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Serum autoantibodies and exploratory molecular pathways in rural miners: A pilot study

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ABSTRACT

Introduction: The Southwestern United States (SWUS) has an extensive history of coal and metal mining, including uranium (U) mining. Lung diseases, including but not limited to, lung cancer and pulmonary fibrosis, have been studied extensively in miners due to occupational, dust-related exposures. However, high-throughput autoimmune biomarkers are largely understudied in miners, despite the fact that ore miners, such as U-miners, are at an increased risk for the development of autoimmune diseases such as systemic sclerosis and systemic lupus erythematosus (SLE). Additionally, there are current gaps in knowledge regarding which signaling pathways may play a role in occupational exposure-associated autoimmunity.

Methods: Most current and former miners in the SWUS live close to their previous workplaces, in remote areas, with limited access to healthcare. In this pilot study, by leveraging a mobile clinical platform for patient care and clinical outreach, we recruited 44 miners who self-identified as either U (n = 10) or non-U miners (n = 34) and received health screenings. Serum IgG and IgM autoantibodies against 128 antigens were assessed using a high-throughput molecular technique, as a preliminary health screening opportunity.

Results: Even when adjusting for age as a covariate, there was a significant ($p < 0.05$) association between self-reported U-mining exposure and biomarkers including IgM alpha-actinin, histones H2B, and H4, myeloperoxidase (MPO) and myelin basic protein. However, adjusting for age did not result in significant associations for IgG autoantibody production in U-miners. Bioinformatic pathway analysis revealed several altered signaling pathways between IgM and IgG autoantibodies among both U and non-U miners.

Conclusions: Further research is warranted regarding the mechanistic connection between U-exposure and autoantibody development, especially regarding histone-related alterations and IgM autoantibody production.

1. Introduction

Uranium (U) mining was a widespread occupation in the Western United States during the Cold War era [1]. U-miners were often exposed to hazardous working conditions, including inhaled mixed, metal (As, V, U, Cd) -based dusts, generated from mining and milling operations. These mining activities affected thousands of former workers in the Four Corners' geographical area of the Western United States (adjoining Utah, Colorado, Arizona, and New Mexico). More than 9100 uranium workers (U-miners, millers and ore haulers) who worked prior to Jan. 1, 1972 have received compensation through the Radiation Exposure Compensation Act (RECA) based on well-characterized, occupational diseases, such as lung cancer, that occurred during the first three decades of mining [2,3]. These awards provided compensation to only about 30% of the estimated 30,000 U-workers in the U.S. [4], as mounting evidence has demonstrated that U-mining results in deleterious health consequences [5–7]. Because these workers with former U-exposures have increased health risks, rural health surveillance and

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screening is an important tool to support former hard-rock miners who also frequently struggle to access regular health care services.

Chronic exposure to U-based dusts has been linked to an increase in autoimmune diseases such as systemic sclerosis and systemic lupus erythematosus (SLE) [8–11] and residential proximity to U-mines is associated with increased, serum-based inflammatory potential [12–15]. In addition, the detection of autoantibodies, specifically IgG, may precede development of autoimmune diseases [16]. Therefore, serum biomarker assessment for occupational settings is beneficial for prevention among vulnerable individuals susceptible to hazardous metal mixture exposures. Previously, U-miners have demonstrated an increased expression of both endothelial and soluble vascular cell adhesion molecule (VCAM-1) compared to other miners [17] which is an indication of both stimulatory molecular pathways as well as inflammatory processes due to U-based dust exposures, relative to other dusts.

Mounting evidence from community-level exposures to U-mine and milling wastes, as documented in the Diné Network for Environmental Health (DiNEH) Project, examined relationships between community health and environmental exposures among 1304 participants in partnership with 20 Navajo communities in New Mexico [18]. The study also included approximately 11% of participants (N = 143) who reported previous U-mine work exposures or jobs related to U-mining (e.g. testing U ore, truck driving in mine sites, radiation technician etc.). This project was the first comprehensive assessment of exposures to abandoned metal, mine-sites on the Navajo Nation. Survey and geospatial data from the 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), and hypertension during the legacy period after mining ceased (1986-present) [19]. This study also indicated that age and the exposure duration to legacy U-mine waste from 100 abandoned U-mine and mill sites in the DiNEH study area were associated with antibodies to denatured DNA, previously known to be an early indicator of xenobiotic-induced autoimmunity. Autoantibodies to native DNA and/or chromatin were also linked to community-level environmental exposures, more specifically, U consumption through drinking water for both men and women. These findings suggest that contaminants derived from U-mine waste enhanced the development of autoantibodies in some individuals, while other metals may exert immunosuppressive, gender-specific effects [20].

