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

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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 of injury.

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

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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, 45% completed.

Major Task 6
Metal Analysis and Tissue Imaging
Year 3/Month 1 to Year 4/Month 12, 50% completed.

Major Task 7
Data Compilation, Statistical Analysis, and Preparation of Final Report
Year 4/Month 1 to Year 5/Month 12, 30% 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, 85% completed.

Major Task 4
6M Experimental Group Microarray analyses
Year 3/Month 5 to Year 4/Month 10, 85% 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, 15% 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, 65% completed.

Major Task 4
Questionnaire analyses
Year2/Month 1 to Year 5 Month 12, 35% completed

Major Task 5
Collection and analyses of urine specimens
Year 1/Month 1 to Year 4/Month 7, 65% completed.

Major Task 6
Collection analyses of PFT and IOS findings
Year 1/Month 1 to Year 4/Month 6, 65% completed.

Major Task 7
Summarize Metal and Renal Findings
Year 2/Month 1 to Year 5/Month 12, 35% completed.

Major Task 8
Summarize PFT and IOS Findings
Year 2/Month 1 to Year 5/Month 12, 35% 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"

During Year 3 of this project, the remaining rats in the 6 month cohort were humanely euthanatized at their experimental endpoints. At euthanasia, tissues were collected and processed. Designated tissues from the experimental group were packaged and shipped to the University of Kentucky for further analysis (Project 2). Weekly health assessment and a pre-euthanasia urine collection were also conducted until the experimental endpoints were reached. The final euthanasia Year 3 euthanasia schedule can be found in the Appendices.

Body weight changes and growth characteristics of the 6M experimental groups are shown in the Appendices (Figures 1 and 2). All 6M groups gained weight at a similar rate except for the nickel-implanted cohort; however, tumor development was observed around the 6-month time point in the nickel-implanted animals necessitating euthanasia suggesting a reason for the decreased weight gain. Hematological assessments of the 6-month cohort showed elevated hemoglobin levels in the nickel-, cobalt-, and depleted uranium-implanted rats as compared to the tantalum-implanted control group (Table 1). Comparison of normalized tissue weights showed no significant differences except of the thymus from aluminum-implanted rats. The reason for this awaits histopathological assessment. A summary of serum analyte changes between the experimental groups is shown in Table 3 of the Appendices and suggests that subtle changes in serum biomarkers may prove to be of benefit in determining potential adverse health effects from embedded metal fragments.

Analysis of changes in protein levels of PSD95 and spinophilin in the frontal cortex region of the brain in rats implanted with metal fragments for 12 months are shown in Figure 3 of the Appendices. Compared to the tantalum control group, levels of PSD95 are increased in the frontal cortex of all of the 12-month experimental groups except for tungsten. Conversely, sphinophilin levels in the frontal cortex of the 12-month cohort are only elevated in the aluminum-implanted group (as compared to tantalum controls). Analysis of expression of other proteins in various regions of the brain (frontal cortex, cerebellum, hippocampus, amygdala, preoptic area) for all experimental groups is currently underway.

Metal imaging of the pellet implantation site in the gastrocnemius muscle was also initiated this year. In Figure 4, representative images from Scanning Electron Microscopy – Energy Dispersive X-ray Spectroscopy (SEM-EDS) are presented to show that this techniques can be used to identify foreign material composition in muscle tissue. In addition, Fourier-Transform Infrared (FTIR) microscopy of gastrocnemius muscle for metal-implanted rats showed secondary protein structural changes as a result of metal exposure (Figure 4). Work in these areas will continue in Year 4.

As noted in last year's report, the implanted copper pellets were not located upon euthanasia of the longer implanted cohorts and we believe the pellets were extruded through the skin. This turned out to be the case. The series of photographs in Figure 5 shows a copper pellet being expelled through the skin. Although we were able to detect the copper pellets in the muscle with a commonly available metal detector, once they were expelled into the bedding they were difficult to locate and sometimes eaten by the rats. In fact, we found one copper fragment in a fecal pellet. The extrusion process was not painful and the area rapidly healed with no adverse health effects

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

During Year 3 of this project we completed microarray analysis of gene expression on all skeletal muscle samples (1, 3, 6 and 12 month time points) for each of the 8 metals and the control metal (tantalum). Comparison of gene expression patterns in 1 and 3 month samples is complete, with 6 and 12 month time points being added for each metal. The initial bioinformatic analysis indicate that nickel and cobalt have the most significant impact on skeletal muscle gene expression at early time points, showing 500-1000 differentially expressed genes (DEGs). Analysis of the response to lead across the entire time point showed nearly 300 differentially expressed genes (DEG) at 1 month, which decreased to 40 DEGs by 12 months, suggesting that the muscle is able to mount an adaptive response. Similar analyses will be performed for all metals. Integrated pathway analysis will then be used to identify processes in muscle modified by each embedded metals.
This year largely focused on isolating good quality RNA from urine and we determined that it was not necessary to first purify exosomes prior to RNA isolation (Figure 1, Appendix). Isolating total RNA directly from urine collected using the LabSand technique, which we optimized in year 2, results in a higher yield and a more diverse array of small RNAs, including microRNAs, tRNAs, snoRNAs, etc, increasing the likelihood that useful biomarkers will be identified. Urine RNA from all samples has been sequenced, which provides more data and requires less RNA than microarrays. Isolation of serum RNA was also completed and has been sent for sequencing.

Dr. McDiarmid continues to conduct quarterly video or in-person conferences with Projects 1 and 2 teams to monitor progress and trouble-shoot any potential barriers.

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 3, we have continued to mail out introduction letters, questionnaires, and reminder postcards to randomly selected eligible participants. To date, mailings have been sent to 7000 eligible participants in batches of 1000 participants at a time. Although our IRB approval allows up to 4 mailings per Veteran, a decision was made this year to mail the questionnaire to a Veteran up to 3 times due to the low response rate from the fourth mailing. We have received a total of 1,791 completed questionnaires. The overall response rate has been approximately 30%. (Major Task 3 -Recruitment and Questionnaire Administration).

