As and U remained significant positive and negative predictors, respectively for oxLDL, and excreted U (urine U) was a positive predictor. Similar results were observed in Model 4, which included an interaction between female gender and urine Cu. BMI, HbA1c, and urine Ni and V did not appear as significant predictors in any model.

Results of the linear regression models for *log* CRP are presented in Table 3. In Models 1 and 3, age was a negative predictor and BMI was a positive predictor for *log* CRP. However, in Model 2 the interaction of gender and BMI was significantly, negatively associated with *log* CRP. Model fit improved in the subset analyses (Models 3 and 4), when urine metals data was included. In Model 4, BMI was again positively associated with for *log* CRP. However, significant interactions were only identified between gender and urine U_{bi} and between age and HbA1c suggesting that women with higher urine U_{bi} possessed higher levels of CRP. Water As_{bi} and U_{bi} and urine As, Cu, Ni, or V did not emerge as significant predictors in any of the models for CRP.

Acellular studies of LDL oxidation

Protein oxidation of LDL cholesterol by As and u

CuSO$_4$ is a known oxidizer of LDL and was used as a positive control. CuSO$_4$-treated LDL demonstrated a time and dose dependent response as expected, for CuSO4 concentrations greater than 50 μM as compared to untreated LDL (Figure 1A) as measured by ELISA for the ApoB 100 protein component of LDL. NaAsO$_2$ appeared to directly oxidize LDL earlier at lower quantities and later at higher concentrations, but the level of oxLDL was similar at each time point (Figure 1B). UA only appeared to oxidize LDL at 3 hr, but inconsistently at only the lowest (0.3 nM) and highest (300 nM) concentrations (Figure 1C). Interestingly, in combined treatments, oxLDL levels were elevated at all time points when both NaAsO$_2$ and UA were present in the reaction mixture (Figure 1D). Only the lowest combined 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 NaAsO2 (0.07 μM) plus UA (0.3 nM) was significantly lower than LDL treated with NaAsO$_2$ alone at 1 and 2 h but was similar at 3 h. LDL treated with 3 nM of UA alone was not significantly altered (Figure 1C), but when the same concentration was combined with NaAsO$_2$, LDL oxidation was elevated at all time points. Data suggest that NaAsO$_2$ directly oxidizes LDL, but when combined with UA, the interaction was unclear as measured by ELISA in this experimental system.

Lipid peroxidation of LDL cholesterol by as and u

CuSO$_4$-induced lipid peroxidation (MDA equivalents) of human LDL as expected with clear concentration- and time-dependent trends observed (Figure 2A). The level of lipid peroxides was not markedly affected by NaAsO$_2$, UA, or combination of both metals (Figure 2B–D) especially in comparison to CuSO$_4$-administered LDL, used repeatedly as a positive control

(Figure 2A). Data suggest that $NaAsO_2$ or UA failed to promote lipid peroxidation 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 may contribute directly to oxidation of the protein (Apo B 100) component and not the lipid components of the LDL particle. U does not appear to be associated with elevation in oxLDL levels.

Discussion

This study examined whether CVD biomarkers, oxLDL and CRP, were associated with annual estimated drinking water intake of As and U, as representative mining-related metal contaminants in this affected community. To establish proof-of-concept whether these metals might directly oxidize LDL was also determined. Both the population and lab results are consistent with the hypothesis that As promotes oxidation of LDL, a crucial step in vascular inflammation and chronic vascular diseases. Outcomes related to U demonstrated negative associations between U intake and oxLDL and U only minimally altered human LDL in direct exposure experiments. Thus cholesterol may not be considered a physiological or chemical target for U. 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 as an interaction term with gender was correlated with CRP, whereas no other metals in water or urine were reliable predictors of this inflammatory marker.