While many environmental factors that can drive immune dysfunction and subsequent autoimmune disease development [21]; growing evidence suggests that particulate matter (PM) exposure may aggravate autoimmunity [22]. Prior literature suggests that silica triggers inflammation and lung ectopic lymphoid neogenesis, in a mouse model of SLE [23]. However, gaps in the literature regarding the type of dust exposure and downstream immune impacts warrant further study. Organic occupational toxicant compounds such as trichloroethylene (TCE) have also been implicated in T-cell-mediated autoimmune development [24] and a link between cardiovascular morbidity in individuals and immune disease was recently established [25]. These exposures and disease morbidities can also play a role in U-miners' health risks and possible adverse health effects detected.

In this pilot study, we compared serum-borne autoimmune biomarkers in two groups of former workers: U-miners relative to non-U miners. In addition, we characterized signaling pathways involved most differentially expressed (6) upregulated and downregulated IgG and IgM autoantibodies in both U- and non-U miners. With the recent increase in metal mining for batteries, electronics and alternative energy sources [26,27], our study is even more pertinent to address long-term occupational risks and to understand potential toxic exposures differentially affecting miners with various metal exposures.

2. Materials and methods

2.1. Patient recruitment

The MiDUS cohort (**Mi**ning **D**ust in the **U**nited **S**tates), a previously-established cohort of miners and millers [17,28] was used to obtain biospecimens from participants with documented mining exposures (both U and non-U mining-related jobs). Miners were recruited using a rural mobile screening platform via Miners' Colfax Medical Center (MCMC), based in Raton, New Mexico, U.S.A. This mobile screening unit consists of a 53-foot-long trailer, specifically designed for patient healthcare visits and supported with adjunct power supply for employing necessary diagnostic measures and health evaluations. The MCMC mobile outreach vehicle travels to remote, rural communities in an effort to reach patients with limited access to healthcare and/or local clinics [17,28,29]. In this study, patients were recruited at three remote, rural sites: Farmington, NM, Questa, NM and Alamogordo, NM locations to ensure enrollment was available for a wide range of miners. All participants who consented to the study were required to read and understand English and agreed to provide biospecimens. Exposure status was self-reported using an occupational health and job survey and defined as any employment within the U-mining industry (U-miners) and other mining operations, such as coal, other metals or non-metal mining (non-U miners). Patient demographics and basic health parameters were previously reported in a simultaneous study assessing biomarkers of cardiovascular disease (CVD) (Table 1) and the average age of non-U miners and U-miners was 55.9 ± 16.2 and 66.9 ± 6.2, respectively [17]. Informed consent was obtained from all participant recruits. This study was approved by the University's Institutional Review Board (IRB) under the Human Research Protection Office (HRPO 14–058).

2.2. Autoantibody arrays and protein profiling

Serum samples collected at the initial visit of the MiDUS study were delivered to the University of Texas Southwestern (UTSW) Microarray Core (Dallas, TX) laboratory and stored at −80^{O}C in appropriate aliquots until experimental use. All autoantigen microarrays were developed by the UTSW Medical Center, Dallas, TX, USA, through strict quality controls of preselected and validated autoantigens. Autoantigens obtained from commercial vendors were selected, based on previous literature and prior known autoantibody involvement in immune-related diseases. Selected antigens were recombinant proteins expressed in *E. coli*, insect or mammalian cell systems. Validation of appropriate antigen recognition by autoantibodies was performed via enzyme-linked immunosorbent assay (ELISA) or Western blot, with correlations in multiple prior published studies [30–32].

Two positive controls, with four varying dilutions (anti-Ig control 1:2, anti-Ig control 1:4, anti-Ig control 1:8, anti-Ig control 1:16, Ig

Table 1
Patient demographics and health data Adapted from Ass'ad et al., 2021 [17].

