AWARD NUMBER: W81-XWH-16-2-0058

TITLE: Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments

PRINCIPAL INVESTIGATOR: Melissa McDiarmid, M.D., MPH, DABT

RECIPIENT: University of Maryland, Baltimore

REPORT DATE: October 2021

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
OCTOBER 2021	Annual	30SEPT2020 - 29SEPT2021

4. TITLE AND SUBTITLE

Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments

CONTRACT NUMBER W81XWH-16-2-0058

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: The 'signature' wound of current and recent conflicts in both Iraq and Afghanistan is that incurred via contact with improvised explosive devices (IEDs) and other high kinetic energy weapons. Beyond the traumatic injury inflicted, health risks from wound contamination with toxic metals must be managed, even as risk from these contaminants is not fully known. To provide a scientific evidence base to refine the clinical management of these patients, a multidisciplinary approach using animal models and patient data will be used. A laboratory rat model system (Project 1) will provide bio-kinetic and toxicological data on a variety of military-relevant metals implanted in the rats. (Project 2) will identify biomarkers of early effect in tissues and body fluids of the implanted animals. Using an existing national VA Embedded Fragment Registry of such injured patients, (Project 3) will assess kidney injury --the presumed target of toxic metal exposure-- and (Project 4) will assess pulmonary injury in these Veterans from both systemic metal absorption and presumed blast-induced -baro-trauma at the time.

15. SUBJECT TERMS

Embedded metal fragments, health effects, military-relevant metals, laboratory rat, toxic metals, transcriptome, registry, exposure

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	84	

Standard Form 298 (Rev. 8-98)

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The 'signature' wound of current and recent conflicts in both Iraq and Afghanistan is that incurred via contact with improvised explosive devices (IEDs) and other high kinetic energy weapons. Beyond the traumatic injury inflicted, health risks from wound contamination with toxic metals must be managed, even as risk from these contaminants is not fully known. To provide a scientific evidence base to refine the clinical management of these patients, a multidisciplinary approach using animal models and patient data will be used. A laboratory rat model system (Project 1) will provide bio-kinetic and toxicological data on a variety of military-relevant metals implanted in the rats. (Project 2) will identify biomarkers of early effect in tissues and body fluids of the implanted animals. Using an existing national VA Embedded Fragment Registry of such injured patients, (Project 3) will assess kidney injury --the presumed target of toxic metal exposure-- and (Project 4) will assess pulmonary injury in these Veterans from both systemic metal absorption and presumed blast-induced-baro-trauma at the time of injury.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Embedded metal fragments, health effects, military-relevant metals, laboratory rat, toxic metals, transcriptome, registry, exposure

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Major Task 1
Experimental Preparation
Year 1/Month 1 to Year 1/Month 6, 100% completed.

Major Task 2
Animal Ordering and Pellet Implantation Surgeries
Year 1/Month 6 to Year 3/Month 8, 100% completed.

Major Task 3
Animal Health Assessments and Urine Collections
Year 1/Month 9 to Year 3/Month9, 100% completed.

Major Task 4*
Euthanasia and Tissue Collection; Transfer of Research Samples to University of Kentucky
Year 2/Month 8 to Year 3/Month 9, 100% completed.

Major Task 5
Histopathology and Immunohistochemical Analyses
Year 2/Month 5 to Year 3/Month 11, 100% completed.

Major Task 6
Metal Analysis and Tissue Imaging
Year 3/Month 1 to Year 4/Month 12, 100% completed.

Major Task 7
Data Compilation, Statistical Analysis, and Preparation of Final Report
Year 4/Month 1 to Year 5/Month 12, 100% completed.

*(See pg. 8)

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Major Task 1
Experimental Preparation
Year 1/Month 1 to Year 1/Month 12, 100% completed.

Major Task 2
3M Experimental Group Microarray analyses
Year 2/Month 4 to Year 3/Month 10, 100% completed.

Major Task 3
12M Experimental Group Microarray analyses
Year 2/Month 8 to Year 4/Month 4, 100% completed.

Major Task 4
6M Experimental Group Microarray analyses
Year 3/Month 5 to Year 4/Month 10, 100% completed.

Major Task 5
1M Experimental Group Microarray analyses
Year 3/Month 9 to Year 5/Month 4, 100% completed.

Major Task 6
Data Compilation, Statistical Analysis, and Preparation of Final Report
Year 5/Month 5 to Year 5/Month 12, 85% completed.

PROJECTS 3 & 4:

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

The Major Tasks for Year 2 are shared by Projects 3 and 4.

Major Task 1
Questionnaire development
Year 1/Month 1 to Year 1/Month 12, 100% completed.

Major Task 2
Obtain regulatory approvals
Year 1/Month 1 to Year 2/Month 1, 100% completed.

Major Task 3
Recruitment and questionnaire administration
Year 1/Month 1 to Year 4/Month 9, 100% completed.

Major Task 4
Questionnaire analyses
Year2/Month 1 to Year 5 Month 12, 80% completed

Major Task 5
Collection and analyses of urine specimens
Year 1/Month 1 to Year 4/Month 7, 100% completed.

Major Task 6
Collection analyses of PFT and IOS findings
Year 1/Month 1 to Year 4/Month 6, 100% completed.

Major Task 7
Summarize Metal and Renal Findings
Year 2/Month 1 to Year 5/Month 12, 50% completed.

Major Task 8
Summarize PFT and IOS Findings
Year 2/Month 1 to Year 5/Month 12, 50% completed.

What was accomplished under these goals?

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

In Year 5 of the project, the remaining items in the major tasks listed above were completed. A manuscript describing the effects of metal fragments embedded in the gastrocnemius muscle of rats on the expression of neurological proteins and metal levels in discrete regions of the brain was published in *NeuroToxicology*. A copy of that publication can be found in the Appendices. Briefly, metal levels and changes in expression of proteins related to blood brain barrier tight junction formation (occludin, ZO-1) and synapse function (PSD95, spinophilin, synaptotagmin) were studied in the frontal cortex, hippocampus, amygdala, and cerebellum of rats implanted with military-

relevant metals for up to 12 months. Although few changes were observed in metal levels and blood brain barrier protein expression, a large number of synapse proteins showed reduced expression levels, particularly within the first 6 months of metal exposure.

In addition, a manuscript on the effects of embedded metal fragments on urinary biomarkers of renal damage was also published in *Toxicology Reports*, a copy of which can be found in the Appendices. Briefly, urine from metal-implanted rats was collected prior to metal exposure and then at 1, 3, 6, 9, and 12 months post-implantation. Urinary metal levels and a variety of biomarkers (albumin, alkaline phosphatase, beta-2-microglobulin, N-acetyl-beta-D- glucosaminidase, neutrophil gelatinase-associated lipocalin, osteopontin, retinal binding protein, and kidney injury molecule-1) were assessed along with total urinary protein and creatinine.

Results showed that implanted metals were rapidly solubilized and excreted in the urine. Although the kidney biomarker results were somewhat inconsistent, and may be due to the relatively low amounts of metal implanted, the data suggest that metal-induced renal effects need to be considered when caring for individuals with embedded metal fragment wounds.

An examination of protein expression changes in the muscle surrounding the embedded fragment has been completed and submitted for publication in the *International Journal of Toxicology*. The results suggest that some embedded metals can induce long-term oxidative damage, as well as affect enzyme systems involved in signal transduction. A table summarizing the salient points of the research is found in the Appendices.

Most of the research in Year 5 focused on the metal analysis of the tissues collected from the experimental rats. After processing, tissue metal levels were assessed using inductively coupled plasma-mass spectrometry. All metal analysis data can be found in the Appendices of this report.

As reported, with few exceptions, all embedded metal fragments eventually solubilize and the metals excreted in the urine at significantly high levels compared to control. Many tissues from metal-implanted rats also show significantly higher metal levels than control. Not unexpectedly, kidney from metal-implanted rats demonstrate high metal levels with the exception of rats implanted with iron, copper, and aluminum. Other tissues showing significantly elevated metal levels include liver (nickel, cobalt, lead, depleted uranium), spleen (tungsten, cobalt), testes (nickel, cobalt, depleted uranium), gastrocnemius (cobalt), lung (cobalt, depleted uranium), tibia (cobalt, lead, depleted uranium), fibula (cobalt, depleted uranium), and femur (cobalt, lead, depleted uranium). A manuscript detailing these results is currently in preparation.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2

"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Year 5 was primarily spent in bioinformatic data analyses, as all microRNAs in urine and serum from all metals at all time points have been sequenced. A paper reporting the results of

microRNA profiling in urine was published in *Biomarkers in Medicine*. Results provide potential tissue targets affected by metal exposure and a list of unique or common urine microRNA biomarkers indicative of exposure to various metals, highlighting a complex systemic response. Analyses of serum microRNA profiles reveals accumulation of unique and common microRNAs across urine and serum. Serum markers suggest kidney involvement, particularly with Al, Pb and DU. As a result, we performed mRNA sequencing of kidney RNA from the 12-month time point and found higher expression of genes involved in oxidative stress and altered cell function in kidney, particularly associated with DU. We also quantified the expression level of 9 microRNAs that were altered in serum at 12 months by RT-PCR of kidney RNA. There appears to be an inverse correlation between abundance of these microRNAs in kidney and serum, suggesting they may be useful biomarkers of adverse effects of embedded metal on kidney function. Integration of the mRNA and microRNA data sets is nearing completion and a manuscript is in preparation. A manuscript on the potential contribution of Ni and Co to tumor formation is also in preparation.

Award PI, Dr. McDiarmid continued quarterly meetings with Project 1 and 2 PIs, Drs. Kalinich and Peterson throughout the year. With the completion of all major tasks in Project 1 and 97% of major tasks in Project 2, the extension year will be spent primarily in manuscript production.

PROJECTS 3 & 4:

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator Project 3
"Biomarker Assessment of Kidney Injury from Metal Exposure in Embedded Fragment Registry Veterans"

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator Project 4
"Respiratory Health in a Cohort of Embedded Fragment Registry Veterans Exposed to Blasts and Metals"

This project consists of two different populations of Veterans who are selected from the VA Toxic Embedded Fragment Registry to either receive an invitation to complete a questionnaire (Study Population #1), or to participate in a clinical assessment visit (Study Population #2).

Study Population #1 – Questionnaire Only Group

During Year 5, we completed the data entry and validation process of all paper surveys. In January 2021, the online survey portal was taken off-line and all results from the online surveys were automatically captured in the study database. In total, questionnaires were sent to 9,000 eligible Veterans. We received a total of 2,396 completed questionnaires. The overall response rate has been approximately 30%. (Major Task 3 -Recruitment and Questionnaire Administration).

We analyzed the data to assess the effect of having an embedded metal fragment on the overall health status of affected Veterans. Using responses to injury-focused questions, we categorized Veterans into high or low risk groups based on their likelihood of having an embedded fragment from a bullet or blast injury. Physical and mental health status were determined using questions from the Veterans-Rand 12-item survey. Our early findings suggested that there were no significant differences in physical health status between the two groups; however, Veterans in the high-risk group for having a retained fragment had significantly higher mental health scores, indicating better general mental health. One potential explanation is that Veterans in the high-risk category may have experienced more severe injuries and therefore received more medical care and support services. These findings were shared during a poster presentation at the Society of Toxicology Conference in March 2021. A copy of

this poster is included in this report. Additional data analyses have been completed and manuscript preparation is underway.

During data analysis, we also found remarkable associations between respiratory symptoms related to self-reported blast using the DOD's Brief Traumatic Brain Injury Screen. We submitted a subset of these findings as an abstract to the 2021 Military Health Systems Research Symposium, and it was accepted for oral presentation. Unfortunately, this conference was cancelled; however, these results were updated and an abstract is being prepared for submission to the American Thoracic Society 2022 Conference. We are also currently preparing a manuscript describing these findings and anticipate submitting to the journal *Chest*.

In May, we presented a virtual, on-demand poster presentation at the American Thoracic Society 2021 conference discussing auto-coding of free text responses about occupational & environmental exposures into US Census-based Industry & Occupational codes among VA TEF (Toxic Embedded Fragment) registry participants who completed the study questionnaire. A copy of this poster is included in this report.

Study Population #2 – Clinical Assessment Group

During Year 5, recruitment continued until our overall targeted recruitment goal of 421 participants was reached in quarter 2. The study team continued to double-enter data from all study questionnaires into the Clinical Assessment database. (Major Task 4 – Questionnaire Analysis).

We continued to review available participant images containing metal fragments and plan to assess the association between imaging findings and metal concentrations in the upcoming year.

During the last quarter, we received the final renal marker results. The laboratory had reported delays in receiving the reagents needed to analyze all remaining samples, but we now have a complete dataset and have started to perform preliminary data analyses. All urine metal results have been entered into the study database and final QC checks were performed by Dr. Gaitens.

Over the past year we have continued to have biweekly data meetings to review the status of data entry, discuss preliminary findings, and outline plans for upcoming analyses.

We also continued to hold quarterly meetings with all VA collaborators, and on September 28, 2021 we held our final quarterly videoconference to review their achievements and to discuss data findings and plans for the coming year. We had initially planned to hold the meeting in-person in Baltimore, but due to the increase in cases of COVID-19, we decided to postpone to June 2022. This will allow us to present all data findings and further discuss study close-out procedures. Biweekly conference calls continued between all site research coordinators.

An additional abstract was presented at the American Thoracic Society 2021 conference in May (held virtually) discussing associations between systemic metal exposure and pulmonary function categorical abnormalities. A copy of the poster is included in this report.

Preliminary findings describing the association between elevated metal concentrations and renal injury markers were also presented virtually during an oral presentation at the Society of Toxicology conference in March 2021. In addition, these findings were included in an oral presentation, held virtually, to medical students at the University of Modena and Reggio Emilia Medical School, Modena, Italy.

What opportunities for training and professional development has the project provided?

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report

PROJECTS 3 & 4:

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator Project 3
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Dr. Katherine Chin and Dr. Maxwell Reback, two post-doctoral research fellows who had been enrolled in a Pulmonary and Critical Care fellowship training program at University of Maryland Medical Center, continued contributing to the project in Year 5. Similar to their predecessor, Dr. Danielle Glick, who remained an active member of the research team during her final year of pulmonary training, they acquired unique expertise and skills in impulse oscillometry testing that they would not have received in their fellowship training otherwise, which will be useful through the rest of their careers. All three research fellows had scientific abstracts accepted for scholarly presentations at national meetings in 2021 and had the opportunity to present the abstracts virtually. Dr. Chin and Dr. Reback completed their programs in July 2021.

Additionally, Jessica Palmer, a third-year medical student at University of Maryland, participated in a summer research internship with our team from May to August 2020. Jessica had successfully applied for and received a stipend award from the University's competitive Program for Research Initiated by Students and Mentors (PRISM) for her work on this project. She used this opportunity to gain experience in research and had the opportunity to present findings in several different forums. In Year 5, she continued to work with Dr. Gaitens and Dr. Hines and presented a poster describing the overall health status of veterans with embedded fragments at the Society of Toxicology Conference in March 2021.

How were the results disseminated to communities of interest?

Nothing to report beyond already cited abstracts, publications, and presentations.

What do you plan to do during the next reporting period to accomplish the goals?

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

The final extension year of the project will be spent writing and submitting final manuscripts from the project.

PROJECTS 3 & 4:

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
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Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Year 6 activities will focus heavily on analyses of complete data sets and preparation of scholarly manuscripts for publication. Drs. Gaitens, Hines, and Brown (statistician) have scheduled and agreed upon timing of analyses and writing tasks to assure that planned publications may be prepared during this year.

For Study Population #1 (Questionnaire Only), we will draft manuscripts on the effect of embedded fragments on overall health status and the correlation between blast exposure and respiratory health. We plan to submit the results of final analyses on blast and reported respiratory symptoms to the national meeting of the American Thoracic Society.

For Study Population #2 (Clinical Assessment), we plan to assess the association between elevated metal concentrations and self-reported renal outcomes. Additionally, we will investigate the association between elevated metal concentrations and concentrations of renal injury markers after adjusting for known chronic kidney disease risk factors. We will submit an abstract describing these results to the Society of Toxicology Conference. We will also assess the correlations between specific metals and renal injury markers and investigate associations between imaging findings and metal concentrations.

In addition, we will examine the association between metal concentrations and respiratory health outcomes, including symptoms, pulmonary function test results, and impulse oscillometry results. We will also evaluate associations between self-reported blast exposure and pulmonary physiological outcomes from pulmonary function tests and impulse oscillometry.

Manuscripts will be drafted describing all study findings.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

The large array of changes in microRNAs in urine and serum in response to different metals suggests that biomarkers will be identified which may have predictive value for long term health consequences in warriors harboring embedded metals. We may also identify specific effects of metals embedded in muscle on kidney function over time.

PROJECTS 3 & 4:

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
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"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Actual or anticipated problems or delays and actions or plans to resolve them.

In late Spring, PI, Dr. Melissa McDiarmid had several discussions with then assigned Award Science Officer, Dr. Zach Zabarsky and Contract Specialist, Lisa Sawyer regarding a request for an extension into year 6, due to some project delays caused by Covid. This request was granted. Work in all four projects in the extension period will primarily focus on data analysis and manuscript writing.

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report.

PROJECTS 3 & 4:

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Study Population #1:

During our preparation of our final data set, we learned that a server of the VA's Cooperative Studies Program – Perry Point (which houses our questionnaire data), had experienced a problem in being able to add additional data received after December 2020. This required us to troubleshoot and create a supplemental data base to capture a limited number of final questionnaire responses that were received via mail. With a series of data management strategies, we have been able to merge all of our data (captured before December 2020) with the limited amount of data received after December 2020, into a single, final data file.

Study Population #2:

We had anticipated having the final set of renal injury marker results in late January 2021, allowing final analyses to begin. However, due to COVID-19 restrictions in Belgium and the analytical laboratory, as well as a shortage of reagents needed to run analysis, the analyses of the samples were delayed. Additionally, delayed mail service was reported to be an ongoing issue, resulting in partial completion of the study protocol for several Veterans as the study team awaited the return of their pending questionnaires.

All of the renal marker results and questionnaires have since been received and analysis has begun. We anticipate completing analysis and drafting of manuscripts by end of year 6.

Changes that had a significant impact on expenditures

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report

PROJECTS 3 & 4:

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Significant changes in use or care of human subjects:

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report. There were no modifications to the protocols in Year 5.

Significant changes in use or care of vertebrate animals:

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications:

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

1) Jessica F. Hoffman, Vernieda B. Vergara, and John F. Kalinich, "Brain region- and metal-specific effects of embedded metals in a shrapnel wound model in the rat". *NeuroToxicology* 83: 116-128 (2021). (doi.org/10.1016/j.neuro.2021.01.001)

2) Jessica F. Hoffman, Vernieda B. Vergara, Anya X. Fan, and John F. Kalinich, "Effect of embedded metal fragments on urinary metal levels and kidney biomarkers in the Sprague-Dawley rat". *Toxicology Reports* 8: 463-480 (2021). (doi.org/10.1016/j.toxrep.2021.02.023)

3) Diane Smith, Todor Todorov, Adrian Defante, Jessica Hoffman, John Kalinich, and Jose Centeno, "Spectroscopic and spectrometric approaches for assessing the composition of embedded metals in tissues". *Applied Spectroscopy* 75: 661-673 (2021). (doi.org/10.1177/0003702820979748).

4) Ivan J. Vechetti, Jr., Yuan Wen, Jessica F. Hoffman, Alexander P. Alimov, Vernieda B. Vergara, John F. Kalinich, Joanna M. Gaitens, Stella E. Hines, Melissa A. McDiarmid, John J. McCarthy, Charlotte A. Peterson, "Urine miRNAs as potential biomarkers for systemic reactions induced by exposure to embedded metal". *Biomarkers in Medicine* 15: 1397-1410. (doi.org/10.2217/bmm-2021-0120).

5) Jessica F. Hoffman, Vernieda B. Vergara, and John F. Kalinich. "Protein Expression in the Gastrocnemius Muscle of Metal-Implanted Sprague-Dawley Rats", *International Journal of Toxicology* (submitted).

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Vechetti, I.J., Y. Wen, J.F. Hoffman, A.P. Alimov, V.B. Vergara, J.F. Kalinich, J.M. Gaitens, M.A. McDiarmid, J.J. McCarthy, C.A. Peterson (2021). Urine miRNAs as potential biomarkers for systemic reactions induced by exposure to embedded metal. *Biomarkers in Medicine*, doi: 0.2217/bmm-2021-0120.

PROJECTS 3 & 4
Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
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Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4

"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Books or other non-periodical, one-time publications.

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report.

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Nothing to report.

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

Other publications, conference papers and presentations.

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

1) Diane Smith, Todor Todorov, Adrian Defante, Jessica Hoffman, John Kalinich, and Jose Centeno, "Spectroscopic and spectrometric approaches for assessing the composition of embedded metals in tissues" 2021 Society of Toxicology Virtual Meeting, March 12-26, 2021.

2) Diane Smith, Todor Todorov, Adrian Lita, Jessica Hoffman, John Kalinich, and Jose Centeno. "Chemical Mapping Study of Trace Metal and Protein Secondary Structure Changes after Metal Implantation" 2021 FDA Science Forum, May 21, 2021.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report.

PROJECTS 3 & 4
Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Abstracts:

1) Palmer J, Hines SE, McDiarmid MA, Brown C, Gaitens J. 2021. Self-Reported General Health Status Among Veterans with Embedded Metal Fragments [Abstract, Poster session]. In: *The Toxicologist:* Supplement to *Toxicological Sciences*, Volume 180(S1), Society of Toxicology Conference, held virtually. Abstract no. 2026.

2) Chin K, McDiarmid MA, Gaitens JM, Marovich S, Purdin J, Schumacher PK, Cornell J, Reback MA, Glick DR, Hines SE. Use of the National Institute for Occupational Safety and Health (NIOSH) Industry & Occupation Computerized Coding System (NIOCCS) to Classify Free Text Occupational Exposure Responses in a US Military Population. Am J Respir Crit Care Med. 2021:203:A3052. American Thoracic Society Conference, held virtually, May 14-19, 2021.

3) Reback MA, McDiarmid M, Gaitens J, Brown CH, Chin KH, Glick DR, Sriram PS, Lawson WE, Cavanaugh K, Beck L, Duch J, Hines SE. Pulmonary Function Categorical Abnormalities in Veterans with Embedded Metal Fragments: An Interim Analysis. Am J Respir Crit Care Med. 2021:203:A3060. American Thoracic Society Conference, held virtually, May 14-19, 2021.

4) Gaitens J, McDiarmid M. 2021. Biomarkers of Exposure and Early Effect in the Medical Surveillance of War-Injured Veterans with Retained Metal Fragments. [Abstract; Symposium session]. In: *The Toxicologist:* Supplement to *Toxicological Sciences*, Volume 180(S1), Society of Toxicology Conference, held virtually. Abstract no. 1116. [This presentation included preliminary renal injury results from this study.]

Research Presentations:

1) Jessica Palmer. "General health status among Veterans with embedded metal fragments", University of Maryland 43rd Annual School of Medicine Medical Student Research Day (MSRD) (virtual), November 12, 2020.

2) Katherine Chin, MD. "Long Term Pulmonary Effects of Primary Blast Injury: The Plot Thickens." University of Maryland School of Medicine, Division of Pulmonary and Critical Care Grand Rounds, June 1, 2021.

3) Stella Hines, MD, MSPH. "Lung Disease in Post 9/11 Military Deployers." Mount Sinai Department of Environmental Medicine, Division of Occupational Medicine Grand Rounds, April 9, 2021.

4) Joanna Gaitens, PhD, MSN/MPH. "The U.S. Veteran Embedded Fragment Registry: Metal Exposure and Potential Adverse Health Effects" presented at University of Modena and

Reggio Emilia Medical School, Modena, Italy, June 2021. [This presentation included preliminary renal injury results from this study.]

- **Website(s) or other Internet site(s)**

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report.

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Nothing to report.

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

- **Technologies or techniques**
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report.

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Nothing to report.

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

- **Inventions, patent applications, and/or licenses**

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report.

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Nothing to report.

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

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- **Other Products**

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

The muscle microarray and RNAseq data were deposited in the NCBI Gene Expression Omnibus (*GEO*) repository. The data can be found in Series GSE157931, which includes both the microarray (GSE157921) and the RNA-seq (GSE157929) data.

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Melissa McDiarmid, M.D., Principal Investigator:
"Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments"

Name: Melissa McDiarmid, M.D.
Project Role: Principal Investigator
Nearest Person Month worked: 2.40
Contribution to Project: Dr. McDiarmid oversaw conduct and progress of all four study projects and participated in quarterly project team calls.

Name: Rachel Coates-Knowles, MSM
Project Role: Finance Manager
Nearest Person Month worked: 6.0
Contribution to Project: Maintained and processed all financial transactions and reporting.

Name: Clayton Brown
Project Role: Statistician
Nearest Person Month worked: 1.2
Contribution to Project: Provided input on data entry processes and conducted preliminary data analyses.

Name: Sheila Williams
Project Role: Administrative Assistant
Nearest Person Month worked: 1.20
Contribution to Project: Assisted with procurement, document preparation, and scheduling teleconferences.

John F. Kalinich, Ph.D., Principal Investigator, Project 1:
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Name: John Kalinich, PhD
Project Role: Principal Investigator, Project 1

Researcher Identifier: 0000-0003-1591-9389
Nearest person month worked: 2 (no change)
Contribution to Project: Responsible for overall functioning of this portion of the project.
Funding Support: Federal Government Employee (Department of Defense)

Name: Christine Kasper, PhD RN, FAAN FACS
Project Role: Co-Investigator,
Research Identifier: 0000-0002-7784-2519
Nearest person month worked: 1 (no change)
Contribution to Project: Responsible for experimental planning
Funding Support: School of Nursing, University of New Mexico.

Name: Jessica Hoffman, PhD
Project Role: Co-Investigator
Researcher Identifier: 0000-0003-1858-8394
Nearest person month worked: 2.5 (decreased from 5)
Contribution to Project: Member of the surgical implantation and euthanasia teams. Responsible for protein analysis of collected tissues.
Funding Support: Federal Government Employee (Department of Defense)
Note: Dr. Hoffman left the project on February 12, 2021.

Name: Vernieda Vergara, BS
Project Role: Research Assistant
Nearest person month worked: 11 (decreased from 12)
Contribution to Project: Responsible for implantation surgeries and animal welfare, sample collection and metal analysis.
Note: Ms. Vergara left the project on August 27, 2021.

Name: Jose Centeno, PhD, FRSC
Project Role: Co-investigator
Nearest Person Month worked: 1 (no change)
Contribution to Project: Oversight of metal imaging experiments and post-doctoral fellow
Funding Support: Retired Federal Government Employee (U.S. Food and Drug Administration)

Name: Diane Smith, PhD
Project Role: Post-doctoral fellow
Nearest person month worked: 7 (decreased from 12)
Contribution to Project: Metal imaging experiments under the direction of Dr. Centeno
Note: Dr. Smith left the project on April 10, 2021.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2:
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Name: Charlotte A. Peterson, PhD
Project Role: Principal Investigator, Project 2
Nearest person month worked: 1.2 (no change)
Contribution to Project: Responsible for overall functioning of this portion of the project.
Funding Support: University of Kentucky

Name: John J. McCarthy, PhD
Project Role: Co-Investigator
Nearest person month worked: 1.2 (no change)
Contribution to Project: Responsible for experimental planning
Funding Support: University of Kentucky

Name: Alexander Alimov
Project Role: Research Scientist II
Nearest person month worked: 12
Contribution to Project: Responsible for exosome isolation and characterization (Western blot analysis) and RNA isolation.

Name: Yuan Wen, MD, PhD
Project Role: Post-doctoral scholar
Nearest person month worked: 12
Contribution to Project: Dr. Wen replaced Dr. Vechetti on the project. He is an accomplished bioinformatician.

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Lead Investigator/ Local Site PI, Project 3:
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Name: Joanna Gaitens, PhD, MSN/MPH
Project Role: Project Lead Investigator/ Local Site PI
Nearest person month worked: 2.4
Contribution to Project: Responsible for overall functioning of this portion of the project, including overseeing recruitment, enrollment, data collection, specimen collection, regulatory protocols, and project team meetings.

Stella Hines, M.D., MSPH, Project Lead Investigator/ Local Site PI, Project 4:
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Name: Stella Hines, MD, MSPH
Project Role: Project Lead Investigator/ Local Site PI
Nearest person month worked: 2.4
Contribution to Project: Responsible for overall functioning of this portion of the project, including overseeing recruitment, enrollment, data collection, pulmonary testing, regulatory protocols, and project team meetings.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. John Kalinich's CDMRP-funded Gulf War Illness grant has been successfully completed.

What other organizations were involved as partners?

Participant Enrollment Sites – Clinical Collaboration

Baltimore VAMC (Site 1)
Joanna Gaitens and Stella Hines are the Local Site Principal Investigators for the Baltimore recruitment site. Their contributions to the projects are listed above.

Name: Kate Agnetti, BS
Project Role: Research Coordinator
Nearest person month worked: 6
Contribution to Project: Interacted with HRPO and regulatory bodies in order to obtain and maintain required approvals; collected data and specimens; organized and participated in quarterly project team calls and biweekly site calls.