Paper versions of completed questionnaires continue to be entered using a two-step validation process into the study database using DataFax, an optical character reader scanning system for questionnaire data entry. Results from the on-line surveys, created by the VA Albuquerque Cooperative Studies Program (CSP) and VA Automated Information and Technology Center, are automatically captured in the study database. We continue to monitor returned questionnaire responses for blast versus no-blast exposure responses to assure

that our sampling strategy was correct to achieve the desired number of bullet-only injuries needed for analytical power. (Major Task 4- Questionnaire Analysis).

Two changes within the VA Maryland Health Care System have resulted in submission of IRB modifications over the past year. The first modification which was approved allowed for phone numbers of the study team to be updated on all study documents. The second modification, also approved, allowed for the participant payment method to be changed from mailing of a paper check to mailing of a ClinCard. ClinCard is a payment method created by Greenphire and used by numerous VAs to pay study participants. It acts like a non-personalized debit card onto which study payment funds can be loaded and used by the participant without the addition of fees.

Study Population #2 – Clinical Assessment Group

During Year 3, recruitment has continued at all sites. As of September 30, 2019, a total of 298 Veterans have completed the clinical assessment. (Major Task 3- Recruitment and Questionnaire Administration). In addition, we received all required approvals to bring on the Phoenix VA, using existing funds, as an additional recruitment site to ensure that recruitment goals are obtained. In early September, Dr. Gaitens, Dr. Hines, and Kate Agnetti, research coordinator for the coordinating site, conducted an in-person meeting with the Phoenix study team (Dr. Permana Paska, Dr. Sam Aguayo, and Kelli Bingham) to train them on the study protocols. It is anticipated that recruitment will begin in Phoenix in October 2019.

We have also continued to double-enter data from all study questionnaires into the Clinical Assessment database and initial analyses of the data have led to refinement of the data entry process. (Major Task 4 – Questionnaire Analysis).

We have supplied local sites with urine collection kits as needed and the local sites continue to collect and send specimens for metal analyses. We have received and stored urine specimens from the local sites for analyses of the renal markers until another shipment is ready to be sent to the Belgium Laboratory. The next shipment is scheduled for 10/7/19. (Major Task 5- Collection and Analyses of urine specimens).

We continue to enter all urine metal results into the study database and QC checks are performed by Dr. Gaitens. In June 2019, we received renal marker results from the first batch of urine samples sent to Belgium (n=119). These data were analyzed, and preliminary findings were presented during our all-investigator meeting which was held September 19-20, 2019. All local site investigators and study coordinators were invited to attend this meeting. Slides presented at this meeting are included in the Appendix. (Major Task 7 – Summarize metal and renal findings).

All sites continue to collect and upload participant PFT and IOS data to the study's Share Point site. These data are then reviewed biweekly by Dr. Hines or one of the study pulmonary fellows to provide test quality feedback to the local study sites. All PFT and IOS data are double-entered into the study database. These data were analyzed, and preliminary findings were also presented during September's all-investigator meeting mentioned above. (Major Task 8- Summarize PFT and IOS findings).

Preliminary findings from all available data were also presented at the FY15 Peer Reviewed Medical Research Program Focused Award Milestone meeting held on January 18, 2019.

During this year, we initiated and continue to have biweekly data meetings, with involvement from the study team's statistician, to review the status of data entry, discuss preliminary findings, and outline plans for upcoming analyses.

Quarterly videoconferences were held with all VA collaborators to review the study protocol and discuss recruitment and any questions that arise. Bi-weekly conference calls continue between all site research coordinators to increase communication and troubleshoot any challenges that arise.

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 ***"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

We have added two additional post-doctoral research fellows to the team (at no cost), Dr. Katherine Chin and Dr. Maxwell Reback, who are both currently enrolled in a Pulmonary and Critical Care fellowship training program at University of Maryland Medical Center. Similar to their predecessor, Dr. Danielle Glick, who is still an active member of the research team during her final year of pulmonary training, they will acquire unique expertise and skills in impulse oscillometry testing that they would not receive in their fellowship training otherwise, which will be useful through the rest of their careers. All three research fellows are currently preparing scientific abstract submission for anticipated scholarly presentations at national meetings in 2020.

How were the results disseminated to communities of interest?

Nothing to report.

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"

During Year 4 of the project, urinary biomarker assays will be completed and correlated to urine and kidney metal levels. Metal analysis of all tissues will be completed and analyzed. Histopathology and metal image analysis on collected tissue will continue. Effects of embedded metal fragments on brain proteins will be completed and assays of serum inflammatory markers resulting from embedded metal fragments will commence.

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

During Year 4 of the project, we will continue bioinformatic analyses of all muscle microarray data to identify differentially expressed genes and pathways at each time point and for each metal sample. We will

also begin bioinformatic analyses of urine and serum RNA sequencing data. Once genes and pathways have been identified, they will be confirmed by real time quantitative polymerase chain reaction (RTqPCR).

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"

In Year 4, for Study Population #1 we will continue to mail invitations and questionnaires to randomly selected Veterans from the Toxic Embedded Fragment Registry and enter data into the study databases.

For Study Population #2, we will continue recruitment and enrollment of Veterans to complete the expanded questionnaire and participate in clinical assessments. This includes collecting and prepping urine specimens, sending urine specimens for metal and renal marker analyses, and performing PFT and IOS testing at VA recruitment sites. Additionally, available imaging records will be reviewed to determine if the presence of fragments have been documented.

For both populations, data entry via primary and secondary validation will continue. We plan to present the results of interim analyses at national meetings in 2020 of the Society of Toxicology and the American Thoracic Society.

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 early microarray analyses indicate differential response to different metals and that in some cases, the induced inflammatory response is later resolved. While these findings are preliminary and need to be confirmed by RTqPCR, they do suggest that we will be able to identify metals likely to have the greatest effect on warriors harboring embedded metals. The large array of changes in small RNAs in urine in response to different embedded metals also suggests that biomarkers will be identified.

