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Metal mixture exposures and multiplexed autoantibody screening in Navajo communities exposed to uranium mine wastes

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ABSTRACT

Background: Environmental exposures to metals in uranium mining wastes and drinking water were documented in more than half of the 1304 Navajo community members of the Diné Network for Environmental Health (DiNEH) Project, the first comprehensive assessment of exposures to these metals and community health on the Navajo Nation.
Objective: Evaluate environmental exposures among participants who provided blood and urine samples using multiplexed autoantibody positivity as an early effect biomarker.
Methods: Survey and geospatial location data, well water quality, and metals biomonitoring were used to assess exposures to mixed-metal wastes from 100 abandoned uranium waste sites.
Results: We observed that the prevalence of multiplexed autoantibody positivity in 239 participants was more than double that reported for the U.S. population (27.2% v. 13.8%) even though the national prevalence was generated using a different assay, the HEp-2 cell-based antinuclear antibody test. Increased risk of multiplexed autoantibody screening positivity (OR = 3.07,95%CI 1.15–8.22) was found among DiNEH study people who lived close to uranium mine and milling wastes and consumed metals in drinking water. Associations for females were even stronger when they lived closed to contaminated uranium mining and milling sites. *Anti*-U1-RNP antibodies were associated with water consumption of nickel.
Conclusion: Proximity to waste sites and consumption of metals in water even below current drinking water standards were associated with perturbations of immune tolerance. These findings are consistent with previous studies of autoimmunity in the local population and demonstrate that multiplexed autoantibody screening method has a potential as sentinel indicator of exposures to environmental metals.
Impact statement: This is the first, community-engaged environmental health study in exposed Navajo communities that applied clinical multiplexed testing in risk assessment of environmental metals associated with abandoned, unremediated uranium mining and milling waste sites. Routine clinical autoimmunity measures could be used as early effect biomarkers of environmental metal exposures.

1. Introduction

The Diné Network for Environmental Health (DiNEH) Project is the first comprehensive assessment of exposures to uranium and other metals from abandoned uranium mine waste sites on the Navajo Nation. This cross-sectional study began in 2004 and broadly examined relationships between community health and environmental exposures among 1304 participants in partnership with 20 Navajo communities in New Mexico (Fig. 1). Initial findings based on survey and geospatial data from the entire study population revealed associations between proximity to legacy waste sites and increased risks of chronic diseases, including kidney disease during the active mining period (1950–1986), hypertension during the legacy period after the mines closed (1986-present), and increased likelihood of multiple chronic diseases including diabetes, kidney disease, and cardiovascular disease [1]. Subsequently, biomonitoring and clinical assessments were implemented in

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partnership with NAIHS to both validate population results and support mechanistic laboratory studies to evaluate exposure associations in a subset of DiNEH participants [2–4].

Concern for environmental exposures impacting the immune system had been raised early in the DiNEH study when initial self-reports indicated that autoimmune diseases appeared to occur more frequently among people living in communities with the largest number of abandoned mines (Fig. 2). A review of participants' medical records at two Navajo Area Indian Health Service (NAIHS) hospitals in the study area confirmed that the concordance between self-reports and recorded diagnoses was greater than 80% [2], and that nearly all cases where discrepancies were observed resulted from diagnoses made subsequent to survey administration. Results examined were responses to "have you now or ever had any of the following health problems?", and included autoimmune disease. (The DiNEH Project survey is reproduced in Supplemental Materials; see Q. 31j.) Only eight of them (3.3%) reported having any autoimmune disease diagnosis from the Phase II participants with biospecimen data. We surmised that the actual prevalence of autoimmune disease may be higher because clinics serving the region have few specialists in the diagnosis and-or management of autoimmune diseases, and participants may not have been aware of or never been diagnosed with frank autoimmune disease, or sought care outside of the NAIHS network. Several studies and toxicological profiles have linked immune system alterations and DNA-repair inhibition to certain environmental metals exposures in both human and animal models [5–10] and to ionizing radiation [11,12], as have serum biomarker studies [13–16].

In our previous analysis of biomarkers of autoimmunity [15], we used four specific autoantibodies (AuAb) determined by in-house ELISA assays in participants' serum samples to examine relationships between environmental metals exposures and immune impairment. Proximity to waste sites and uranium in drinking water were significantly associated with not only xenobiotic-induced specific autoantibodies, but also with idiopathic AuAbs (anti-native DNA and anti-chromatin) as well. Furthermore, proximity to abandoned uranium mines and waste sites was strongly associated with environmentally induced serum AuAbs (i.e., anti-denatured, single-stranded DNA and anti-histone) in both male and female Diné participants [15].

In the study reported here, we investigated the hypothesis that environmental metal-mixture exposures to uranium mining and milling wastes were predictive of autoantibody positivity based on a multiplexed bead assay, a screening method that has gained wide use in clinics across the U.S. and on Navajo Nation. We rigorously reviewed multiplexed autoantibody results provided by a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory that is one of the official contractors to NAIHS. We confirmed multiplexed bead assay results in 239 participants who donated specimens and their samples were examined. We further examined the relationships between metal-mixture exposures and autoantibodies identified by the multiplexing panel that could be used as potential early effect biomarkers. We discuss here how the multiplexed bead autoantibody screening information can be applied as feasible immune alteration outcome measure in this exposed population.

2. Materials and methods

2.1. Survey information

A questionnaire (see, Supplemental Materials), designed in partnership with community members to provide a culturally appropriate assessment of environmental exposures and health, was administered by Navajo-speaking trained field staff to 1304 individuals over a six-year period (2004–2010) during Phase I of the DiNEH study [1–4,15]. Demographic information, water and land uses, history of both active mining exposures and ongoing environmental contact with uranium through various pathways, and personal health histories were recorded.