Name: Christopher Crayton
Role on Project: Database Manager
Nearest person month worked: 6
Contribution to Project: Data entry and database maintenance

Nashville (Site 2):

Name: Kerri Cavanaugh, MD MHS
Project Role: Local Site Investigator
Nearest person month worked: 1.2
Contribution to Project: Acquired and maintained required approvals; oversaw local recruitment, enrollment, specimen collection; participated in quarterly project team calls.

Name: William Lawson, MD
Project Role: Local Site Investigator
Nearest person month worked: 0.6
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls.

Name: Audrey Tesi
Project Role: Local Study Coordinator
Nearest person month worked: 5 (Audrey left the project in May 2021)
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls, attended biweekly site calls; recruited and enrolled participants; collected data and specimens.

Gainesville (Site 3):

Name: Perevumba Sriram, MD
Project Role: Local Site Investigator
Nearest person month worked: 0.6
Contribution to Project: Acquired and maintained required approvals; oversaw local recruitment, enrollment, specimen collection; participated in quarterly project team calls.

Name: Nataliya Kirichenko
Project Role: Local Study Coordinator
Nearest person month worked: 1.5 (Decreased from 4.5 months)
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls and biweekly site calls; recruited and enrolled participants; collected data and specimens.
Note: Nataliya left the project in June, 2021.

Name: Katherine Solis
Project Role: Local Study Coordinator
Nearest person month worked: 4.5
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls, attended biweekly site calls; recruited and enrolled participants; collected data and specimens.

Name: Paige Webb (Formerly Gustad)
Project Role: Local Regulatory Assistant
Nearest person month worked: 2 (increased from 1.2 months)
Contribution to Project: Interacted with local HRPO and regulatory bodies

Oklahoma City (Site 4):

Name: Lisa Beck, MD
Project Role: Local Site Investigator
Nearest person month worked: 1.8
Contribution to Project: Acquired and maintained required approvals; oversaw local recruitment, enrollment, specimen collection; participated in quarterly project team calls.

Name: Vickie Phillips
Project Role: Local Study Coordinator
Nearest person month worked: 7.2
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls, attended biweekly site calls; recruited and enrolled participants; collected data and specimens.
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San Antonio (Site 5):

Name: John Duch, MD
Project Role: Local Site Investigator
Nearest person month worked: 1.2
Contribution to Project: Acquired and maintained required approvals; oversaw local recruitment, enrollment, specimen collection; participated in quarterly calls.

Name: Antonio Anzueto, MD
Project Role: Local Site Investigator
Nearest person month worked: 1.2
Contribution to Project: Acquired and maintained required approvals.

Name: Alex Aguilera
Project Role: Local Study Coordinator
Nearest person month worked: 1.2
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls and biweekly site calls; recruited and enrolled participants.

Name: Myra Mireles
Project Role: Local Study Coordinator
Nearest person month worked: 12

Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls, attended biweekly site calls; recruited and enrolled participants; collected data and specimens.

Phoenix (Site 6):

Name: Paska Permana, PhD
Project Role: Local Site Investigator
Nearest person month worked: 1.2
Contribution to Project: Acquired and maintained required approvals; oversaw local recruitment, participated in quarterly calls.

Name: Samuel Aguayo, MD
Project Role: Local Site Investigator
Nearest person month worked: 1.2
Contribution to Project: Acquired and maintained required approvals; oversaw local recruitment, participated in quarterly calls.

Name: Kelli Bingham
Project Role: Local Study Coordinator
Nearest person month worked: 12
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls, attended biweekly site calls; recruited and enrolled participants; collected data and specimens.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to report.

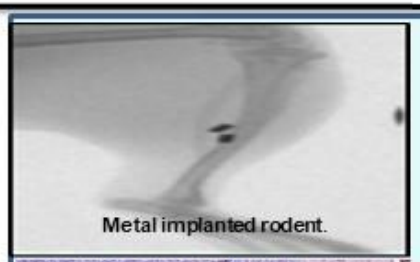
QUAD CHART

Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments
ERMS/Log Number PR151808
W81XWH-16-2-0058

PI: Melissa McDiarmid, M.D. , M.P.H. **Org:** University of Maryland, Baltimore **Award Amount:** $7,967,578

Study/Product Aim(s)
To provide a scientific evidence base to refine the clinical management of the Veteran or Service member with retained, embedded metal fragments.

Approach
A multidisciplinary approach using animal models and patient data will be used. Simulated metal fragment wounds will be studied using rodents surgically implanted with various metals of toxic concern. In **Project 1**, tissues surrounding the implant will be studied for histopathology, immunochemistry and neoplastic change. **Project 2** will attempt to identify early biomarkers of potential malignant transformation in skeletal muscle, urine and serum from these implanted animals. **Project 3** will assess kidney injury (the presumed target of toxic metal exposure) in Embedded Fragment Registry Veterans and **Project 4**, will assess pulmonary injury in these Veterans both from systemic metal absorption and presumed blast-induced –baro-trauma at the time of injury.



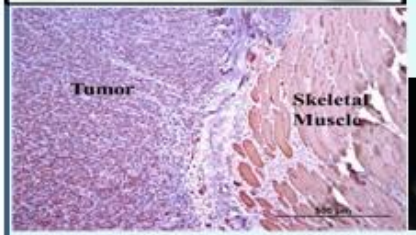


Fig. 2: Desmin-staining of tumor and muscle From metal implant (Project1)

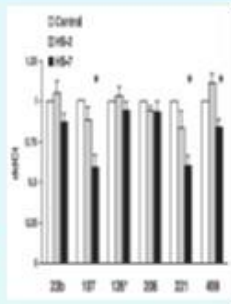
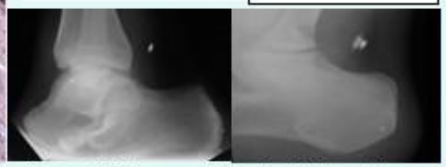


Figure 5. qRT-PCR validation of differentially expressed microRNAs in rat skeletal muscle implanted with metals **(Project 2).** Asterisk denotes significantly ($p < 0.05$) different from control.



X-ray of Veteran with embedded metal fragment de-forming (Projects 3 & 4).

Timeline and Cost

Activities CY	2017	2018	2019	2020	2021
PRJ 1: Health Effects of Embedded Fragments of Military-Relevant Metals	100%				
PRJ 2: Biomarkers for Assessing Return-to-Duty Potential of Personnel	97%				
PRJ 3: Biomarker Assessment of Kidney Injury from Metal Exposure	85%				
PRJ 4: Respiratory Health in Cohort of Embedded Fragment Registry Veterans					
Estimated Budget ($Mil)	$1.0	$1.8	$1.9	$1.8	$1.2

Updated: October 30, 2021

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Goals/Milestones
Project 1: Project complete.
Project 2: : Manuscript on microRNA biomarkers of embedded metal exposure in urine is under review. Manuscript on microRNA biomarkers in serum is in preparation.
Projects 3 & 4: Twenty-two new participants were enrolled (totaling 421; 100% of targeted number). Received a total of 2,396 completed surveys (96% of targeted number). Final data analyses have begun. Final quarterly call held on September 28, 2021.
Comments/Challenges/Issues/Concerns: Projects 3& 4- Recruitment was limited due to delays in manufacturing of urine collection kits and significant delays involving the postal service.

Budget Expenditure to Date (9/30/16 – 09/29/2021)
Projected Expenditure: $ 7,967,579
Actual Expenditure: $6,834,987

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

John F. Kalinich, Ph.D., Principal Investigator, Project 1:
"Health Effects of Embedded Fragments of Military-Relevant Metals"

APPENDICES (continued):

Projects 3 & 4
Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Lead Investigator/ Local Site PI, Project 3:
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Stella Hines, M.D., MSPH, Project Lead Investigator/ Local Site PI, Project 4:
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Poster Presentations:

1) Palmer J, Hines SE, McDiarmid MA, Brown C, Gaitens J. 2021. Self-Reported General Health Status Among Veterans with Embedded Metal Fragments [Abstract, Poster session]. In: *The Toxicologist:* Supplement to *Toxicological Sciences,* Volume 180(S1), Society of Toxicology Conference, held virtually. Abstract no. 2026.

2) Chin K, McDiarmid MA, Gaitens JM, Marovich S, Purdin J, Schumacher PK, Cornell J, Reback MA, Glick DR, Hines SE. Use of the National Institute for Occupational Safety and Health (NIOSH) Industry & Occupation Computerized Coding System (NIOCCS) to Classify Free Text Occupational Exposure Responses in a US Military Population. Am J Respir Crit Care Med. 2021:203:A3052. American Thoracic Society Conference, held virtually, May 14-19, 2021.

3) Reback MA, McDiarmid M, Gaitens J, Brown CH, Chin KH, Glick DR, Sriram PS, Lawson WE, Cavanaugh K, Beck L, Duch J, Hines SE. Pulmonary Function Categorical Abnormalities in Veterans with Embedded Metal Fragments: An Interim Analysis. Am J Respir Crit Care Med. 2021:203:A3060. American Thoracic Society Conference, held virtually, May 14-19, 2021.

Table 1: Summary of Protein Expression Changes in Metal-Implanted Rat Gastrocnemius Muscle

		Time Post-Implantation			
Marker		1M	3M	6M	12M
Desmin	59 kDa		**Co, Fe, Cu**		
eNOS	97 kDa			Pb, DU	
iNOS	100 kDa			**Co, Cu**, Al, Pb, DU	Pb, DU
MMP-2	93 kDa			Pb, DU	
	58 kDa	**Fe**			
	47 kDa	**Fe, Cu**		Pb	**Al, DU**
MMP-9	92 kDa		**W**		**Ni**
HNE-modified	35 kDa	Cu		**DU**	**Co, Cu**
	40 kDa			**Fe**	**Fe, Pb, DU**
	55 kDa		**Fe**	**Co, DU**	**Fe, Cu, Al**
	62 kDa				**Cu, Pb**
	95 kDa		**Fe, DU**	**Fe**	**Fe, Pb**
	144 kDa		**Fe, Pb**		**Fe, Pb**

Where there is a significant post-hoc value difference of metal-implanted versus tantalum, **black** = increased over Ta-implanted animals, gray = decreased over Ta-implanted animals.

Table 2: Kidney metal levels in 1-, 3-, 6-, and 12-month experimental groups.

1 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.44 ± 0.13	1.43 ± 0.13	11.08 ± 0.98	5629.74 ± 658.59	399.01 ± 33.93	7426.37 ± 779.98	0.42 ± 0.03	0.26 ± 0.03
Implanted Metal	1.99 ± 0.27*	63.63 ± 5.09*	39.47 ± 6.92*	5258.43 ± 489.11	479.11 ± 61.80	8241.63 ± 672.40	1.67 ± 0.25*	49.75 ± 8.83*

3 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.19 ± 0.07	0.82 ± 0.07	10.62 ± 0.80	6528.28 ± 694.91	515.77 ± 41.58	6208.83 ± 487.62	0.34 ± 0.02	0.21 ± 0.02
Implanted Metal	2.16 ± 0.29*	73.95 ± 7.98*	54.21 ± 3.93*	8392.16 ± 887.89	599.35 ± 226.53	11393.4 ± 3189.85*	1.64 ± 0.27*	59.79 ± 16.13*

6 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.82 ± 0.19	1.32 ± 0.16	12.85 ± 0.62	14293.25 ± 1019.62	902.27 ± 48.91	10257.71 ± 585.00	0.46 ± 0.20	0.32 ± 0.01
Implanted Metal	3.88 ± 0.62*	35.24 ± 5.25*	54.83 ± 3.04*	12306.82 ± 1221.95	857.28 ± 81.43	14549.48 ± 890.64	2.99 ± 0.32*	54.04 ± 12.62*

12 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.51 ± 0.12	0.70 ± 0.05	7.15 ± 0.41	15129.96 ± 1618.03	626.84 ± 59.83	5408.22 ± 469.46	0.24 ± 0.01	0.15 ± 0.00
Implanted Metal	1.76 ± 0.16*	ND	53.92 ± 4.60*	15185.11 ± 737.34	541.246 ± 86.09	7887.81 ± 1138.18	1.26 ± 0.09*	143.03 ± 24.45*

Data are the mean of 8 individual determinations and expressed as ng metal/gm tissue. Errors are standard error of the mean. An * indicates a result statistically different than the tantalum control at $P < 0.05$ using two-way ANOVA followed by Sidak's multiple comparisons test. ND – Not Determined (nickel-implanted rats in the 12-month group did not reach their experimental endpoint due to tumor formation necessitating early euthanasia).

Table 3: Liver metal levels in 1-, 3-, 6-, and 12-month experimental groups.

1 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.65 ± 0.12	0.37 ± 0.11	2.31 ± 0.15	10929.23 ± 755.90	330.65 ± 16.97	8160.39 ± 566.77	0.31 ± 0.02	0.11 ± 0.01
Implanted Metal	0.34 ± 0.03	3.34 ± 0.35*	22.69 ± 4.15*	11270.80 ± 1358.80	338.03 ± 30.56	11644.98 ± 2677.26	0.47 ± 0.04*	0.70 ± 0.20*

3 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.32 ± 0.12	2.14 ± 0.36	3.14 ± 0.21	13344.15 ± 1117.86	326.408 ± 15.45	8624.57 ± 545.97	0.48 ± 0.03	0.22 ± 0.02
Implanted Metal	0.30 ± 0.04	7.08 ± 1.43*	26.63 ± 3.17*	14740.75 ± 1234.46	202.54 ± 28.29*	9939.86 ± 1515.50	0.61 ± 0.07*	0.64 ± 0.09

6 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.90 ± 0.23	0.72 ± 0.17	1.91 ± 0.18	16477.45 ± 1413.79	337.76 ± 22.44	8929.13 ± 642.31	0.27 ± 0.02	0.17 ± 0.01
Implanted Metal	0.44 ± 0.05*	2.56 ± 0.69	18.92 ± 3.34*	14862.39 ± 1982.20	288.47 ± 16.45	15245.1 ± 1143.91*	0.30 ± 0.02	0.76 ± 0.20*

12 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.59 ± 0.13	1.09 ± 0.26	2.22 ± 0.17	17991.93 ± 955.41	324.17 ± 5.78	12366.68 ± 734.24	0.31 ± 0.01	0.15 ± 0.01
Implanted Metal	0.43 ± 0.05	ND	24.96 ± 2.81*	15531.54 ± 1108.95	336.46 ± 15.51	12294.17 ± 1011.98	0.45 ± 0.01*	1.23 ± 0.22*

Data are the mean of 8 individual determinations and expressed as ng metal/gm tissue. Errors are standard error of the mean. An * indicates a result statistically different than the tantalum control at $P < 0.05$ using two-way ANOVA followed by Sidak's multiple comparisons test. ND – Not Determined (nickel-implanted rats in the 12-month group did not reach their experimental endpoint due to tumor formation necessitating early euthanasia).

Table 4: Spleen metal levels in 1-, 3-, 6-, and 12-month experimental groups.

1 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.40 ± 0.05	5.66 ± 0.64	1.57 ± 0.04	41605.85 ± 3095.71	121.36 ± 3.33	16583.20 ± 662.57	0.97 ± 0.05	0.36 ± 0.01
Implanted Metal	2.31 ± 0.15	14.09 ± 1.14*	5.79 ± 0.49*	54904.83 ± 6190.13	134.88 ± 12.97	44951.70± 12781.57*	1.26 ± 0.05	2.42 ± 0.99

3 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	1.32 ± 0.51	5.55 ± 1.17	1.24 ± 0.11	143648.98 ± 17082.8	124.42 ± 8.07	11202.52 ± 1205.56	0.89 ± 0.10	0.41 ± 0.08
Implanted Metal	7.11 ± 1.28*	9.79 ± 0.51*	8.48 ± 0.65*	15396.33± 1598.39*	122.38 ± 5.24	19447.63 ± 2110.79	0.99 ± 0.07	6.20 ± 3.56*

6 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	3.16 ± 0.76	7.27 ± 1.15	1.49 ± 0.09	222361.81± 37083.41	123.89 ± 4.71	21552.85 ± 3520.31	1.05 ± 0.13	0.64 ± 0.11
Implanted Metal	11.20 ± 1.25*	7.64 ± 1.85	9.41 ± 0.48*	174919.06± 23423.36	115.01 ± 7.97	11441.06 ± 1233.50	1.36 ± 0.13	3.98 ± 1.68

12 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	3.56 ± 0.81	0.97 ± 0.45	0.91 ± 0.08	218531.78± 32698.57	90.69 ± 8.12	3554.57 ± 480.69	0.39 ± 0.02	0.10 ± 0.01
Implanted Metal	17.81 ± 2.74*	ND	12.72 ± 1.63*	205274.82± 35645.25	99.83 ± 5.01	7587.83 ± 1320.54	0.78 ± 0.09*	2.90 ± 0.32

Data are the mean of 8 individual determinations and expressed as ng metal/gm tissue. Errors are standard error of the mean. An * indicates a result statistically different than the tantalum control at $P < 0.05$ using two-way ANOVA followed by Sidak's multiple comparisons test. ND – Not Determined (nickel-implanted rats in the 12-month group did not reach their experimental endpoint due to tumor formation necessitating early euthanasia).

Table 5: Testes metal levels in 1-, 3-, 6-, and 12-month experimental groups.

1 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.31 ± 0.06	0.24 ± 0.02	0.35 ± 0.03	1649.28 ± 173.39	108.89 ± 7.87	1264.37 ± 121.06	0.10 ± 0.01	0.03 ± 0.00
Implanted Metal	0.18 ± 0.02	2.75 ± 0.24*	1.28 ± 0.15*	1930.94 ± 222.43	128.16 ± 10.49	5295.43 ± 2168.08*	0.14 ± 0.01*	0.09 ± 0.01

3 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.10 ± 0.04	0.27 ± 0.03	0.40 ± 0.02	2642.79 ± 229.13	137.35 ± 7.55	1012.01 ± 178.51	0.05 ± 0.01	0.03 ± 0.01
Implanted Metal	0.11 ± 0.01	3.02 ± 0.30*	1.25 ± 0.09*	2934.59 ± 276.52	166.58 ± 24.95	1452.47 ± 354.60	0.04 ± 0.01	0.12 ± 0.02*

6 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.27 ± 0.08	0.17 ± 0.01	0.40 ± 0.02	3303.66 ± 164.82	129.73 ± 3.86	709.15 ± 52.65	0.06 ± 0.00	0.02 ± 0.00
Implanted Metal	0.17 ± 0.01	2.03 ± 0.42*	1.34 ± 0.10*	3207.36 ± 215.84	124.33 ± 5.16	1503.14 ± 320.84	0.08 ± 0.01	0.19 ± 0.02*

12 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.33 ± 0.09	0.19 ± 0.01	0.45 ± 0.02	4340.08 ± 122.21	127.89 ± 1.64	551.58 ± 48.18	0.07 ± 0.01	0.01 ± 0.00
Implanted Metal	0.23 ± 0.03	ND	2.39 ± 0.12*	4061.45 ± 175.81	122.02 ± 6.91	886.49 ± 129.70	0.09 ± 0.01	0.46 ± 0.06*

Data are the mean of 8 individual determinations and expressed as ng metal/gm tissue. Errors are standard error of the mean. An * indicates a result statistically different than the tantalum control at $P < 0.05$ using two-way ANOVA followed by Sidak's multiple comparisons test. ND – Not Determined (nickel-implanted rats in the 12-month group did not reach their experimental endpoint due to tumor formation necessitating early euthanasia).

Table 6: Gastrocnemius metal levels in 1-, 3-, 6-, and 12-month experimental groups.

1 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.98 ± 0.32	0.81 ± 0.11	0.26 ± 0.03	1183.01 ± 124.71	104.71 ± 10.54	3613.35 ± 525.13	0.26 ± 0.04	0.10 ± 0.02
Implanted Metal	0.46 ± 0.25	2.56 ± 0.44*	1.80 ± 0.26*	1170.82 ± 139.50	80.63 ± 7.22	3008.44 ± 505.76	0.26 ± 0.02	2.09 ± 1.08

3 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.58 ± 0.22	2.30 ± 0.32	0.21 ± 0.04	1236.74 ± 81.00	90.29 ± 4.66	2402.65 ± 216.43	0.35 ± 0.06	0.10 ± 0.04
Implanted Metal	0.31 ± 0.16	3.24 ± 0.50	1.73 ± 0.28*	1142.28 ± 107.04	72.41 ± 7.20	3363.47 ± 434.36	0.31 ± 0.04	6.22 ± 3.56*

6 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.48 ± 0.15	0.86 ± 0.27	0.24 ± 0.03	1562.84 ± 129.78	89.13 ± 7.97	3418.29 ± 410.25	0.21 ± 0.03	0.10 ± 0.01
Implanted Metal	0.15 ± 0.4	1.42 ± 0.14	1.96 ± 0.16*	1464.79 ± 114.40	81.03 ± 10.04	3203.91 ± 402.44	0.33 ± 0.07	0.47 ± 0.02

12 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.65 ± 0.15	0.43 ± 0.20	0.32 ± 0.03	2183.66 ± 127.39	93.27 ± 4.65	7240.82 ± 891.74	0.39 ± 0.04	0.15 ± 0.02
Implanted Metal	0.22 ± 0.70	ND	2.91 ± 0.21*	1804.73 ± 185.49	86.19 ± 6.18	6176.14 ± 190.87	0.31 ± 0.02	5.17 ± 2.38

Data are the mean of 8 individual determinations and expressed as ng metal/gm tissue. Errors are standard error of the mean. An * indicates a result statistically different than the tantalum control at $P < 0.05$ using two-way ANOVA followed by Sidak's multiple comparisons test. ND – Not Determined (nickel-implanted rats in the 12-month group did not reach their experimental endpoint due to tumor formation necessitating early euthanasia).

Table 7: Lung metal levels in 1-, 3-, 6-, and 12-month experimental groups.

1 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	1.84 ± 0.31	1.10 ± 0.18	0.57 ± 0.04	13660.27 ± 1200.92	141.03 ± 6.17	3622.99 ± 720.24	0.67 ± 0.09	0.10 ± 0.01
Implanted Metal	0.50 ± 0.06*	0.58 ± 0.11	3.17 ± 0.49*	11859.52 ± 1392.53	137.65 ± 8.74	2696.88 ± 575.70	0.41 ± 0.04	0.26 ± 0.01

3 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.43 ± 0.22	4.02 ± 0.54	0.62 ± 0.06	10507.24 ± 299.90	155.61 ± 11.50	4457.49 ± 370.35	0.71 ± 0.10	0.14 ± 0.43
Implanted Metal	0.19 ± 0.02	12.78 ± 1.85*	3.88 ± 0.33*	12452.15 ± 836.29	131.00 ± 3.82	4408.95 ± 572.94	0.64 ± 0.09	0.33 ± 0.03

6 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	2.00 ± 0.76	4.11 ± 0.85	0.63 ± 0.06	12562.87 ± 424.01	122.01 ± 1.86	4093.65 ± 832.63	0.67 ± 0.11	0.26 ± 0.13
Implanted Metal	0.27 ± 0.02*	6.34 ± 0.76	4.37 ± 0.38*	12455.92 ± 343.41	118.70 ± 4.35	6326.94 ± 1371.99	1.26 ± 0.77	0.53 ± 0.07*

12 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	1.69 ± 0.53	2.99 ± 0.81	0.81 ± 0.05	10219.11 ± 404.94	118.40 ± 6.50	4244.46 ± 2144.65	0.63 ± 0.20	0.13 ± 0.07
Implanted Metal	0.58 ± 0.10	ND	6.72 ± 0.30*	10903.58 757.54	129.16 ± 7.70	5502.56 ± 938.70	0.68 ± 0.05	0.84 ± 0.07*

Data are the mean of 8 individual determinations and expressed as ng metal/gm tissue. Errors are standard error of the mean. An * indicates a result statistically different than the tantalum control at $P < 0.05$ using two-way ANOVA followed by Sidak's multiple comparisons test. ND – Not Determined (nickel-implanted rats in the 12-month group did not reach their experimental endpoint due to tumor formation necessitating early euthanasia).

Table 8: Tibia metal levels in 1-, 3-, 6-, and 12-month experimental groups.

1 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	2.37 ± 0.42	4.98 ± 1.37	0.46 ± 0.02	2134.23 ± 106.24	20.38 ± 1.09	192.51 ± 16.64	0.41 ± 0.06	0.08 ± 0.02
Implanted Metal	1.77 ± 0.18	5.38 ± 0.88	1.13 ± 0.13*	1581.24 ± 161.61	17.47 ± 4.39	197.46 ± 46.08	0.95 ± 0.15	5.01 ± 0.77*

3 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	4.04 ± 0.95	2.77 ± 1.61	0.83 ± 0.06	4743.87 ± 271.29	31.65 ± 0.99	472.11 ± 39.11	0.32 ± 0.03	0.11 ± 0.02
Implanted Metal	4.46 ± 0.21	2.19 ± 0.67	3.86 ± 0.03*	4824.94 ± 222.63	26.86 ± 0.35	281.38 ± 11.05*	4.35 ± 0.13*	14.30 ± 1.10*

6 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	8.89 ± 2.46	4.48 ± 1.38	0.46 ± 0.04	3019.02 ± 341.10	BD	322.29 ± 27.35	0.42 ± 0.28	0.07 ± 0.01
Implanted Metal	4.07 ± 0.16	6.34 ± 1.64	1.84 ± 0.23*	2879.91 ± 205.61	BD	348.53 ± 93.76	2.70 ± 0.35*	7.03 ± 0.98*

12 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	13.18 ± 4.59	2.70 ± 0.66	0.40 ± 0.06	2044.40 ± 334.93	30.60 ± 11.15	382.75 ± 52.86	0.84 ± 0.39	0.06 ± 0.02
Implanted Metal	4.51 ± 0.54*	ND	2.59 ± 0.24*	1592.35 ± 260.40	5.48 ± 2.90*	309.08 ± 43.70	3.64 ± 0.70*	11.42 ± 3.22*

Data are the mean of 8 individual determinations and expressed as ng metal/gm tissue. Errors are standard error of the mean. An * indicates a result statistically different than the tantalum control at $P < 0.05$ using two-way ANOVA followed by Sidak's multiple comparisons test. BD – Below the limit of detection. ND – Not Determined (nickel-implanted rats in the 12-month group did not reach their experimental endpoint due to tumor formation necessitating early euthanasia).

Table 9: Fibula metal levels in 1-, 3-, 6-, and 12-month experimental groups.

1 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	11.98 ± 3.53	232.43 ± 156.85	0.50 ± 0.24	1525.18 ± 145.64	BD	2103.15 ± 369.80	BD	0.95 ± 0.54
Implanted Metal	12.03 ± 1.30	46.18 ± 17.86	1.25 ± 0.32	3254.79 ± 240.51*	183.33 ± 92.14*	7144.02 ± 982.38*	14.33 ± 1.54*	16.42 ± 1.37*

3 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	21.20 ± 4.80	38.01 ± 12.04	2.26 ± 0.34	3537.63 ± 147.24	122.02 ± 8.37	2026.62 ± 243.12	0.72 ± 0.11	0.44 ± 0.07
Implanted Metal	14.08 ± 3.49	48.54 ± 25.41	4.82 ± 0.48*	3888.80 ± 504.75	112.78 ± 5.53	723.59 ± 329.21	2.43 ± 0.31	6.03 ± 1.17

6 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.56 ± 0.26	38.15 ± 15.97	1.39 ± 0.39	1791.01 ± 79.60	BD	1958.30 ± 304.39	BD	2.32 ± 1.18
Implanted Metal	10.90 ± 1.93*	84.54 ± 32.94	3.42 ± 0.35*	1799.77 ± 300.50	3.66 ± 3.66	1874.06 ± 253.53	BD	21.43 ± 1.91*

12 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.13 ± 0.13	15.76 ± 7.67	1.07 ± 0.02	2138.82 ± 322.18	BD	681.24 ± 129.47	BD	0.37 ± 0.13
Implanted Metal	5.73 ± 0.27	ND	5.52 ± 0.46*	1776.67 ± 117.81	BD	2899.28 ± 691.83*	BD	33.52 ± 3.07*

Data are the mean of 8 individual determinations and expressed as ng metal/gm tissue. Errors are standard error of the mean. An * indicates a result statistically different than the tantalum control at $P < 0.05$ using two-way ANOVA followed by Sidak's multiple comparisons test. BD – Below the limit of detection. ND – Not Determined (nickel-implanted rats in the 12-month group did not reach their experimental endpoint due to tumor formation necessitating early euthanasia).

Table 10: Femur metal levels in 1-, 3-, 6-, and 12-month experimental groups.