In our studies, there was a limited correlation between our modeled estimates of metals uptake and measured urine levels in our cohort. Evidence indicates that this as an important contrast between a metric of chronic exposure (annual intake) and a more acute reflection of exposure (urine output). It is important to consider that our water intake calculations were based upon a survey tool wherein participants provided estimates of where they obtained water and for what purpose. Thus, while previously Calderon et al. (1999) studies found an immediate correlation between drinking water and urine levels of As, our estimate incorporates a longer history of water usage, albeit with likely increased error due to recall inaccuracies. Consistently, data demonstrated that annual estimate of As intake was best associated with oxLDL. Numerous epidemiological studies reported that As contributes to CVD development (Rahman et al. 1999; Tseng et al. 2003; Wang et al. 2002; Yang 2006), but limited evidence is available for As effects on the intermediate biochemical changes associated with atherogenesis. Chronic As exposure from drinking water was noted to be associated with biomarkers of inflammation and endothelial cell activation including oxLDL, the ratio of oxLDL with HDL, intercellular adhesion molecule-1 (Karim et al. 2013), and vascular cell adhesion molecule-1 (F. Wu et al. 2012). In contrast to other investigations, an association between As exposure and elevated plasma CRP levels was not found, although concentrations of As in the Eastern Agency region of Navajo Nation were lower than those detected in severely contaminated regions of Bangladesh (Karim et al. 2013; Peters et al. 2015).

OxLDL contains heterogeneous lipid and protein components that vary in degree and type of oxidation which induce a variety of pro-atherogenic effects (Reis et al. 2015). Data suggest

that As, in direct contact with LDL particles, might modify the ApoB 100 component in a manner that makes it recognizable by the 4E6 antibody. While As was shown to promote chronic vascular remodeling, there is conflicting evidence whether As elevates lipid peroxide levels in ApoE$^{-/-}$ mice (Simeonova et al. 2003; Srivastava et al. 2009). In the present study, As exerted 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). However, the direct interaction of As with ApoB may be sufficient to drive minimally oxidized form of oxLDL and thereby play a role in systemic vascular disease. As the more chronic measure of annual As intake was found to be a more reliable predictor of oxLDL than the acute snapshot of urine As, the relevance of the ApoB100 target, rather than untargeted lipid peroxidation, becomes more important from a pathophysiologic standpoint.

It remains unknown how metals such as As and U may influence oxidative modifications of LDL. As is redox inactive but possesses 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 may potentially be modified by As. As is also known to bind hemoglobin on erythrocytes as well as other proteins (especially thiol-containing moieties) in the plasma, including thioredoxin and glutathione (Hughes et al. 2011; Schmidt, Fahlbusch, and Otto 2009), which may influence the bioavailability and pharmacokinetics of As as well as affect the behaviors of the bound proteins. Little is known regarding the effects of chronic low-level exposure to U, particularly in terms of CVD prevalence and pathogenesis. Limited studies on endothelial cell toxicity are generally negative (Dobson et al. 2006). Our results suggest that in an isolated acellular system, low concentrations of U do not directly modify either protein or lipid components of LDL. The concentrations of As and U that were employed reflect realistic levels that are detected in blood, albeit without addition of blood components that may reduce the overall bioavailability of the free metals. The interaction of U and NaAsO$_2$ on ApoB-100 oxidation was unclear, and the level of oxidation was not greater than NaAsO$_2$ treatment alone. Further, measures of U in drinking water were negatively associated with oxLDL and not at all with CRP levels in the Navajo population. This observed molecular antagonism in the acellular assays may help explain this association. An important consideration for mine wastes is the mixture of metal contaminants, not simply the residual ores of interest, which may 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 including As, V, Ni, and Cu may be omitted 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 reduced antioxidant capacity were noted in plasma from humans exposed to As in drinking water (Wu et al. 2001). Changes in redox status or enhanced free radical production (Lankin et al. 2014) and inflammatory factors may be induced in the presence of toxic metals in plasma or atherosclerotic lesions (Stadler, Lindner, and Davies 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 population-based study to demonstrate a correlation between As and LDL oxidation in an exposed population in the U.S.A. The relatively high rate of hypertension and obesity in this cohort, along with other major effect modifiers such as socioeconomic status, education, smoking status, and access to health care, all are known contributors to CVD and affect many biomarkers (Harmon et al. 2016). Selection bias is a possibility in this volunteer population; however, no apparent biases in exposure risks were 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). Urine values of metals were measured without correction for urine dilution, which might increase the variability of this measure depending upon the hydration levels of participants. Blood samples were non-fasting, and medication use was not included in this initial analysis, which might influence LDL modifications. Because of the long storage times, it was not possible to assess TBARS in the banked serum from the DiNEH study as a parallel to the acellular assays. Finally, the lack of complete data for urine metals values and overall small size of the cohort limits the strength of conclusions from this community-based study.