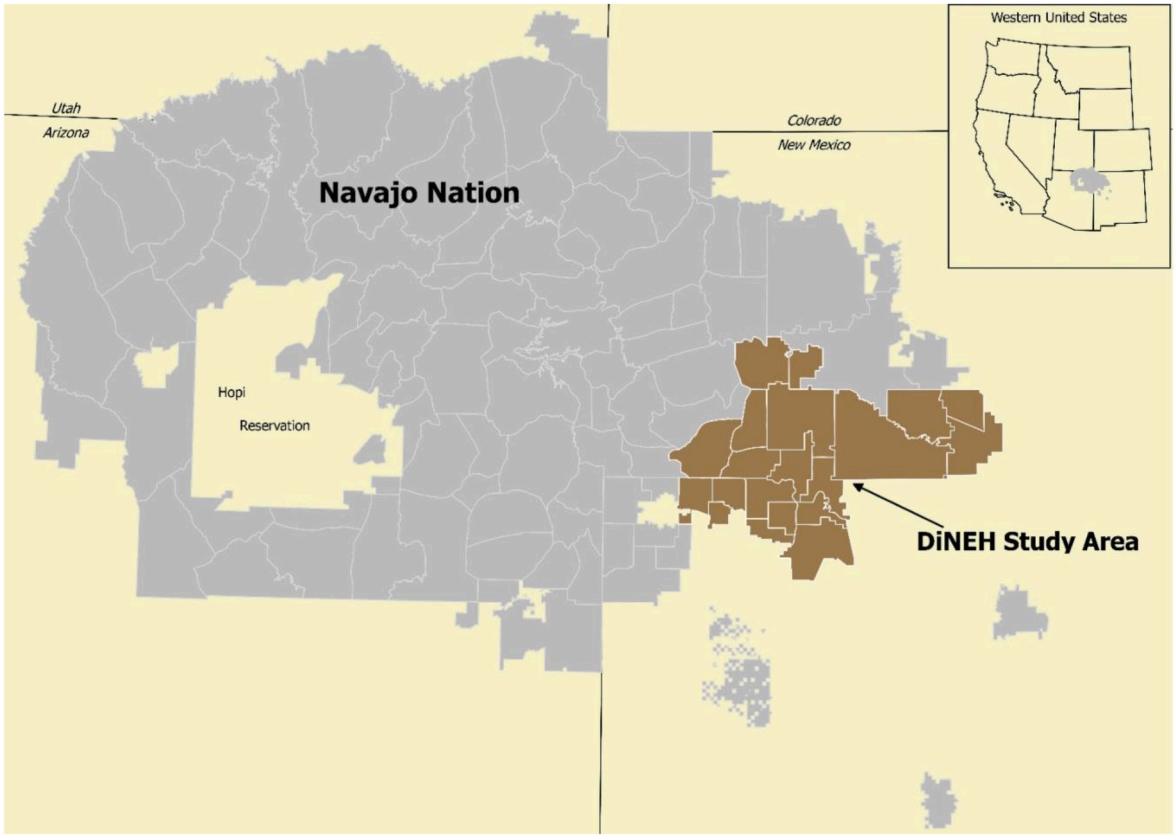

Fig. 1. DiNEH project geographical area in the State of New Mexico, USA and relationship to the Navajo Nation.

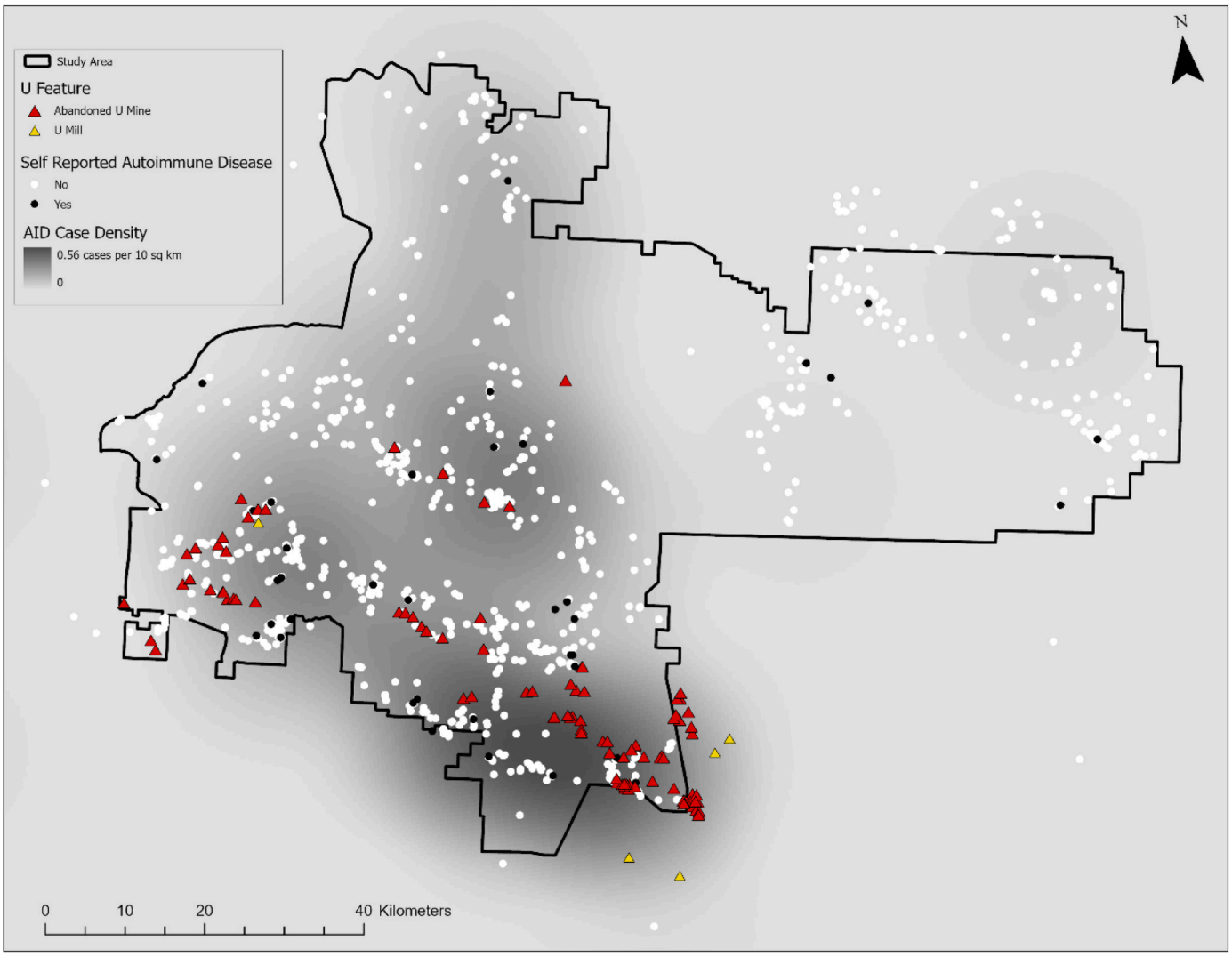

Fig. 2. Map of phase II DiNEH project participants' locations relative to abandoned uranium mines and mills and self-reported autoimmune disease diagnosis (kernel density cases) across the study area.