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"

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:

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:

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

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
"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"

In order to improve our probability of success in achieving our targeted enrollment, we have added The Phoenix VA as a sixth recruitment site. This was approved by the DOD and our amended protocol approved by VA C-IRB.

Protocol Deviations:
(Study Population #2)- Protocol #A-19735.2

During Year 3, there was one deviation related to the pulmonary function testing at the Nashville site on January 11, 2019. The participant arrived at the VA for his study visit, but instead of meeting the coordinator in the lobby as instructed, he inquired about his appointment with the receptionist at the information desk. She instructed the participant to go directly to the PFT lab. When the participant arrived at the PFT lab, the staff performed the research PFTs prior to obtaining his written informed consent. The coordinator located the participant at the lab where they completed the informed consent process and the rest of the study testing. The event was reported to VA C-IRB within 5 days of discovering it and the IRB determined that the event was not serious since the participant clearly intended to take part in the study. This determination was sent to the DoD HRPO on 2/12/19.

At Nashville and at all of our sites, we have created mechanisms to assure that patients will meet with the coordinator first and sign the consent form before undergoing PFT lab testing. Our PFT lab collaborators also know that these study patients shall not undergo testing unless they have signed consent forms. No additional deviations have occurred since then.

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:

The Phoenix VA was added as a sixth recruitment site during Year 3. The purpose for adding this site is to assure attainment of the needed number of Veteran participants for the study. The Phoenix VA site will be supported in years 3-5 with unexpended funds from the existing sub-recipient site budget across all four of the projects. Project 1 experienced savings prior to Year 3 due to changes in personnel and lab renovations that impacted the implantation schedule. Project 2 had time savings from innovations in the urine collection methods used in Project 1. This resulted in reduced staff time and subsequent savings in personnel costs. For projects 3 and 4, some funds budgeted in Year 2 for UMB urine metal testing were allocated, as this cost was reduced due to methods changes in metal testing for Embedded Fragment Registry participants, for which our DOD project participants are eligible. Also, the sub-recipient patient enrollment sites all had some savings due to a late start and delayed hire of research coordinators in Year 1. Ultimately, these savings allowed creation of a budget that would allow inclusion of the Phoenix VA site.

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

Significant changes in use or care of <u>human subjects:</u>

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

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

Amendments submitted to IRB and USAMRMC HRPO
(Study Population #1)- Protocol #A-19735.1

- **Modification 1** submitted to University of Maryland HRPO 8/18/17 (prior to approval by subsequent IRBs); approved on 9/1/2017; Submitted to DoD HRPO 9/27/17; acknowledged 12/14/17. This modification was submitted due to a change in letterhead logos on recruitment letters and was included in the initial USAMRMC HRPO submission form.
- **Modification 2** submitted to University of Maryland HRPO 3/12/18; approved on 3/28/2018; Submitted to DoD HRPO 3/28/18; acknowledged 4/3/18. This modification was submitted due the change from use of VA REDCap to VA Cooperative Studies Program-Austin Information Technology Center (CSP-AITC) for capture of electronic survey data.
- **Modification 3** submitted to University of Maryland HRPO on 5/11/2018; approved 6/1/18; Submitted to DoD HRPO 6/5/18; acknowledged 6/13/18. This modification included a slight change to the appearance of the cover letter accompanying the questionnaire providing a better visual representation of instructions for completing the questionnaire online, and information about how an access code and PIN number will be used to log onto the electronic survey site.
- **Modification 4** submitted to University of Maryland HRPO on 7/20/2018 and approved on 7/30/2018; Submitted to DoD HRPO; acknowledged on 8/3/2018. This modification was submitted to replace CTRIC and VA Teleforms with the VA Perry Point Cooperative Studies Program and Datafax for the paper questionnaires.
- **Modification 5** submitted to University of Maryland HRPO on 11/1/2018; approved on 11/25/2018; Submitted to DoD HRPO 11/30/18; acknowledged 12/17/18. This modification was required to update the study coordinator's new extension on all recruitment documents and electronic survey webpage.
- **Modification 6** submitted to University of Maryland HRPO on 7/3/19; approved on 7/12/19; Submitted to DoD HRPO 7/16/19. This modification changed the method of payment to ClinCard by Greenphire, rather than checks. Two new pulmonary fellows were added to the protocol.
- **Modification 7** submitted to University of Maryland HRPO on 8/20/19; approved on 8/21/19; Submitted to DoD HRPO 9/3/19. This modification was required to include a new mandatory VA warning on the electronic survey homepage.

*Please see the "Submissions Tracking Table" in the appendices for a summary of these amendments

Amendments submitted to IRB and USAMRMC HRPO
(Study Population #2)- Protocol #A-19735.2

- **Modification 1**: Submitted to VA C-IRB on 6/5/2018; Approved 6/19/18; Submitted to DoD HRPO 6/25/18 and approved on 6/26/18. This modification grants some flexibility in the event that all study procedures cannot be completed during a single visit. In such an event, (i.e. equipment malfunctions in the pulmonary function lab and test not able to be completed), we will be able to bring participants back to complete the protocol. This modification also allows for additional travel pay for the second visit and includes language in the consent form describing this.

- **Modification 2**: Submitted to VA C-IRB on 8/15/18; Approved 11/5/18; Submitted to DoD HRPO 11/20/18; Acknowledged 12/17/18. This modification gives the participant the opportunity to be re-contacted for future studies.
- **Modification 3**: Submitted to VA -CIRB on 9/4/18; Approved 11/5/18; Submitted to DoD HRPO 11/20/18; Acknowledged 12/17/18. This modification was submitted per the IRB's recommendation in the determination of a deviation that occurred at the San Antonio site. This deviation stemmed from a scheduling error which resulted in a study participant performing a research PFT prior to being consented. In this modification, the HIPAA authorization was revised to indicate that PHI will be used to schedule pulmonary function tests prior to obtaining consent and HIPAA.
- **Modification 4**: Submitted to VA -CIRB on 3/27/19; Approved 4/9/19; Submitted to DoD HRPO 4/18/19; Approved 6/13/19. The Phoenix VA was added as 6th recruitment site.
- **Modification 5**: Submitted to VA -CIRB on 5/2/19; Approved 5/28/19; Submitted to DoD HRPO 5/28/19; Approved 7/9/19. The San Antonio LSI, Dr. Catherine Do, was replaced by Dr. John Duch. The Eligibility Criteria were updated to specify location and era in which injury occurred resulting in fragment, and the participant must be able to provide urine specimen without the use of a catheter.