	All		Non-U Miner (n = 34)		U-Miner (n = 10)	
	n	(%)	n	(%)	n	(%)
Gender						
Male	42	(95)	34	(100)	8	(80)
Female	2	(5)	0	0	2	(20)
Ethnicity						
White	11	(25)	9	(26)	2	(20)
Hispanic	27	(61)	19	(56)	8	(80)
American Indian	6	(14)	6	(18)	0	0
Current Smoker						
Yes	5	(11)	4	(12)	1	(10)
No	39	(89)	30	(88)	9	(90)
Body Mass Index						
BMI <25	6	(14)	4	(12)	2	(20)
BMI ≥ 25	38	(86)	30	(88)	8	(80)

control 1:2, Ig Control 1:4, Ig control 1:8, Ig control 1:16), were also used on the arrays. Similarly, controls were run for IgM (anti-Ig control 1:2, anti-Ig control 1:4, anti-Ig control 1:8, anti-Ig control 1:16, Ig control 1:2, Ig Control 1:4, Ig control 1:8, Ig control 1:16). Initially, serum samples were treated with DNAse I enzyme to remove free-DNA and then applied to the autoantigen array (1:50 dilution). The autoantibodies binding to the specific antigen were detected with cy3-labeled anti-human IgG and cy5-labeled anti-human IgM, and the array slides were scanned with a Genepix440A scanner at 532 nm for cy3 and 635 nm for cy5. Images were generated, and GenePix Pro 7.0 software was used to analyze the images and generate GenePix report (GPR) files. The net fluorescent intensity (NFI) of individual antigens was generated by subtracting the background and negative control (phosphate buffered saline (PBS)) from the positive signal. The signal-to-noise ratio (SNR = (Foreground Median-Background median)/standard deviation (Background)) was also generated for each antigen. SNR was used as a quantitative measure testing the resolving ability of the true signal from the background, with a higher SNR indicating a higher signal over background noise. Autoantibody score (Ab-score), which was defined by log 2 ((NFI*SNR)+1, was used for downstream analysis. NFI was normalized using a robust linear model using Ig control with multiple dilutions [32].

2.3. Statistical analysis

Differences in detectable levels of autoantibodies between U-miners and other miners (non-U miners) was assessed using Wilcoxon rank sum test. Multivariable linear analyses were then conducted for each antibody with regard to U-mining status, while adjusting for study participants' age. To adjust for multiple testing in the regression models, we used false discovery rate (FDR) correction following a previously established method [33]. Statistical significance was set at FDR adjusted $p < 0.05$. Data were analyzed using the statistical software R 4.2.1. version [34].

2.4. Pathway network analysis of autoantibody targets

The most relevant signaling pathways associated with the most differentially expressed up and down regulated autoantibodies were assessed according to upregulated and downregulated autoantibodies in the U-miners and non-U miners group. Autoantibody targets were individually evaluated and IgG and IgM levels were normalized to mean and standard deviation to 0 and 1, respectively. The data were thus cross-partitioned into 8 groups of up/down regulated, with/without U-mining and per immunoglobulin targets IgG/IgM of the detectable autoantibodies. Within each group, in order to assess the most highly differentially expressed biomarkers, the top 6 up or down regulated