1 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	12.28 ± 2.30	BD	0.32 ± 0.02	1093.88 ± 123.98	6.33 ± 1.73	316.58 ± 38.56	0.48 ± 0.08	0.06 ± 0.00
Implanted Metal	3.77 ± 0.29*	BD	1.27 ± 0.19*	1230.75 ± 56.07	3.90 ± 3.49	433.82 ± 88.62	2.10 ± 0.22*	5.53 ± 0.53*

3 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	1.57 ± 0.39	6.96 ± 2.76	0.75 ± 0.03	4822.57 ± 288.21	28.47 ± 1.52	828.53 ± 120.82	0.24 ± 0.02	0.06 ± 0.01
Implanted Metal	3.65 ± 0.19	5.51 ± 0.53	3.81 ± 0.51*	4777.35 ± 1847.61	24.98 ± 1.80	743.77 ± 215.49	5.44 ± 0.95*	16.72 ± 2.22*

6 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.89 ± 0.50	1.39 ± 1.39	0.23 ± 0.02	1109.57 ± 96.91	1.14 ± 0.74	51.62 ± 13.95	0.38 ± 0.09	0.02 ± 0.01
Implanted Metal	1.30 ± 0.19	BD	1.77 ± 0.19*	1359.14 ± 159.93	2.68 2.22	185.05 ± 15.63	3.02 ± 0.33*	7.53 ± 0.62*

12 Month Groups

	Tungsten	Nickel	Cobalt	Iron	Copper	Aluminum	Lead	Depleted Uranium
Tantalum Control	0.88 ± 0.25	1.07 ± 0.13	0.23 ± 0.04	1281.24 ± 184.96	9.06 ± 2.04	250.37 ± 25.89	0.11 ± 0.03	0.03 ± 0.00
Implanted Metal	1.78 ± 0.16	ND	1.84 ± 0.09*	1294.29 ± 96.32	8.44 ± 1.30	161.99 ± 11.57	1.26 ± 0.19	6.66 ± 0.64*

Data are the mean of 8 individual determinations and expressed as ng metal/gm tissue. Errors are standard error of the mean. An * indicates a result statistically different than the tantalum control at $P < 0.05$ using two-way ANOVA followed by Sidak's multiple comparisons test. BD – Below the limit of detection. ND – Not Determined (nickel-implanted rats in the 12-month group did not reach their experimental endpoint due to tumor formation necessitating early euthanasia).

Neurotoxicology 83 (2021) 116–128

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro

Brain region- and metal-specific effects of embedded metals in a shrapnel wound model in the rat

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ARTICLE INFO

Edited by: Dr. Michael Aschner

Keywords:
Brain
Frontal cortex
Hippocampus
Amygdala
Cerebellum
Rat
Embedded metal
Shrapnel

ABSTRACT

The health effects of prolonged exposure to embedded metal fragments, such as those found in shrapnel wounds sustained by an increasing number of military personnel, are not well known. As part of a large collaborative effort to expand this knowledge, we use an animal model of shrapnel wounds originally developed to investigate effects of embedded depleted uranium to investigate effects of military-relevant metals tungsten, nickel, cobalt, iron, copper, aluminum, lead, and depleted uranium compared to an inert control, tantalum. Rats are surgically implanted with pellets of one of the metals of interest in the gastrocnemius (leg) muscle and tracked until 1 month, 3 months, 6 months, or 12 months from the time of implant, at which point they are euthanized and multiple organs and tissue samples are collected for inspection. Here we focus on four regions of the brain: frontal cortex, hippocampus, amygdala, and cerebellum. We examined changes in accumulated metal concentration in each region as well as changes in expression of proteins related to blood brain barrier tight junction formation, occludin and ZO-1, and synapse function, PSD95, spinophilin, and synaptotagmin. We report few changes in metal accumulation or blood brain barrier protein expression, but a large number of synapse proteins have reduced expression levels, particularly within the first 6 months of exposure, but there are regional and metal-specific differences in effects.

1. Background

Metals can be internalized by one of three main mechanisms: inhalation, mixed in particulate matter (dust) or within aerosols; ingestion, such as in contaminated food and water; or wounds, either directly as embedded metal fragments such as those found in blast, bullet, or shrapnel wounds, by contamination of an open wound, or even implanted medical devices with metal components. A majority of research into the effects of internalized metals has been focused on inhalation or ingestion, particularly in populations with contaminated environments, and much of this has focused on systemic health effects like kidney disease or cancer development. Research into adverse effects of embedded metals has been ongoing for many years, but with a focus on the safety of implanted medical devices (IARC, 1999; Jacobs et al., 2003; Keegan et al., 2007). Internalized metal fragments from ballistics injuries in war are not new, but have become more survivable due to parallel advances in ammunition design and type as well as surgical techniques that rarely result solely in amputation (Manring et al., 2009; Dougherty and Eidt, 2009). A growing percentage of military personnel are returning from conflicts with retained metal fragments. Between ballistic wounds, improvised explosive devices (IEDs), rocket-propelled grenades (RPGs), and secondary blasts through vehicle armor, the variety of metals potentially involved in retained fragments is expanding as well as the frequency with which they occur.

In the past, due to the belief that most embedded metal fragments remained inert in the body and the higher risk of damage to muscle and blood vessels in the removal of fragments, only large, easily accessible, or dangerously-positioned fragments were surgically removed, leaving more military personnel with retained metals for longer periods of time than ever before (Maggio et al., 2008). Embedded metals are not as inert as initially thought, however; over time case reports of medical issues associated with retained fragments started appearing years after the

Abbreviations: AAALAC-I, Association for Assessment and Accreditation of Laboratory Animal Care International; Al, aluminum; Amyg, amygdala; BBB, blood brain barrier; Cb, cerebellum; Co, cobalt; Cu, copper; DoD, Department of Defense; DU, depleted uranium; FC, frontal cortex; Fe, iron; Hip, hippocampus; IACUC, Institutional Animal Care and Use Committee; ICP-MS, inductively coupled-plasma mass spectroscopy; LoD, limit of detection; Ni, nickel; Pb, lead; PSD95, post-synaptic density protein 95; Ta, tantalum; W, tungsten; ZO-1, zonula occludens-1.

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https://doi.org/10.1016/j.neuro.2021.01.001
Received 13 November 2020; Received in revised form 3 January 2021; Accepted 3 January 2021
Available online 14 January 2021
0161-813X/Published by Elsevier B.V.

initial injury (Schenck and Kronman, 1977; Knox and Wilkinson, 1981; Lindeman et al., 1990; Ligtenstein et al., 1994; Eylon et al., 2005) along with an increase in understanding of carcinogen risk from metals and foreign-bodies in tissues in recent decades (International Agency for Research on Cancer (IARC), 2012; Tang and Eaton, 1999; Schetter et al., 2010; Okada, 2007; Moizhess, 2008). After a "friendly fire" incident during Operation Desert Storm in 1991 where multiple US troops sustained embedded fragment wounds with fragments of depleted uranium (DU) too small and numerous to remove efficiently (Office of the Special Assistant for Gulf War Illness (OSAGWI), 2000a), concern focused on the long-term effects of the toxicity as well as potential for radiological effects of permanently retained DU (AEPI, 1995), beginning with setting up long-term surveillance of this cohort that is still ongoing today (Hooper et al., 1999; McDiarmid et al., 2000, 2001; McDiarmid et al., 2004, 2007; McDiarmid et al., 2013, 2017; McDiarmid et al., 2018).

The development of a model of shrapnel injury using rodents with surgically implanted metal pellets in the gastrocnemius (leg) muscle has been key in studying health effects of retained fragments of different metal types (Castro et al., 1996). Early research focused on biokinetics and toxicology of DU in the rodent model alongside the Operation Desert Storm military personnel cohort in order to provide insight into risks they faced (Pellmar et al., 1999a; Hahn et al., 2002), which was then expanded to investigate potential health risks of alternative munitions materials for DU at the Department of Defense (DoD)'s request. When one tungsten alloy (tungsten, nickel, and cobalt) resulted in highly aggressive rhabdomyosarcomas which metastasized to other organs, but another tungsten alloy (tungsten, nickel, and iron) did not (Kalinich et al., 2005; Schuster et al., 2012; Emond et al., 2015), it became clear that more research into the health effects of a variety of retained metals was necessary. The DoD fragment removal policy was also reassessed, and guidelines for the use of the shrapnel injury model in assessing potential health effects of retained metals was developed (OTSG/MEDCOM, 2011; Kane et al., 2009). The DoD and Department of Veterans Affairs (DVA) also developed a list of militarily-relevant "metals of concern" for retained metal fragments (Health Affairs Policy Letter 07-029, 2007), but the biokinetic and toxicological properties of these metals as embedded fragments, such as if and how much of the metals may solubilize from a fragment into the blood stream, where throughout the body they may be deposited, and what organs they may affect, is not well understood.

There is growing evidence that some metals internalized by any of the mechanisms mentioned earlier can cross the blood brain barrier (BBB) from the blood into the brain interstitial space and disrupt the balance of endogenous metals, accumulate in the brain, and disrupt normal neuronal function (Pellmar et al., 1999a; Ong et al., 2006; Chrosniak et al., 2006; Linares et al., 2007; Bensoussan et al., 2009; Legrand et al., 2016; Dinocourt and Culeux, 2017; Arnal et al., 2014; Dorman et al., 2001; Avila-Costa et al., 2006; Taylor et al., 2006; Radcliffe et al., 2009; Lucchini et al., 2012; Calderon-Garciduenas et al., 2013; Barber et al., 2005; Monleau et al., 2005; Fitsanakis et al., 2006; Kalinich and Kasper, 2014; Chen et al., 2006; Jones et al., 2008) and may have a role in the development of neurological diseases (Zheng et al., 2003; Levenson, 2005; Weiss et al., 2009; Rosenberg, 2012). Iron and copper are essential metals for neuronal function, requiring strict regulation of the BBB, and deficiency or excess can lead to neurodegenerative disorders, including loss of cognition (Vallée, 2017; Thirupathi and Chang, 2019; Zheng and Monnot, 2013; Squitti et al., 2019). Inhalation of cobalt dust can cross into the brain and cause neurotoxicity and memory deficits (Persson et al., 2003), but there is also concern of chronic cobalt exposure from metal-on-metal joint replacement prosthetics associated with neurologic damage and motor deficits (Clark et al., 2014). Lead is a toxin well-known to have adverse effects on neurodevelopment and cognition when exposure occurs in early childhood. Acute and chronic lead exposure across a variety of ages can lead to a wide array of adverse systemic effects, including cognitive deficits (Santa Maria et al., 2018; Mitra et al., 2017). Even aluminum, with a very low acute toxicity, has been associated with Alzheimer's disease and breast cancer after high chronic exposure from multiple environmental sources (Klotz et al., 2017).

Our lab, which was involved in the development of the rodent shrapnel model, is working in close collaboration with the University of Maryland School of Medicine, the Baltimore Department of Veterans' Affairs Medical Center (MDVA), the U.S. Food and Drug Administration (FDA), and the University of Kentucky (UKY) to investigate the health effects and potential associated biomarkers of multiple metals from the "metals of concern" list in the rodent shrapnel model alongside an expanded human investigation into military personnel with a wider array of retained metal fragments. We hypothesize that tungsten, nickel, cobalt, iron, copper, aluminum, lead, and DU, as embedded metals will demonstrate vastly different biokinetic, toxicological, and carcinogenic properties and thus different patterns of health effects. In our arm of the collaboration we use our rat embedded metal model and analyze urine, serum, and multiple tissue samples over various periods of implant exposure, up to a year, with the goals to 1) identify what those specific health risks are and 2) identify a set of biomarkers indicative of those health risks at a stage early enough to implement the most effective medical intervention for the wounded individual. This manuscript will focus on effects of embedded metals over multiple implant periods on several regions of the brain.

2. Materials and methods

2.1. Animals and housing conditions

Experiments in this study were conducted at the Armed Forces Radiobiology Research Institute (AFRRI, Bethesda, MD). Male Sprague–Dawley rats (*Rattus norvegicus*; age, approximately 30 d; weight, 75–100 g) were purchased from Envigo (Barrier 208A, Frederick, MD). Rats were allowed to acclimate in the AFRRI vivarium for at least 2 weeks prior to the start of experiments. The room was maintained at standard temperature (21 ± 2 °C) and humidity (30%–70%), with a 12:12-h light:dark cycle (lights on, 0600) and access to standard rat chow (Teklad Global Rodent Diet 8604, Envigo) and water *ad libitum*. Rats were pair-housed in plastic microisolation cages (23.8 × 45.4 cm) with filter tops and bedding (Teklad Sani-Chips, Envigo), changed 2 or 3 times weekly. All procedures involving animals were conducted to achieve maximal possible wellbeing of the rats, were IACUC-approved (protocol no. 2016-05-006) prior to the start of the study, and were performed in compliance with the guidelines set forth in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011) in an AAALAC-I-accredited facility.

2.2. Experimental design and metals of interest

Using a model of embedded metals ("shrapnel wounds"), we investigated the effects of eight militarily relevant metals: tungsten (W), nickel (Ni), cobalt (Co), iron (Fe), copper (Cu), aluminum (Al), lead (Pb), and depleted uranium (DU). The control group followed all the same procedures, but utilized the inert metal tantalum (Ta) to serve as the surgery sham, which would control for changes such as inflammatory response to surgery and embedded foreign material. No naïve animals were used as a control because previous work with this embedded metal model found no differences between naïve animals and the tantalum sham (Pellmar et al., 1999a; Hahn et al., 2002; Kalinich et al., 2005) and thus reducing the total number of animals needed for this study and keeping with the spirit of the ARRIVE Guidelines (Percie du Sert et al., 2020). Rats were randomly assigned to one of the nine metal implant groups with an n of 8 per metal, and the metal groups were conducted in 4 different cohorts – 1, 3, 6, or 12 months (M) post-implant surgery ("Time from Implant"). A total of 288 rats (n = 8 × 9 metal groups x 4 timed cohorts) were used in this study.

All metal "pellets" were 1 mm in diameter x2 mm in length. DU was

purchased from Aerojet Ordnance (Jonesborough, TN) and all other metals were purchased from Alfa Aesar (Ward Hill, MA) with purities greater than 99.99 %. Pellets were surgically implanted into the gastrocnemius muscle, and each animal received 2 pellets per limb for a total of 4 pellets per rat. Prior to use, pellets were cleaned and chemically sterilized as previously described (Emond et al., 2015).

After arrival and the two-week acclimation, pre-surgery urine was collected. A week after urine collection (at appx 50 days old) weights were determined and animals underwent surgery to implant an ID chip transponder and metal pellets, and health status was monitored daily for two weeks. Body weight and temperature were determined once a week for the duration of each cohort. At the end of each time point rats were humanely euthanized and tissues collected for analysis.

2.3. Metal pellet implantation procedure

Anesthesia was induced by administration of isoflurane in an induction chamber and then maintained via nose cone for the duration of the surgery, using an open circuit system with a scavenger/recapture system. The surgical sites were clipped and cleansed with 70 % isopropyl alcohol then betadine (Purdue Pharma, LP, Stamford, CT), followed by administration of a prophylactic dose of an analgesic (Buprenorphine, 0.05 – 0.1 mg/kg, s.c., Rickitt and Colman, Hull, UK). A small transponder (Electronic Lab Animal Monitoring System, Bio-Medic Data Systems, Seaford, DE) with a unique ID code was injected subcutaneously in the mid-dorsal thoracic region, and ear punch was performed as a secondary mode of identification. Under aseptic technique, a small incision (approximately 5 mm in length) was made through the skin of each hind leg to reveal the gastrocnemius muscle. Two sterile pellets were implanted in each gastrocnemius muscle spaced approximately 1.5 mm apart on the lateral side of each leg by placing the sterilized pellet in a 14−16-gauge needle, putting a specially designed plunger inside that needle, pushing the needle into the rat muscle, then depressing the plunger, forcing the pellet out of the needle and into the rat muscle. The incision sites were sealed with tissue adhesive (VetBond; 3 M Corp, St Paul, MN). Rats were closely monitored following implantation until ambulatory. The implantation sites were examined daily for signs of inflammation, infection, and local metal toxicity for two weeks following the procedure and weekly thereafter throughout the study. The transponders were programmed with a unique animal identification number using a personal computer, which can then be read using a low-power radio frequency scanner.

2.4. Euthanasia and tissue collection

Upon reaching their experimental end point or when indicated by guidelines approved by the IACUC, rats were humanely euthanized under deep isoflurane anesthesia by exsanguination via transection of the caudal vena cava and confirmatory pneumothorax per the guidelines of the American Veterinary Medical Association (American Veterinary Medical Association (AVMA), 2007). Criteria for euthanasia included tumor size greater than 1 cm in diameter, loss of greater than 10 % of baseline body weight, or other indications of approaching moribundity as determined by animal health assessments. After euthanasia, a complete gross pathology examination was conducted, and a variety of tissues isolated for analysis, including brain, liver, heart, lung, popliteal lymph node, tibia, fibula, femur, gastrocnemius and triceps muscle, spleen, thymus, kidney, bladder, testes, urine, blood, and a fecal sample. For brain tissue collection, the skull cap was removed with surgical scissors and the whole brain lifted out of the skull. Hemispheres were separated sagittally; one hemisphere of the brain was prepared for histopathological analysis, fixed in zinc-buffered formalin and stored at 4 °C with other prepared tissues until processing. The other hemisphere was micro-dissected using a large rat brain slicer matrix (Zivic Instruments, Pittsburgh, PA, cat#BSRLS001-1) with 1.0 mm coronal section slice intervals. Using razor blades chilled on ice, blades were placed relative to the optic chiasm to collect slices containing frontal cortex, preoptic area, hippocampus, amygdala, and cerebellum, which were then specifically separated out of the slice using gross anatomical markers and flash frozen in isopropyl alcohol on dry ice for protein and metal analyses. Results in this manuscript focus on analysis of brain tissues collected, but it is important to note that analyses from other tissues of the same animals are also underway.

2.5. Seizure marker S100B in serum

The S100 family of proteins plays a role in the regulation of various cellular processes. In particular, S100B has been shown to be a marker of blood-brain barrier disruption and potential neuronal damage (Misler et al., 1997; Wunderlich et al., 1999; Vajtr et al., 2009; Golmohammadi et al., 2019). Observation of seizure episodes in several of the experimental groups led us to measure S100B levels in the serum, obtained at euthanasia, of these rats. The S100B Rat ELISA Kit from BioVision Inc. (Catalog #: E4755-100, Milpitas, CA) was used for determining serum levels. Below is a listing of the rats with known seizures, as well as additional animals that were assayed. Where available, we tested cage mates of the seizure animals, as well as animals delivered in the same shipment and belonging to the same experimental implantation group. As per the vendor's literature, the detection range of the kit is 62.5–4000 pg/ml, with a lower sensitivity limit of 37.5 pg/mL.

2.6. Metal analysis by inductively coupled plasma mass spectroscopy (ICP-MS)

All compounds used in this study were of the highest grade available. Plastic ware and other disposables were also obtained from Thermo Fisher Scientific. Samples were dissolved in ultrapure nitric acid (Fisher Scientific, Newark, DE) and metal content determined using an inductively coupled plasma-mass spectrometer (XSeries 2 ICP-MS System, ThermoFisher, Madison, WI) equipped with a Cetac ASX520 Autosampler (Cetac Technologies, Omaha, NE). High-pressure liquid argon, 99.997 %, was used for the plasma gas. The instrument was calibrated with external standards of the appropriate metal standard (SPEX CertiPrep, Metuchen, NJ) in 2% HNO_3. The sample probe was washed with a constant flow of 2% nitric acid between measurements to prevent carryover. Quantitative analysis was obtained by reference to the slope of the calibration curve (counts per second / ng per liter) as well as an internal standard. Homogenates of the dissection brain regions (prepared as described below) were resuspended in 2% nitric acid prior to metal analysis. Results were normalized to protein content. ICP-MS operating conditions and parameters can be found in Table S1. Limit of Detection (LoD)/Limit of Quantitation (LoQ), in ppb, are as follows: Ta - 0.50/0.91; W - 0.12/0.15; Ni - 0.17/0.21; Co - 0.03/0.06; Fe - 1.08/1.85; Cu - 0.24/0.54; Al - 0.38/0.44; Pb - 0.02/0.04; U - 0.02/0.07.

2.7. Protein expression in tissues

Tissue samples were suspended in RIPA buffer (Thermo, Waltham, MA, cat#89901) plus Halt Protease and Phosphatase Inhibitor Cocktail (Thermo cat#78442) and homogenized in a Bullet Blender (Next Advance, Troy, NY) with 1.0 mm zirconium oxide beads (NextAdvance, cat#ZROB10) (settings speed 6, 5 min x 3 runs) and then centrifuged at 1340 x g for 10 min. Total protein from each sample supernatant was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA, cat #500-0006), in triplicate, against a BSA standard curve, on a spectrophotometer (BioTek Synergy Model H1 M Multimodal Plate Reader with GEN5 Software, BioTek Instruments, Winooski, VT), and read at 595 nm. Proteins of interest were quantified using an automated capillary-based size sorting chemiluminescent system 'WES' from ProteinSimple (San Jose, CA). All procedures were performed with manufacturer's reagents (12−235 kDa kit, cat#SM-W004-1 and PS-ST01EZ-8) according to the user manual with some adjustments: samples are

aliquoted to 1 μg/μl before mixing 4 μl with 1 μl fluorescent master mix, then denatured at 94 °F (34.4 °C) for 4 min, given a quick spin, and loaded on the plate at 4 μl per well. In run settings, stacking time was changed to 18 s, separation time to 31 min, and antibody diluent time to 30 min. Antibody information is listed in Table S2, along with any additional changes specific to a particular antibody. Peak values were determined using Compass Software (ProteinSimple). Results are in arbitrary units, and presented as a ratio of the target protein expression normalized to within-sample β-actin expression. Since the ProteinSimple Wes system uses capillary size separation and chemiluminescent detection, an example of the raw data for one of the 6 M cerebellum ZO-1 and β-actin runs is shown in Figure S1A, and a traditional Western blot image derived from that data is in Figure S1B.

2.8. Histopathology

Microdissections of brain regions were combined in the same processing cassette and were fixed in zinc buffered formalin (Z-Fix; Anatech Ltd, Battle Creek, MI), processed and embedded in paraffin, sectioned at 5–6 mm, and stained with hematoxylin and eosin (H&E). The 12 M samples were processed in-house through USU's histopathology lab, but due to facility renovations, all 1 M, 3 M, and 6 M samples were processed through HistoServ (Germantown, MD). Histopathological assessments were conducted by board-certified veterinary pathologists.

2.9. Statistical analyses

Analyses were performed using GraphPad Prism Software (version 8.4.2, La Jolla, CA, USA). Protein and metal concentrations were analyzed as a two-way ANOVA using the variables of time from implant (1 M, 3 M, 6 M, or 12 M) and implanted metal (Ta, W, Ni, Co, Fe, Cu, Al, Pb, DU) followed by Sidak's multiple comparisons test where each metal within a time from implant is compared back to the corresponding Ta-implanted group value. Samples below the limit of detection are noted as are any changes to statistical analysis. Data are presented as mean ± SD. In all cases P values <0.05 were considered significant.

Of note for tables: please refer to the online version of this manuscript for color-coding. Green indicates a mean value increased over the compared control group, and magenta indicates a mean value decreased compared to the control group. A green-magenta scheme was chosen to better accommodate red-green colorblind readers.

3. Results

3.1. Spontaneous convulsions and early deaths

An unexpected phenomenon we encountered during weekly health checks was spontaneous seizure/convulsion activity in some rats. Convulsion events consisted of mild to violent shaking, freezing, and face twitching, lasting anywhere from 15 s to nearly 2 min. Most convulsions were observed during the longer period of observation during the weekly health checks where rats were weighed and scanned for their temperature, and animals either actively being handled started to convulse or a commotion was heard in the cages and animals immediately observed. No convulsions were observed in the 1 M group. Only one animal in the 3 M group, a Pb-implanted rat, had an observed convulsion; this was the first one we noted in the study, and after recovery from the event it appeared normal with no further convulsion events and continued to the study endpoint. In the 6 M group, one Ta-implanted rat had two observed convulsion events, and there was one observed event for one each of the Cu-, Co-, and W-implanted groups. In the 12 M group, two Co-implanted rats had observed convulsion events, one with a single event and the other with two events. A single DU-implanted rat had 5 observed events over several months and was finally euthanized after a prolonged event. A single Fe-implanted rat had 3 separate observed convulsions, as did a single W-implanted rat. One Al-implanted rat and one Ni-implanted rat also had only a single observed convulsion event each. It is difficult to know exactly how many convulsive events actually occurred, and in which animals, as they were not monitored 24 h a day for the duration of the study. We increased observations as we realized convulsions were more wide-spread, but such short events could easily be missed. Animal husbandry staff members were asked to report any observed events to the PI as well, but cooperation was inconsistent. We inspected the housing area for any chemical or toxin exposure but found none. We contacted the supplier, Envigo, to attempt to track breeding cohorts and determine if this was a particular line of animals, but were not able to determine relationship status between rats in our cohorts. Subsequent histopathology did not reveal anything overtly different about the rats that had experienced convulsions. Other research has reported spontaneous convulsions in Charles River Wistar rats (0.7 % in males), at 16 weeks of age or later, with single or multiple convulsions, and no histopathological abnormalities of the brain (Nunn and Macpherson, 1995). Spontaneous convulsions in Sprague-Dawley rats from Charles River were similarly reported as single or multiple convulsions as early as 25 weeks but more commonly around 66 weeks, with frequency in males of 2.9 % occurrence (Satomoto et al., 2012). Our observed frequency of rats experiencing seizures of 12/288 (0.04 %) falls well within this range, and we postulate the observed spontaneous convulsions are not related to embedded metals. Further, our investigation into seizure-marker S100B levels in serum from rats who experienced known seizures and their matched cage mates did not provide any further insight as none of the serum samples tested reached the lower sensitivity limit (data not shown; details of animal selections in Table S3). The only sample that reached a detectable level, at 30.5 pg/mL, was a 12 M Fe-implanted control cage mate (no known seizures).

All 1 M and 3 M rats survived to the end of their implant period. In the 6 M group (full implant period 24–25 weeks), only one animal had to be euthanized early: a Ni-implanted rat at approximately 21 weeks, which had developed a large mass on its front limb. In the 12 M group (full implant period 51–52 weeks), one Pb-implanted animal was found dead at 27 weeks due to a massive abdominal tumor. One 12 M Fe-implanted animal was found dead at 31 weeks, cause unknown, but multiple seizure events were suspected as contributing to the cause of death. One 12 M Co-implanted animal was euthanized at 48 weeks due to a large leg tumor. Two 12 M Cu-implanted animals were found dead; one at 32 weeks, one at 36 weeks, neither with a known cause of death or history of observed seizures. Three DU-implanted animals were euthanized early: one at 36 weeks who was found bleeding from the nose and minimally responsive, and necropsy revealed an abnormal kidney and distended bladder, another euthanized at 45 weeks due to a swollen foot, and the third euthanized at 50 weeks after a particularly long seizure and a history of repeated observed seizure events. Additionally, and important to note, all of the 12 M Ni-implanted animals were euthanized early at between 20 and 28 weeks from implant due to tumors around the implant site; tissues and samples were taken in most cases prior to euthanasia, but since this group did not survive much farther than the 6 M time period, all analyses of 12 M group data comparing 12 M Ni-implanted animals and the 12 M Ta-implanted animals must be viewed with the caveat that the actual age of these animals is different.

Additionally, data from Cu-implanted animals must also be interpreted with a caveat. Full details are discussed in Wen, et al., 2020 [77 - supplemental methods and figure S1], where we focus on changes in muscle tissue from the same animals in this study, and describe an interesting phenomenon where the majority of copper pellets were self-extruded from the muscle between 4.5 weeks and 6.5 weeks after implantation, such that copper is only present in the body for the full implant period for the 1 M implant group, and the 3 M, 6 M, and 12 M groups only have copper present in the body for a relatively short period of time.

3.2. Histopathology

In addition to the analysis of seizure-related brains, all brain tissues were examined for gross anatomical changes. Nothing of significance was noted in any metal/time period in the H&E stained tissues.