*Please see the "Submissions Tracking Table" in the appendices for a summary of these amendments

Significant changes in use or care of <u>vertebrate animals:</u>

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

Nothing to report

<u>Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2</u>
"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:

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

  Jessica Hoffman, Ivan Vechetti Jr, Alexander Alimov, John Kalinich, John McCarthy, and Charlotte Peterson. Hydrophobic sand is a viable method of urine collection from the rat for extracellular vesicle biomarker analysis. Molecular Genetics and Metabolism Reports (in press, 2019) (pre-print provided in Appendices)

  <u>Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2</u>
  "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"

Preliminary findings were presented at the FY15 Peer Reviewed Medical Research Program Focused Award Milestone meeting held on January 18, 2019 as well as the study all-investigator meeting held September 19-20, 2019 in Baltimore, MD.

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"

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.

- **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"

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.

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 call.

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: Assist with procurement, travel arrangements, and document preparation.

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
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
Contribution to Project: Responsible for experimental planning
Funding Support: Federal Government Employee (Department of Veterans Affairs)

Name: Anya Fan, MS
Project Role: Research Assistant
Nearest person month worked: 12
Contribution to Project: Responsible for implantation surgeries, urine collection, and animal welfare. Ms. Fan left the project in August 2019.

Name: Jessica Hoffman, PhD
Project Role: Co-Investigator
Researcher Identifier: 0000-0003-1858-8394
Nearest person month worked: 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)

Name: William Danchanko, PhD, CDR, USN
Project Role: Local Site Investigator
Nearest person month worked: 1
Contribution to Project: Member of the surgical implantation and euthanasia teams.
Funding Support: U.S. Navy (active duty)

Name: Vernieda Vergara, BS
Project Role: Research Assistant
Nearest person month worked: 12
Contribution to Project: Responsible for implantation surgeries and animal welfare, sample collection and metal analysis.

Name: Jose Centeno, PhD, FRSC
Project Role: Co-investigator
Nearest Person Month worked: 1
Contribution to Project: Oversight of metal imaging experiments and post-doctoral fellow
Funding Support: Federal Government Employee (U.S. Food and Drug Administration)

Name: Diane Smith, PhD
Project Role: Post-doctoral fellow
Nearest person month worked: 12
Contribution to Project: Metal imaging experiments under the direction of Dr. Centeno

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: Ivan Vechetti
Project Role: Postdoctoral Scholar
Nearest person month worked: 6
Contribution to Project:

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?

Drs. Peterson and McCarthy were awarded a 5 year NIH R01DK119619 on Sept 19, 2018. Each will spend 1.2 calendar months on that project but that will not affect the current project. No overlap.

Dr. Gaitens has been awarded a NIOSH contract to develop data collection tools for a mesothelioma registry. She will spend 1.8 calendar months on this new project but that will not affect the current project. No overlap.

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: 12
Contribution to Project: Interacted with HRPO and regulatory bodies in order to obtain and maintain required approvals; assisted in developing recruitment, enrollment, and scheduling strategies; recruited and enrolled participants, collected data and specimens; organized and participated in quarterly project team calls and biweekly site calls, organized and participated in All-Investigator meeting.

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 and attended the All-Investigator meeting.

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: 12
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls, attended the All-Investigator meeting, and 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 (5% effort)

Contribution to Project: Acquired and maintained required approvals; oversaw local recruitment, enrollment, specimen collection; participated in quarterly project team calls, and attended the All-Investigator meeting.

Name: Nataliya Kirichenko
Project Role: Local Study Coordinator
Nearest person month worked: 4.5 (37.5% effort)
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.

Name: Katherine Solis
Project Role: Local Study Coordinator
Nearest person month worked: 4.5 (37.5% effort)
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls, biweekly site calls, attended the All-Investigator meeting; recruited and enrolled participants; collected data and specimens.

Name: Paige Webb (Formerly Gustad)
Project Role: Local Regulatory Assistant
Nearest person month worked: 1.2 (10% effort)
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, and attended the All-Investigator meeting.

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 the All-Investigator meeting, and biweekly site calls; recruited and enrolled participants; collected data and specimens.

San Antonio (Site 5):

Name: Catherine Do, MD
Project Role: Local Site Investigator
Nearest person month worked: 1.2 (20% effort; Left LSI role as of May 1, 2019.)
Contribution to Project: Acquired and maintained required approvals; oversaw local recruitment, enrollment, specimen collection; participated in quarterly project team calls.

Name: John Duch, MD
Project Role: Local Site Investigator
Nearest person month worked: 1.2 (20% effort; New LSI as of May 1, 2019.)

Contribution to Project: Acquired and maintained required approvals; oversaw local recruitment, enrollment, specimen collection; participated in quarterly project team calls and attended All-Investigator meeting.

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 the All-Investigator meeting, and 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 (15% effort annually; Person months reflects site approved as of April 9, 2019.)
Contribution to Project: Acquired and maintained required approvals; participated in impulse oscillometry and PFT training; participated in Baltimore team site visit; oversaw initiation of local recruitment.

Name: Samuel Aguayo, MD
Project Role: Local Site Investigator
Nearest person month worked: 1.2 (15% effort annually; Person months reflects site approved as of April 9, 2019.)
Contribution to Project: Acquired and maintained required approvals; participated in Baltimore team site visit; oversaw initiation of local recruitment.