Navajo language use documented in the survey was used as a proxy measure for participation in cultural practices that can mitigate or potentiate exposures to mine waste. The degree of traditional language use was calculated based on the frequency and exclusivity of Navajo language use at work, at home, with friends or in a combination of all three. Self-reported demographic, exposure and health data for participants who took part in the biomonitoring program came from the same survey administered to all participants in the first phase of the DiNEH Project.

2.2. Environmental exposures to uranium wastes

Using survey responses, we separated historic exposures to mining (since 1940s up until 1986 when all mines ceased activity) from more recent community-level exposures to *existing* waste sites during the legacy period since 1986. This information allowed estimation of exposure risk from both high-dose, occupational-level exposures to uranium and associated toxic metals and lower dose, more chronic, community-level chronic exposures and activities that bring people contact with wastes [1,15]. Furthermore, biomonitoring of five common metals in spot urine samples allowed confirmation of the most recent exposures to environmental metals.

Participants' proximity to uranium mining and milling waste sites was established geospatially [1,3,15]. A cumulative "proximity" variable was established as the mean of the inverse of the distance of the participants to each of 100 uranium waste sites, weighted by the size of each site. In the modeling process, a log-transformed proximity variable (logProx) was applied as this variable was right-skewed. (See list of abbreviations.)

Variables for self-reported "mining era exposures" (*M*) and for "environmental legacy exposures" (*E*) were developed by Hund and colleagues [1] to account for the differences in exposures to uranium during the period of active mining as compared to the long-term, chronic nature of ongoing exposures to nearby populations once the wastes were abandoned in place. *M* exposures (1950–1986) included having worked in a mine, mill or reclamation project, lived in a mining community, or washed the clothes of a uranium worker. *E* exposures included such practices as using mine wastes in home construction, herding or sheltering livestock in or near abandoned mines, playing on or near waste sites, or being exposed to contaminated mine water.

2.3. Exposure to contaminants in water sources

Sampling and analytical methods used to ascertain water quality in drinking water sources used by DiNEH participants were summarized in Erdei et al. [15]. As reported, study participants were matched to the

water sources they drank from based on their responses to survey questions about current and past water consumption with respect to both source and volume. Mean and median concentrations for seven metals present in local water sources were calculated from field data and compared with national drinking water standards (called Maximum Contaminant Levels, or MCLs [16]). Five contaminants – arsenic, copper, mercury, total radium and uranium – have MCLs; two contaminants, nickel and vanadium, do not. In their place, we used a numerical value proposed by the U.S. Environmental Protection Agency (USEPA) for nickel [17] and a California Water Board Notification Level for vanadium [18]. The mean of multiple method detection limits (MDLs) reported by laboratories as "non-detects" were used to establish "baseline" concentrations for the seven contaminants to avoid using zero in annual intake calculations.

The seven contaminants were selected based on their known presence in local water supplies (principally, arsenic and uranium) [19–21], and on being constituents (particularly, uranium, arsenic, vanadium and radium) of the mined ore throughout the Navajo Nation and in the study area [21,22]. The biomonitoring program generated data on arsenic, copper, nickel, uranium and vanadium in urine samples donated by participants. Investigation of several of the selected contaminants-mercury, arsenic, copper and nickel-was also based on *a priori* knowledge of their potential immune system toxicities and reported immune modulatory effects [7,8,23–26].

Estimated annual consumption (EAC) of each contaminant from each drinking water source was calculated as the measured concentration (or the average of multiple measurements of the same contaminant) or the average MDL multiplied by 2 L of water per day multiplied by 365 days in a year. The daily water intake of 2 L was based on USEPA recommendations [16]. Some participants hauled water for human consumption from up to four different sources, in which case the EAC was calculated as the sum of one-fourth of the EAC from each source. For participants who reported drinking exclusively from a public water supply (PWS), the number of water sources used was considered to be one.

To gauge the magnitude of these annual intakes, we calculated annual consumptions of each of the seven water contaminants as if a person drank water containing a concentration equal to the MCL for each contaminant for a year (i.e., concentration in micrograms per liter x 2 L per day x 365 days per year).

2.4. Biological sampling and biomonitoring

Of the 1304 participants in the first phase of the study, 263 provided blood and urine samples between June 2010 and May 2011. Navajo team members living in the study area advertised the collection events in radio announcements, newspaper postings, at outreach meetings in chapters affected by mining wastes and with local and regional grassroots organizations.

The biomonitoring program generated estimates of exposures to five metals – arsenic, copper, nickel, uranium and vanadium – for the purpose of assessing possible health effects in the communities. Blood and urine samples were processed initially on site and then transported to the UNM Health Sciences Center College of Pharmacy where specimen aliquots were stored at −80 °C until analysis. Metals concentrations in participants' urine samples (N = 200) were determined by inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer, Inc., Houston, TX) at the UNM Earth and Planetary Sciences Analytical Geochemistry Laboratory. Method Detection Limits (MDLs) in urine were 0.15 μg/l for total arsenic, 1.17 μg/l for copper, 0.16 μg/l for nickel, 0.09 μg/l for uranium, and 0.10 μg/l for vanadium.