3.3. Metal distribution in brain regions

For each brain region collected (frontal cortex, hippocampus, amygdala, and cerebellum), the concentration all metals of interest (W, Ni, Co, Fe, Cu, Al, Pb, and U) was determined for each and every sample within each ICP-MS run. In this way, the Ta-implanted animals serve as the control for evaluation of each implanted metal of interest, where the data for the concentration for the metal of interest is pulled from the Ta-implanted animals and compared against the same metal concentration data for each particular implanted metal group. Thus, for example, the concentration of lead in the frontal cortex of Ta-implanted animals from each of the four time-from-implant groups (1 M, 3 M, 6 M, and 12 M) is compared to the concentration of lead in the frontal cortex of Pb-implanted animals from each of the four time-from-implant groups;

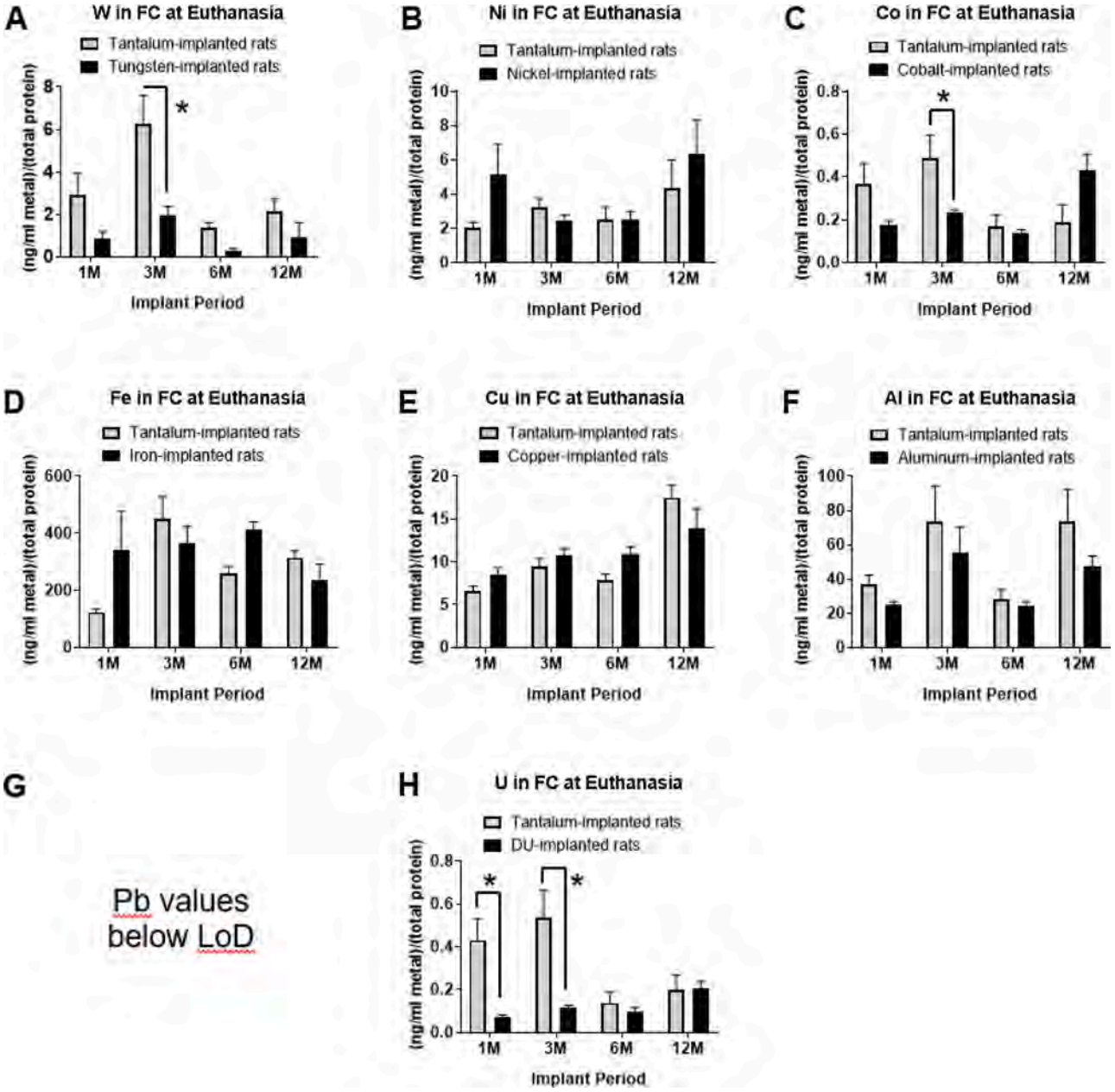

Fig. 1. Metal concentrations in Frontal Cortex. Frontal Cortex samples from metal implanted rats from four different groups of implant periods: 1 month (M), 3 M, 6 M, or 12 M from implant. In all cases gray bars indicate metal values in samples from the Ta-implanted (control) animals, and black bars indicate metal values in samples from target metal-implanted animals, with metal expression presented as ng/mL of metal normalized to total protein of the sample evaluated. Cases where data is unavailable or metal values were below the limit of detection (LoD) are indicated within the graph placeholder. A) concentration of tungsten (W) in samples from Ta- or W-implanted animals. B) concentration of nickel (Ni) in samples from Ta- or Ni-implanted animals. C) concentration of cobalt (Co) in samples from Ta- or Co-implanted animals. D) concentration of iron (Fe) in Ta- or Fe-implanted animals. E) concentration of copper (Cu) in samples from Ta- or Cu-implanted animals. F) concentration of aluminum (Al) in samples from Ta- or Al-implanted animals. G) concentration of lead (Pb) in samples from Ta- or Pb-implanted animals. H) concentration of uranium (U) in samples from Ta- or DU-implanted animals. * indicates significant post-hoc p-value between Ta- and target metal-implanted animals within an implant period.

the concentration of cobalt in the frontal cortex of Ta-implanted animals from each time-from-implant group is compared to the concentration of cobalt in the frontal cortex of Co-implanted animals from each time-from-implant group, and so on.

Metal concentrations in frontal cortex samples are presented in Fig. 1: A) tungsten, F_{metal} (1, 48) = 14.12, B) nickel, F_{metal} (1, 53) = 1.798, C) cobalt, F_{metal} (1, 56) = 1.394, D) iron, F_{metal} (1, 54) = 1.215, E) copper, F_{metal} (1, 53) = 0.757, F) aluminum, F_{metal} (1, 56) = 3.223, G) lead, no statistical analysis because values were below limit of detection (LoD), and H) uranium, F_{metal} (1, 55) = 18.500. The only comparisons to survive post-hoc significance comparing the metal concentration between the Ta-implanted animals and the target metal-implanted animals within the matched implant period were tungsten 3 M (*p = 0.0006), cobalt 3 M (*p = 0.0438), and DU 1 M (*p = 0.0013) and 3 M (*p = 0.0001). In all cases, surprisingly, the sample metal concentration was lower in the target metal-implanted group than the matched Ta-implanted control group.

Metal concentrations in hippocampus samples are presented in Fig. 2: A) tungsten, F_{metal} (1, 53) = 6.350, B) nickel, F_{metal} (1, 53) = 0.112, C) cobalt, F_{metal} (1, 56) = 0.018, D) iron, F_{metal} (1, 42) = 9.126, E) copper, F_{metal} (1, 54) = 0.001, F) aluminum, F_{metal} (1, 55) = 3.104, G) lead, F_{metal} (1, 45) = 1.781, and H) uranium, F_{metal} (1, 56) = 5.064. The only comparisons to survive post-hoc significance comparing the metal concentration between the Ta-implanted animals and the target metal-implanted animals within the matched implant period were iron 1 M (*p = 0.0451) where the concentration of iron in the hippocampus was

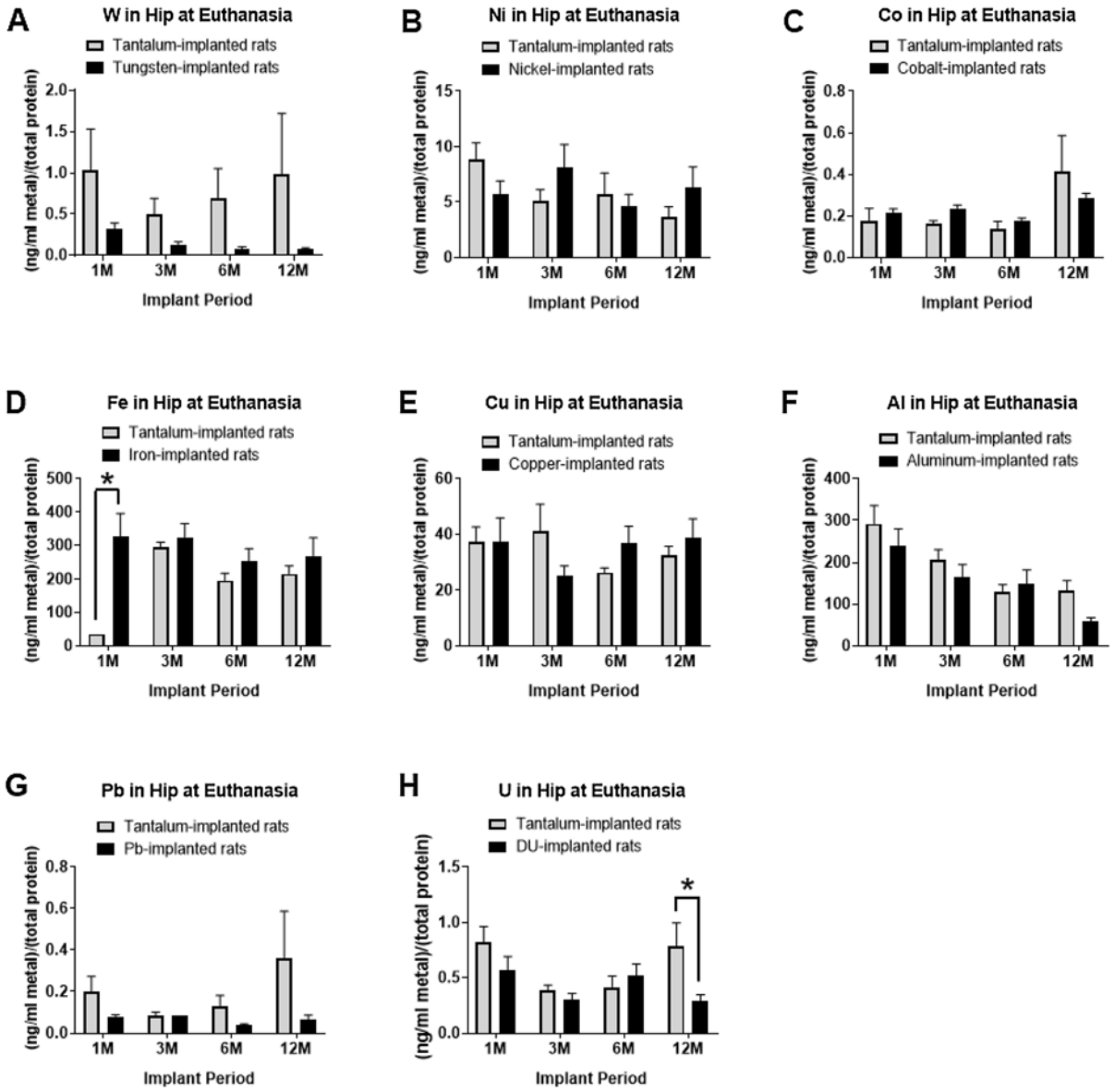

Fig. 2. Metal concentrations in Hippocampus. Hippocampus samples from metal implanted rats from four different groups of implant periods: 1 month (M), 3 M, 6 M, or 12 M from implant. In all cases gray bars indicate metal values in samples from the Ta-implanted (control) animals, and black bars indicate metal values in samples from target metal-implanted animals, with metal expression presented as ng/mL of metal normalized to total protein of the sample evaluated. Cases where data is unavailable or metal values were below the limit of detection (LoD) are indicated within the graph placeholder. A) concentration of tungsten (W) in samples from Ta- or W-implanted animals. B) concentration of nickel (Ni) in samples from Ta- or Ni-implanted animals. C) concentration of cobalt (Co) in samples from Ta- or Co-implanted animals. D) concentration of iron (Fe) in Ta- or Fe-implanted animals. E) concentration of copper (Cu) in samples from Ta- or Cu-implanted animals. F) concentration of aluminum (Al) in samples from Ta- or Al-implanted animals. G) concentration of lead (Pb) in samples from Ta- or Pb-implanted animals. H) concentration of uranium (U) in samples from Ta- or DU-implanted animals. * indicates significant post-hoc p-value between Ta- and target metal-implanted animals within an implant period.

higher in the Fe-implanted animals than the matched 1 M Ta-implanted animals, and uranium 12 M (*p = 0.0152), where, again surprisingly, the concentration of uranium was lower in the DU-implanted animals than the matched 12 M Ta-implanted animals.

Metal concentrations in amygdala samples are presented in Fig. 3: A) tungsten, data not available, B) nickel, F_{metal} (1, 39) = 0.1258, p = 0.7247, C) cobalt, F_{metal} (1, 50) = 0.6296, p = 0.4312, D) iron, F_{metal} (1, 23) = 1.9560, p = 0.1752, E) copper, F_{metal} (1, 31) = 0.0404, p = 0.8420, F) aluminum, no statistical analysis from a combination of some data unavailable, G) lead, below limit of detection (LoD), and H) uranium, F_{metal} (1, 37) = 1.682, p = 0.2027. The only comparison to survive post-hoc significance comparing the metal concentration between the Ta-implanted animals and the target metal-implanted animals within the matched implant period was with DU-implanted animals, where uranium was higher in the DU-implanted animals at 3 M than the matched Ta-implanted group (*p = 0.0002). Data for tungsten values was not available for any implant period due to an issue with sample drift in the ICP-MS process, data for aluminum was unavailable due to a combination of processing errors and many values being below the LoD, and other metal implant time periods were removed from statistical analysis due to processing errors.

Metal concentrations in cerebellum samples are presented in Fig. 4: A) tungsten, data not available, B) nickel, F_{metal} (1, 42) = 0.744, C) cobalt, F_{metal} (1, 40) = 1.427, D) iron, F_{metal} (1, 33) = 8.651, E) copper, F_{metal} (1, 40) = 1.984, F) aluminum, F_{metal} (1, 39) = 18.71, G) lead, no statistical analysis because values were below limit of detection (LoD), and H) uranium, F_{metal} (1, 34) = 0.240. The only comparisons to survive post-hoc significance comparing the metal concentration between the Ta-implanted animals and the target metal-implanted animals within the matched implant period were nickel 6 M (p = 0.0319), where, again

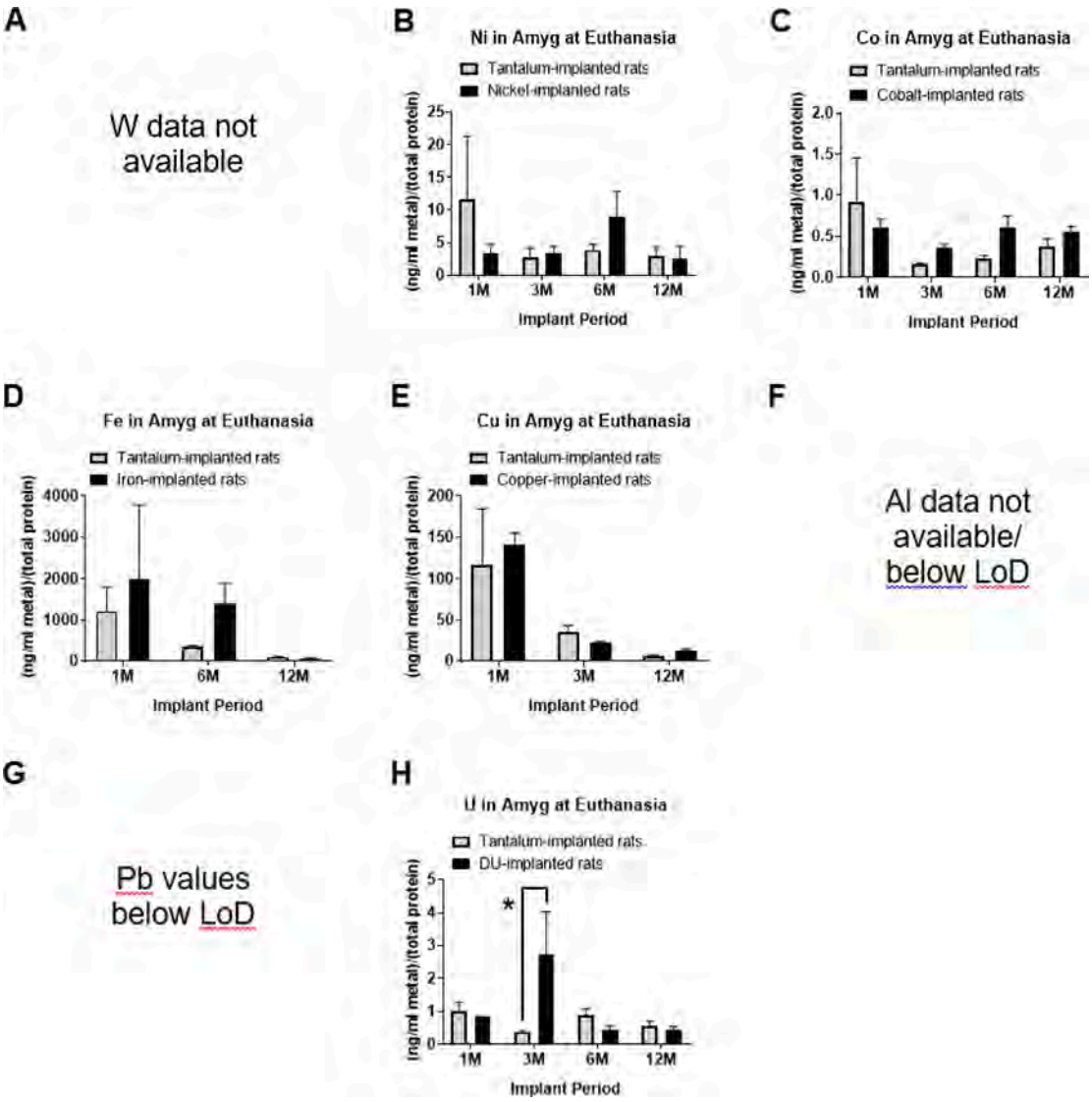

Fig. 3. Metal concentrations in Amygdala. Amygdala samples from metal implanted rats from four different groups of implant periods: 1 month (M), 3 M, 6 M, or 12 M from implant. In all cases gray bars indicate metal values in samples from the Ta-implanted (control) animals, and black bars indicate metal values in samples from target metal-implanted animals, with metal expression presented as ng/mL of metal normalized to total protein of the sample evaluated. Cases where data is unavailable or metal values were below the limit of detection (LoD) are indicated within the graph placeholder. A) concentration of tungsten (W) in samples from Ta- or W-implanted animals. B) concentration of nickel (Ni) in samples from Ta- or Ni-implanted animals. C) concentration of cobalt (Co) in samples from Ta- or Co-implanted animals. D) concentration of iron (Fe) in Ta- or Fe-implanted animals. E) concentration of copper (Cu) in samples from Ta- or Cu-implanted animals. F) concentration of aluminum (Al) in samples from Ta- or Al-implanted animals. G) concentration of lead (Pb) in samples from Ta- or Pb-implanted animals. H) concentration of uranium (U) in samples from Ta- or DU-implanted animals. * indicates significant post-hoc p-value between Ta- and target metal-implanted animals within an implant period.

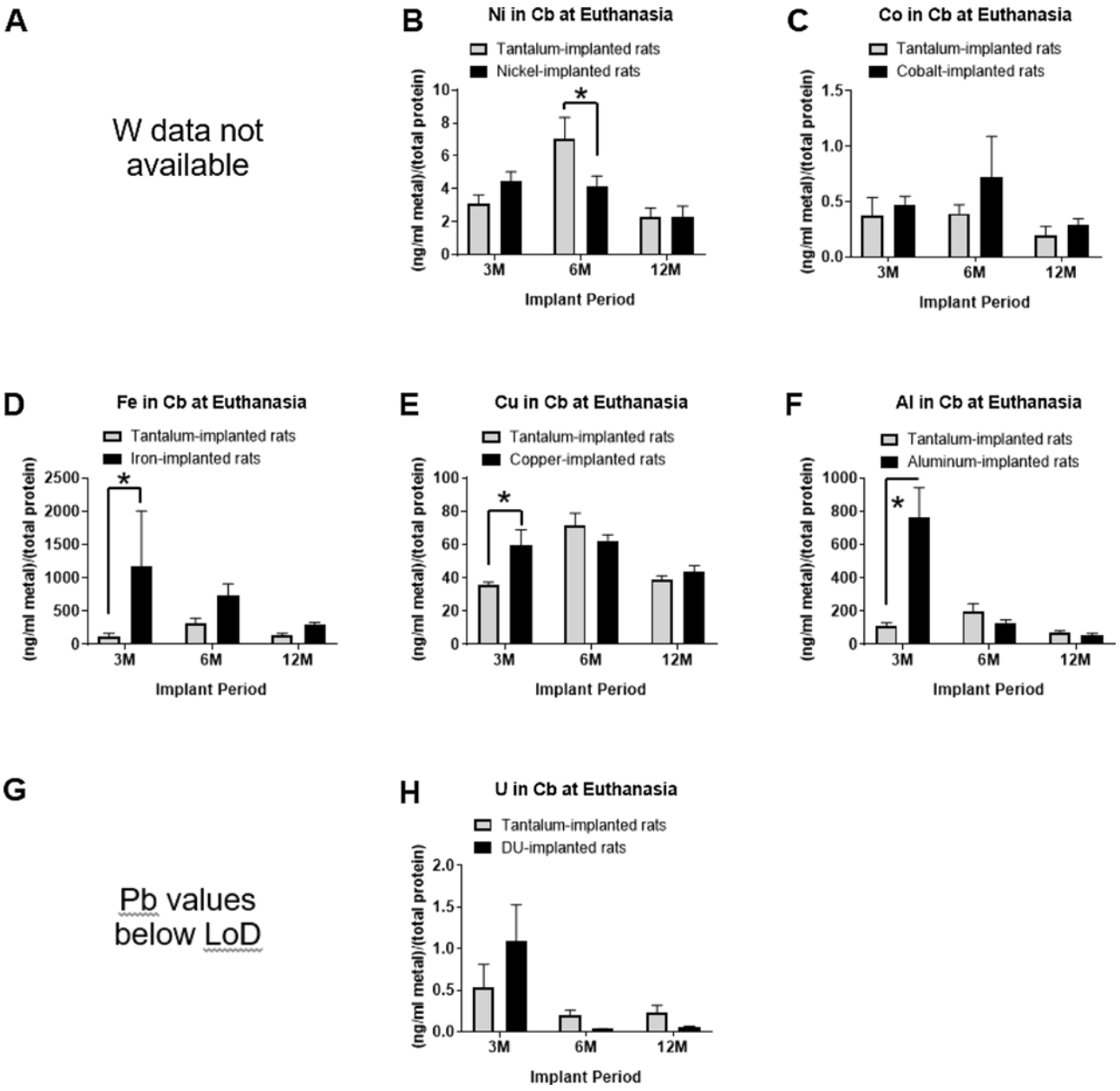

Fig. 4. Metal concentrations in Cerebellum. Cerebellum samples from metal implanted rats from four different groups of implant periods: 1 month (M), 3 M, 6 M, or 12 M from implant. In all cases gray bars indicate metal values in samples from the Ta-implanted (control) animals, and black bars indicate metal values in samples from target metal-implanted animals, with metal expression presented as ng/mL of metal normalized to total protein of the sample evaluated. Cases where data is unavailable or metal values were below the limit of detection (LoD) are indicated within the graph placeholder. A) concentration of tungsten (W) in samples from Ta- or W-implanted animals. B) concentration of nickel (Ni) in samples from Ta- or Ni-implanted animals. C) concentration of cobalt (Co) in samples from Ta- or Co-implanted animals. D) concentration of iron (Fe) in Ta- or Fe-implanted animals. E) concentration of copper (Cu) in samples from Ta- or Cu-implanted animals. F) concentration of aluminum (Al) in samples from Ta- or Al-implanted animals. G) concentration of lead (Pb) in samples from Ta- or Pb-implanted animals. H) concentration of uranium (U) in samples from Ta- or DU-implanted animals. * indicates significant post-hoc p-value between Ta- and target metal-implanted animals within an implant period.

surprisingly, the concentration of nickel was lower in the Ni-implanted animals than the matched 6 M Ta-implanted animals, and iron 3 M ($p = 0.0254$), copper 3 M ($p = 0.0132$), and aluminum 3 M ($p < 0.0001$), where in all three cases the sample metal concentration was higher in the target metal-implanted group than the matched Ta-implanted control group. Data for all 1 M cerebellum samples was not included in statistical analyses because of technical issues with determining total protein for the 1 M Ta-implanted animal samples, preventing the ability to normalize metal concentration. Data for tungsten values was not available for any implant period due to an issue with sample drift in the ICP-MS process.

3.4. Protein expression in brain regions

The BBB functions to heavily restrict movement of molecules from the circulatory system into the central nervous system primarily through formations of tight junctions between endothelial cells, which include occludin and zonula occludens-1 (ZO-1) (Daneman, 2012; Daneman and Prat, 2015; Zlokovic, 2008). Post-synaptic density protein 95 (PSD95) interacts with other related proteins to form a multimeric scaffold for organizing receptors, ion channels, and signaling proteins in the post-synaptic density of neurons, with an important role in synaptic plasticity and synapse stabilization (Stathakis et al., 1997). Spinophilin

is a protein highly enriched in dendritic spines and plays an important role in their formation and stabilization (Hsieh-Wilson et al., 1999). Synaptotagmins are a family of proteins involved in calcium sensors and regulation of neurotransmitter transport and release (Dean et al., 2012). All three of these proteins are important for the stabilization and proper function of neuronal synapses. Due to their role in BBB or synapse integrity and function, expression values of these five proteins (occludin, ZO-1, PSD95, spinophilin, and synaptotagmin) were analyzed for the four brain regions examined here.

In the interest of space, all statistical results are presented in table format, with the mean and standard deviation for protein values of the Ta-implanted groups followed by the two-way ANOVA F-statistic value for the implanted metals comparisons and its p-value, and the post-hoc corrected p-value and corresponding group mean and standard deviation, but only for those with significant post-hoc results. Protein for frontal cortex samples are in Table S4, hippocampus samples in Table S5, amygdala samples in Table S6, and cerebellum samples in Table S7. A visual summary of the significant differences is presented in Table 1, where green numbers indicate the expression value for that implant period/metal group is increased over the Ta-implanted group, and magenta numbers indicate the expression value is decreased from the Ta-implanted group. For ease of visualization we are also presenting data for two select data sets, the frontal cortex (Fig. 5A–E) and cerebellum (Fig. 5F–J), looking only at the Ta-implanted animals compared with the DU-implanted animals. A few patterns emerge that are of note: across the board if there is a significant difference between a Ta-implanted group and matched metal-implanted group, it is much more common for the protein expression to be decreased in the metal implanted group compared to the Ta controls. Across brain regions, the hippocampus has far fewer significantly affected BBB or synapse proteins, across all implant time periods, compared to the frontal cortex, amygdala, or cerebellum. Across metals, the amygdala has more late-term effects, with protein expression lower than controls in many more 12 M groups than for other brain regions. In the frontal cortex and cerebellum, there is a pattern of decreased expression, for many of the proteins, most frequently in the 3 M and 6 M time from implant groups, which then seems to resolve by the 12 M period. Overall tungsten, nickel (keeping in mind the caveat that the 12 M nickel-implanted animals did not actually experience the full 12 M time period the Ta-implanted group did), and cobalt had fewer significant effects on protein expression regardless of brain region, whereas iron, aluminum, lead, and DU have a much broader adverse effect on protein expression in each brain region. Also interestingly, with two exceptions occludin expression was never affected, and ZO-1 expression in the cerebellum was increased in the 1 M period after every metal except for tungsten and Ni, but this pattern did not hold true in other brain regions.

4. Discussion

In our study, the presence of embedded metals had relatively little impact on the concentration of metal ions in specific brain regions. We would expect accumulation of metals in brain regions would not change if the metal pellet does not solubilize enough to increase circulating levels of foreign metal or if the BBB is not damaged enough to allow the metal to cross into the brain region and accumulate. Conversely, if the foreign metal did increase in circulation and bioavailability and the BBB was damaged, we would expect to see an increase in metal concentration in the target metal-implanted rats compared to the Ta-implanted controls. This is exactly what we did see with iron in the cerebellum and hippocampus, with copper and aluminum in the cerebellum, and depleted uranium in the amygdala, but none of those increases in accumulation were sustained beyond one or three months post-implant (keeping in mind that the copper pellets were extruded by the 3 month period, so it is unknown whether brain accumulation would have been sustained as well if the pellet were present for longer periods of time). However, other metals had no effect on brain regions, or in several cases, resulted in lower concentrations of the target metal in the target implanted animals compared to the tantalum implanted controls. We can say with confidence that many of these metals do in fact solubilize and circulate throughout the body, based on similar previous studies and data from our ongoing research using this particular cohort of rats, where we examine changes in metal concentration of multiple tissues and urine [77, and data to be included in forthcoming publications]. It is also not surprising that we did not see much change in metal accumulation in specific brain regions because previous studies, while using a similar pellet implantation model, either had the same number and size of pellets that we used but used mice instead of rats, thus providing a much higher body-burden of embedded metals, or used rats but included

Table 1
Summary of protein expression in specific brain regions by metal.

Brain Region	Protein	W	Ni*	Co	Fe	Cu[a]	Al	Pb	DU
Frontal Cortex	Occludin								
	ZO-1		3 6			3		6	3
	PSD95	6		12	6	6	12	6	6
	Spinophilin			6	3 6	3 6	3 6	3 6	3 6
	Synaptotagmin			6	1	1 6	1	1	
Hippocampus	Occludin	1							
	ZO-1					1	1		
	PSD95								
	Spinophilin					6			
	Synaptotagmin	1		1	1				
Amygdala	Occludin	3	3					3	3 6
	ZO-1		12		12	12	3	3 6 12	3 12
	PSD95			12	12		12		
	Spinophilin		3	12	3 12		3	3 6	3 12
	Synaptotagmin			3 6	1 3 6	3	1 3	1 3 6	1 3 6
Cerebellum	Occludin		6						
	ZO-1			1	1	1	1	1	1
	PSD95		1	3	1 3	3	3 6	3 6	3 6
	Spinophilin	6		1	1 6	1 6	1 3 6	3 6	6
	Synaptotagmin		1	1 3	1 3 6	1 3 6	3	1 3	1 3 6

Data here is presented in summary format in order to gain an overview picture of changes in different brain regions over time for each type of embedded metal. Statistics behind this table are presented in supplementary tables. Where there is a significant post-hoc value for a within-time group difference of implanted metal compared to tantalum, green = increased over Ta-implanted animals, magenta = decreased from Ta-implanted animals.
Footnotes(* and [a]): *Ni early deaths of 12 M animals confounding factor and, [a]notes that copper pellets extrude between the 1 M and 3 M time periods.