Name: Kelli Bingham
Project Role: Local Study Coordinator
Nearest person month worked: 3 (100% annually; Person months reflects delay in receiving IOS equipment and required IOS trainings necessary to begin recruitment.)
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls and biweekly site calls; participated in IOS and PFT training; participated in Baltimore team site visit; attended All-Investigator meeting; initiated recruitment.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to report.
QUAD CHART

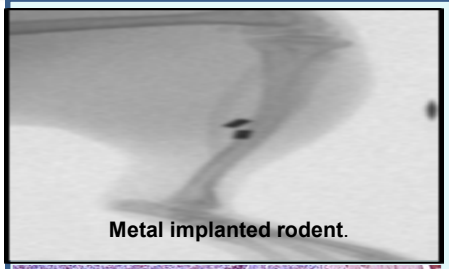
Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments
ERMS/Log Number PR151808
W81XWH-16-2-0058

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.



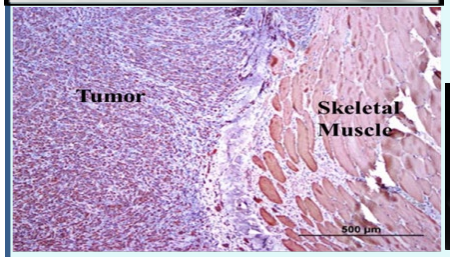


Fig. 2: Desmin-staining of tumor and muscle From metal implant (Project 1)

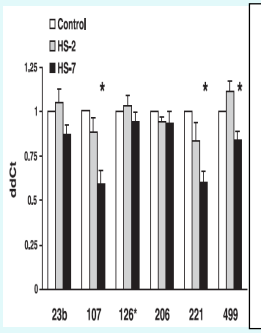
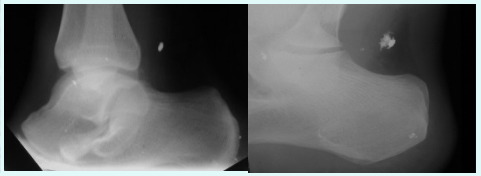


Figure 5. qRT-PCR validation of differentially expressed microRNAs in rat skeletal muscle implanted with metals **(Project 2).** Asterisk denotes significantly ($p < 0.05$) different from control.



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	81%				
PRJ 2: Biomarkers for Assessing Return-to-Duty Potential of Personnel	81%				
PRJ 3: Biomarker Assessment of Kidney Injury from Metal Exposure	63%				
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 16, 2019

Goals/Milestones

Project 1: Animals from all groups have reached their experimental endpoints and been humanely euthanized.

Project 2: Microarray analyses on all muscle RNA samples at all time points have been completed. Sequencing of all urine RNA samples is complete and serum RNA samples have been sent for sequencing.

Projects 3 & 4: Enrolled 298 participants across 5 sites. Amendment to add Phoenix as sixth recruitment site was approved. Mailed invitation letters to 7,000 Veterans to participate in survey. Received 1,791 completed surveys. Year 3 "All-investigators" meeting was held in Baltimore in September 2019.

Comments/Challenges/Issues/Concerns

- Nothing to report

Project Expenditures to Date (9/30/16 – 9/29/19)

Projected Expenditure: $4,872,360
Actual Expenditure: $3,391,319

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

Year 3 Euthanasia Schedule

Figure 1 - Weight Change in 6M Experimental Animals

Figure 2 - Growth Over Time in 6M Experimental Animals

Table 1 – Hematological Results from 6M Experimental Animals

Table 2 – 6M Experimental Animal Tissue Weights

Table 3 – Serum Analyte Patterns between Experimental Groups

Figure 3: Frontal Cortex Protein Changes in Metal-Implanted Rats

Figure 4: Metal Imaging in Metal-Implanted Gastrocnemius Muscle

Figure 5: Time Course of Copper Pellet Expulsion from Rat Muscle

Preprint of *Molecular Genetics and Metabolism Reports* manuscript:
"Hydrophobic sand is a viable method of urine collection from the rat for extracellular vesicle biomarker analysis"

FDA Scientific Forum Poster: Assessing the Effect of Embedded Metals in Tissues Using Novel Spectroscopic Techniques

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

Figure 1. Validation of isolation of total RNA from urine compared to EV purification followed by RNA isolation

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"

1. Submission Approvals Table
2. Agenda from All-Investigator Meeting, September 2019
3. Slide set from All-Investigator meeting, September 2019

OCTOBER 2018 (YR 3 Euthanasia Schedule- - John Kalinich, PhD, Project 1)

Sun	Mon	Tues	Wed	Thur	Fri	Sat
	1 Euthanasia – Fe/6M (4)	2 Euthanasia – Fe/6M (4) LabSand – Al/6M	3 Euthanasia – Cu/6M (4)	4 Euthanasia – Cu/6M (4) LabSand – Pb/6M	5	6
7	8 **COLUMBUS DAY**	9 Euthanasia – Al/6M (4) LabSand – DU/6M	10 Euthanasia – Al/6M (4)	11 Euthanasia – Pb/6M (4)	12 Euthanasia – Pb/6M (4)	13
14	15 Euthanasia – DU/6M (4)	16 Euthanasia – DU/6M (4)	17	18	19	20
21	22	23	24	25	26	27
28	29	30	31			