2.5. Laboratory examinations of antinuclear antibodies by multiplexed screening assay

Serum samples were processed at community collection events by Navajo Area Indian Health Service (NAIHS) clinical personnel, and immediately transported at 4 °C to the clinical laboratory (LabCorps, a CLIA-certified Indian Health Service contract laboratory in Phoenix, AZ) within 6 h of collection. Sera were assessed for several commonly used clinical laboratory measurements, including the widely used serum multiplexed screening test [27–29]. Twenty-four samples had no multiplexed autoantibody results due to problems with serum volume or quality. Screening for autoantibody positivity (dilution 1:100) was determined by a set of fluorescent beads multiplexed (Bio-Rad, BioPlex® ANA Screen platform, Hercules, CA) in which nine common antigens: double stranded DNA (*anti*-dsDNA, or "*anti*-DNA"), small nuclear ribonucleoprotein (U1-RNP, or "*anti*-U1-RNP"), chromatin, Ro/SSA (SS-A, Ro60), La/SSB antigens (SS–B), topoisomerase I (SCl-70), Smith antigen (Sm), Jo-1 (histidyl tRNA synthetase) and centromere B. The assay applies verification and an external standard (the World Health Organization w/o 80 Standard, validated by WHO International Standards (IS) established by the Expert Committee on Biological Standardization, Geneva, Switzerland) to quantify fluorescence intensity and generate IU/ml concentration value to each sample. A serum sample was considered positive when the above-listed antigen bead's fluorescence intensity was detected above 0.2 International Units per milliliter (IU/ml) fluorescence intensity threshold. This was true for all beads in the array except for *anti*-dsDNA, for which the laboratory detection positivity threshold was higher, above ≥5 IU/ml established by the laboratory bead detection parameters. This concentration informs clinical diagnosis, therefore it was applied accordingly in this analysis as well. The detected autoantibodies potentially demonstrate both DNA- and RNA-containing macromolecular complexes as target self-antigens [27]. We employed the summative bead detection results in exposure modeling and examined separately some of the frequently found, such as *anti*-U1-RNP and *anti*-dsDNA autoantibodies in association with metal exposures. Autoantibodies to Jo-1 and was not detected in the laboratory, hence, that was not analyzed statistically.

2.6. Statistical methods

To evaluate whether the 263 DiNEH participants who donated blood and urine samples were representative of all study participants, we compared their attributes to those of individuals who did not participate in the biomonitoring phase of the study (N = 1074). Fisher tests were applied to binary data comparisons (i.e., yes-no answers, such as female or male) and a Wilcoxon nonparametric test was used to examine continuous variables, such as mining era exposures, environmental legacy exposures and proximity of homes to uranium waste sites. Results are shown in Supplemental Materials Table S1.

Autoantibody positivity determined using all detectable fluorescence bead results among 239 participants was verified by laboratory reports, and results were evaluated by gender, age (at Phase II biospecimen collection), duration of residency, proximity to abandoned uranium mining and milling sites, NavajoUse and *M* and *E* exposures. Clinically relevant thresholds of fluorescence bead positivities provided by the laboratory were used to generate a binary multiplexed autoantibody positivity variable ('ANA positive' variable: yes/no). In addition, we separately modeled the frequently detected autoantibody responses recorded in the multiplexed screening assay to investigate individual autoantigen responses. All other bead positive results – including *anti*-dsDNA – were modeled individually as well, but other than *anti*-U1-RNP autoantibody positivities, no statistical approach rendered reliable models due to small sample sizes. Two-sided t-tests were used to examine any significant differences in autoantibody positivity between Navajo females and males or by age of participants (at Phase II sampling).

As previously described, estimated annual metals consumption from drinking water was based on average concentrations of the seven metals in 62 water sources used by the 239 participants who provided blood and urine samples. Metals concentrations in urine specimens from the

same participants were log-transformed when they showed skewed distributions. When concentrations of metals in urine were below the MDLs (>30% of samples) metals concentrations were calculated as the MDL divided by the square root of 2 [30]. Biomonitoring data for arsenic, copper, nickel, and vanadium were modeled as continuous variables. Excreted uranium was coded as a binary variable based on distribution of observed concentrations ('yes' if ≥ 0.09 μg/l and 'no' if ≤ 0.09 μg/l), resulting in a likely more conservative estimate of high exposure. Spearman correlations were then performed to assess bivariate association strength between metals consumed and metals excreted.

For modeling composite multiplexed autoantibody responses (yes-no binary variable), logistic regression was applied to environmental exposure variables, and their interactions with age and gender were included for the entire participant cohort. Potential covariates of the models were age, NavajoUse, *M, E,* logProx, log V, log Ni, log As, log Cu, U-binary, logAs.EAC, logHg.EAC, logNi.EAC, logU.EAC, logCu.EAC, and sqrtRa.EAC. (See list of abbreviations for definitions.) Two models were fitted. The first used all covariates without interactions. A second model was used all covariates listed above, interaction of age with other covariates, and three-way interaction of age, *E* and logProx. Model reduction was applied to each full model to simplify the main contribution of variables using stepwise selection. Reduced model results were expressed as estimated odds ratios of multiplexed autoantibody positivity. Akaike Information Criterion (AIC) was applied to minimize information loss associated with stepwise removal of predictor variables. If an AIC-reduced model as a final model had more than 15 predictor variable parameters, a Bayesian Information Criterion (BIC) method that tends to find smaller models with smaller *p*-values, was also used as an alternate model selection technique.

Linear regression models were also applied to some frequently detected and relevant autoantibodies, *anti*-U1-RNP and *anti*-dsDNA. Serum positivity of these autoantibodies were also evaluated for associations with environmental contaminants and classic risk factors, such as gender and age. Since serum autoantibody values may be potentially influenced by serum lipid interference at diagnosis [31], we also used study participants' serum total cholesterol, high-density lipoprotein and triglyceride concentrations as predictors of *anti*-U1-RNP serum autoantibody responses.