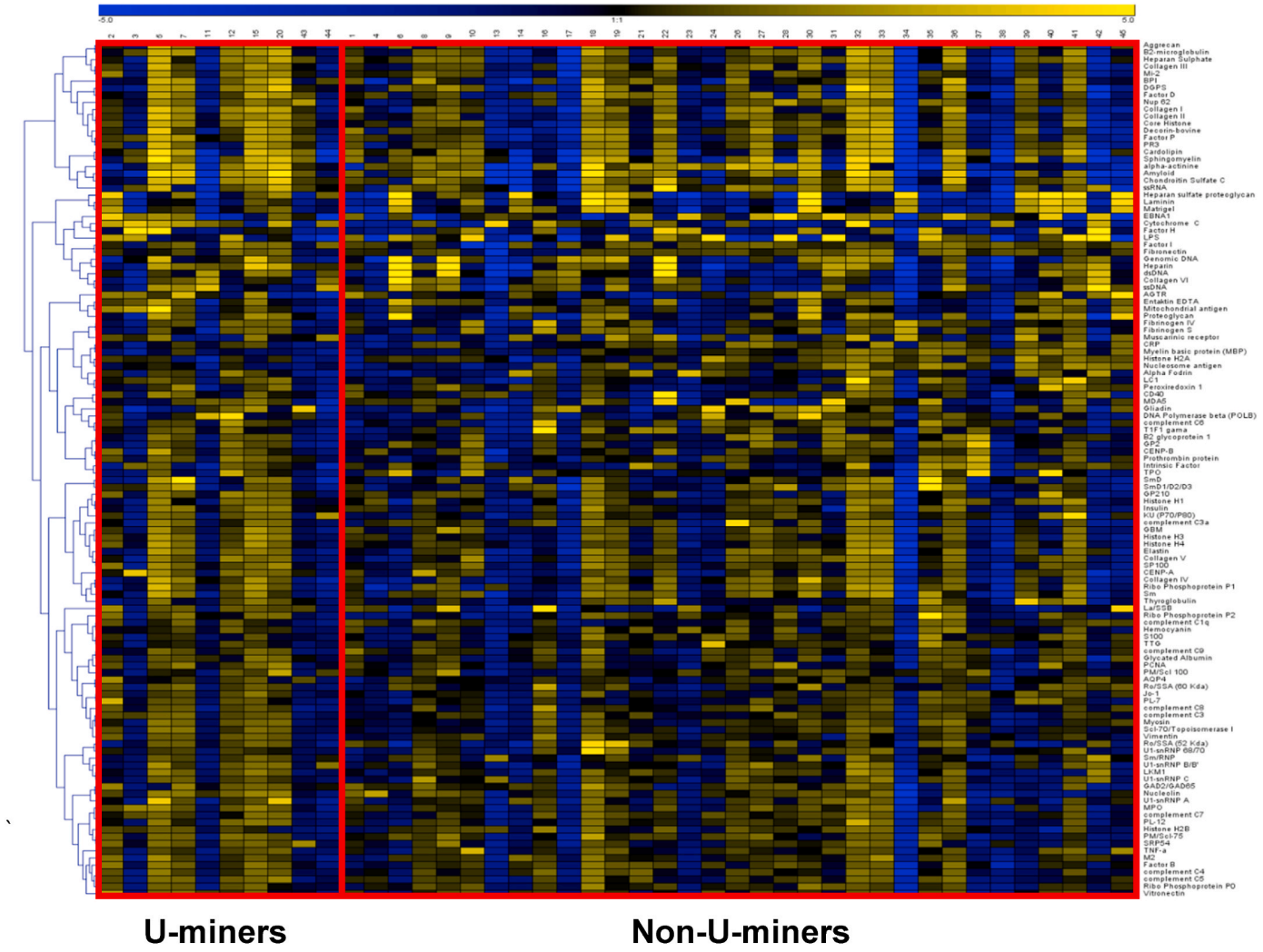

Fig. 1. Serum IgG autoantibodies High through-put screening of autoantibodies in U (left) and non-U miners. Abs = $\log_2$(NFI*SNR +1) Abs = $\log_2$(NFI*SNR +1) The terms are defined as follows: Antibody signal = Abs, Net fluorescent intensity = NFI, Signal-to-noise ratio (SNR).

targets were included in network analysis for both IgG and IgM. Pathway enrichment and network analysis were performed by R (v4.1.3) package ReactomePA (v1.38.0).

3. Results and discussion

Demographics and initial health data were previously reported for study participants from the three New Mexican study recruitment sites [17] (Table 1). Briefly, 10 U-miners and 34 non-U miners were recruited (total n = 44, 25% White, 61% Hispanic and 14% Native American). The pilot cohort of this analysis included former U-miners and other (mostly coal) miners, which represents historical trends of the New Mexico mining industry. The majority of participants were non-smokers, did not report having hypertension, did not have high cholesterol or self-report coronary artery disease and were not actively prescribed statin drugs.

Figs. 1 and 2 (enlarged: Supplemental Figs. 1 and 2) show patterns of detected serum-specific autoantigen expression according to each mining group (U vs. non-U miners). This study indicates that while there were only a few significantly altered IgG autoantibodies across mining groups, U-miners exhibited more significantly increased IgM expressions compared to non-U miners, even when adjusting for age (Table 2). The diminished IgM autoantibody expression, including histone-related IgM target autoantibodies such as histone H1, H2B, H4, H3 and IgM SmD1/D2/D3 was detectable among U-miners compared to non-U miners (Figs. 1 and 2). These data indicate possible long-term alteration in IgM autoantibodies following occupational exposures. IgM autoantibodies generally play a protective role in autoimmune disease development [35–37]. The observed IgM autoantibody downregulation is an interesting finding because extracellular, pro-inflammatory histones may be released upon NETosis production [38]. Histones that are produced by NETosis may serve as DAMPs (damage-associated molecular patterns) [39]. As described in our findings, several histone-related

Table 2
Statistically significant IgM targets. Multivariable linear regression model (adjusted for age) predicting IgM targets of specific AuAb detections among MiDUS study U miners (comparison group non U-miners).