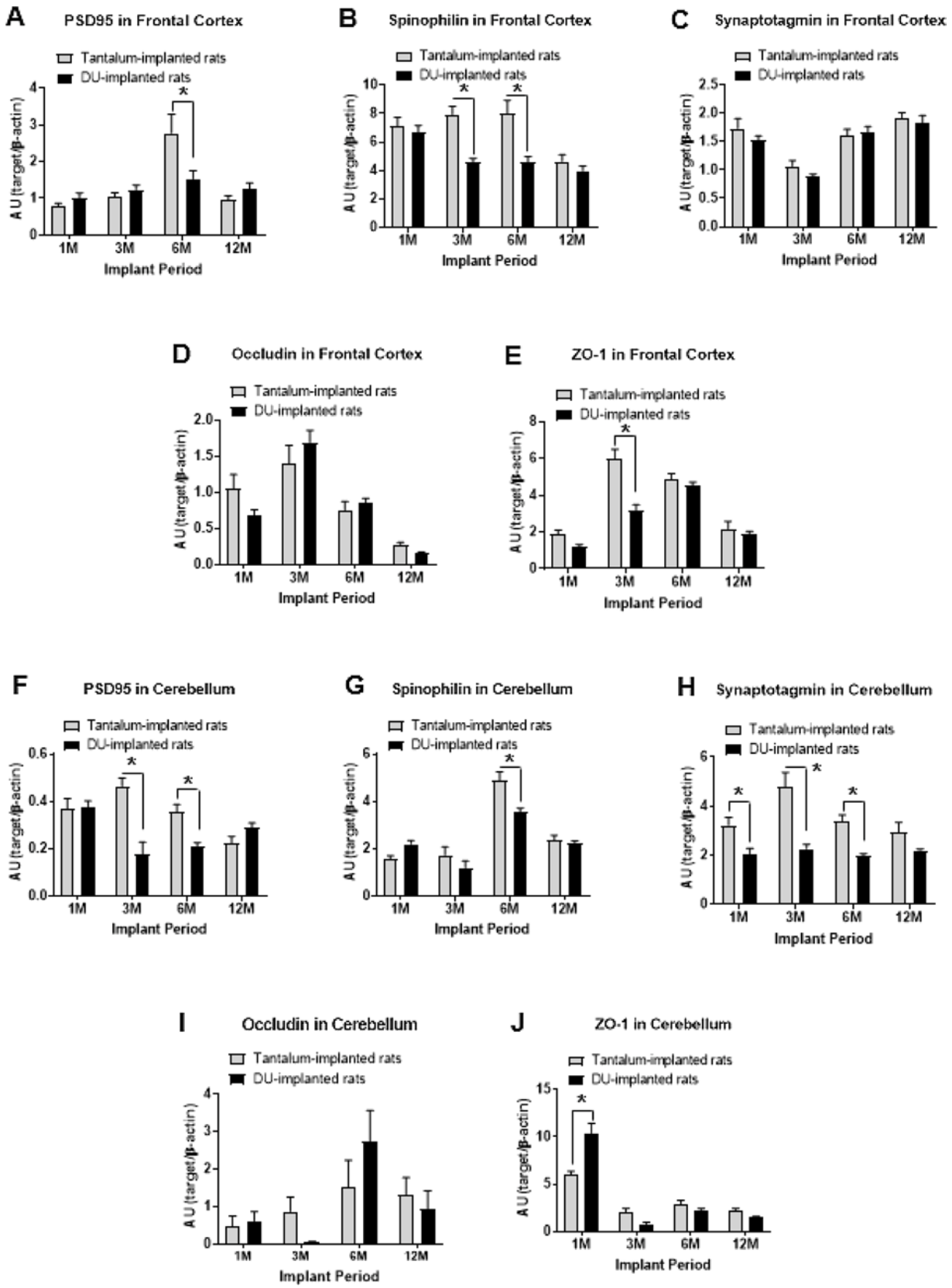

Fig. 5. Changes in protein expression in select data sets – frontal cortex and cerebellum comparing Ta-implanted rats to DU-implanted rats. In the interest of space not all protein changes for all brain regions and all metals tested are graphed in this manuscript. Instead, they are presented in summary form in Table 1 and statistics in tables S4-S7. Here we show changes in the five proteins of interest for Ta-implanted animals vs DU-implanted animals across the four implant periods for the frontal cortex (A-E) and cerebellum (F-J).

groups with much a higher number of pellets, 10 or 20 per animal as opposed to our 4, and only saw increases in metal accumulation at the higher metal body burden (Pellmar et al., 1999a; Emond et al., 2015). Additionally, it is important to note that those studies looked at metal concentrations of the entire brain, whereas here we micro-dissected specific sub-regions of the brain, thus possibly missing higher accumulation of metal in regions we did not examine here.

While the specifics of embedded metal x time from implant x brain region x specific BBB or synapse protein is complex, it is clear that not all brain regions respond to metals equally, or that every metal will have the same effect on the same brain regions or even specific proteins. BBB proteins occludin and ZO-1 were minimally affected in all brain regions, suggesting the BBB integrity is not strongly disrupted by the relatively low body burden of embedded metal in our rat cohort, which also may explain why we didn't see much of an increase in metal concentration within collected brain regions. Interestingly, some studies show zinc is involved in improving tight junction function in the intestine by increasing occludin and ZO-1 protein expression in intestinal endothelial cells in pigs (Zhang and Guo, 2009; Miyoshi et al., 2016), but it's unclear if that is also true for brain endothelial cells. While we didn't look at effects of zinc in this cohort, similar mechanisms may explain why we see an early and transient increase in ZO-1 in the cerebellum with many metals.

The majority of the work investigating effects of internalized metals on the brain comes from inhalation or oral ingestion studies. Effects of internalized metals through embedded metal, such as that from a shrapnel wound, are not as well studied. Similarly, the mechanism of action is not always clear. Tungsten has been reported to induce oxidative stress in several brain regions (cerebral cortex, hippocampus, and cerebellum) (Sachdeva et al., 2015) and morphological changes in the rat brain, correlated with neurobehavioral and memory disruptions, seen in both rats and humans (Agarwal et al., 2011; McInturf et al., 2008; Chen et al., 2005). Studies have shown that overexposure to Ni via oral ingestion alters cognitive and locomotor behaviors and leads to neurodegeneration in rat brains, and neural structural changes were reported in hippocampus and striatum (Ijomone et al., 2018a, b). Chronic exposure to cobalt and chromium from properly functioning joint replacements is associated with subtle structural change in basal ganglia in asymptomatic patients (Clark et al., 2014). DU has been shown to cross the BBB and accumulate in the brain in multiple animal models (Bolt et al., 2018; Simon et al., 2018), particularly in the frontal cortex, hippocampus, and cerebellum in rats and mice under chronic exposure conditions and multiple exposure routes (Linares et al., 2007; Barber et al., 2005; Monleau et al., 2005; Fitsanakis et al., 2006; Pellmar et al., 1999b). Interestingly, in a study using the same implanted metal rat model as we used in this study, hippocampal electrophysiological recordings in rats implanted with DU for 6 M and 12 M time periods showed reduced synaptic potentials which were no longer seen by the 18 M time point (obscured by age and natural deterioration), indicating the hippocampus may be sensitive to metals in ways that are different than other brain regions, instead of unaffected as our observed changes in protein expression could suggest. The changes in synaptic structure and function protein expression that we do see, however, could be part of the mechanism involved in the neurodegenerative diseases and adverse cognitive effects associated with so many of these metals, and the biokinetic pathways of embedded metal toxicity could be different from ingested or inhaled pathways. Part of the larger exploration of this rat cohort is examining biomarkers of adverse effects, and changes in microRNA in muscle around the pellets (Wen et al., 2020) and microRNA in exosomes in serum and urine may shed some light on those mechanisms, especially since some of the pathway algorithms include the cerebellum [data not shown, publications forthcoming].

Reports on effects of DU on behavior in rodents are mixed, with some reporting no changes (Pellmar et al., 1999a; Arfsten et al., 2007) and others reporting increased anxiety, depressive-like behavior, and disrupted spatial working memory (Lestaevel et al., 2015, 2016), likely due to differences in study design regarding timing (neonatal, early postnatal, young adult) and ranges in dose in addition to internalization route. Investigated structural changes from uranium exposure are subtle, consisting of mostly of reported differences in cell proliferation numbers during early development in rats (Legrand et al., 2016; Dinocourt and Culeux, 2017). In humans, no consistent differences in cognitive performance related to internal DU dose have been reported in the longitudinal studies of the Operation Desert Storm cohort (McDiarmid et al., 2017, 2018). Without behavioral analysis, which was unfortunately beyond the scope of this animal experimental cohort, it is difficult to say what clinical relevance, if any, the observed alterations in expression of BBB tight junction and synapse markers may have.

4.1. Limitations and future investigations

As mentioned before, behavioral tasks were outside the scope of this grant and thus are not available to correlate with changes in brain metals or BBB and synapse related proteins, limiting the interpretation of clinical relevance of observed changes. Further, due to various sources of sample loss described in relevant results sections, we do not have the statistical power to run correlation analyses between changes in metal concentration and changes in protein expression by brain region. In addition, the experimental design set out to identify potential differences in a wide variety of biomarkers relevant to specific embedded metals, not necessarily to determine mechanistic actions of those metals in various tissues. This manuscript focuses on changes observed in several brain regions, but ongoing work in samples from the same animals will assess changes in metal concentrations of multiple tissues (including gastrocnemius, thymus, heart, kidney, lung, liver, spleen, bone, feces, urine, and blood serum) as well as miRNA expression changes in serum and urine, and a wide variety of biomarker panels related to inflammatory cytokines, oxidative stress, kidney damage, and muscle damage. As more of this data is processed we plan to create a full-system assessment of the impact of different types of embedded metals over time in the rat model, and compare this with urine and blood serum markers observed in the human military personnel investigated concurrently by our partners in the Baltimore Department of Veterans' Affairs (DVA) Medical Center.

4.2. Conclusions

The expanded human retained-fragment investigational cohort being conducted by our co-investigators may reveal subtle differences in cognitive function associated with different types of retained metal. Meta-analysis of those results plus other health assessments and tissue biomarkers of this rat cohort will hopefully provide greater insight into the overall health risks of a variety of embedded metals. For now, we conclude that even relatively low body burdens of metal can have adverse effects of protein expression related to blood brain barrier structure and several proteins involved in synapse structure and function, which could play a role in adverse cognitive effects observed in some military personnel with retained metal fragments.

Funding and ethical approval

The project described was supported by the grant Health Effects of Blast Injuries and Embedded Metal Fragments (W81XWH-16-2-0058) from the Congressionally Directed Medical Research Program (CDMRP) Peer-reviewed Medical Research Program. All procedures involving animals were (a) conducted with maximal possible well-being of the rats, (b) approved by the AFRRI IACUC prior to the start of the study under protocol 2016-05-006, and (c) performed in compliance with the guidelines set forth in the *Guide for the Care and Use of Laboratory Animals* in an AAALAC-I-accredited facility. The views expressed in the paper are those of the authors and do not reflect the official policy or position of the Armed Forces Radiobiology Research Institute,

Uniformed Services University, Department of Defense, or US Government.

Data availability

All data obtained in support of this submission have been presented. Due to instrument malfunction, metal data were not obtained for tungsten levels in amygdala and cerebellum and could not be reanalyzed due to lack of sample. In addition, in those cases where metal concentration results were below the limit of detection, that fact is denoted in the figures.

CRediT authorship contribution statement

Jessica F. Hoffman: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **Vernieda B. Vergara:** Data curation, Investigation, Methodology, Software, Validation, Visualization, Writing - review & editing. **John F. Kalinich:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

The authors would like to thank Raisa Marshall, Anya Fan, and William Danchanko for their expertise in the pellet implantation surgeries, animal welfare checks, and tissue collection. The authors would also like to thank W. Louis Wilkins for histopathology support.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2021.01.001.

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Toxicology Reports 8 (2021) 463–480

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Effect of embedded metal fragments on urinary metal levels and kidney biomarkers in the Sprague-Dawley rat

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ARTICLE INFO

Edited by Dr. A.M Tsatsaka

Keywords:
Embedded metals
Rat
Urine
Kidney
Biomarker

ABSTRACT

Background: Wounds with embedded metal fragments are an unfortunate consequence of armed conflicts. In many cases the exact identity of the metal(s) and their long-term health effects, especially on the kidney, are not known.
Aim of study: The aim of this study was to quantitate the urinary levels of metals solubilized from surgically implanted metal pellets and to assess the effect of these metals on the kidney using a battery of biomarker assays.
Materials and methods: Using a rodent model system developed in our Institute to simulate embedded fragment injuries, eight metals considered likely components of an embedded fragment wound were individually implanted into the gastrocnemius muscle of male Sprague-Dawley rats. The rats were followed for 12 months post-implantation with urine collected prior to surgery then at 1-, 3-, 6-, 9-, and 12-months post-implantation to provide a within-subjects cohort for examination. Urinary metal levels were determined using inductively coupled plasma-mass spectrometry and urinary biomarkers assessed using commercially available kits to determine metal-induced kidney effects.
Results: With few exceptions, most of the implanted metals rapidly solubilized and were found in the urine at significantly higher levels than in control animals as early as 1-month post-implantation. Surprisingly, many of the biomarkers measured were decreased compared to control at 1-month post-implantation before returning to normal at the later time points. However, two metals, iron and depleted uranium, showed increased levels of several markers at later time points, yet these levels also returned to normal as time progressed.
Conclusion: This study showed that metal pellets surgically implanted into the leg muscle of Sprague-Dawley rats rapidly solubilized with significant levels of the implanted metal found in the urine. Although kidney biomarker results were inconsistent, the changes observed along with the relatively low amounts of metal implanted, suggest that metal-induced renal effects need to be considered when caring for individuals with embedded metal fragment wounds.

1. Introduction

Embedded metal fragment wounds are not a new phenomenon. They have been a potential battlefield injury since the invention of gunpowder. However, because of the ballistic properties of the ammunition used in early weapons, most injuries resulted in death or traumatic amputation [1,2]. It wasn't until the development of the full metal-jacketed bullet around the time of the Spanish-American War that the survivability from battle wounds improved and the probability of embedded metal fragments increased [3]. The health risk of embedded fragments was considered low because they were considered to be inert once in the body. However, there began appearing in the scientific literature occasional individual case reports on medical issues associated with embedded fragment wounds [4–9]. In most instances, these wounds were suffered during wartime many years prior to the manifestation of the adverse health effect.

Abbreviations: AAALAC-I, Association for Assessment and Accreditation of Laboratory Animal Care International; AFRRI, Armed Forces Radiobiology Research Institute; Al, Aluminum; ALB, Albumin; ALP, Alkaline phosphatase; B2m, Beta-2-microglobulin; Co, Cobalt; Cu, Copper; DoD, Department of Defense; DU, Depleted uranium; Fe, Iron; IACUC, Institutional Animal Care and Use Committee; ICP-MS, Inductively coupled plasma-mass spectroscopy; IL-18, Interleukin-18; KIM-1, Kidney injury molecule-1; LoD, Limit of detection; LoQ, Limit of quantitation; NAG, N-acetyl-beta-D-glucosaminidase; NGAL, Neutrophil gelatinase-associated lipocalin; Ni, Nickel; OPN, Osteopontin; Pb, Lead; RBP, Retinal binding protein; Ta, Tantalum; W, Tungsten.

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https://doi.org/10.1016/j.toxrep.2021.02.023
Received 22 December 2020; Received in revised form 20 February 2021; Accepted 23 February 2021
Available online 1 March 2021
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While laboratory investigations into the health effects of embedded metals have been ongoing for many years, these studies have focused primarily on the safety of implanted medical devices [10]. Little had been done to assess the long-term health effects of military-relevant metals and metal mixtures [11]. However, that changed after the First Persian Gulf War in the early 1990′s when, as a result of friendly-fire incidents, several U.S. military personnel were wounded by depleted uranium (DU) fragments. Standard medical advice at that time was to leave embedded fragments in place. However, due to the unique chemical and radiological properties of DU, questions were raised over the wisdom of leaving these fragments in place for the life of the individual. Because of the lack of available information on the long-term effects of embedded DU, preliminary investigations into the biokinetics and toxicology of embedded DU fragments were initiated [12,13]. Furthermore, due to continuing concerns over the health and environmental effects of DU munitions, replacement materials were sought, and several tungsten-based compositions were proposed. However, when one of these compositions (tungsten/nickel/cobalt) was tested in a rodent embedded-fragment model system, it was discovered that this composition induced malignant highly-aggressive rhabdomyosarcomas at the implantation sites [14]. Conversely, a material composed of tungsten/nickel/iron did not result in any tumor formation [15,16]. These findings further underscore our dearth of knowledge with respect to the health effects of embedded fragments of military-relevant metals. Compounding the urgency of this issue is the fact that the recent conflicts in Iraq and Afghanistan have resulted in over 45,000 wounded U.S. personnel, with an estimated two-thirds of these individuals having retained metal fragments. Since standard surgical guidelines recommend leaving embedded fragments in place in an attempt to balance the potential long-term health risk of the embedded fragment with the risk of morbidity that extensive surgery brings, these wounded warriors could carry these fragments for the rest of their lives. Adding to this dilemma is that due to advances in vehicle armor and weapons design and the insurgent use of improvised explosive devices (IEDs), the list of metals and metal mixtures that may potentially be found as embedded fragments is extensive. As a result, the U.S. Department of Defense (DOD) and the Department of Veterans Affairs (DVA) have promulgated a list of “metals of concern” with respect to embedded fragments [17,18]. However, the biokinetics and toxicological properties of many of these metals when embedded as fragments are not known.

One *in vivo* property of most embedded metal fragments, regardless of the system, is that they tend to degrade over time with the resulting solubilized metal eventually excreted in the urine [12,14,19–23]. This raises a concern about both acute and chronic effects on the kidney as the solubilized metal is excreted. Although our group is focused on embedded metal fragments such as those found in shrapnel wounds, implanted medical devices have also been found to degrade and release metals *in vivo*. For example, cobalt from metal-on-metal hip replacements [24], titanium from dental implants [25], and aluminum from titanium/aluminum/vanadium joint prostheses [26] have been found in serum and urine. Previously published studies examining metal effects on kidney function focused primarily on environmental exposures, particularly through drinking water. These studies showed that a variety of non-essential metals including aluminum [27,28], nickel [29], lead [30,31], and uranium [32,33] can adversely affect kidney health. Little is known concerning the *in vivo* overload of endogenous metals on renal health, with iron being the most studied [34–36]. In addition, exposure to environmental metal pollutants via inhalation or dermal routes can also affect metal excretion patterns as well as kidney well-being [37–40]. Unfortunately, in many cases these types of exposures disproportionally affect poorer populations lacking the financial or political ability to induce change [41]. However, metals are not alone in disrupting renal health. Exposure to other toxins, including both biological and chemical, by a variety of exposure routes have been shown to induce adverse renal effects [42–45].

This work is part of a larger collaborative effort with the University of Maryland School of Medicine, the Department of Veterans' Affairs Medical Center in Baltimore, the U.S. Food and Drug Administration, and the University of Kentucky to investigate the potential health effects of embedded fragments from the “metals of concern” list in our rodent shrapnel model alongside an expanded human investigation into military personnel with a wider array of retained metal fragments. In this particular study, we followed rodents embedded with metal fragments for up to 12 months to determine urinary metal levels as a result of solubilization of the embedded fragments, as well as a series of urinary biomarkers designed to assess metal-induced renal damage.

2. Materials and methods

2.1. Animals and husbandry

All animal research in this study was approved prior to initiation by the Armed Forces Radiobiology Research Institute IACUC under protocol number 2016−05-006. All procedures involving animals were conducted in compliance with the guidelines found in the *Guide for the Care and Use of Laboratory Animals* [46] in an AAALAC-I-accredited facility. Male Sprague-Dawley (*Rattus norvegicus*) rats were utilized in these experiments and were obtained from Envigo (Barrier 208A, Frederick, MD) when approximately 30 days old and 75−100 g. After arriving at the AFRRI vivarium, rats were allowed to acclimate for at least 2 weeks prior to the start of experiments. Rats were pair-housed throughout the study in plastic microisolator cages (23.8 × 45.4 cm) with filter tops and bedding (Teklab Sani-Chips, Envigo). Bedding was changed 2–3 times per week. Animal rooms were maintained at 21 ± 2 °C with 30–70% humidity and a 12:12-h light:dark cycle (lights on at 0600). Rats were fed a standard rat chow (Teklad Global Rodent Diet 8604, Envigo) with access to water *ad libitum*.

2.2. Experimental design

Earlier work in our Institute developed a rodent model for the study of the health effects of embedded metals such as shrapnel wounds [47]. Utilizing that model, we assessed the effects of eight metals of military relevance including tungsten (W), nickel (Ni), cobalt (Co), iron (Fe), copper (Cu), aluminum (Al), lead (Pb), and depleted uranium (DU). The inert metal tantalum (Ta) was used as a control for any changes due to the surgical procedure or presence of a foreign material in the muscle. Previous work by us and others [12–14] have shown no differences between naïve rats and tantalum-implanted sham rats. As a result, the total number of animals needed for the study was reduced and the goals of the ARRIVE Guidelines met [48]. A within-subjects experimental design was used with rats (n = 8) randomly assigned to one of the nine metal implantation groups.

Metal pellets were 1 mm in diameter x 2 mm in length and were purchased from Alfa Aesar (Ward Hill, MA) with purities greater than 99.99 %, with the exception of DU pellets which were purchased from Aerojet Ordnance (Jonesboro, TN). Prior to surgical implantation, pellets were cleaned and chemically sterilized as previously described [16].

2.3. Metal implantation procedures

Metal pellets were surgically implanted into the gastrocnemius muscle of the rats as previously described [49]. Briefly, rats were initially anesthetized by administration of isoflurane (Baxter Healthcare Corp., Deerfield, IL) in an induction chamber and then maintained for the surgical period using a nose cone with a scavenger/recapture system. The surgical sites were clipped then swabbed with 70 % isopropanol followed by betadine (Purdue Pharma LP, Stamford, CT). A prophylactic dose of the analgesic buprenorphine (0.05−0.1 mg/kg, s.c., Rickitt and Colman, Hull, UK) was administered. For identification purposes, a small transponder (Electronic Lab Animal Monitoring

System, BioMedic Data Systems, Seaford, DE) was injected subcutaneously in the mid-dorsal thoracic region. The transponders were programmed with a unique animal identification number which can then be read with a low-power radio frequency scanner. An ear punch was also performed to serve as a secondary mode of identification in case of transponder failure. Using aseptic technique, an incision approximately 5 mm in length was made through the skin of each hind leg to reveal the gastrocnemius muscle. Using a 16-gauge needle and specially designed plunger, two sterile pellets were implanted into the lateral side of each gastrocnemius spaced approximately 1.5 mm apart. Following metal implantation, the incisions were sealed with tissue adhesive (VetBond, 3 M Corp, St Paul, MN). Rats were closely monitored following surgery until ambulatory. The surgical sites were examined daily for signs of inflammation, infection, and localized metal toxicity for two weeks post-surgery and then weekly thereafter for the duration of the study.

2.4. Urine collection with lab sand

Prior to pellet implantation and then at 1, 3, 6, and 9 M post-implantation, as well as immediately prior to scheduled euthanasia at 12 M post-implantation, urine samples were collected using a previously published method [50–52]. The hydrophobic sand method is less stressful than metabolic cage methods and does not introduce metal contaminants or otherwise affect urine biomarkers. Briefly, for each rat, 300-g (single pack) of hydrophobic sand (LabSand, Coastline Global, Palo Alto, CA) was spread across the bottom of a mouse plastic micro-isolation cage (15.2 cm × 25.4 cm, surface area 386 cm^2) with a filtered lid. Rats were singly placed in the prepped cage without food or water for 2 h with no further restriction on movement. Urine pooled on top of the sand and was collected immediately by pipette during each 2 h session. Samples were stored at −80 °C until analyzed.

2.5. Euthanasia and tissue collection

Upon reaching their experimental end point or when indicated by guidelines approved by the IACUC, rats were humanely euthanized by isoflurane overdose followed by exsanguination and confirmatory pneumothorax per the guidelines of the American Veterinary Medical Association [53]. Criteria for euthanasia included tumor size greater than 1 cm in diameter, loss of greater than 10 % of baseline body weight, or other indications of approaching moribundity as determined by animal health assessments. After euthanasia, a complete gross pathology examination was conducted, and a variety of tissues isolated for analysis, including brain, liver, heart, lung, popliteal lymph node, tibia, fibula, femur, gastrocnemius and triceps muscle, spleen, thymus, kidney, bladder, testes, and a fecal sample. The wet weights of liver, spleen, thymus, kidney, and testes were determined and normalized to body weight. Tissues for metal analysis were weighed and stored at −80 °C until analysis. Tissues destined for histopathology were fixed in zinc-buffered formalin and stored at 4 °C. In addition, immediately prior to euthanasia, a blood sample was collected from the abdominal aorta of the anesthetized animal to provide a sample for hematological assessment, as well as isolation of serum for clinical chemistry and metal analysis. This study focuses on results of urine sample analyses.

2.6. Metal analysis by inductively coupled plasma-mass spectroscopy (ICP-MS)

All reagents used in this study were of the highest grade available. Plastic ware and other disposables were obtained from Thermo Fisher Scientific (Pittsburgh, PA). Urine samples were diluted in ultrapure nitric acid (Optima Ultrapure Grade, Fisher Scientific, Newark, DE) and metal content determined using an inductively coupled plasma-mass spectrometer (XSeries 2 ICP-MS System, ThermoFisher, Madison, WI) equipped with a Cetac ASX520 Autosampler (Cetac Technologies, Omaha, NE). High-pressure liquid argon, 99.997 %, was used for the plasma gas. The instrument was calibrated with external standards of the appropriate metal standard (SPEX CertiPrep, Metuchen, NJ) in 2% HNO_3. The sample probe was washed with a constant flow of 2% nitric acid between measurements to prevent carryover. Quantitative analysis was obtained by reference to the slope of the calibration curve (counts per second / ng per liter) as well as an internal standard. Urine data were normalized to creatinine levels (see below). Limit of Detection (LoD)/ Limit of Quantitation (LoQ), in ppb, are as follows: Ta - 0.50/0.91; W - 0.12/0.15; Ni - 0.17/0.21; Co - 0.03/0.06; Fe - 1.08/1.85; Cu - 0.24/ 0.54; Al - 0.38/0.44; Pb - 0.02/0.04; U - 0.02/0.07.

2.7. Urine markers

2.7.1. Creatinine

Urine creatinine levels were determined by utilizing a modified Jaffe reaction [54,55] with a commercially available colorimetric kit (catalog no. CR01, Oxford Biomedical Research, Oxford, MI). Reactions were read at 490 nm in a plate reader (BioTek Synergy Model H1 M Multimodal Plate Reader with GEN5 Software, BioTek Instruments, Winooski, VT). Creatinine concentrations were determined against a standard curve (0–10.0 mg/dL) and corrected for dilution.

2.7.2. Total protein

Total protein from each urine sample was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, cat #500−0006), in triplicate, against a BSA standard curve, in a plate reader (BioTek Synergy) at 595 nm.

2.7.3. Alkaline phosphatase

Urinary alkaline phosphatase levels were determined using a colorimetric kit (kit # ab83369, Abcam, Cambridge, MA). The kit uses p-nitrophenyl phosphate (pNPP) as a phosphatase substrate which turns yellow when dephosphorylated by alkaline phosphatase. Briefly, samples and pNPP are added to a 96-well plate and incubated at room temperature for 60 min. After adding stop solution, the plate is read at 405 nm in a spectrophotometer (BioTek Synergy). Alkaline phosphate concentration is read from a standard curve and expressed as μmol/min/mL or μU/mL with an assay range of 10–250 μU.

2.7.4. Beta-2-microglobulin

Urinary beta-2-microglobulin (B2m) was measured using an ELISA kit (kit # 80666, Crystal Chem Inc., Elk Grove Village, IL). The kit uses a double antibody sandwich technique where the B2m in the sample binds to antibodies against B2m bound to the surface of a 96-well plate. Use of a second anti-B2m antibody conjugated to horseradish peroxidase (HRP) allows for the determination of beta-2-microglobulin levels against a standard curve. Briefly, diluted samples are added to the antibody-coated plate and incubated at room temperature for 60 min. After washing, the B2m antibody-HRP conjugate is added and the plate incubated in the dark at room temperature for 10 min. After washing the plate, HRP Substrate Solution, contained in the kit, is added and the plate incubated in the dark at room temperature for 10 min. After stopping the reaction, the plate is read at both 450 and 630 nm in a plate reader (BioTek Synergy). After subtracting the 630 nm reading from the 450 nm reading to correct for background, the amount of B2m in the sample can be determined from a standard curve as ng/mL, with an assay range of 0.156–5 ng/ml.