3. Results

1. Demographic and exposure differences

In comparing participants who donated blood and urine samples (Phase II - N = 263) with those who did not (Phase 1 only - N = 1074), those donating samples generally reported more environmental exposures than the Phase I only group (Supplemental Table S1.). Proximity to waste sites and having worked in a mine, lived in a mining camp and washed the clothing of workers were significantly greater among the Phase II group. Similarly, all six *E* exposures – from playing on mine wastes as a child to using mine materials in home construction – were also significantly greater among the Phase II participants. Among other self-reported exposures, only two occupations — gold- and silversmithing — were more likely to be reported by Phase II participants. These findings suggest that those who volunteered to participate in biological specimen collections may have had increased awareness of and concern about their exposures to uranium mine wastes both during the *M* and *E* periods.

2. Multiplexed autoantibody positivity and environmental exposures: Descriptive results

The overall prevalence of a detectable positive multiplexed serum autoantibody test was 27.2% (65 of 239) (Table 1) — almost two times the national average prevalence rate of 13.8% reported in the 2012 National Health and Nutrition Examination Survey (NHANES), however

Table 1
Prevalence of multiplexed autoantibody positivity and selected autoantibody bead target positivity among DiNEH Phase II participants. Results shown as stratified by gender, age of participants (at Phase II sampling), duration of residency, and mining era and environmental legacy era exposures.

	Female	Male	All	*p*-value[a]
N	138	101	239	
Autoantibody positive (%) *p*-value difference in positivity between females and males	39 (28.3)	26 (25.7)	65 (27.2)	0.59
Selected Individual Autoantibody Positivities (> standard reference value)				
Anti-dsDNA (%) (≥5.0) *p*-value for difference in antigen (means)	7 (63.6)	4 (36.3)	11	0.22
Anti-U1-RNP (%) (>0.2) *p*-value for difference in antigen (means)	19 (54.3)	16 (45.7)	35	0.84
Anti-chromatin (%) (>0.2) p-value for difference in antigen (means)	2 (66.7)	1 (33.3)	3	1.0
Anti-SSA (%) (>0.2) *p*-value for difference in antigen (means)	14 (73.7)	5 (26.3%)	19	0.57
Anti-SSB (%) (>0.2) *p*-value for difference in antigen (means)	8 (61.5)	5 (38.5)	13	0.40
Anti-SLC-70 (%) (>0.2) *p*-value or difference in antigen (means)	6 (43.9)	8 (57.1)	14	0,54
Anti-Sm (%) (>0.2) *p*-value or difference in antigen (means)	5 (62.5)	3 (37.5)	8	1.0
Age (yrs), mean ± SD	55.1 ± 14.2	55.0 ± 14.6	55.0 ± 14.3	
p-value difference in age by sex				0.996
Residency (yrs), mean ± SD	34.6 ± 22.5	35.48 ± 21.9	34.60 ± 22.0	
p-value difference in duration by sex				0.757
Navajo Use = 0 (%)	11	5	16 (6.7)	
Navajo Use =>1 (%)	124	99	223 (93.3)	
p-value difference by gender				0.217
Mining Era = 0 (%)	89	57	146 (61.1)	
Mining Era = >1 (%)	46	47	93 (38.9)	
p-value difference in *M* by gender				*0.023*
Environmental Legacy = 0 (%)	83	59	142 (59.4)	
Environmental Legacy = >1 (%)	52	45	97 (40.6)	
p-value difference in *E* by gender				0.102

Italics indicate significant finding at α level of ≤0.05.

[a] All *p*-values calculated using Wilcoxon rank sum test, except otherwise noted.

that comprehensive and national data were generated using a different testing methodology, HEp-2 cell-based microscopic slide assay [32]. No significant difference in DiNEH samples was observed in multiplexed autoantibody positivity prevalence across the sexes ($p = 0.59$); 28.3% of females and 25.7% of males had positive screening tests. Similarly, there was no significant difference in the detection of serum *anti*-DNA and *anti*-U1-RNP between females and males. The average age of Phase II participants was 55 years (Table 1), and there was no significant difference in ages between females and males with a positive multiplexed autoantibody test.

Participants with multiplexed autoantibody screening test results (N = 239) had continuous environmental exposures over many years as shown in Table 1. The average duration of residency, 34.6 years, and the large percentage of participants who frequently use the Navajo language in their daily interactions (93%) indicate that participants were generally older, more traditional, and connected to their communities. Indeed, 41% of participants reported one or more *E* (mining legacy environmental) exposures and 39% reported *M* (active mining, occupational) exposures, some three decades before the biological samples were collected for this study. No metal mixture exposures or mining site proximity measures have been obtained yet in association with HEp-2

ANA assays among NHANES participants [32].

3. Drinking water exposures among DiNEH Phase II participants

Phase II participants with multiplexed autoantibody screening test results consumed water from a combination of both "piped-in" water from regulated public water systems and unregulated water from such sources as windmills and developed springs (Supplemental Table 2A). Overall, median, mean and upper confidence limit (UIC) concentrations (95% CI) of the seven water contaminants were well below their respective MCLs. Only arsenic had a UIC greater than its drinking water standard (13.6 μg/l versus 10 μg/l). Three contaminants – arsenic (9), radium (9) and uranium (7) – were detected in concentrations of at least one-half of their MCLs. Radium, a human carcinogen [11], was detected in concentrations exceeding its MCL of 5 pCi per liter in three sources and at concentrations exceeding one-half of its MCL in six other water sources. One PWS had a 5-year average radium concentration that exceeded the MCL by 10%. Uranium in excess of its MCL was detected in four water sources. Median concentrations reported here are consistent with published values for unregulated water sources throughout the Navajo Nation [19,20].

The annual intake of metals shown in Supplemental Table 2B represents participants' estimated total exposures from drinking water. To estimate the magnitude of these annual intakes, and to provide a point of comparison, we calculated annual consumptions of each of the seven water contaminants as if a person drank water containing a concentration equal to the MCL for each contaminant for a year (i.e., concentration in micrograms per liter x 2 L per day x 365 days per year). Twelve participants had actual annual consumptions that exceeded the MCL-derived comparison value, and of those, three participants had EACs exceeding more than one MCL-derived comparison value. As shown in Supplemental Table 2B, EACs of four contaminants – arsenic, radium, uranium and vanadium – exceeded the MCL-derived comparison values; EACs for copper, mercury and nickel did not. Generally, annual intakes of the metals examined here were reflective of the relatively low median concentrations in the source waters from which they drank.