John Kalinich, PhD, Project 1

Figure 1: Weight Change in 6-Month Experimental Animals

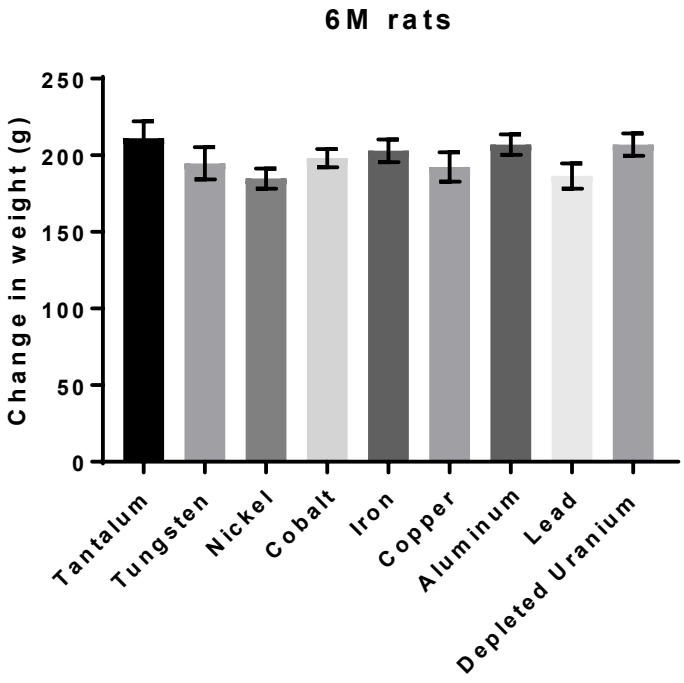
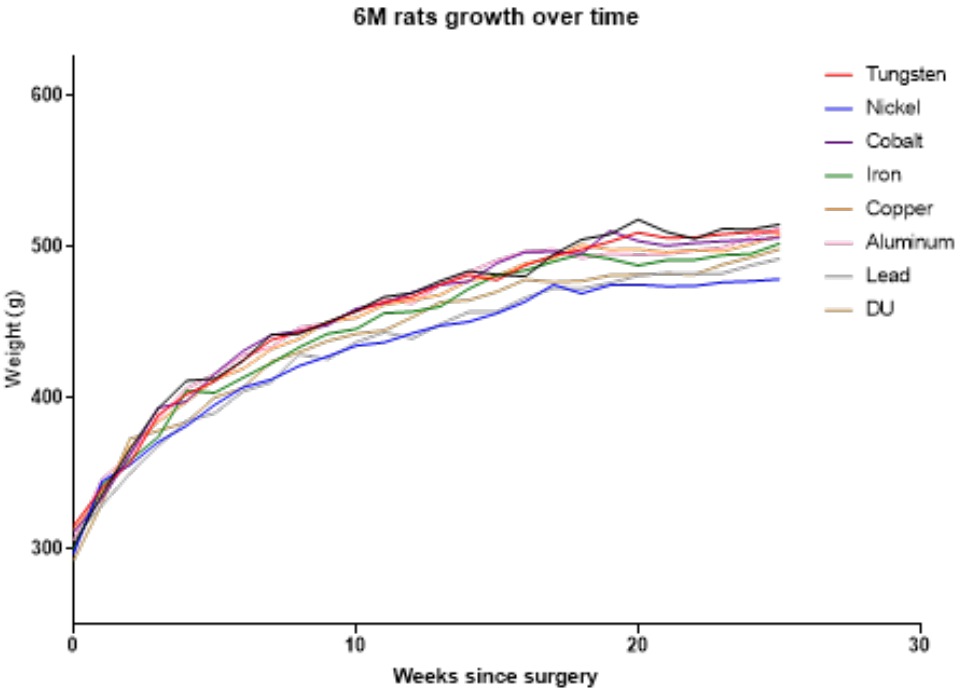


Figure 2: Growth Over Time in 6M Experimental Animals



John Kalinich, PhD, Project 1

Table 1: Hematological Assessment of 6M Experimental Animals

6M Rats			
	WBC (10^3/mm^3)	**RBC (10^6/mm^3)**	**HGB (g/dl)**
	F(8,63)=0.957 p=0.4771	F(8,63)=0.335 p=0.949	F(8,63)=2.518 *p=0.019
Tantalum	6.588 (1.030)	8.318 (0.146)	14.5 (0.214)
Tungsten	6.588 (0.808)	8.153 (0.344)	15.35 (0.325)
Nickel	7.275 (1.074)	8.296 (0.377)	**15.60 (0.370)**
Cobalt	7.013 (2.004)	8.321 (0.255)	**15.68 (0.345)**
Iron	6.413 (1.180)	8.190 (0.199)	15.15 (1.263)
Copper	6.063 (2.326)	8.174 (0.571)	15.16 (0.980)
Aluminum	7.323 (1.311)	8.165 (0.440)	15.35 (0.609)
Lead	6.738 (1.387)	8.31 (0.285)	15.36 (0.293)
Depleted Uranium	7.625 (1.185)	8.186 (0.425)	**15.64 (0.526)**

HGB: Tantalum vs Nickel *p=0.0073
(HGB is higher in Nickel animals compared to tantalum)
Tantalum vs Cobalt *p=0.0036
(HGB is higher in Cobalt animals compared to tantalum)
Tantalum vs DU *p=0.0051
(HGB is higher in DU animals compared to tantalum)
One-way ANOVA comparing each metal back to Tantalum (control)
Data presented as mean(SD), bold indicates significant difference when compared to Tantalum

John Kalinich, PhD, Project 1

Table 2: Tissue Weights at Time of Euthanasia

Normalized to body weight, reported as % of body weight (tissue weight/body weight x 100)
One-way ANOVA comparing each metal back to Tantalum (control)

6M Rats					
	Thymus	**Liver**	**Spleen**	**Kidney**	**Testes**
	F(8,62)=3.689 *p=0.0014	F(8,63)=1.291 p=0.2644	F(8,63)=0.645 p=0.737	F(8,63)=0.468 p=0.874	F(8,63)=1.592 p=0.145
Tantalum	0.0422 (0.007)	3.202 (0.223)	0.146 (0.009)	0.6001 (0.041)	0.8747 (0.069)
Tungsten	0.0364 (0.003)	3.187 (0.271)	0.148 (0.014)	0.5947 (0.049)	0.8348 (0.073)
Nickel	0.0454 (0.010)	3.288 (0.186)	0.163 (0.029)	0.5972 (0.041)	0.9110 (0.035)
Cobalt	0.0356 (0.006)	3.323 (0.304)	0.149 (0.019)	0.5965 (0.040)	0.8419 (0.063)
Iron	0.0422 (0.009)	3.219 (0.203)	0.147 (0.008)	0.6020 (0.026)	0.8235 (0.076)
Copper	0.0402 (0.008)	3.198 (0.126)	0.149 (0.016)	0.6111 (0.017)	0.8748 (0.060)
Aluminum	**0.0266 (0.010)**	3.453 (0.205)	0.151 (0.027)	0.5832 (0.038)	0.8482 (0.056)
Lead	0.0373 (0.008)	3.173 (0.191)	0.148 (0.015)	0.5852 (0.036)	0.8721 (0.068)
Depleted Uranium	0.0421 (0.009)	3.273 (0.244)	0.150 (0.008)	0.6019 (0.021)	0.8853 (0.050)