Statistically significant IgM targets	β-coefficient estimate	SE	Test statistic (t-value)	p-value	p.- value (adjusted)
alpha-actinin	2.05	0.54	3.83	**0.00046**	**0.018**
Entactin	0.91	0.37	2.47	**0.01814**	0.218
Histone H1	−5.61	2.22	−2.53	**0.01572**	0.218
Histone H2B	−2.25	0.41	−5.48	**0.00000**	**0.000**
Histone H4	−4.52	1.30	−3.49	**0.00121**	**0.029**
MPO	−1.13	0.33	−3.41	**0.00154**	**0.031**
Myelin basic protein (MBP)	−2.04	0.39	−5.31	**0.00000**	**0.000**
SmD	−2.19	0.61	−3.61	**0.00087**	**0.026**
ssRNA	2.34	0.94	2.49	**0.01733**	0.218
U1-snRNP B/B′	−0.80	0.32	−2.49	**0.01697**	0.218
U1-snRNP C	−0.90	0.39	−2.32	**0.02587**	0.282

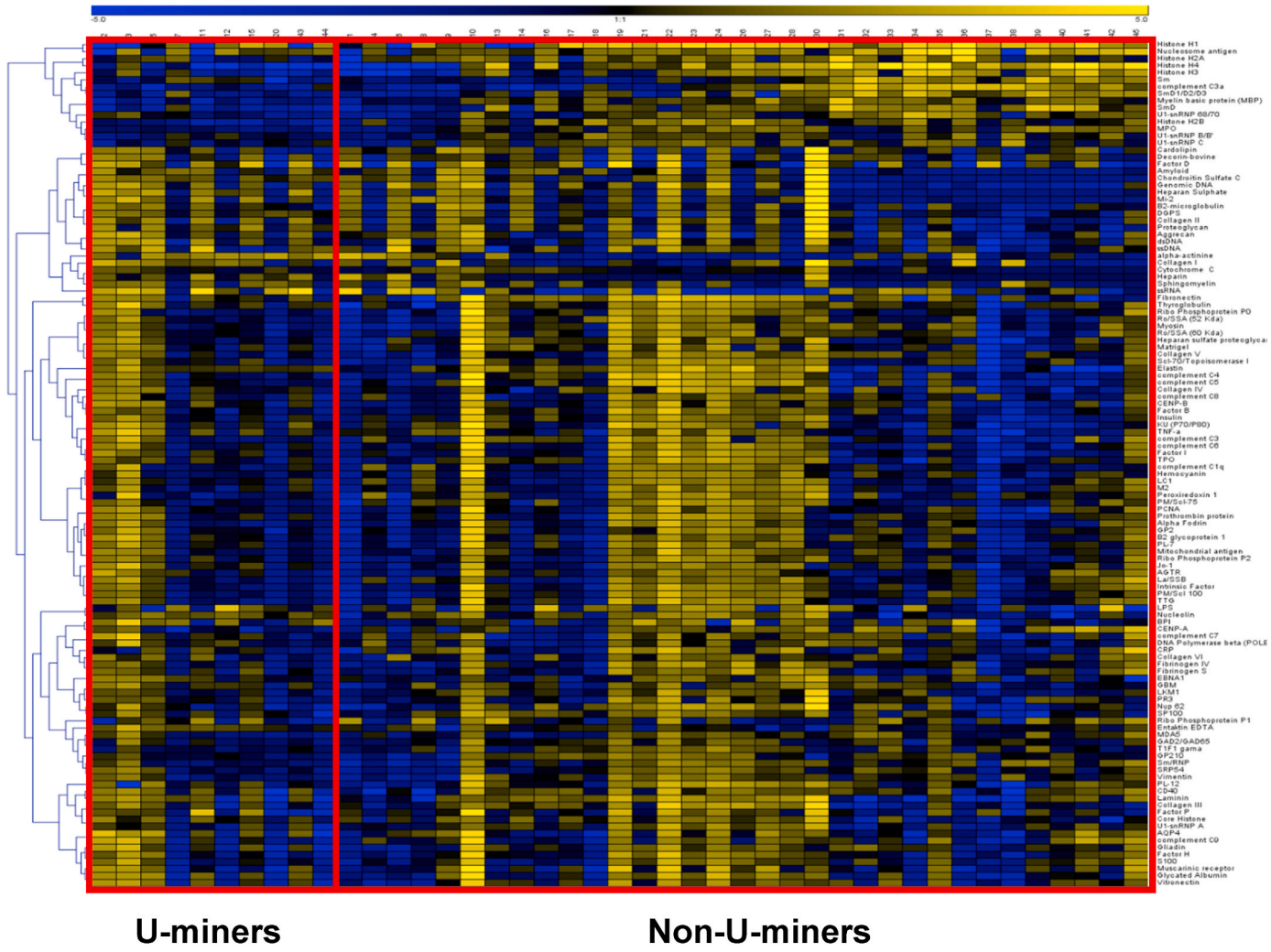

Fig. 2. Serum IgM autoantibodies High through-put screening of autoantibodies in U (left) and non-U miners.

autoantibodies exhibited a diminished response in the U-miners group, compared to other miners (Table 2). In addition, alpha-actinin IgM autoantibodies were increased in U-miners relative to non-U miners (Fig. 2, Table 2) potentially indicating autoreactivities in U-miners related to the intracellular matrix and nucleus of immune cells [40].

In the unadjusted statistical model, autoantibodies against cellular and nuclear protein expressions were significantly different between miner groups, exemplified by changes in IgG CENP-A, cytochrome C, and myelin basic protein (Table 3). Similarly, entactin IgM response was also significantly upregulated in U-miners in unadjusted model, but not when statistically adjusting for age.

The importance of examining both IgG and IgM targets of autoantibodies in these samples reflect their clinical importance and applicability to immune dysfunction [41,42]. The detectable presence of IgG autoantibodies is regularly used in clinical immunology practice to identify pathogenic expressions that are progressing toward an autoimmune phenotype [43]. However, there is not as much evidence in the current literature regarding IgM presence in autoimmune conditions, compared to IgG, with the exception of autoantibodies such as IgM rheumatoid factor [44,45]. Autoantibodies respond to certain antigens, and may serve as markers for disease or exposures [46]. Furthermore, signaling pathway