EACs for the 239 participants were used in the correlation analyses to explore relationships between metals consumed in drinking water (Supplemental Table 2B) and metals excreted in urine (Supplemental Table 3). The Spearman correlations revealed that U consumption in water was positively correlated with arsenic, copper and nickel consumption, reflecting the observed co-occurrence of these metals in Eastern Navajo water sources [20]. Nickel, vanadium, and uranium consumption was correlated with urine-uranium excretion. Radium consumption was positively correlated with copper excretion. Strong correlations were observed in excreted vanadium with copper and nickel.

4. Modeling associations of multiplexed autoantibody summative response with environmental exposures

Living close to abandoned uranium mine and/or milling waste sites was the strongest predictor of a positive serum autoantibody response generated by the multiplexed assay (OR = 3.073; 95%CI 1.15–8.22) (Table 2A). Summative serum antibody positivity was also significantly predicted by age (at Phase II sample collection) and male gender of the respondents. The every-year increase above the mean age of study participants represented a 7% increase in the risk of having a positive multiplexed autoantibody result (OR = 1.07, 95% CI 1.011–1.124) (Table 2). Furthermore, mercury consumption – even at the low levels observed in water sources - was a significant predictor of a summative autoantibody positivity. Copper consumption was negatively associated with serum autoantibodies. There was significant interaction variable term found between living close proximity to abandoned uranium mine sites and female gender, explaining that close proximity location of the homes to uranium mine wastes was an important variable among those

Table 2
Odds ratios derived from multiple logistic regression model presenting the likelihood of having a clinically defined, increased serum multiplexed autoantibody response among DiNEH Phase II participants (summative response N = 239).

Covariates	OR	95% CI	*p*-value
Age	1.070	1.011–1.124	***0.018***
Gender[a]	0.0007	6.098e-06 – 0.088	***0.003***
NavajoUse	0.799	0.498–1.283	0.353
logProx	3.073	1.149–8.219	***0.025***
logCu.EAC	0.748	0.619–0.905	***0.003***
logHg.EAC	2.343	1.248–4.398	***0.008***
logNi.EAC	0.582	0.290–1.168	0.128
sqrtRa.EAC	1.065	0.975–1.164	0.161
Interactions			
Age: logNi.EAC	1.010	0.998–1.022	0.116
Age: sqrtRa.EAC	0.999	0.997–1.000	0.090
Gender:NavajoUse	1.531	0.860–2.723	0.148
Gender:logProx	***0.206***	***0.059–0.713***	***0.013***
Gender:sqrtRa.EAC	1.041	0.995–1.090	0.083
AIC			279.2
BIC *p*-value of overall X^2 statistic			327.7
Deviance			*0.0003*
R^2 (Cox-Snell)			264.2
R^2 (McFadden)			0.098
Tjur's COD			0.085
			0.154

Shading denotes significant covariate at α level of ≤0.05.
[a] Gender comparison was coded as males compared to females as women have documented increased risk for both ANA and autoimmune disease diagnosis. Small OR indicates male risk increase.

DiNEH study women who had multiplexed bead autoantibodies.

5. Modeling individual multiplexed autoantibody responses with environmental exposures

Logistic and linear regression models were applied to the two common nuclear autoantibodies, *anti*-dsDNA (data not shown in individual table) and *anti*-U1-RNP (Tables 3a and 3b). When *anti*-dsDNA antibodies were modeled with a final multivariable logistic regression model, the estimated annual water consumption of mercury contamination and the binary urinary uranium excretion stayed in the model and were both positive predictors of an increased serum *anti*-dsDNA antibodies (binary variable: yes/no). These variables were final in the logistic regression model, but non-significant predictors.

Linear regression models showed nickel consumption in drinking water significantly predicted increased serum *anti*-U1-RNP production, while radium consumption had a significant negative effect (Table 2A).

Table 3a
Anti-U1-RNP serum response as *continuous* variable (log values applied): full model, no interactions, no biomonitoring (N = 35).

Covariate	β Estimate	SE	*p*-value
Age	−0.014	0.020	0.665
E	0.186	0.143	0.208
Gender	0.253	0.474	0.599
logAs.EAC	−0.319	0.373	0.403
logCu.EAC	0.164	0.124	0.199
logHg.EAC	−0.536	0.374	0.167
logNi.EAC	0.314	0.132	***0.027***
logProx	−3.59	8.35	0.672
logU.EAC	0.285	0.192	0.152
logV.EAC	0.31	0.526	0.562
M	0.241	0.234	0.315
NavajoUse	0.00013	0.207	0.9995
sqrtRa.EAC	−0.043	0.019	***0.0324***
AIC			114.3
Adjusted R^2			0.09818
p-value of overall F statistic			0.2952

Shading denotes significant covariate at α level of ≤0.05.

Table 3b
Anti-U1-RNP serum response as *continuous* variable (log values applied): reduced model; no interactions, no biomonitoring (N = 35).

Covariate	Estimate	SE	*p*-value
E	0.144	0.098	0.153
logNi.EAC	0.314	0.094	***0.0021***
AIC			101.7
Adjusted R^2			0.2246
p-value of overall F statistic			*0.006*

Shading denotes significant covariate at α level of ≤0.05.

When further reduced models were applied, the *E* variable again stayed in the final model, but was not a statistically significant predictor (data not shown).

Serum lipid values from clinical results were also modeled to assess whether markers of inflammatory effects may contribute to false positive serum multiplexed autoantibody detections as described in the literature [31]. None of the measured serum lipid components were predictors of any of the ANA bead screening positivities, not even when age interactions were also included in both general linear regression and in logistic models (not shown).