Data presented as mean (SD) of normalized body weight, bold indicates significant difference when compared to Tantalum

Thymus: Tantalum vs Aluminum *p=0.0021 (thymus is smaller in aluminum animals compared to tantalum)

One-way ANOVA
F(8,63) = 1.269, p=0.2757

Multiple comparisons, each metal compared only with Tantalum (Control)

Tungsten – p=0.6303
Nickel – p=0.1561
Cobalt – p=0.8298
Iron – p=0.9832
Copper – p=0.4829
Aluminum – p=0.9995
Lead – p=0.2067
DU – p=0.9995

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Table 3: Summary of Analyte Patterns (where there is a difference compared to Tantalum; green = increased, red = decreased)

Analyte	W	Ni*	Co	Fe	Cu	Al	Pb	DU
Sodium	1	3 6 12	3	3 6 12	3 6 12	3	3	
Potassium			6	12	6			
Chloride		3	3 12	1 3 12	3 12	3 12	3	
Carbon Dioxide			12				3	3
Glucose						12	12	
Urea Nitrogen						12	12	12
Creatinine							12	
Calcium			6	6	6 12	12		
Phosphorous						3	3	
Total Protein			3		3	12	12	
Albumin			3				1 12	
ALKP				3			3	
LDH					3	3	3	
AST	12							
Lipase	1				1			
Cholesterol		12					12	
Dir HDLC		12		12	12		12	
Uric Acid							12	12

Total Bilirubin, CK, ALT, GGT, and Amylase had no significant differences between the target metal and tantalum for any time group

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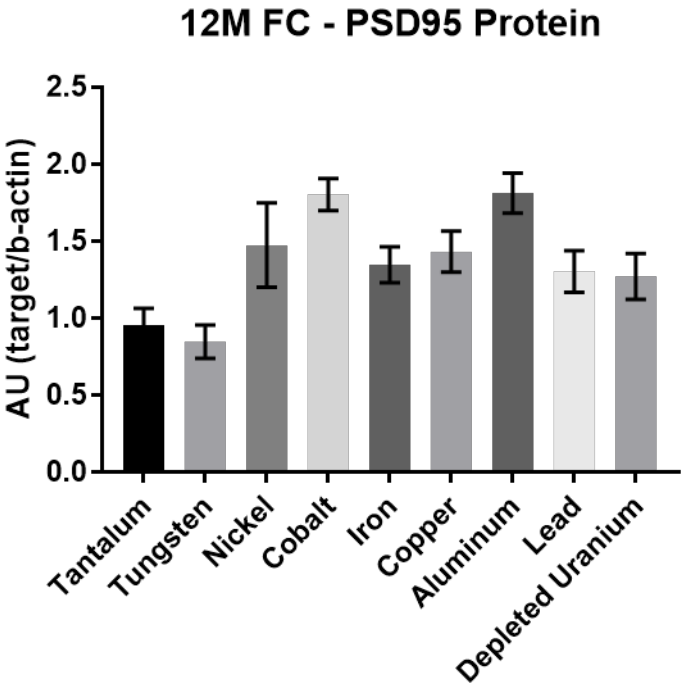
Figure 3: Changes in Frontal Cortex Levels of PSD95 and Spinophilin in Metal-Implanted Rats