analyses may give valuable insight to the impact and mechanism of uranium exposure and differences between IgG/IgM in the U-miners and non-U miners groups. Pathway analysis revealed several significant impacts with regard to molecular signaling and differential U-mining history (Supplemental Figs. 3 and 4). In IgG autoantibody signaling pathways, proteins including somatic cytochrome C (CYCS), C-Reactive Protein (CRP) and nucleoporin 210 (NUP210) were consistently involved in both U- and non-U miners (Supplemental Fig. 3) indicating overall inflammatory reactions and cellular component reactivities in miners. Only the U-miners group demonstrated involvement in pathways related to pancreatic secretory granule membrane major glycoprotein 2 (GP2) potentially showing metabolic damage and GI tract susceptibilities previously demonstrated in pre-clinical toxicological examinations of U exposures [47]. Both U- and non-U miners' IgM autoantibodies demonstrated signal pathway changes involving nidogen (NID-1) protein (formerly known as entactin) and histones (H1-0, H2B), as well as myeloperoxidase enzyme (MPO) (Supplemental Fig. 4). Both U- and non-U miners' pathways for IgM autoantibodies indicated involvement in extracellular matrix proteins and DNA damage. Only in non-U miners were decreased IgM expression pathways involved in the Rho-GTPase cycle, compared to U-miners. This seminal work may suggest that these key signaling pathways could be influenced by metal-specific occupational or environmental exposures.

3.1. Study strengths

Historically, the MiDUS cohort embodies a racially diverse population, with roughly one-third Native American, Hispanic and White participants, respectively. The mobile recruitment strategy allows for routine health screening in rural mining communities with limited access to healthcare initiatives. Individuals who enrolled in our study are typically less inclined to seek medical care in a hospital or fixed-clinic site. In addition, the high-throughput nature of autoantibody assessment is a novel way to examine immune dysfunction in an occupational cohort. High-throughput molecular assessments can be conveniently used in future studies and applied to much larger cohorts.