2.7.5. N-Acetyl-beta-D-glucosaminidase

N-Acetyl-beta-D-glucosaminidase (NAG) urinary levels were determined using a colorimetric kit (kit # 80390, Crystal Chem Inc.) based on the ability of NAG to hydrolyze 2-methoxy-4-(2′-nitrovinyl)-phenyl-2-acetoamido-2-deoxy-β-D-glucopyranoside to 2-methoxy-4-(2′-nitrovinyl)-phenol. Making the reaction mixture alkaline results in color development of the product that can be detected at 505 nm. Briefly, the assay involves mixing two of the kit reagents and adding that to diluted

urine samples in a 96-well plate. The plate is then incubated at 37 °C on a plate shaker (VWR International, Radnor, PA) at 200 rpm for 5 min. After an initial absorbance reading at 505 nm in a plate reader (BioTek Synergy), alkaline color development reagent from the kit is added and the plate incubated for 1 min at 37 °C and 200 rpm on a plate shaker before a second absorbance reading at 505 nm. Subtracting the initial reading from the second reading at 505 nm gives the change in absorbance per min. This result is compared to the calibration standard supplied in the kit to obtain NAG concentration in the sample expressed as IU/L. The linear assay range of the procedure is 0–200 IU/L.

2.7.6. Retinol binding protein

Urinary levels of retinol binding protein (RBP) were determined using a sandwich ELISA method (kit # ab203362, Abcam). Briefly, diluted urine samples and the Antibody Cocktail mixture, consisting of anti-RBP antibody and HRP-conjugated detection antibody, provided in the kit are added to an antibody-coated plate and incubated for 1 h at room temperature on a plate shaker (VWR International) set at 400 rpm. After extensive washing, the HRP substrate 3, 3′, 5, 5′-tetramethylbenzidine (TMB) is added and the plate incubated with shaking (400 rpm) for 10 min in the dark at room temperature to allow for color development. After stopping the reaction, the plate is read at 450 nm in a plate reader (BioTek Synergy). The amount of RBP in the sample is determined using a standard curve and is expressed as pg/mL. The reported assay linear range is 13.3–850 pg/ml.

2.7.7. Interleukin-18

Interleukin-18 (IL-18) levels in the collected rat urine were assessed using a sandwich ELISA (kit # EKF57851, Biomatik, Wilmington, DE). Briefly, after an initial plate washing, samples and standards are added and the plate incubated at 37 °C for 90 min on a plate shaker (VWR International) set at 200 rpm. After washing, biotin-labeled antibody is added, and the plate incubated at 37 °C for 60 min on a plate shaker set at 200 rpm. After extensive washing, HRP-streptavidin conjugate is added to the plate and again incubated at 37 °C and 200 rpm; this time for 30 min. After additional washes, TMB substrate is added and the plate incubated at 37 °C and 200 rpm, in the dark, for up to 30 min. After the addition of Stop Solution, the plate is read at 450 nm in a plate reader (BioTek Synergy). The amount of IL-18 present is determined from a standard curve. Reported linear range of the assay is 31.25–2000 pg/ml.

2.7.8. Kidney Injury Molecule-1, neutrophil gelatinase-associated lipocalin, albumin, and osteopontin

Urinary levels of Kidney Injury Molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), albumin, and osteopontin (OPN) were determined using the rat kidney injury panel 1 kit from Meso Scale Discovery (kit # K15162C, Rockville, MD). The technique uses a special electrode-containing 96-well plate coated with the appropriate capture antibodies to bind the analytes of interest. Addition of detection antibodies conjugated to a proprietary chemiluminescence tag allow quantitation of the targeted proteins using a Meso Scale Discovery QuickPlex Reader. The instrument applies a voltage to the electrodes in the plate that causes the tagged detection antibodies to emit light. The intensity of the light provides a quantitative measure of the analytes in the urine sample, with concentrations determined by comparison to a standard curve. Reported linear ranges of the procedure are as follows: KIM-1 (0.135–9.86 ng/ml); NGAL (0.316–230 ng/ml); albumin (67.2–49000 ng/ml); and OPN (0.0711–51.8 ng/ml).

2.8. Histopathology

Tissues for histopathology were fixed in zinc-buffered formalin (Z-Fix; Anatech Ltd, Battle Creek, MI), processed and embedded in paraffin, sectioned at 5–6 mm, and stained with hematoxylin and eosin. Histopathological assessments were conducted by a board-certified veterinary pathologist.

Table 1
ICP-MS Operating Conditions and Parameters.

Instrument Parameters	
Nebulizer type	Concentric
Spray chamber	Conical, with impact bead
Sampler cone	Platinum, 1 mm orifice diameter
Skimmer cone	Platinum, 0.7 mm orifice diameter
Sample uptake rate	1.0 mL/min
Sample read delay	45 s
Plasma conditions	
RF power	1400 W
Plasma argon gas flow	13.0 L/min
Auxiliary argon gas flow	0.80 L/min
Nebulizer gas flow	0.91 L/min
Mass spectrometer settings	
Scanning mode	Peak jump
Sweeps	100
Dwell time	500 μs
Channels/mass	1
Acquisition time	10 s
Number of readings/replicate	3
Number of replicates	2

2.9. Statistical analysis

The 12 M-implant group had urine collected at every time point (pre-surgery (PS), 1 M, 3 M, 6 M, 9 M, and 12 M) using LabSand, and thus metal and biomarker analysis using these samples were analyzed as within-subjects repeated measures (across time): two-way ANOVA, with Sidak's multiple comparisons test post-hoc. Mixed-model analysis is used instead of ANOVA if data is missing. Analyses were performed using GraphPad Prism Software (version 8.0.1, La Jolla, CA). In all cases P values < 0.05 were considered significant.

3. Results

In this study, male Sprague-Dawley rats were surgically implanted in the gastrocnemius muscle with pellets of military-relevant metals (2 pellets per leg). Using the LabSand technique, urine was collected prior to surgery, then at 1 M, 3 M, 6 M, 9 M, and 12 M post-implantation to provide urine for a within-subjects evaluation of metal excretion and urinary biomarkers.

3.1. Urinary metals

Operating conditions and parameters for the ICP-MS are found in Table 1. Urine metal concentrations, normalized to creatinine levels, are presented in Fig. 1. Tungsten values are presented in Panel A, comparing Ta-implanted (control) rats to W-implanted rats; $F_{time}(560) = 45.27$, $p < 0.0001$, $F_{metal}(114) = 156.4$, $p < 0.0001$, $F_{interaction}(560) = 20.37$. At all times from implant with the exception of pre-surgery (PS), W was higher in urine from the W-implanted animals than from Ta-implanted animals (1 M, 3 M, 6 M, 9 M, 12 M, *$p < 0.0001$ for all). Nickel values are presented in Panel B, comparing Ta-implanted rats to Ni-implanted rats; $F_{time}(332) = 33.47$, $p < 0.0001$, $F_{metal}(114) = 124.8$, $p < 0.0001$, $F_{interaction}(332) = 30.14$, $p < 0.0001$, noting that analysis drops the 9 M and 12 M groups because Ni-animals were euthanized prior to this point due to tumor formation and no data exists for comparison. Ni was higher in urine from the Ni-implanted animals compared to the Ta-implanted animals at 1 M, 3 M, and 6 M collection times (*$p < 0.0001$ for all). Cobalt values are presented in Panel C, comparing Ta-implanted rats to Co-implanted rats; $F_{time}(557) = 5.032$, $p = 0.0007$, $F_{metal}(114) = 54.67$, $p < 0.0001$, $F_{interaction}(557) = 4.760$, $p = 0.0011$. Co was higher in urine from the Co-implanted rats than from Ta-implanted rats at all

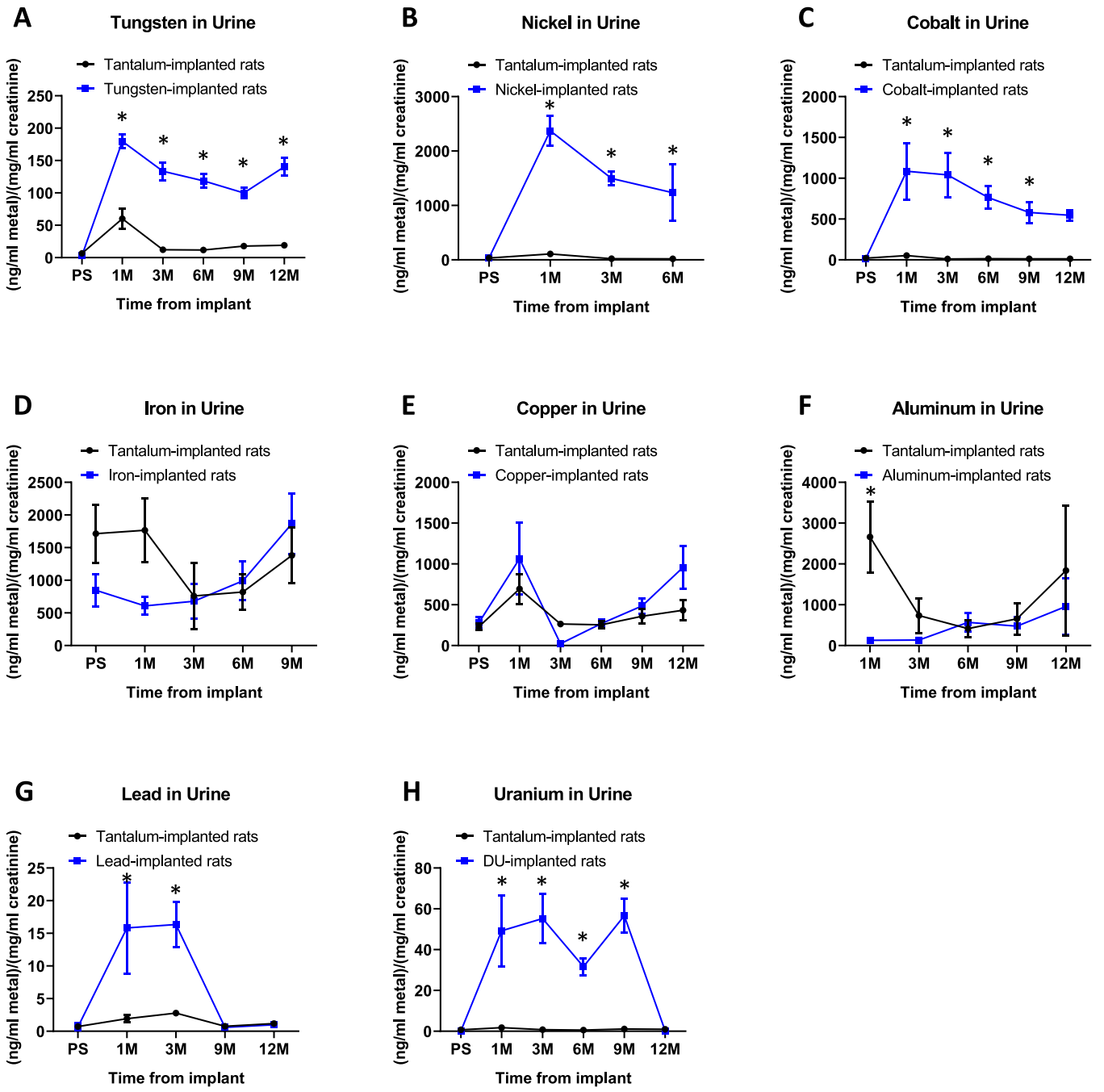

Fig. 1. Urinary metal concentrations.
Data presented as target metal concentration normalized to creatinine values in urine collected from multiple time points from individual animals. Each panel compares values in urine from the Ta-implanted animals as controls (black circles/line) vs specific target metal-implanted animals (blue squares/line). (A) tungsten, (B) nickel, (C) cobalt, (D) iron, (E) copper, (F) aluminum, (G) lead, (H) uranium. Error bars represent standard error of the mean. An * indicates a post-hoc test between Ta- and target metal-animals at that time point, $p < 0.05$.

collection times except PS and 12 M (1 M and 3 M *$p < 0.0001$, 6 M *$p = 0.0005$, 9 M *$p = 0.0094$). Iron values are presented in Panel D, comparing Ta-implanted rats to Fe-implanted rats; $F_{time}(442) = 1.662$, $p = 0.1769$, $F_{metal}(114) = 1.248$, $p = 0.2828$, $F_{interaction}(442) = 2.327$, $p = 0.0718$, noting that the 12 M data set was dropped from analysis due to lack of adequate urine samples. There were no significant post-hoc differences between metal and control at any time period.

Copper values are presented in Panel E, comparing Ta-implanted rats to Cu-implanted rats; $F_{time}(555) = 6.143$, $p = 0.0001$, $F_{metal}(114) = 1.393$, $p = 0.2576$, $F_{interaction}(555) = 1.264$, $p = 0.2927$. There were no significant post-hoc differences between metal and control at any time period. Aluminum values are presented in Panel F, comparing Ta-implanted rats to Al-implanted rats; $F_{time}(442) = 1.848$, $p = 0.1377$, $F_{metal}(114) = 4.076$, $p = 0.0631$, $F_{interaction}(442) = 2.876$, $p = 0.0342$, noting the PS data set was dropped from analysis due to errors measuring Al for this set. Aluminum in urine from the control Ta-implanted animals is higher than in Al-implanted animals at 1 M (*$p = 0.0023$) but is not significantly different at any other time point. Lead values are presented in Panel G, comparing Ta-implanted rats to Pb-implanted rats; $F_{time}(452) = 6.829$, $p = 0.0002$, $F_{metal}(114) = 10.71$, $p = 0.0019$, $F_{interaction}(452) = 4.554$, $p = 0.0031$, noting the 6 M data set was dropped from analysis due to lack of adequate urine samples. Pb

Table 2
Potential Urinary Biomarkers of Kidney Injury.

Nephron Location	Marker(s)
Glomerulus	Albumin, β2m
Proximal Tubule	Albumin, β2 m, IL-18, KIM-1, NGAL, NAG, OPN, RBP
Loop of Henle	OPN
Distal Tubules	OPN, NGAL, NAG, IL-18, ALP
Collecting Duct	NGAL

was higher in urine from Pb-implanted animals compared to Ta-implanted animals at 1 M (*p = 0.0016) and 3 M (*p = 0.0008) after implant. Uranium values are presented in Panel H, comparing Ta-implanted rats to DU-implanted rats; $F_{time}(553) = 8.153$, $p < 0.0001$, $F_{metal}(114) = 40.49$, $p < 0.0001$, $F_{interaction}(553) = 7.881$, $p < 0.0001$. Uranium was higher in urine from DU-implanted animals compared to Ta-implanted animals for all time points except pre-surgery (PS) and 12 M (1 M, 3 M, and 9 M *<0.0001, 6 M *p = 0.0028).

3.2. Urinary biomarkers

An overview of urinary markers associated with the site of potential nephron damage is given in Table 2. As can be seen, most nephron locations are represented by multiple markers and markers can be linked to more than one location. The compiled urinary marker data is

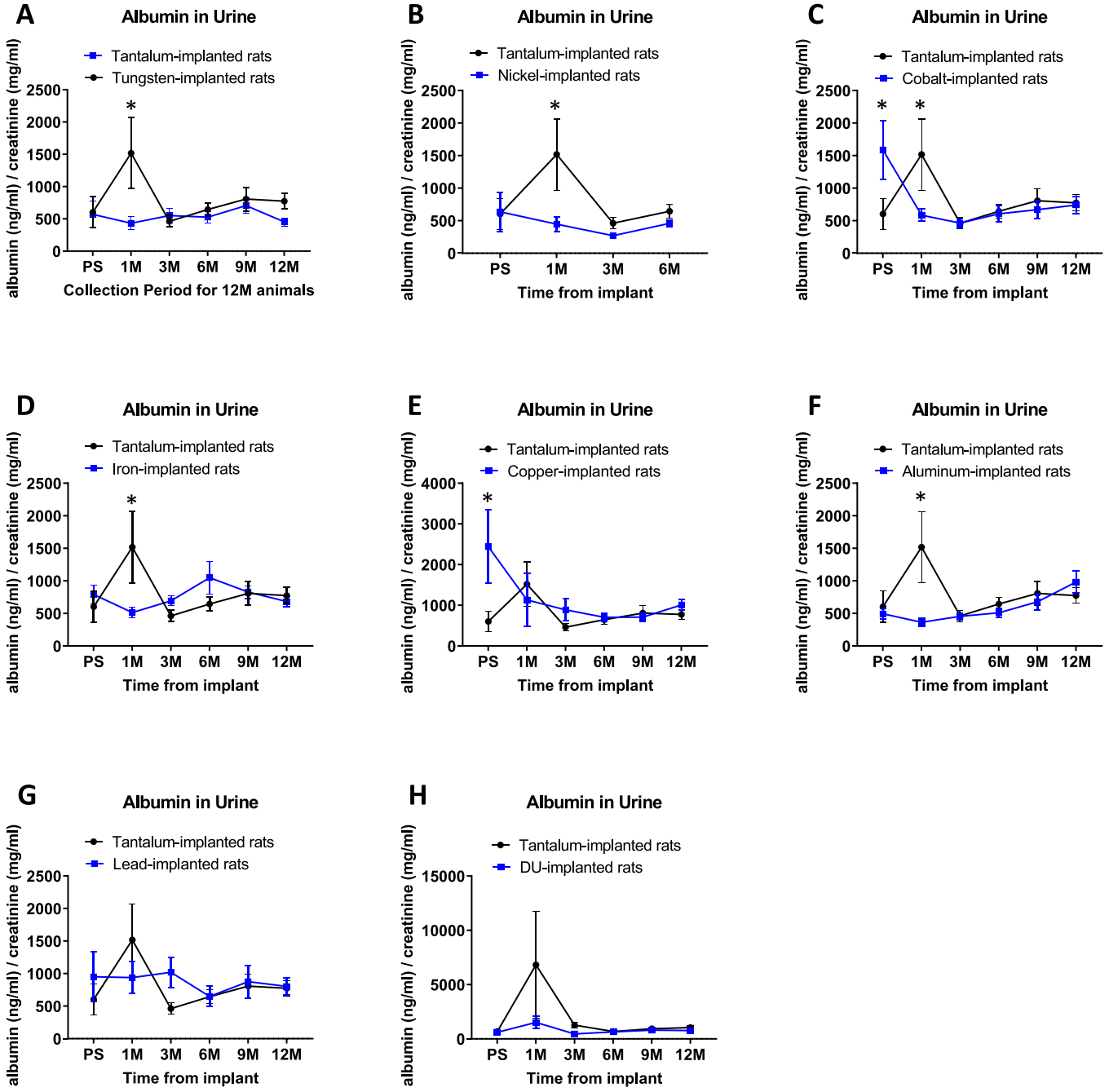

Fig. 2. Urinary albumin levels.
Data presented as albumin normalized to creatinine values in urine collected from multiple time points from individual animals. Each panel compares values in urine from the Ta-implanted animals as controls (black circles/line) vs specific target metal-implanted animals (blue squares/line). Error bars represent standard error of the mean. An * indicates a significant difference between Ta- and target metal-animals at that time point, $p < 0.05$.

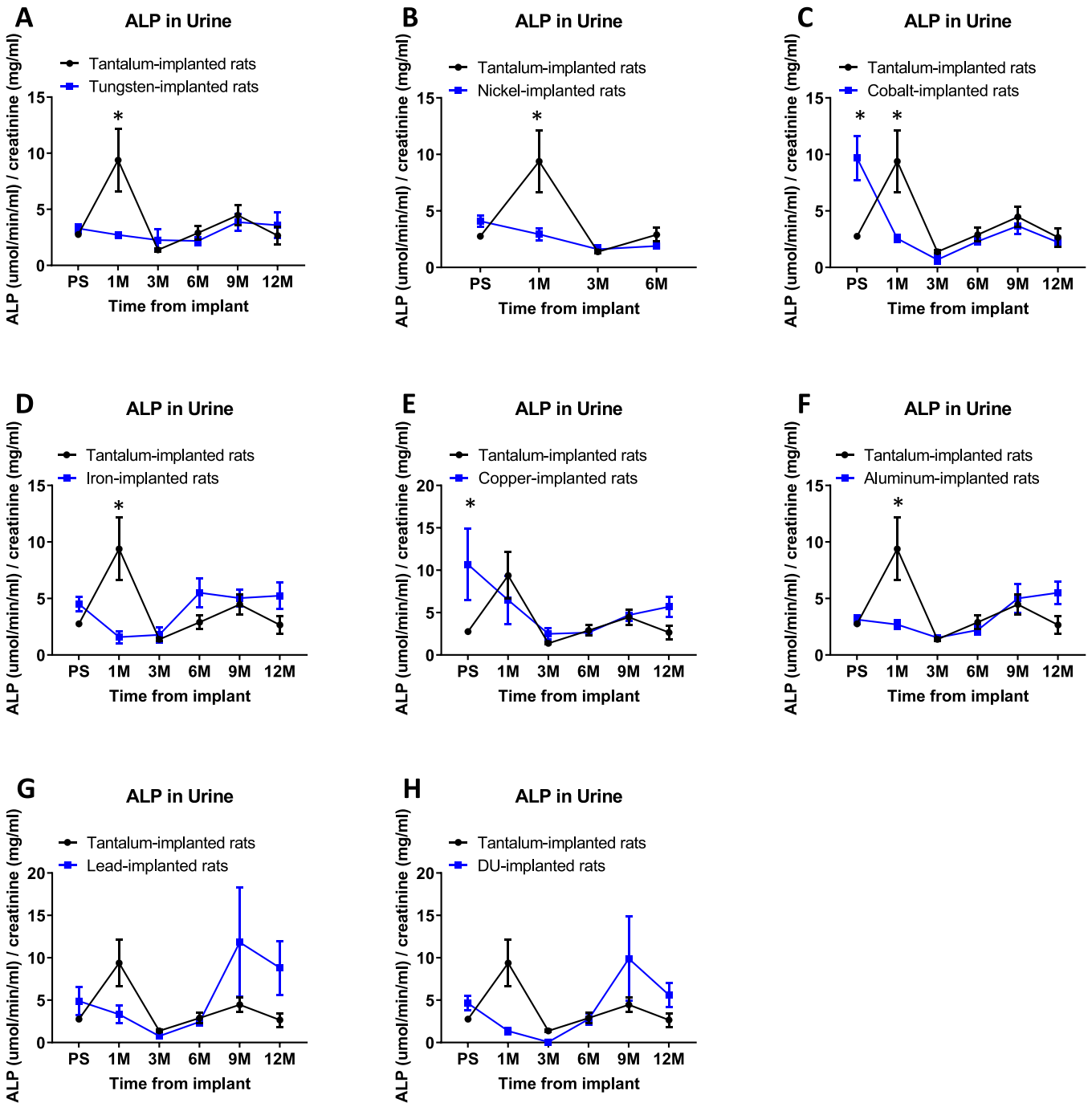

Fig. 3. Urinary ALP levels.
Data presented as ALP normalized to creatinine values in urine collected from multiple time points from individual animals. Each panel compares values in urine from the Ta-implanted animals as controls (black circles/line) vs specific target metal-implanted animals (blue squares/line). Error bars represent standard error of the mean. An * indicates a significant difference between Ta- and target metal-animals at that time point, $p < 0.05$.

presented as a figure for each individual urinary marker. Within each figure, data is always presented as the black circles/line as the values for the Ta-implanted (control) animals, and the blue squares/line as the values for the test metal-implanted animals. Panels follow the pattern of (A) tungsten, (B) nickel, (C) cobalt, (D) iron, (E) copper, (F) aluminum, (G) lead, and (H) DU. F-value statistics for the metal, time from implant, and interaction variables are listed in the figure legend. Just like with metal analysis, all biomarker analyses in panel B (Ta-implanted vs Ni-implanted) drop the 9 M and 12 M groups because Ni-animals were euthanized prior to this point due to tumor formation at the pellet implantation site and therefore no data exists for comparison. In addition, assay values for IL-18 were below the limit of detection (data not shown).

Albumin values are presented in Fig. 2. In the pre-surgery urine collection, even though no embedded metal is present, albumin is higher in urine from Co-implanted animals (*$p = 0.0128$) and Cu-implanted animals (*$p = 0.0277$) than Ta-implanted animals. At the 1 M collection time point, albumin is lower in urine from W-, Ni-, Co-, Fe-, Cu-, and Al-implanted animals than from Ta-implanted animals (*$p = 0.0006$, *$p = 0.0041$, *$p = 0.0301$, *$p = 0.0038$, and 1 M *$p = 0.0002$, respectively). There were no other significant differences in urine albumin concentration between Ta- and other metal-implanted animals.

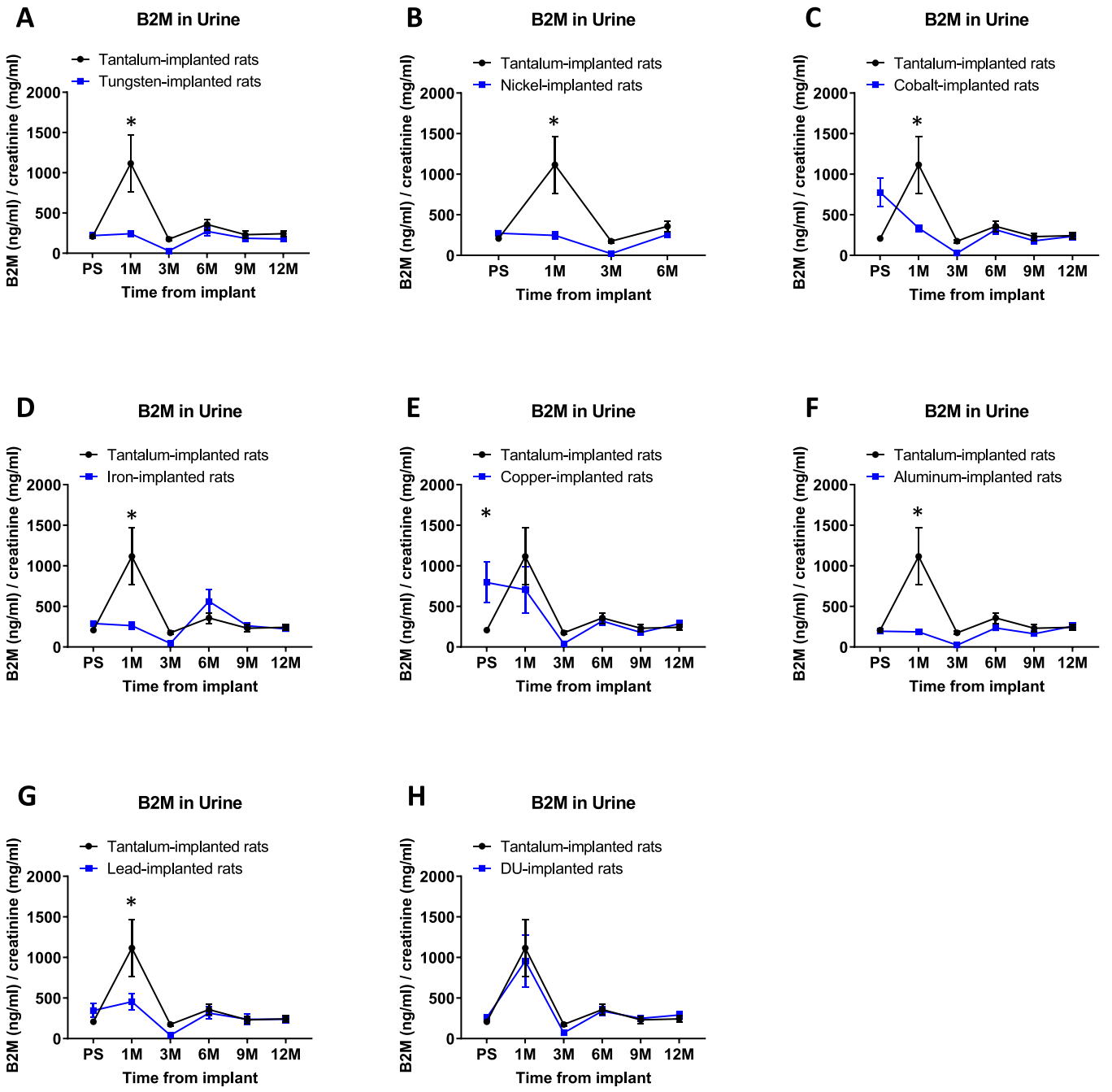

Fig. 4. Urinary B2m levels.
Data presented as B2m normalized to creatinine values in urine collected from multiple time points from individual animals. Each panel compares values in urine from the Ta-implanted animals as controls (black circles/line) vs specific target metal-implanted animals (blue squares/line). Error bars represent standard error of the mean. An * indicates a significant difference between Ta- and target metal-animals at that time point, $p < 0.05$.

ALP values are presented in Fig. 3. Similar to albumin, ALP is higher in urine from Co-implanted animals (*$p < 0.0001$) and Cu-implanted animals (*$p = 0.0002$) than from Ta-implanted animals at the pre-surgery collection period. At the 1 M collection time point, ALP is lower in urine from W-, Ni-, Co-, Fe-, and Al-implanted animals than from Ta-implanted animals (*$p < 0.0001$, *$p = 0.0002$, *$p = 0.0003$, *$p < 0.0001$, and *$p < 0.0001$, respectively). There were no other significant differences in urine ALP concentration between Ta- and other metal-implanted animals.

B2m values are presented in Fig. 4. In the pre-surgery collection period B2m is higher in urine from Cu-implanted animals than from Ta-implanted animals (*$p = 0.0415$). In the 1 M collection time point B2m is lower in urine from W-, Ni-, Co-, Fe-, and Al-implanted animals than from Ta-implanted animals (*$p < 0.0001$, *$p < 0.0001$, *$p < 0.0001$, *$p < 0.0001$, *$p < 0.0001$, and *$p = 0.0011$, respectively). There were no other significant differences in urine B2m concentration between Ta- and other metal-implanted animals.