To establish biological plausibility, proof-of-concept acellular assays were performed, which were selected to represent direct oxidation of LDL by As and U in the circulatory system, thereby contributing to the atherosclerotic process. However, this might also represent what may be occurring within developing lesions. It is postulated that most modifications to LDL probably occur *in situ* within the atherosclerotic lesion (Itabe, Obama, and Kato 2011), and more research is needed to assess whether As and/or U accumulate in vulnerable vascular lesions. Several metals including As were detected in atherosclerotic lesions and cardiovascular tissue (Shi, Shi, and Liu 2004; Simeonova et al. 2003). Importantly, our acellular data are in agreement with findings observed in human serum, in terms of ApoB modifications. Finally, although the buffers used in these experiments (PBS and HEPES) are stable and nonreactive, these reagents do not share the same milieu of factors that may contribute to the atherosclerotic process or affect metal-LDL interactions.

Conclusions

Our findings suggest that As intake from contaminated drinking water may influence oxidative modifications of LDL in the Navajo population. Direct oxidation of LDL by environmental metals such as U and As may be an overlooked contributing mechanism to explain chronic CVD health outcomes, although future research needs to explore such relationships in more physiologically relevant experimental systems. Specific exposure levels and temporal dynamics of oxidative effects of environmental metals require to be more comprehensively elucidated in future studies. For the Navajo community, there remains a dearth of literature exploring the potential adverse health effects of unremediated wastes and tailings from legacy and future mining operations in the region. Continued research into mining waste-related community health outcomes, and pathophysiological basis thereof, is essential to ensuring that mineral resources may be extracted with minimal

negative impacts on public health. Exposure assessments and health outcomes need to be detailed in a more rigorous manner to permit such investigations.