4. Discussion

4.1. Classical risk factors

That age (at Phase II sampling) was a significant predictor of multiplex autoantibody positivity in the DiNEH cohort is consistent with NHANES national sample results [32]. NHANES reported a greater prevalence of ANA positivity among people in the 50–59 year age group compared with the all-ages NHANES sample (17.4% versus 13.2%). The overall prevalence (27.2%) of multiplexed autoantibodies in DiNEH participants reflects a similarly older population (mean age = 55 years; Table 1), however, laboratory assay differences likely underestimated the DiNEH sample positivities making this comparison weaker. Using NHANES data is important for comparison in the context of our Navajo Nation DiNEH cohort as NHANES applies random sampling and population-based, representative biospecimen collections. Furthermore, the lack of First Nation, population-based, autoimmune marker measures do not allow direct comparisons to our results yet. First Nation and Alaska Native autoimmune disease publications were based on clinical cohorts and family members' analyses that resulted in increased prevalence of ANA test positivities, therefore they do not reflect accurately community-based autoimmunity examinations [33,34].

The mean duration of residency of the DiNEH cohort of nearly 34 years also reflects an older and more stable population. No ANA national database provides for direct comparison of decades of continuous metal/metalloids and other chemical exposures. In our tribal community setting, where geographic mobility is limited, participants' advanced ages represent chronic, cumulative exposures over their lifetimes.

A recent article also advocates for an urgent call to action for improved understanding, diagnosis, treatment, and prevention of autoimmunity and autoimmune diseases. Major probable causes of increased autoimmunity were considered to be changes in food, contacts with xenobiotics, air pollution, infections, stress, and climate change [35] supporting our exposed community-focused research approach.

While there was no statistical difference in multiplexed autoantibody positive test prevalence between Native men and women (Table 1), the elevated prevalence among Navajo males (25.7%) was unexpected in light of previous studies that have shown autoantibody positivity was usually much higher among females than males [36–38]. The higher prevalence of ANA positivities - carried out using HEp-2 assay-in Navajo men has also been observed by us among much younger Navajo fathers (mean age 29 years) enrolled in our ongoing Navajo Birth Cohort Study [39]. Furthermore, men who were uranium workers (about 10% of DiNEH Project participants [1]) may be impacted by estrogen-mimicking properties of uranium and radium [40,41]. Other heavy metals (e.g., arsenic, mercury, manganese, copper and lead) present in mine wastes in the Eastern Navajo region have also been identified as endocrine modulators and disruptors in the literature [24, 42–44].

4.2. Environmental factors

Although it has been assumed clinically for decades that complex genetic traits are responsible for idiopathic autoreactive responses in various ethnic groups [45], more attention has been paid recently to understanding associations between production of markers of autoimmune diseases and environmental contaminant exposures, especially among vulnerable populations [13–15,46]. The results reported here provide further evidence that immune impairment and autoimmunity may be associated with various environmental exposures in Native American and indigenous populations [7,15,33,34,35].

We previously reported that breaking of immune tolerance may result from proximity to mine wastes [15], and that more broadly, proximity and contacts with mine wastes are consistent predictors of chronic diseases – kidney disease during the mining period and cardiovascular disease and autoimmunity in the legacy era – in the Navajo communities participating in the DiNEH Project [1,3,15]. In the current study, close proximity to abandoned uranium mine and milling waste sites yielded a *threefold increase* in the likelihood of having a positive serum multiplexed autoantibody screening response (Table 2A).

Drinking water exposures also appear to play a role in alterations of immune tolerance. As we previously reported [15], uranium and arsenic consumption in drinking water was associated with idiopathic autoantibodies (anti-native DNA and anti-chromatin autoantibodies). Here, the second largest detected OR (2.34 [95% CI: 1.248–4.389]) of producing increased multiplexed autoantibody detection was found in association with Hg consumption in drinking water sources. Mercury exposure that may induce break in immune tolerance has been identified previously in various exposure pathways both in animal immunotoxicological research [26,47,48] and in U.S. female populations represented in NHANES [36]. Our team previously also detected increased prevalence of ANA positivity using both HEp-2 substrate IIF method and ELISA technique that was associated with fresh water fish consumption, a common dietary source of mercury exposure in another U.S. tribal community exposed to mine waste [7].

Several environmental factors were predictors of autoantibody production against specific nuclear antigens, dsDNA and U1-RNP in DiNEH participants (Tables 3A and 3B). Radium's negative effect on *anti*-U1-RNP production is in agreement with literature data describing RNA metabolism changes and decreases in mRNA productions after radiation exposures in mammalian cells [49]. Nickel consumption through drinking water, again, at average concentrations below drinking water standards in our study, was associated with increased *anti*-U1-RNP positivity. Nickel's autoimmune potential was revealed in animal studies [17] and further supported by its capacity to modulate Toll-like receptors (TLRs) [50]; however, its autoimmune toxicological effect is not well-studied [17].

4.3. Multiplexed bead autoantibody assay as effective autoimmunity assessment tool in environmental health research

While the use of autoantibody testing using HEp-2 cell IIF assay is well-established as a diagnostic tool and biomarker of various autoimmune diseases, especially for systemic lupus erythematosus and mixed connective tissue diseases [46,51], some disagreement still exists about the most appropriate laboratory assays to use as a screening test [52,53]. The HEp-2 IIF test is expected to detect a much broader spectrum of autoantibodies than the multiplexed array platform. In reality, however, multiplexing gained significant diagnostic popularity in recent years

over the time-consuming and low specificity HEp-2 cell test [51,54]. Indeed, the multiplexed autoantibody test is used for diagnosis across NAIHS hospitals, including those in the study area. We propose that the multiplexed test can be applied effectively in studies assessing risks of exposure to environmental contaminants as an effective environmental health research tool.

Research has demonstrated the usefulness of serum autoantibody testing in detecting associations with different environmental and chemical exposures nationwide as well as ANA was used to explore unexplained increasing trends especially in U.S. males with heightened autoimmune marker production [55,35,56]. Since self-reported autoimmune disease prevalence in the DiNEH study were within the normal U.S. national ranges among both Phase I (3.1%) and Phase II (3.3%) participants, and we did not identify participants with active lupus and/or extremely high *anti*-dsDNA autoantibodies, the observed 27.2% serum multiplexed autoantibody positivity prevalence was an unexpected result that merits further investigation. The large difference between the self-reported prevalence of autoimmune diseases and the lab-derived prevalence of multiplexed autoantibodies suggests that both autoimmune diseases and autoimmunity may be underdiagnosed and-or under-observed in the study population.