NAG values are presented in Fig. 5. In the 1 M collection time point NAG is lower in urine from Ni-implanted animals than from Ta-implanted animals (*$p = 0.0015$). At the 9 M collection time point, NAG is lower in urine from Co- and Cu-implanted animals than from Ta-implanted animals (*$p < 0.0001$ and *$p = 0.0436$, respectively), but higher in DU-implanted animals compared to Ta-implanted controls (*$p < 0.0001$). There were no other significant differences in urine NAG

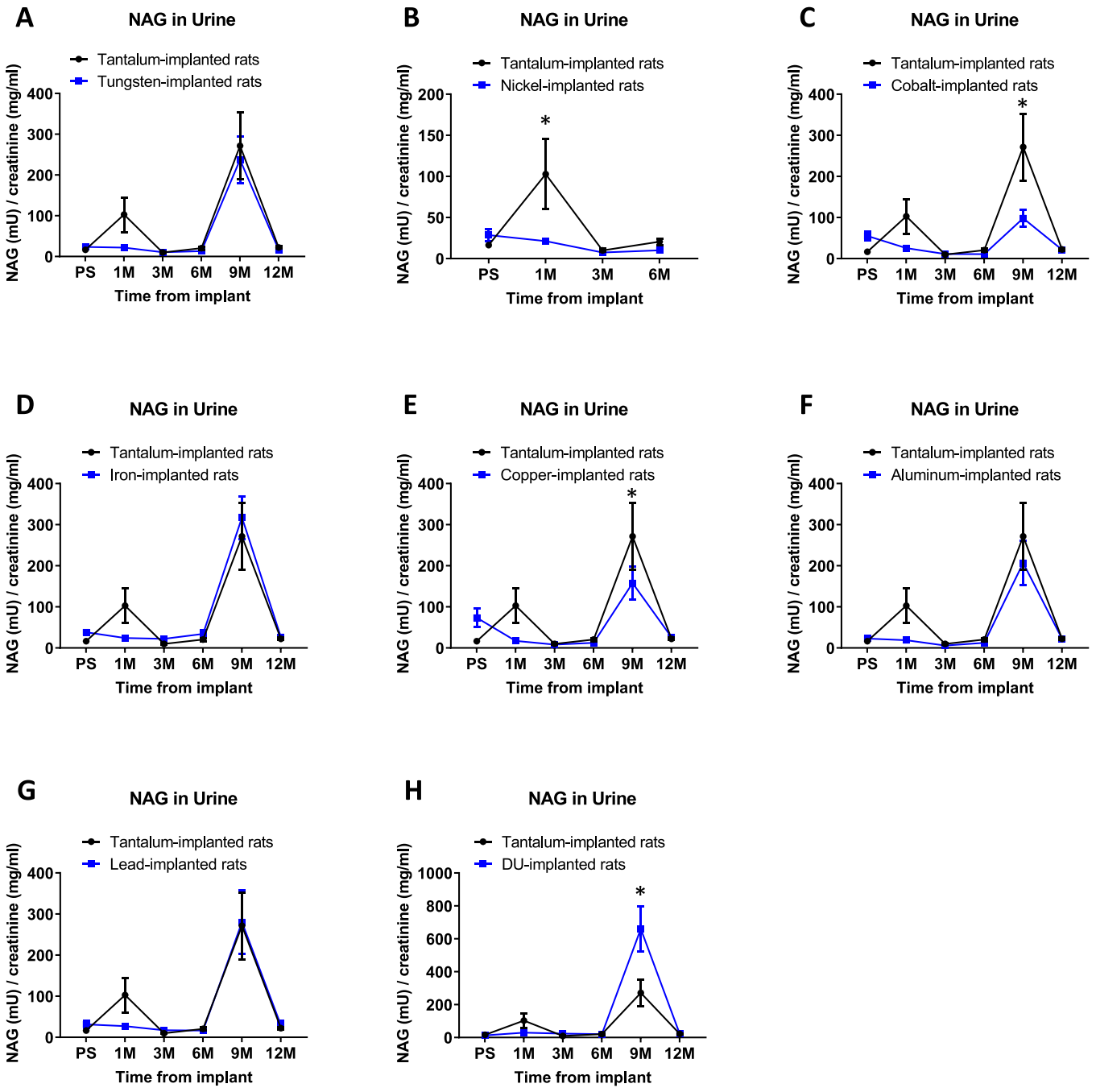

Fig. 5. Urinary NAG levels.
Data presented as NAG normalized to creatinine values in urine collected from multiple time points from individual animals. Each panel compares values in urine from the Ta-implanted animals as controls (black circles/line) vs specific target metal-implanted animals (blue squares/line). Error bars represent standard error of the mean. An * indicates a significant difference between Ta- and target metal-animals at that time point, $p < 0.05$.

concentration between Ta- and other metal-implanted animals.

RBP values are presented in Fig. 6. RBP is higher in urine from W-implanted animals than Ta-implanted animals at the 9 M time point (*$p < 0.0001$), higher in urine from Fe-implanted animals than Ta-implanted animals at the 6 M time point (*$p = 0.0308$), and higher in urine from DU-implanted animals than Ta-implanted animals at the 3 M time point (*$p = 0.0193$). There were no other significant differences in urine RBP concentration between Ta- and other metal-implanted animals.

NGAL values are presented in Fig. 7. In the pre-surgery urine collection, even though no embedded metal is present, NGAL is higher in urine from Co-implanted animals (*$p = 0.0182$) and Cu-implanted animals (*$p = 0.0024$) than Ta-implanted animals. At the 1 M collection time point, albumin is lower in urine from W-, Ni-, Co-, Fe-, and Al-implanted animals than from Ta-implanted animals (*$p < 0.0001$, *$p = 0.0003$, *$p = 0.0083$, *$p = 0.0101$, and *$p < 0.0001$, respectively). At the 6 M collection time point, NGAL is higher in urine from Fe-implanted animals than from Ta-implanted animals (*$p = 0.0003$). There were no other significant differences in urine NGAL concentration between Ta- and other metal-implanted animals.

OPN values are presented in Fig. 8. The only significant difference in OPN expression was at the 3 M collection time point, OPN is higher in urine from DU-implanted animals than Ta-implanted animals (*$p = 0.0003$).

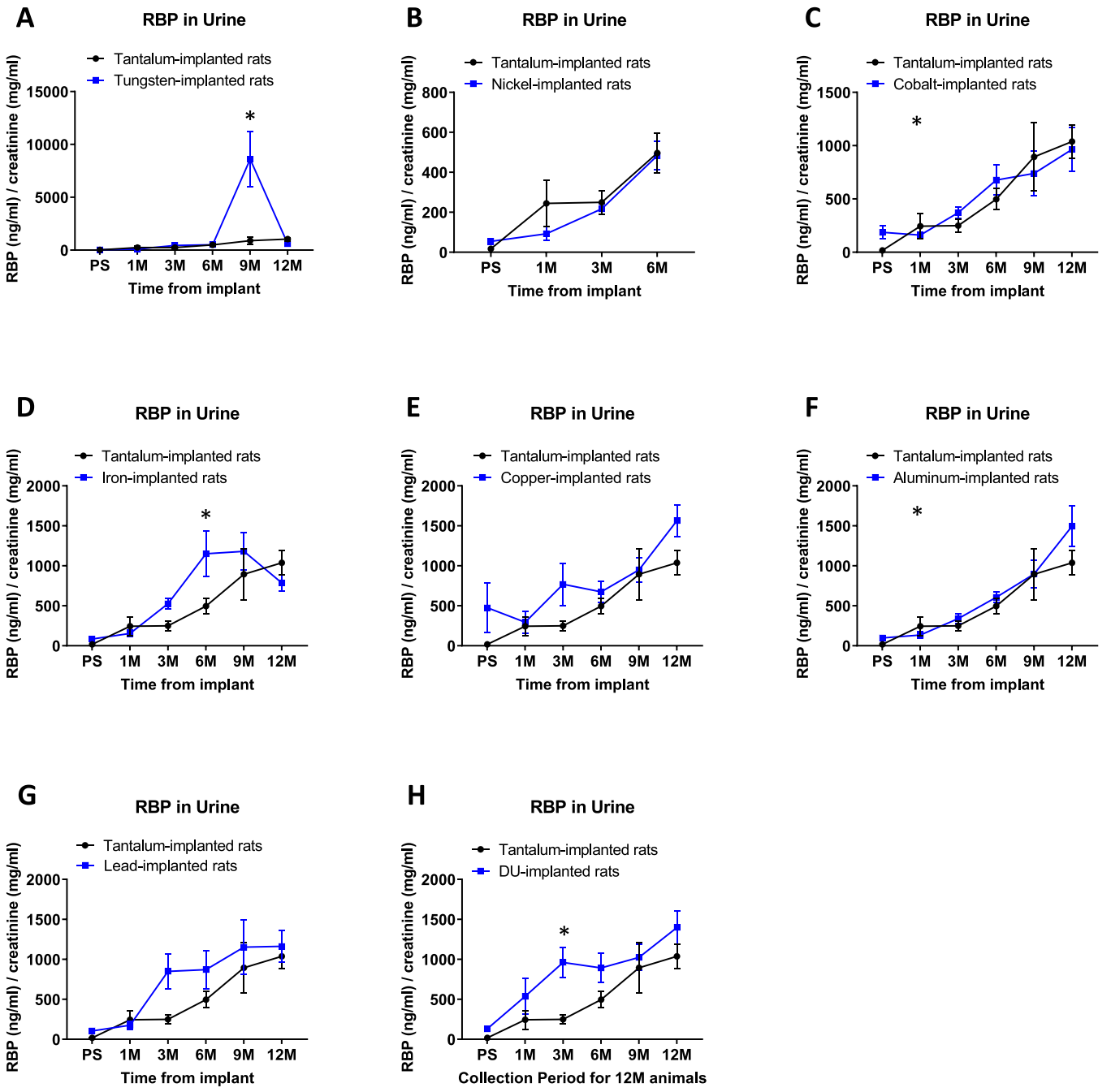

Fig. 6. Urinary RBP levels.
Data presented as RBP normalized to creatinine values in urine collected from multiple time points from individual animals. Each panel compares values in urine from the Ta-implanted animals as controls (black circles/line) vs specific target metal-implanted animals (blue squares/line). Error bars represent standard error of the mean. An * indicates a significant difference between Ta- and target metal-animals at that time point, $p < 0.05$.

KIM-1 values are presented in Fig. 9. In the pre-surgery urine collection KIM-1 is higher in urine from Co-, Cu-, and Al- implanted animals than Ta-implanted animals (*p = 0.0009, *p = 0.0018, and *p = 0.0189, respectively). At the 1 M collection time point, KIM-1 is lower in urine from W- and Ni-implanted animals than from Ta-implanted animals (*p = 0.0049 and *p = 0.0003). KIM-1 is higher in urine from Fe-implanted animals than Ta-implanted animals at the 6 M collection time point (*p = 0.0011).

Total protein values are presented in Fig. 10. In the pre-surgery urine collection total protein is higher in urine from Co- and Cu- implanted animals than Ta-implanted animals (*p = 0.0071 and *p = 0.0153, respectively), and at the 1 M urine collection time point total protein is lower in urine from W- and Ni-implanted animals compared to urine from Ta-implanted animals (*p = 0.0463 and *p = 0.0041, respectively).

Creatinine values are used to normalize other urine biomarker values because it is a commonly accepted value to correct for changes in urine volumes, especially in spot tests like the collections with LabSand. Raw values of creatinine collected from all urines are shown in Fig. 11. There is some variation within animals across time as well as between implant treatment groups. Two points of note is that the creatinine in urine from the Ta-implanted 1 M time from implant collection period is the lowest of all the Ta-implanted animal urines, as well as the lowest of all of the urines collected during this time period. This could explain why so many

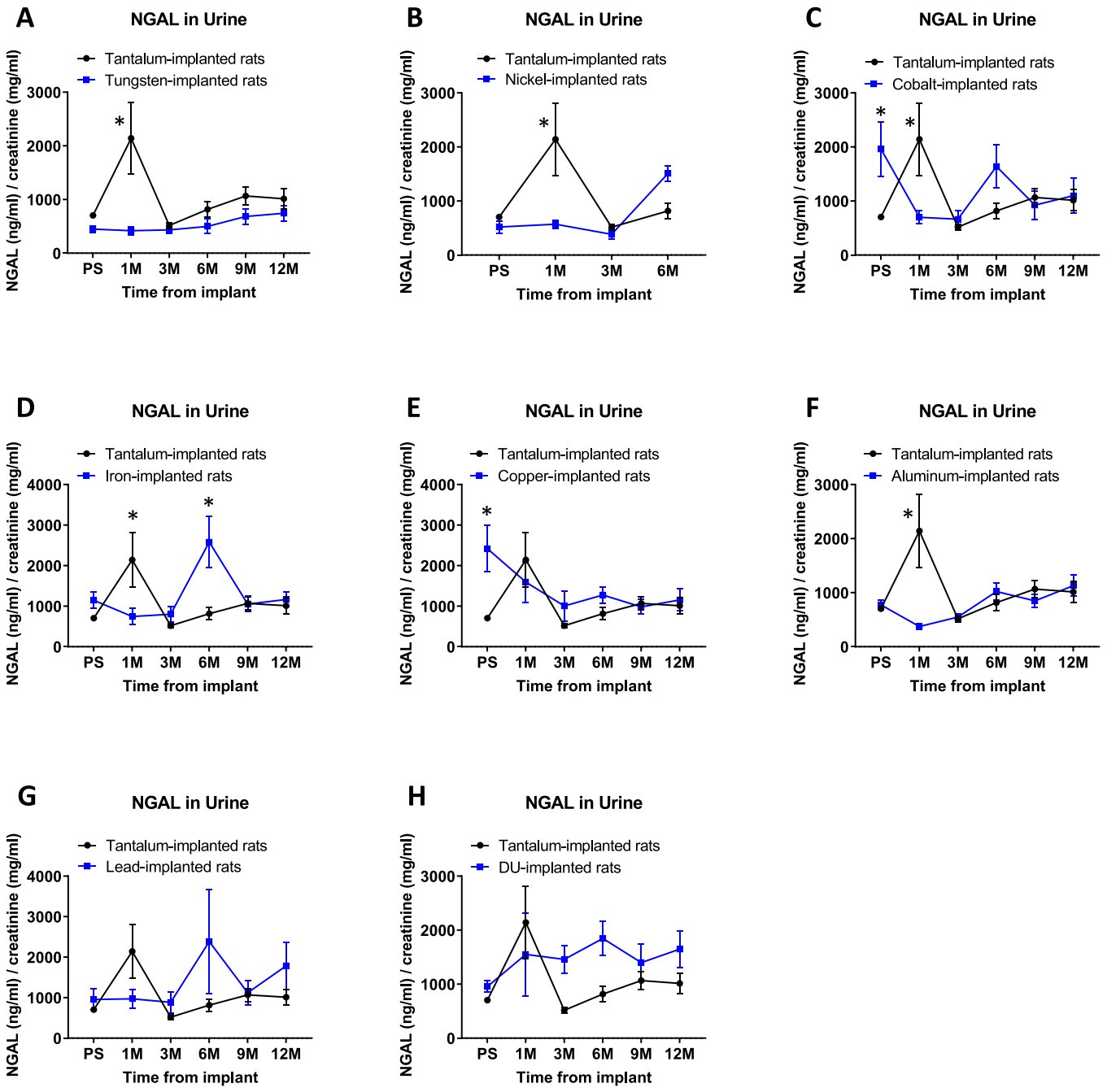

Fig. 7. Urinary NGAL levels.
Data presented as NGAL normalized to creatinine values in urine collected from multiple time points from individual animals. Each panel compares values in urine from the Ta-implanted animals as controls (black circles/line) vs specific target metal-implanted animals (blue squares/line). Error bars represent standard error of the mean. An * indicates a significant difference between Ta- and target metal-animals at that time point, $p < 0.05$.

biomarkers are higher in the Ta-implanted animals compared to the other metal-implanted animals at the 1 M time point. Similarly, the Ni-implanted animals had a very high level of creatinine compared to all the other groups at the 3 M time period.

To examine the overall variation in the urinary biomarkers, a "heat map" depicting biomarker changes is displayed in Fig. 12. Significant decreases (red squares) are seen in many of the biomarkers at the 1 M post-implantation time point for several of the metals most notably W, Ni, Co. Fe, and Al. These markers were then not statistically different than control values (yellow squares) at subsequent assay points. Both Fe- and DU-implanted rats showed significantly increased excretion of several biomarkers over control (green squares) at later time points, although this increase was transient. However, the biomarkers that were increased are indicative of proximal tubule damage.

3.3. Histopathology

Histopathological examination of kidneys from the metal-implanted rats showed no metal-specific abnormalities. Chronic progressive nephropathy was observed in all experimental groups; however, this is a common age- and strain-related condition.

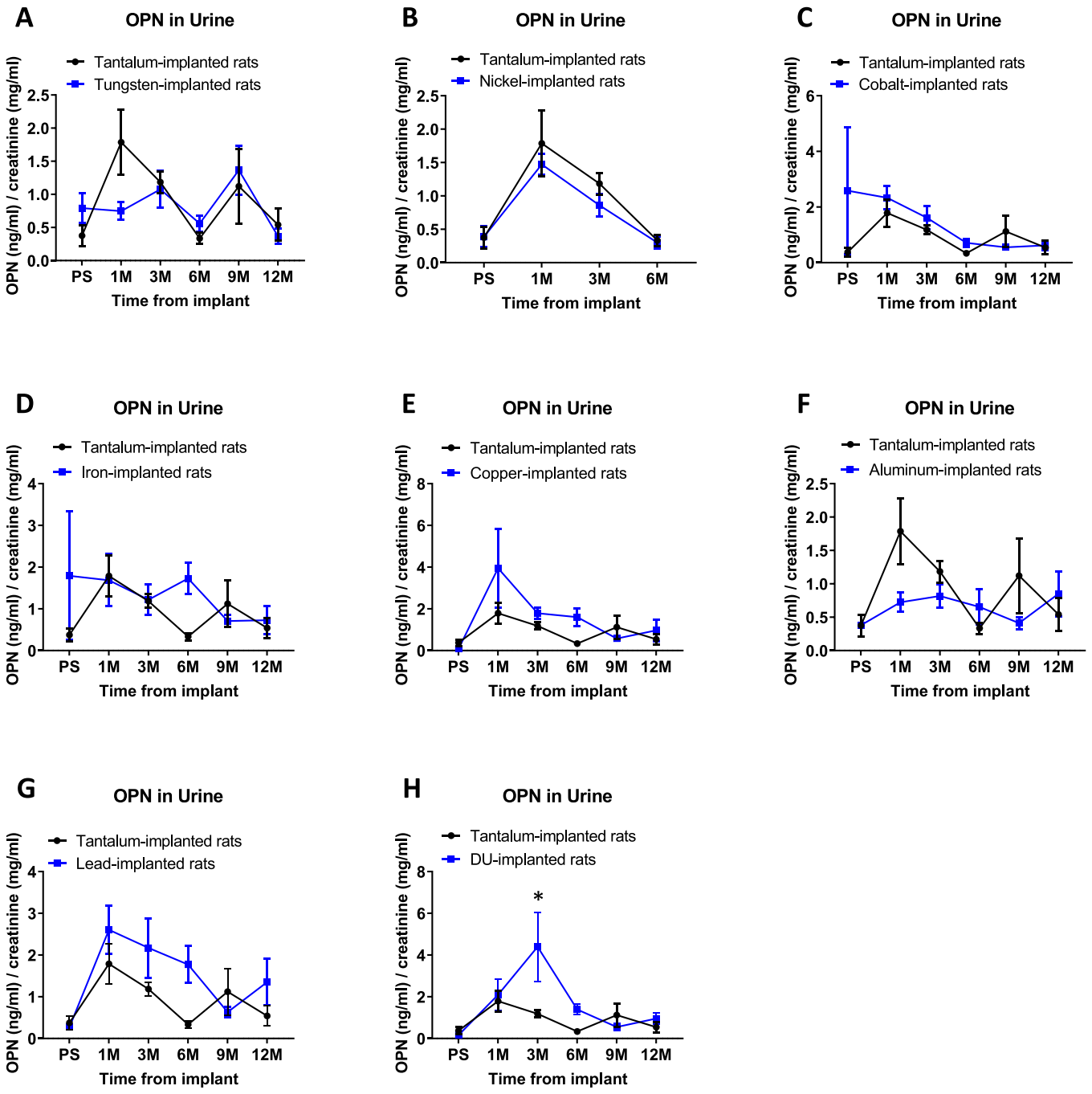

Fig. 8. Urinary OPN levels.
Data presented as OPN normalized to creatinine values in urine collected from multiple time points from individual animals. Each panel compares values in urine from the Ta-implanted animals as controls (black circles/line) vs specific target metal-implanted animals (blue squares/line). Error bars represent standard error of the mean. An * indicates a significant difference between Ta- and target metal-animals at that time point, $p < 0.05$.

4. Discussion

Injuries with embedded metal fragments are an inevitable result of armed conflicts. In some cases, the embedded fragment is the primary injury sustained, while in other instances it might be secondary to a more significant trauma. Regardless, the short- and long-term health effects of embedded metal fragments are an emerging medical concern. Compounding the issue is the fact that there is no limit (other than the periodic table) of what metals might be found in an embedded fragment wound and, for the most part, the toxicological and carcinogenic effects of many metals are not yet known. Previous research on embedded metal fragments suggests that many are capable of solubilizing with the released metal ions being excreted in the urine and, in some cases, sequestering in specific organs of the body [12,14,16,21–23,56]. Because of the recent conflicts in Iraq and Afghanistan, the U.S. Department of Defense and the Department of Veterans' Affairs have developed a list of "metals of concern" with respect to embedded fragment injuries [17,18]. In this study we selected metals from that list and, using a rodent model to study the effects of embedded metals, assessed the associated health effects. We report here the urinary levels of these metals as well as their effects on a battery of renal biomarkers using a within-subjects experimental setup.

For most of the metals tested, significantly elevated levels were measured in the urine starting at 1 M post-implantation with elevated

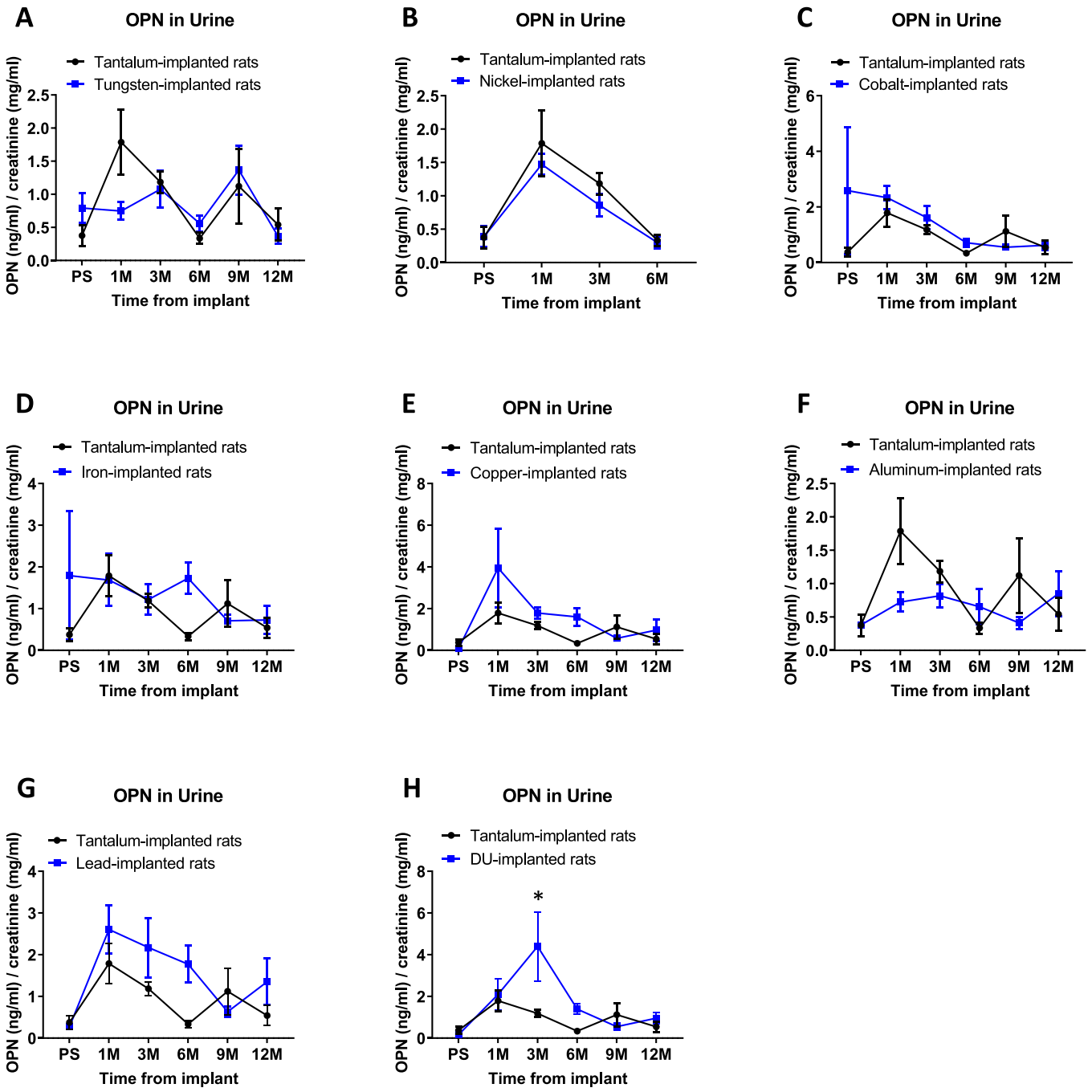

Fig. 9. Urinary KIM-1 in urine.
Data presented as KIM-1 normalized to creatinine values in urine collected from multiple time points from individual animals. Each panel compares values in urine from the Ta-implanted animals as controls (black circles/line) vs specific target metal-implanted animals (blue squares/line). Error bars represent standard error of the mean. An * indicates a significant difference between Ta- and target metal-animals at that time point, $p < 0.05$.

levels continuing for the life of the animal. However, three of the metals tested showed no significant increase in urinary levels. Urinary levels of iron, copper, and aluminum in the metal-implanted rats were not different than that of control animals. It should be noted that there are significant levels of these metals in the tap water used for animal care in our facility [50,51] and excretion of these endogenous metals may have masked any subtle changes in metal excretion due to solubilization of the implanted pellets. It should also be noted that to minimize collection time and thus eliminate any undue stress on the rats, urine collections were limited to a two-hour period with the hydrophobic sand. Basically, this represents a spot collection rather than the historical standard of a 24-h collection in a confined-space metabolic cage. However, we had previously demonstrated no difference in urinary parameters between equal-time collections using hydrophobic sand versus metabolic cage [50–52].