Acknowledgments

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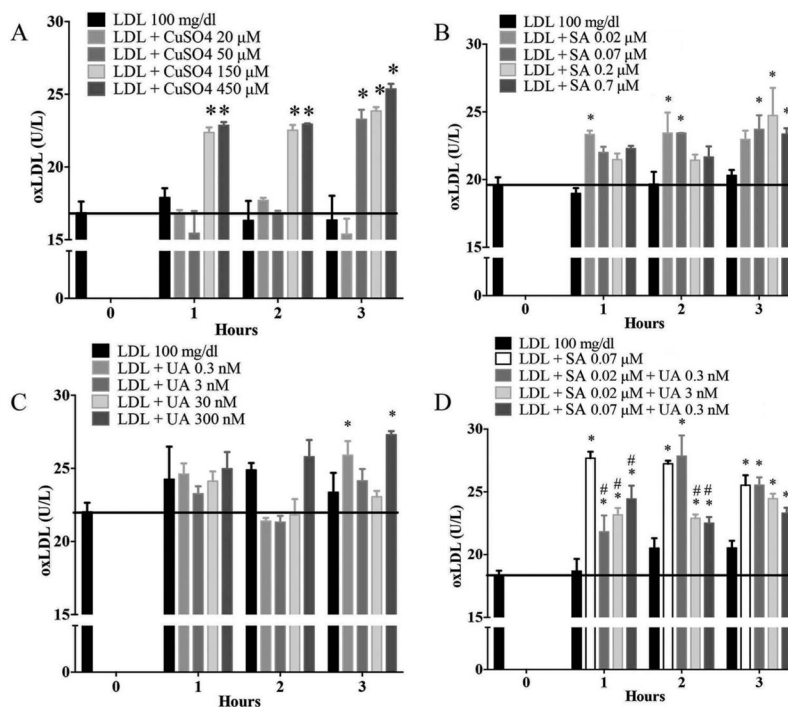
Figure 1.
Sodium arsenite directly oxidizes the apolipoprotein B component of LDL cholesterol. (A) CuSO$_4$ was used as a positive control. Effects of different concentrations of (B) Sodium arsenite (SA); (C) Uranyl acetate (UA), or (D) the combination of SA and UA on the apoprotein B (ApoB) of human purified LDL as measured by the 4E6 ELISA antibody. * Indicates significant difference from LDL untreated. # represents significant difference from LDL treated with SA at 0.07 μM. Error bars show SEM. *P* < 0.05 was considered statistically significant.
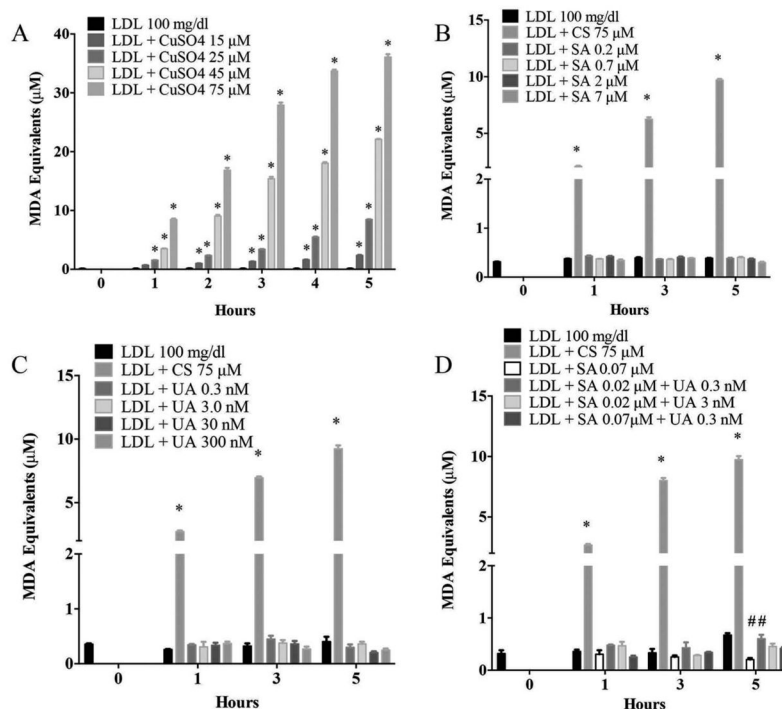
Figure 2.
Sodium arsenite and uranyl acetate do not increase lipid peroxidation of LDL as measured by TBARS (malondialdehyde equivalents). (A) $CuSO_4$ (CS) was used as a positive control. Effects of different concentrations of (B) SA, (C) UA, or (D) the combination of SA and UA on lipid peroxidation of human purified LDL. * Indicates significant difference from LDL untreated; # represents significant difference from LDL treated with SA at 0.07 μM. Error bars show SEM. $P < 0.05$ was considered statistically significant.

Table 1

Navajo demographic and clinical characteristics by gender.