4.4. Limitations

Biomonitoring data were not obtained for 39 of the 239 participants for whom multiplexed autoantibody laboratory results were reported. We also did not assess urinary concentrations of arsenic and uranium as representations of distance from one or more abandoned mines [20]. Some metals (radium and mercury) were not assessed in urine even though water contamination information for them was applied in models, resulting in some information loss. Furthermore, this analysis did not cover other possible routes of exposures (dietary and/or respiratory) to mixed-metal mine wastes.

Laboratory limitations included the smaller set (albeit common) autoantibody targets used in the laboratory and the lack of known metals such as mercury & silver exposure-associated and also specific autoimmune disease-associated autoantibody targets in the multiplexed array. These likely resulted in an underestimation of the prevalence of positive sera in the study population. Furthermore, future examinations should investigate in a parallel fashion the use and results of both the HEp-2 IIF assay and a multiplexed assay platform for comparison similarly as we applied that in a smaller set of Navajo participants' samples before [39].

4.5. Policy implications

The unexpected increase in serum multiplexed autoantibody positivity (27.2%) among Eastern Navajo community members exposed to complex metal mixtures in uranium mine wastes indicates an unmet need for immune dysregulation surveillance and research in the region. In its current clinical application, the multiplexed autoantibody serum measures are recorded and kept within clinical settings and are not yet incorporated to any risk assessment efforts in the Navajo Nation. Since minority groups have been shown to have increased rates of autoimmune diseases [38,33,34], and many Tribal communities are exposed to a variety of mine waste-associated metals, autoimmunity markers in an improved and targeted fashion could directly be used to inform both exposure assessments and community health research.

Furthermore, the protectiveness of national drinking water standards applicable to public water systems for immune system alternations should be carefully examined. Possible health impacts of long-term exposure of multiple metals even at low concentrations need to be considered. While the vast majority of water supplies on the Navajo Nation meets or exceeds national standards, unregulated and regulated water supplies remain at risk in areas where natural conditions and mixed-metal mining wastes have contributed to localized contamination of land and water [19,20,22]. Consideration of and research into these cumulative and mixture exposures to environmental contaminants in Navajo communities are warranted.

Credit author statement

EE- Analytical design, clinical laboratory data management, evaluation, interpretation of data, data curation, risk estimation analysis, IRB reporting, original writing, writing review and editing. CS – Methodology (Navajo partner involvement, community outreach, liaison training, survey development, water contamination data management, evaluation), project administration, data presentation, original writing, writing review and editing. CM – Formal analysis, statistical modeling and interpretation. JH - GIS analytical support, data visualization. MC - Data cleaning, database management. JL- Funding acquisition, project administration, project oversight, supervision, and review.

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Ethical approval

Research reported herein was approved by the University of New Mexico Human Research Protections Office and the Navajo Nation Human Research Review Board, and renewed annually. No human or animal experiments were conducted for this study.

Financial interest disclosures

The authors state that they have no competing financial interests associated with the study and/or the analysis presented here.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtauto.2023.100201.

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TABLE OF ABBREVIATIONS

age: Age as an exposure factor
age:E: Interaction of age and environmental legacy exposures
age:M: Interaction of age and mining era exposures
age:E:logProx: Interaction of age and environmental legacy exposures with the log of the participant's home's distance from all 100 uranium waste sites
age:logProx: Interaction of age with the log of the participant's home's distance from all 100 uranium waste sites
age:logNi.EAC: Interaction of age with the log of the estimated annual consumption of nickel in drinking water
age:sqrtRa.EAC: Interaction of age with the square root of the estimated annual consumption of radium in drinking water
AIC: Akaike Information Criterion
ANA: Antinuclear antibodies
As.EAC: Estimated annual consumption of arsenic in drinking water
AuAb: Autoantibody, specific autoimmune marker produced against nuclear, cellular or tissue antigens
BIC: Bayesian Information Criterion
CLIA: Clinical Laboratory Improvement Amendments
Cu.EAC: Estimated annual consumption of copper in drinking water
DiNEH: Diné Network for Environmental Health Project
E: Environmental legacy exposures
Gender: Gender (male and female) as an exposure factor
gender:E: Interaction of gender with environmental legacy exposures
gender:logAs.EAC: Interaction of gender with the log of the estimated annual consumption of arsenic in drinking water
gender:logCu.EAC: Interaction of gender with the log of the estimated annual consumption of copper in drinking water
gender:NavajoUse: Interaction of gender with environmental legacy exposures
gender:sqrtRa.EAC: Interaction of gender with the square root of the estimated annual consumption of radium in drinking water
Hg.EAC: Estimated annual consumption of mercury in drinking water
logAs.EAC: Log of estimated annual consumption of arsenic
logCu.EAC: Log of estimated annual consumption of copper
logNi.EAC: Log of estimated annual consumption of nickel
logProx: Log of the participant's home's distance from all 100 uranium waste sites
logU.EAC: Log of estimated annual consumption of uranium
M: Mining era exposures
NavajoUse: Ordinal values for not speaking Navajo, speaking Navajo at home, or speaking Navajo at work
Ni.EAC: Estimated annual consumption of nickel in drinking water
oxLDL: Oxidized low-density lipoprotein
Ra.EAC: Estimated annual consumption of radium in drinking water
sqrtRa.EAC: Square root of the estimated annual consumption of radium in drinking water
U.EAC: Estimated annual consumption of uranium in drinking water
UrinaryAs: Biomonitoring of arsenic concentrations in urine
UrinaryCu: Biomonitoring of copper concentrations in urine
UrinaryNi: Biomonitoring of nickel concentrations in urine
Ubinary: Biomonitoring of uranium in urine; binary assessment of 0 = no, 1 = yes at a concentration cutoff of 0.09 μg/l
UrinaryV: Biomonitoring of vanadium concentrations in urine