Using a bioinformatics approach to assessing signaling pathways related to the presence of up- or downregulated IgM and IgG autoantibodies, we have determined that autoantibody alterations may be implicated in histone-specific signaling pathways and are potentially implicated in some enzymatic involvement, such as myeloperoxidase (MPO, Table 2). With this pilot study, we have taken preliminary steps to characterizing immune biomarkers and related signaling pathways in a rural, occupational, human mining cohort with limited access to healthcare.

Table 3
Significant results generated using multivariable linear regression model (also adjusted by age) predicting IgG targets among MiDUS study U miners (comparison group non U-miners).

Statistically significant IgG targets	β-coefficient estimate	SE	Test statistic (t-value)	p-value	p.- value (adjusted)
CENP-A	1.08	0.53	2.04	**0.04802**	0.941
Cytochrome C	1.85	0.74	2.50	**0.01680**	0.941
Myelin basic protein (MBP)	−0.95	0.31	−3.06	**0.00399**	0.475

3.2. Study limitations

The most significant limitation of this study was the small sample size, which decreases statistical power. However, this was expected given the exploratory, pilot nature of the study. In addition, this is an elderly cohort and U as well as non-U exposures, occurring years prior to biospecimen collection. While determining associations with molecular markers in a historical, cohort may have limitations, it is worthwhile noting that autoimmune diseases demonstrate a latency of decades following exposure [48,49]. In addition, there is a growing body of literature investigating epigenetic features of various toxicant exposures [21,50] which indicate possible long-term effects on the immune system that are not fully investigated in the context of occupational exposures to U and other metals.

Furthermore, our analyses did not investigate the *radioactive composition* of the ore (radioactive isotopes, U decay pathway elements, and radium) nor considered radon gas exposures during mining activities. The majority of miners recruited through the MCMC mobile unit were non-U miners. Future studies will recruit from both the MCMC mobile clinic and the UNM Hospital-based clinic setting to create a larger sample size and a more equitable representation of U- and non-U miners. In addition, future approaches will include detailed biomonitoring examinations (serum/whole blood and urine samples), as well as a more comprehensive exposure analysis, including mining tenure as a variable of interest, and modeling approach with geospatial and proximity-focused statistics [51]. Detailed health outcome studies using organ- and tissue-specific autoantibodies (e.g. thyroid, liver and pancreatic autoimmune markers) are also warranted with corresponding endocrinological examinations.

4. Conclusions

These findings suggest that U-miners have differential autoantibody expression and serological patterns from non-U miners, with predominant alterations in IgM autoantibody expression. Moreover, histone-related proteins and signaling pathways may be implicated in autoimmune development [52]. Serum detection of histone-specific autoantibodies has been used in clinical laboratories for autoimmune disease diagnoses [53–55].

Although this study focused solely on the high-throughput autoantibody surveillance methodology to generate hypothesis for future research approaches, a larger environmental analytical work will also incorporate more complex multivariable modeling to adjust for several biological confounding variables (age, gender, body mass index), and socioeconomic factors impacting overall workers' health.

Routine screening of serum-specific autoantibodies among miners is a feasible way to identify autoimmune biomarkers in a long-term, occupationally cohort with potential immune dysfunction. According to our analysis, long-term alterations are detectable in serum specimens even after miners are not routinely exposed to U and other metals, even after adjusting for age. Implementing serum autoantibody surveillance in metal-exposed workers would benefit risk assessment efforts and can

improve clinical care for miners.

Further research is warranted to identify specific mechanisms of autoantibody-induced disease development following occupational exposures to dust and metal mixtures, including U, especially as the societal and technology-driven demand for energy-related metal-mining continues to grow.

Credit author statement

Esther Erdei: conceptualization, writing original draft; Xixi Zhou: visualization, formal analysis; Chris Shuey: writing- original draft; Deborah Kanda: formal analysis; Li Luo: formal analysis; Nour Ass'ad: conceptualization, data curation, project administration; Kimberly Page: methodology, project administration; Bobbi Gore: project administration; Chengsong Zhu: data curation; Akshay Sood: conceptualization, investigation; Katherine E. Zychowski: conceptualization, investigation, funding acquisition, writing and editing original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Katherine Zychowski reports financial support was provided by National Institutes of Health. Katherine Zychowski reports financial support was provided by Centers for Disease Control and Prevention.

Data availability

Data will be made available on request.

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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.2023.100197.

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