Variable	Female (n = 145)	Male (n = 107)	Value above, below median for AS	Value above, below median for U
Age (years)	55.5 ± 14	55.1 ± 14.7	54.0 ± 14.0, 56.2 ± 14.1	55.7 ± 14.1, 54.6 ± 14.4
BMI, kg/m²	31.2 (27.7–34.9)	28.2 (26.1–31.5)	29.0 ± 5.5, 29.0 ± 5.0	28.7 ± 5.4, 29.3 ± 5.1
Total Cholesterol, mg/dL	182.5 (163.3–201.8)	182 (159.5–204.5)	184.1 ± 36.8, 186.9 ± 33.5	186.6 ± 36.2, 184.5 ± 34.6
LDL, mg/dL	105 (90.3–123.8)	104 (88–122)	109.0 ± 31.0, 107.1 ± 32.4	113.0 ± 28.9, 103.1 ± 33.2
HDL, mg/dL	47.5 (39–57)	42 (34–49.5)	47.6 ± 13.4, 47.8 ± 18.2	47.6 ± 14.0, 47.8 ± 17.8
Triglycerides, mg/dL	177 (127.5–247.3)	191 (125–261.5)	204.9 ± 141.7, 224.5 ± 136.8	198.6 ± 114.0, 231.0 ± 166.4
Systolic BP (mmHg)	126 (114–142)	132 (121.5–145.5)	128.8 ± 20.1, 134.0 ± 20.3	133.5 ± 21.0, 129.8 ± 19.6
Diastolic BP (mmHg)	76 (70–85)	81 (73–88.5)	77.6 ± 12.9, 80.6 ± 11.3	79.2 ± 13.0, 79.2 ± 11.3
Glucose (non-fasting), mg/dL	90 (77–122.8)	92 (79–120)	120.0 ± 75.4, 129.1 ± 89.2	119.9 ± 78.2, 129.3 ± 87.1
Median HbA1c, %	6.3 (5.9–7.3)	6 (5.6–6.9)	6.83 ± 1.97, 7.08 ± 2.10	6.74 ± 1.76, 7.17 ± 2.26
% 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)	48.8 ± 18.43, 48.1 ± 15.2	49.7 ± 18.3, 47.3 ± 15.5
CRP, mg/L	2.5 (0.9–5.2)	1.8 (0.9–4.2)	3.59 ± 3.83, 4.96 ± 12.69	4.02 ± 11.8, 4.54 ± 6.69
CRP > 3.0 mg/L, %	44	32		
Mean Water Arsenic, ppb (10–90%ile)	2.48 (1.00–6.00)	4.80 (1.00–4.85)		
Mean Water Uranium, ppb (10–90%ile)	5.35 (0.38–13.23)	6.04 (0.38–13.30)		

Data are presented as median (IQR) or %; age was reported as mean (SD). BMI: body mass index; LDL: low-density lipoprotein; HDL: high-density lipoprotein; BP: blood pressure; HbA1c: glycated hemoglobin; CRP: C-reactive protein.

Table 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, AIC = 2121, 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, AIC = 2123, 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, AIC = 800.7, 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, AIC = 797, p = 0.002				

$P < 0.05$ was considered statistically significant.

Reduced models are shown. All full models included demographic (age, gender) and physiologic variables (BMI, HbA1c), and estimated annual water intakes of U and As as predictors.

[*] Models 1 and 2 included data from 249 participants. Models 3 and 4 include a subset of 94 participants with available data for urinary Cu. Models 3 and 4 also included continuous concentrations of urinary copper (Cu), vanadium (V), and nickel (Ni), As (log transformed), and U (binary). Models 2 and 4 included second order interactions and significant interactions are reported.

Table 3

Reduced regression models showing coefficient estimates for CRP.

Coefficient	Estimate	Standard error	P-value	N*
Model 1				249
Age (years)	−0.015	0.005	0.002	
BMI, kg/m^2	0.038	0.014	0.007	
Adjusted R^2 = 0.085, AIC = 750.4, p = < 0.0001				
Model 2				249
Age (years)	−0.016	0.005	0.001	
Female x BMI	−0.059	0.03	0.049	
Adjusted R^2 = 0.092, AIC = 750.4, p = < 0.0001				
Model 3				94
Age (years)	−0.019	0.007	0.011	
Female	−0.327	0.205	0.115	
BMI, kg/m^2	0.049	0.022	0.026	
Adjusted R^2 = 0.157, AIC = 263.1, p = 0.001				
Model 4				94
BMI, kg/m^2	0.051	0.021	0.017	
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, AIC = 258.7, p = 0.0003				

$P < 0.05$ was considered statistically significant.

Reduced models are shown. All full models included demographic (age, gender) and physiologic variables (BMI, HbA1c), and estimated annual water intakes of U and As as predictors.

*Models 1 and 2 included data from 249 participants. Models 3 and 4 include a subset of 94 participants with available data for urinary Cu. Models 3 and 4 also included continuous concentrations of urinary copper (Cu), vanadium (V), and nickel (Ni), As (log transformed), and U (binary). Models 2 and 4 included second order interactions and significant interactions are reported.