The same spot-collection caveat applies when considering the analysis of the urinary markers of kidney damage. Because of the complex composition (structure/function) of the kidney, it is generally agreed that multiple biomarkers will be required to adequately assess adverse effects induced by a potential toxin [57–59]. Of the urinary biomarkers measured, many were decreased at 1 M post-implantation for practically all the metals tested, except for depleted uranium. Whether this is a result of decreased expression or excretion of the various proteins, an increase in reabsorption in the kidney, or only an artifact of normalizing

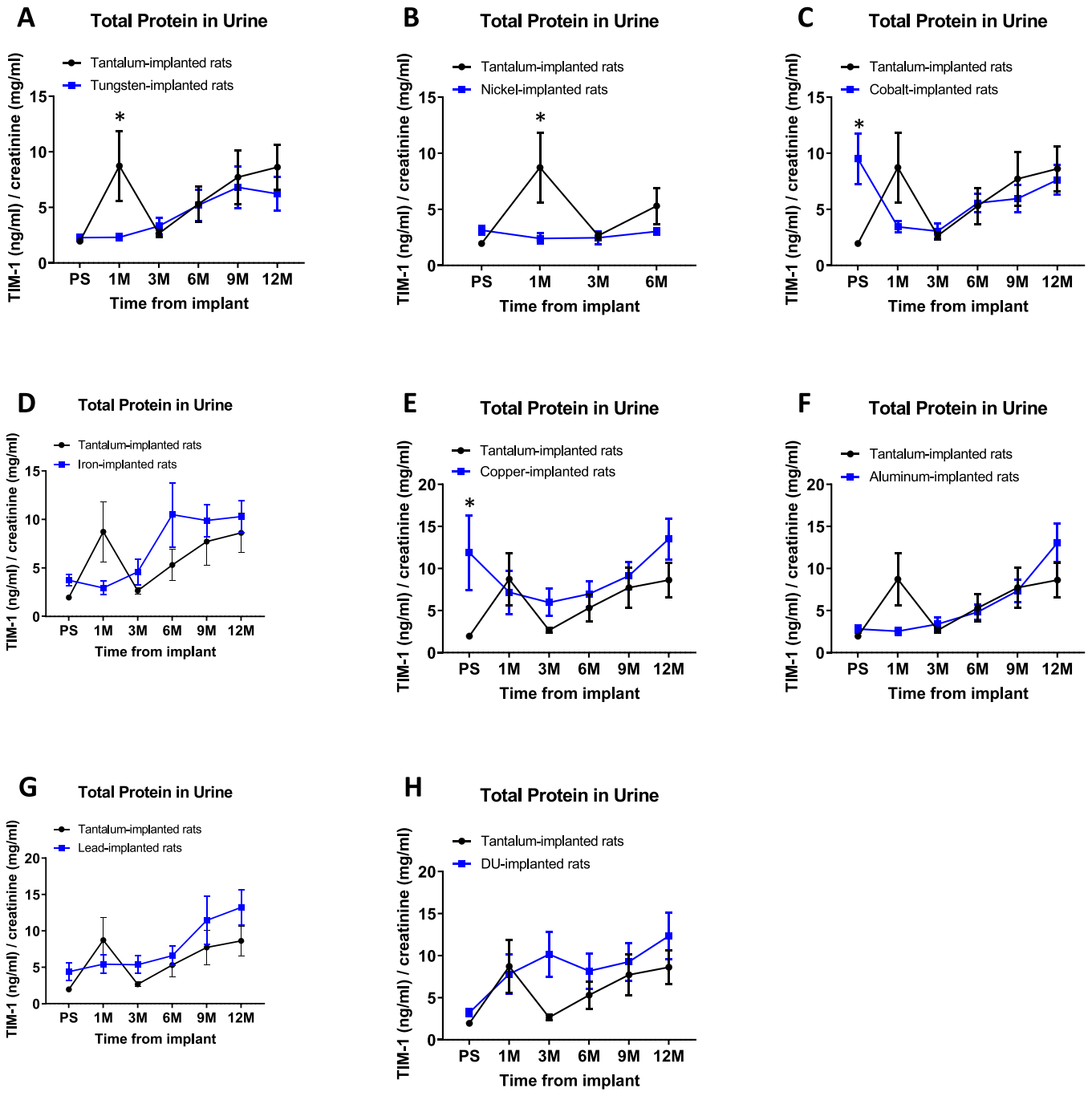

Fig. 10. Total protein levels in urine.
Data presented as total protein normalized to creatinine values in urine collected from multiple time points from individual animals. Each panel compares values in urine from the Ta-implanted animals as controls (black circles/line) vs specific target metal-implanted animals (blue squares/line). Error bars represent standard error of the mean. An * indicates a significant difference between Ta- and target metal-animals at that time point, $p < 0.05$.

to low creatinine expression in the 1 M control (Ta-implanted) animals is not known at this time. After the 1 M time point, all markers assessed return to control values. For the iron group at the 6 M post-implantation point, RBP, NGAL, and KIM-1 were all significantly higher than control indicating potential damage to the proximal tubule region of the kidney even though urinary iron levels were not significantly different. However, this biomarker increase was transient, as values returned to control by 9 M. Similar increases in several proximal tubule markers were seen at 3 and 6 M post-implantation in the DU group. Again, over time these markers returned to control levels. Uranium is a known kidney toxin that damages the proximal tubule area and embedded fragments of DU have been shown to affect several proximal tubule markers [60]. It should be noted however that the amount of DU implanted in those rats far exceeded the amount implanted in this study, indicating that even low amounts of embedded DU, once solubilized, can adversely but transiently affect the kidney. In addition, it has been proposed that the kidney has a substantial capacity to repair its tubular epithelial regions [61] and that repaired epithelium appears more resistant to further uranium-induced damage [62]. It should also be noted that along with being indicators of renal damage, some of the urinary biomarkers also participate in the repair of damage. For example, KIM-1 is a biomarker of both acute and chronic renal injury [63]. However, its

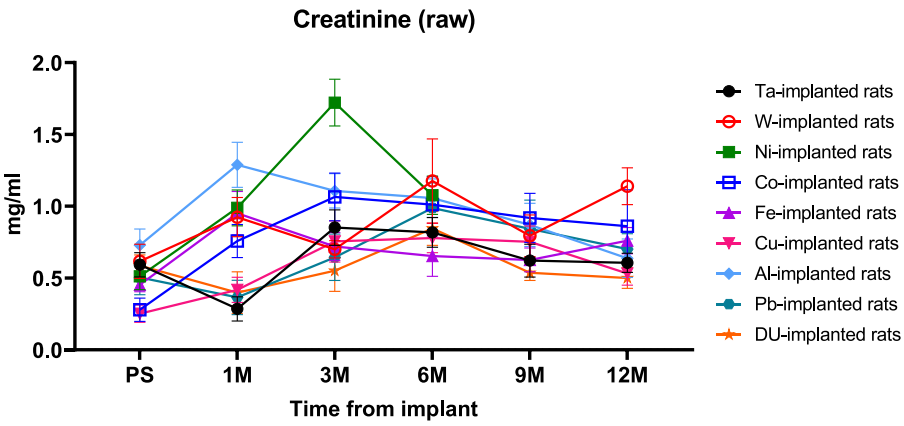

Fig. 11. Urinary creatinine values.
Data presented as creatinine (mg/mL) in urine samples without normalization, since this data is used to normalize metal and other biomarker concentrations. Within subject values are presented as connected lines for a particular metal implant group over urine collection times. Ta – black circle, W – red open circle, Ni – green square, Co – blue open square, Fe – purple triangle, Cu – pink upside down triangle, Al – light blue diamond, Pb – teal hexagon, DU – orange star.

over-expression in proximal renal tubular epithelial cells induces the transformation of these cells into those with phagocytic ability. These cells in conjunction with KIM-1 can identify and eliminate apoptotic bodies resulting from the programmed death of damaged cells [64]. Further, by activation of the ERK/MAPK pathway, over-expression of KIM-1 promotes the proliferation of renal tubular epithelial cells which helps to initiate renal repair [65]. Although there were limited significant differences in urinary levels of KIM-1 as a result of metal implantation, it is not known whether even low levels of KIM-1 could initiate renal repair thus preventing more extensive damage as metal-exposure times increase. This could also explain the return to "normal" values of many of the urinary markers that showed deviations from control at the 1 M post-implantation time point but showed no significant differences at the later time points. Most notably, histopathological examination indicated no overt signs of long-term renal damage. Although well beyond the scope of this study, an investigation into KIM-1 expression changes as a result of metal implantation and its concomitant role in the repair of damaged renal tubular epithelial cells, especially with respect to the signaling pathways involved, would be worthwhile.

During the development and validation of this rodent embedded fragment method an attempt was made to correlate urine uranium levels found in U.S. military personnel wounded with DU fragments with embedded DU pellets in the rat. It was found that from 4 to 20 implanted pellets implanted per rat gave a reasonable correlation with respect to urine uranium levels in wounded individuals [47]. It is not known whether these assumptions would hold for other military-relevant metals. In fact, to minimize potential adverse health effects due to excessive metal loads and based upon our earlier studies, we implanted a limited number of pellets. As such, apart from nickel, there were no metal-associated adverse health issues observed. Most notable was the absence of overt renal effects as determined by histopathological examination of the kidney. Nickel, a known carcinogen, did result in 100 % tumor formation at the metal implantation site [49] resulting in the early euthanasia of this cohort. Cobalt also induced tumors at the implantation site (87 % incidence), as did copper (13 % incidence); however, in neither case were the tumors severe enough to warrant euthanasia under our IACUC protocol [66]. It can be difficult to compare surgically implanted metal pellets in rodents to metal loads in wounds suffered by humans due to the paucity of case reports. However, a recent publication reporting on the metal mobilization from embedded fragments removed from an Iraq War veteran included a thorough description of the physical characteristics, including size, of the surgically excised fragments [21]. Although the wounded individual had many retained fragments, only three were surgically removed because of discomfort. Based on the reported size, we calculated the volume of these fragments which ranged from 0.5 mm^3 to 200 mm^3. The volume of a single pellet (1 mm x 2 mm) used in our study is 1.57 mm^3. Calculating the "human equivalent" of this one pellet gives an estimated volume range of 190 to 366 mm^3 assuming an average rat pre-surgery mass of 300 gm or average pre-euthanasia mass of 580 gm, respectively, as well as the standard "reference man" mass of 70 kg [67]. Therefore, the volume of one rat-implanted pellet is comparable to the volume of a reported excised fragment from a wounded individual.

5. Conclusion

This study demonstrated that pellets of military-relevant metals implanted in the gastrocnemius muscle of rats to simulate a shrapnel wound solubilized over time. With few exceptions, the metals were excreted in the urine of the implanted rats at significantly higher levels than in control animals. While the implanted metals had limited effects on urinary kidney biomarkers, we would expect that increased metal loads would result in much more extensive renal damage based upon our metal excretion results. This study suggests the need for continued surveillance of individuals wounded with metal fragments with urinary metal levels and kidney biomarker assays of particular importance.

Funding

The project described was supported by the grant Health Effects of Blast Injuries and Embedded Metal Fragments (W81XWH-16-2-0058) from the Congressionally Directed Medical Research Program (CDMRP) Peer-reviewed Medical Research Program. All procedures involving animals were (a) conducted with maximal possible well-being of the rats, (b) approved by the AFRRI IACUC prior to the start of the study under protocol 2016−05-006, and (c) performed in compliance with the guidelines set forth in the *Guide for the Care and Use of Laboratory Animals* in an AAALAC-I-accredited facility. The views expressed in the paper are those of the authors and do not reflect the official policy or position of the Armed Forces Radiobiology Research Institute, Uniformed Services University, Department of Defense, or U.S. Government. The use of the LabSand brand of hydrophobic sand in this work does not represent an endorsement of the product or company by the U. S. Government.

CRediT authorship contribution statement

Jessica F. Hoffman: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - review & editing. **Vernieda B. Vergara:** Data curation, Investigation, Methodology, Software, Validation, Writing - review & editing. **Anya X. Fan:** Data curation, Investigation, Methodology, Validation, Visualization, Writing - review & editing. **John F. Kalinich:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization,

A: Tungsten

	1	3	6	9	12
ALB					
ALP					
B2M					
NAG					
RBP					
NGAL					
OPN					
KIM-1					
Protein					

B: Nickel

	1	3	6	9	12
ALB					
ALP					
B2M					
NAG					
RBP					
NGAL					
OPN					
KIM-1					
Protein					

C: Cobalt

	1	3	6	9	12
ALB					
ALP					
B2M					
NAG					
RBP					
NGAL					
OPN					
KIM-1					
Protein					

D: Iron

	1	3	6	9	12
ALB					
ALP					
B2M					
NAG					
RBP					
NGAL					
OPN					
KIM-1					
Protein					

E: Copper

	1	3	6	9	12
ALB					
ALP					
B2M					
NAG					
RBP					
NGAL					
OPN					
KIM-1					
Protein					

F: Aluminum

	1	3	6	9	12
ALB					
ALP					
B2M					
NAG					
RBP					
NGAL					
OPN					
KIM-1					
Protein					

G: Lead

	1	3	6	9	12
ALB					
ALP					
B2M					
NAG					
RBP					
NGAL					
OPN					
KIM-1					
Protein					

H: Depleted Uranium

	1	3	6	9	12
ALB					
ALP					
B2M					
NAG					
RBP					
NGAL					
OPN					
KIM-1					
Protein					

Fig. 12. Comparison of biomarker levels from metal-implanted rats.
"Heat map" depiction of renal biomarkers in urine of metal-implanted rats after 1, 3, 6, 9, and 12 M post-implantation. Blue block indicates a statistically significant result that is lower than control; yellow block indicates no statistical difference between the experimental group and control; red block indicates a statistically significant value that is greater than control value.

Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Acknowledgements

The authors would like to thank Raisa Marshall and William Danchanko for their expertise in the pellet implantation surgeries, animal welfare checks, and tissue collection. The authors would also like to thank W. Louis Wilkins for histopathology support.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2021.02.023.

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Self-reported General Health Status among Veterans with Embedded Metal Fragments

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2021-A-3390-SOT

Background

- Over 40,000 wounded personnel from Iraq and Afghanistan conflicts may have retained metal fragments, primarily from contact with improvised explosive devices (IEDs).
- Studies have indicated that metal fragments can oxidize in situ, thereby releasing metal ions into the systemic circulation and potentially affecting target organs far from the site of injury.
- While chronic health effects of other metal-exposed populations have been investigated, long-term outcomes resulting from metal fragments embedded in soft tissue have not been fully elucidated.
- As a result, the Department of Veterans Affairs (VA) established the Toxic Embedded Fragment (TEF) Surveillance Center and Registry in 2008 to identify and provide on-going medical surveillance for Veterans at-risk for embedded fragments.
  - Registry-enrolled Veterans are identified using a series of screening questions incorporated in the VA's electronic medical record system.
- The goal of this study was to evaluate the effect of having an embedded metal fragment, related to a bullet or blast injury, on the overall health status in a subset of affected Veterans.

Methods

Data Collection

- 9,000 Iraq and Afghanistan Veterans enrolled in the VA's Embedded Fragment Registry were randomly selected to receive a mailed questionnaire.
- The questionnaire asked about the nature of their injury, military exposure history, and chronic health conditions. It also included the Veterans RAND-12 Item Health Survey (VR-12), a well-validated instrument for assessing overall physical and mental health.

Statistical Analyses

- Using responses to injury-focused questions, Veterans were categorized into high or low risk groups based on their likelihood of having an embedded fragment from a bullet or blast injury.
  - Data from the TEF Registry support the use of these categories as an indicator of relative metal exposure.
- Veterans not injured by a bullet or blast or injured before 2001 were excluded from analyses.
- Using a standardized scoring algorithm, a Physical Component Score (PCS) and Mental Component Score (MCS) were generated from the VR-12 data. Higher scores indicate better health.
- A linear regression model was used to evaluate if Veterans at high risk for having an embedded fragment had worse physical or mental health scores compared to those at low risk while controlling for:
  - Age
  - Race
  - Branch of service
  - Severity of injury (treatment at Landstuhl, Germany defined as severe)
  - Smoking status

Results

- 2,331 Veterans responded. Of the 2,112 who reported a bullet or blast injury during the Iraq and Afghanistan wars, 2,086 had complete data to permit determination of risk categories.
- Overall, the population was mostly white (81%), male (96%), and served in the Army (70%). 46% were never smokers. Mean age was 40.5 ± 8.1.
- Those in the high-risk category were more likely to have been treated at Landstuhl Regional Medical Center, speaking to the severity of their injury (Table 1).

Results (continued)

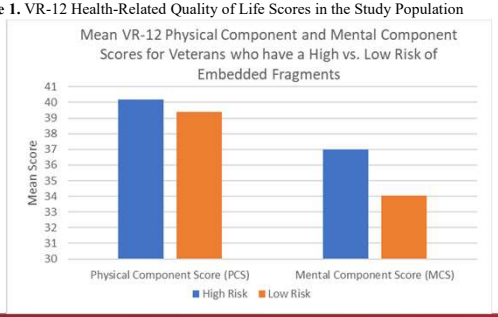
Table 1. Demographic Characteristics of Study Population

		High Risk (n =1608)	Low Risk (n = 478)	P-value**
Mean age, years		40.24	41.40	0.020
Demographic Variable		**Number (%)**	**Number (%)**	
Sex	Male	1549 (96.5)	463 (97.1)	0.516
	Female	57 (3.6)	14 (2.9)	
Service branch	Army	1135 (70.6)	332 (69.5)	0.068
	Marine Corps	341 (21.2)	91 (19.0)	
	Other	132 (8.2)	55 (11.5)	
Race	White	1314 (82.4)	364 (76.8)	0.024
	Black/AA*	110 (6.9)	43 (9.1)	
	Other	171 (10.7)	67 (14.1)	
Smoking status	Never	770 (47.9)	206 (43.1)	0.135
	Former	527 (32.8)	178 (37.2)	
	Current	311 (19.3)	94 (19.7)	
Treatment facility for injury	Landstuhl	575 (35.8)	56 (11.7)	<0.0001
	Other	1033 (64.2)	422 (88.3)	

*AA=African American; **comparison between high and low risk categories

- Physical Component Scores (PCS)
  - The mean PCS score in the high-risk group was 40.20 ± 11.34 and 39.39 ± 10.74 in the low-risk group (Figure 1), both lower than the general population norm of 50 ± 10.
  - There was no statistically significant difference in PCS scores between the two risk groups when controlling for other factors (Table 2).
- Mental Component Scores (MCS)
  - The mean MCS score in the high-risk group was 37.01 ± 13.82 and 34.03 ± 13.59 in the low-risk group in the low-risk group (Figure 1), both lower than the general population norm of 50 ± 10.
  - Mean MCS scores were significantly higher in the high-risk group compared to the low-risk group, even after controlling for other factors (Table 2).

Figure 1. VR-12 Health-Related Quality of Life Scores in the Study Population



Results (continued)

Table 2. Adjusted Statistical Comparisons of VR-12 Scores for Selected Variables of Interest

		PCS Adjusted Model		MCS Adjusted Model	
Variables of Interest		**Estimate (SE)**	**P-value**	**Estimate (SE)**	**P-value**
Risk category	High	0.75 (0.62)	0.226	2.08 (0.78)	0.008**
	Low	REF*		REF	
Age		-0.22 (0.03)	<0.0001**	0.08 (0.04)	0.041 **
Service branch	Marine Corps	1.53 (0.64)	0.017**	-0.59 (0.80)	0.460
	Other	2.30 (0.89)	0.010**	0.83 (1.12)	0.456
	Army	REF		REF	
Race	Black/AA	-0.83 (0.99)	0.398	-2.90 (1.24)	0.020**
	Other	-2.68 (0.79)	0.0007**	-3.08 (0.99)	0.002**
	White	REF		REF	
Smoking status	Former	-0.093 (0.58)	0.872	-2.04 (0.73)	0.005 **
	Current	-2.89 (0.63)	<0.0001**	-3.25 (0.85)	0.0002**
	Never	REF		REF	
Treatment facility for injury	Landstuhl	-1.67 (0.57)	0.003**	2.36 (0.71)	0.0009**
	Other	REF		REF	

*indicates the reference group in the analysis; **defined as a statistically significant difference

Discussion/Conclusion

- Compared to mean U.S. population norms of 50 (SD 10) for MCS and PCS scores, this Veteran cohort had poorer mental and physical health.
  - Compared to other Veteran populations, mean PCS scores were slightly higher, but mean MCS scores were lower in both risk-groups (Table 3).

Table 3. Reported PCS and MCS Scores in Past Veteran Population Studies

Study	Survey Population	Mean PCS	Mean MCS
Borzecki et al., 2005	U.S. Veterans, 1998 Veterans Health Study with specific health behaviors	36.9	48.9
Kazis et al., 2004	U.S. Veterans, 1998 Veterans Health Study with specific health conditions	37.1	48.0
Selim et al., 2002	U.S. Veterans, 1998 National Survey of Ambulatory Care Patients	32.4	42.1

- Physical health status was comparable between the two risk groups; however, Veterans with a high likelihood of having an embedded metal fragment had higher MCS scores than their lower risk counterparts, indicating better general mental health.
- Possible explanations for these findings include:
  - Systemic metal concentrations from embedded metal fragments have not reached toxicity thresholds that would affect general health-related quality of life.
  - Veterans with severe injuries may receive more intense medical care or connections with more support services, positively affecting overall health status.
- Our findings highlight the need for future research on the effects of embedded metal fragments, and whether healthcare services that Veterans receive influence their ability to recover from these fragment injuries physically and mentally.

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Acknowledgement: This work is funded by the Department of Defense Congressionally Directed Medical Research Program

Use of the National Institute for Occupational Safety and Health (NIOSH) Industry & Occupation Computerized Coding System (NIOCCS) to Classify Free Text Occupational Exposure Responses in a US Military Population

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Background

- Accurate occupational classification is important in epidemiologic surveillance to identify possible exposure-outcome relationships.
- NIOSH developed the NIOSH Industry & Occupation Coding System engine to classify industry and occupation data from datasets into census-based standard Industry & Occupation (I&O) codes.
- NIOCCS utilizes machine learning technology to classify responses into I&O codes
- These I&O codes have successfully classified civilian occupations in epidemiologic surveillance, but their utility in classifying military occupations is unknown

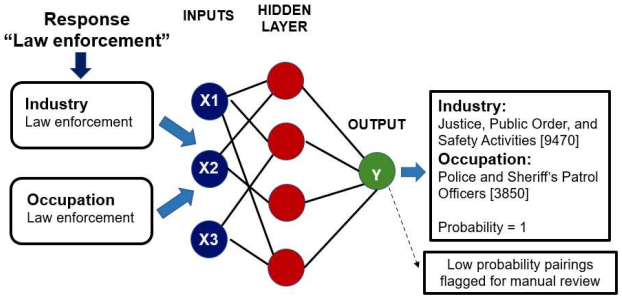

Figure 1: NIOCCS creates vector representations of words from responses as inputs into neural networks to categorize I&O entries into I&O codes

Methods

- Veterans enrolled in the Veterans' Affairs (VA's) Toxic Embedded Fragment (TEF) registry completed clinical questionnaires regarding military service, exposures, renal and respiratory symptoms and conditions.
- Veterans who reported working ≥1 year in a dusty job or exposure to gas or chemical fumes at work were invited to specify the occupation in which they were exposed using free text
- We uploaded free-text data into NIOCCS engine to produce I&O code pairs (Census 2010 coding scheme)
- If respondent specified multiple occupations, only the first occupation was selected for input into NIOCCS
- A NIOSH I&O coding expert then reviewed the dataset for accuracy.
- I&O code pairs were manually reconciled if the NIOCCS auto-coder could not classify the entry while the I&O coding expert could, or if the auto-coded I&O pair was inaccurate

Results

Table 1: Summary of veterans reporting exposure to fumes/chemicals and dusty occupation (total respondents n=2338) and the more common census groups reported after review by I&O coding expert

	Fume/Chemical	Dust
Respondents reporting exposure	1048	1152
Entries accurately auto-coded by NIOCCS	643 (61.4%)	893 (77.5%)
Broad Census Occupation Groups		
General Military	443 (42%)	695 (60%)
Production Occupation	267 (26%)	87 (7.6%)
Construction and Extraction Occupation	62 (5.0%)	160 (14%)
Installation, Maintenance, and Repair Occupation	95 (9.1%)	50 (4.3%)
Transportation and Material Moving Occupation	79 (7.5%)	42 (3.6%)
Unclassified	31 (3%)	17 (2%)

Common reasons for I&O code reassignment by the I&O coding expert

- Military-specific duty response that has civilian census I&O code with similar job duties (e.g. "road construction Army")
- Clear reference to exposure on military service (e.g. "Army Iraq") that could not be assigned to a more specific occupation

Results

Table 2: Examples of survey responses classified into census occupation codes (e.g. 9900) by NIOCCS and subsequently by expert I&O coder review

	Survey response	NIOCCS assignment	Final assignment after I&O coding expert review
Successful	"Diesel mechanic"	Bus and Truck Mechanics and Diesel Engine Specialists [7210]	Bus and Truck Mechanics and Diesel Engine Specialists [7210]
	"Demolition"	Construction laborers [6260]	Construction laborers [6260]
Unsuccessful	"Gypsum plant"	Insufficient information [9900]	Production workers, other [8965]
	"Operating on the streets in Iraq"	Operating engineering and other construction equipment operators [6320]	Military, rank not specified [9830]

Discussion

- The NIOCCS auto-coding engine is a powerful tool in classifying free-text responses from this military dataset into census I&O codes when responses related to military occupations have an analogous civilian occupation
- Specificity in language to elicit I&O responses from respondents may help improve data quality prior to classification by NIOCCS
- The unique nature of military occupations (e.g. one occupation may include multiple duties or lack of analogous civilian occupation) presents challenges in classification in the US Census I&O system

Future Directions

- Examination of associations between respiratory and renal health outcomes in this population related to these self-reported occupational exposures classified by NIOCCS

Acknowledgments

- Funded by the US Department of Veterans Affairs and the Department of Defense Congressionally Directed Medical Research Program, award #W81-XWH16-2-0058

Pulmonary Function Categorical Abnormalities in Veterans with Embedded Metal Fragments: An Interim Analysis

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Background

- Veterans who deployed to Southwest Asia since 2001 have increasingly reported respiratory symptoms.
- The signature post-2001 military service injury results from detonation of improved explosive devices (IEDs), including embedded metal fragment injury.
- Veterans with embedded metal fragments may mobilize metal from the fragments, resulting in systemic metal circulation.
- Given that systemic exposure to certain metals has been associated with decreased lung function, we hypothesized that veterans with significantly elevated urine metal concentrations were more likely to have abnormal PFTs than veterans without elevated metal levels.

Methods

- Drawing from the Department of Veterans Affairs' (VA) Toxic Embedded Fragment (TEF) registry, 242 veterans were evaluated during a 1-day visit to one of five VA medical centers.
- Veterans completed medical history and exposure questionnaires, along with PFTs and spot urine collection.
- PFT abnormalities were defined as in Table 1. Reference values used were Global Lung Initiative (GLI; 2012) for spirometry, Quanjer (1993) for lung volumes, and GLI (2017) for DLCO.
- Concentrations of 13 urine metals were measured by the Joint Pathology Center using inductively coupled plasma mass spectrometry and dichotomized as elevated or normal based on established reference values (Table 3).
- The relationship between elevated metal concentration and PFT abnormality was assessed via linear regression, while controlling for smoking history, weight, and race.

Table 1. Classification criteria for PFT categories

Outcome	Criteria
Ventilatory Pattern	
Normal	FEV1/FVC ≥ LLN AND LLN ≤ TLC ≤ ULN AND RV ≤ ULN
Obstructive	FEV1/FVC < LLN OR TLC > ULN OR RV > ULN
Restrictive	FEV1/FVC ≥ LLN AND TLC < LLN AND RV ≤ ULN
Gas Exchange Pattern	
Normal	DLCO ≥ LLN
Reduced	DLCO < LLN

LLN (lower limit of normal) is the 5th percentile of the reference distribution

ULN (upper limit of normal) is the 95th percentile of the reference distribution

FEV1: forced expiratory volume in 1 second | *FVC: forced vital capacity*

TLC: total lung capacity | *RV: residual volume*

DLCO: diffusing capacity for carbon monoxide

Results

Table 2. Select characteristics of the study population (N = 242).

Demographics	
Gender, male, N (%)	234 (96.7)
Age, years (SD)	42.1 (7.6)
Race, N (%)	
White	145 (59.9)
Black/African American	20 (8.3)
Hispanic	42 (17.4)
Other	35 (14.5)
Smoking History, N (%)	
Current	48 (19.8)
Former	81 (33.5)
Never	113 (46.7)

Military History	
Service Branch, N (%)*	
Army	186 (76.9)
Navy	14 (5.8)
Air Force	7 (2.9)
Marines	43 (17.8)
Active Duty, N (%)	226 (93.4)
Location of Injury, N (%)*	
Iraq	190 (78.5)
Afghanistan	64 (26.5)
Time Since Injury, years (SD)	12.7 (3.2)

**Percentages may add up to more than 100% due to multiple deployments.*

Table 3. Urine metal concentrations in µg/g creatinine (N = 242). 63 (26%) subjects had a significant elevation in at least one urine metal.

Metal	Upper limit of normal	Subjects above upper limit, N (%)	Mean (SD)
Aluminum	30	36 (14.9)	18.9 (12.5)
Arsenic	60	3 (1.2)	7.1 (12.1)
Cadmium	1	2 (0.8)	0.2 (0.2)
Chromium	2	7 (2.9)	0.6 (0.8)
Cobalt	1	6 (2.5)	0.4 (0.4)
Copper	40	2 (0.8)	5.7 (15.4)
Iron	250	0	18.4 (20.0)
Lead	2	5 (2.1)	0.4 (0.7)
Manganese	2	4 (1.7)	0.5 (0.4)
Molybdenum	122	2 (0.8)	31.3 (22.8)
Nickel	8	0	1.7 (0.8)
Tungsten	0.4	11 (4.5)	0.2 (0.5)
Uranium	0.04	3 (1.2)	0.01 (0.02)

Highlight = known pulmonary toxicants

Table 4. Odds of PFT abnormality in subjects with vs without elevated urine metals. There was no significant difference between the odds of any measured pulmonary outcomes with metal levels, even after adjusting for smoking history.

PFT abnormality, N (%)		Elevated urine metal Yes (N=63)	Elevated urine metal No (N=179)	Adjusted Odds Ratio (95% CI)
Ventilatory pattern				
Normal	150 (62%)	43	107	
Obstructive	27 (11%)	7	20	0.7 (0.3-1.9)
Restrictive	65 (27%)	13	52	0.6 (0.3-1.3)
Gas exchange pattern				
Normal	203 (84%)	54	149	
Reduced	39 (16%)	9	30	0.8 (0.4-1.5)
Any abnormality 44%		***	***	1.2 (0.7-2.1)

Discussion

- Approximately one-fourth of those veterans reporting embedded metal fragments had elevated urine metal levels. Reasons for this discrepancy may include a low body metal burden or metal fragment stability over the time period assessed.
- There was a high prevalence of PFT abnormalities in the examined TEF cohort, supporting prior observations of respiratory system dysfunction in post-2001 deployers.
- PFT abnormalities were not associated with elevated urine metal content in this interim analysis.
- Future analyses with a larger cohort will explore the impact of these metals and factors such as blast exposure on lung function.

Acknowledgement: This work is funded by the U.S. Department of Veterans Affairs and the Department of Defense Congressionally Directed Medical Research Program award #W81-XWH16-2-0058.

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