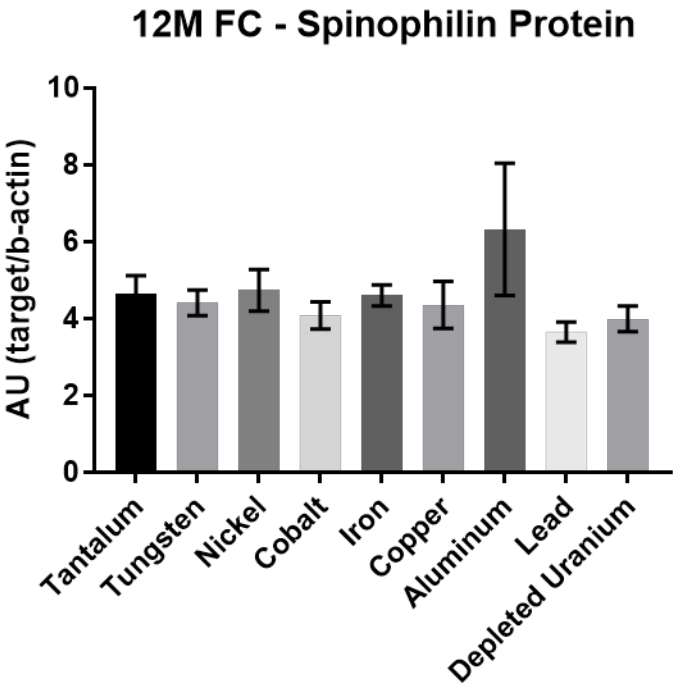


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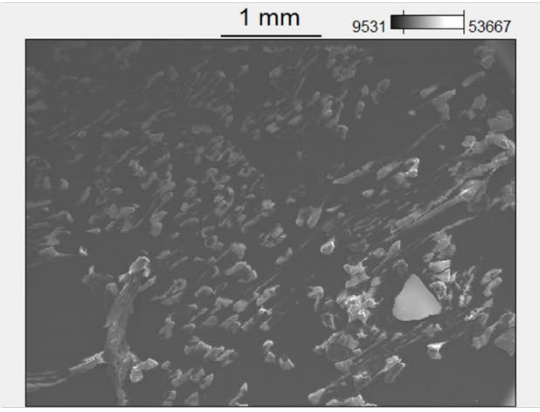
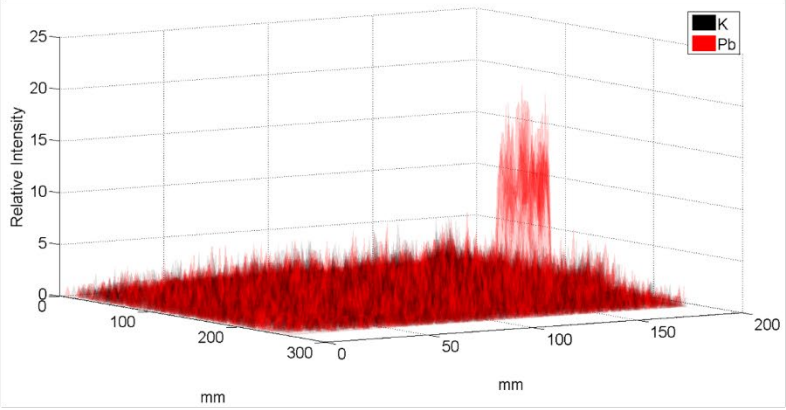
Figure 4: Metal Imaging in Metal-Implanted Gastrocnemius Muscle by SEM-EDS and FTIR

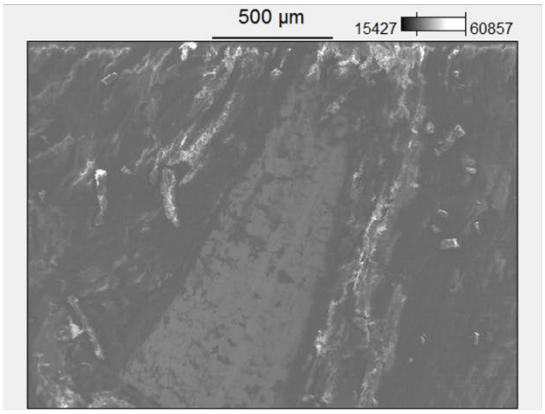
1. SEM-EDS: Elemental mapping of rat gastrocnemius tissue containing exposed metal can be used to verify the foreign material composition

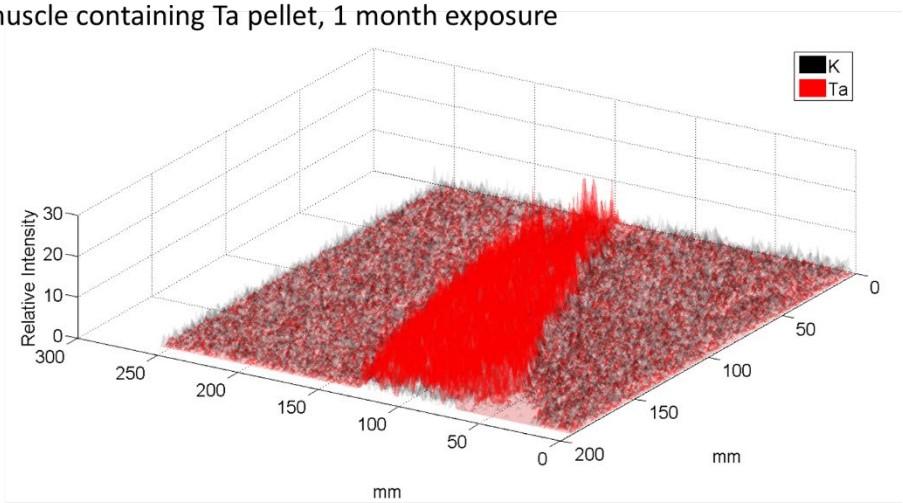
Gastrocnemius muscle containing Pb pellet fragment, 12 month exposure



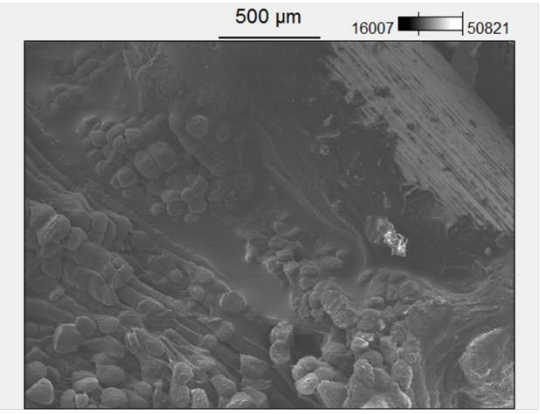


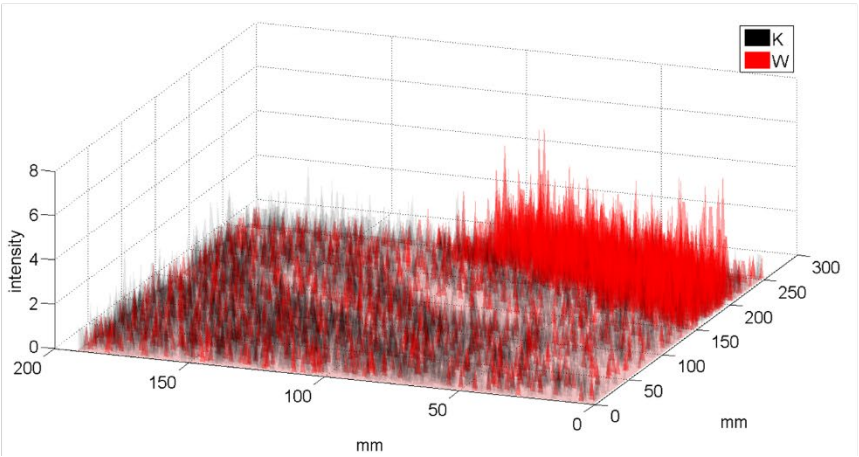
Gastrocnemius muscle containing Ta pellet, 1 month exposure





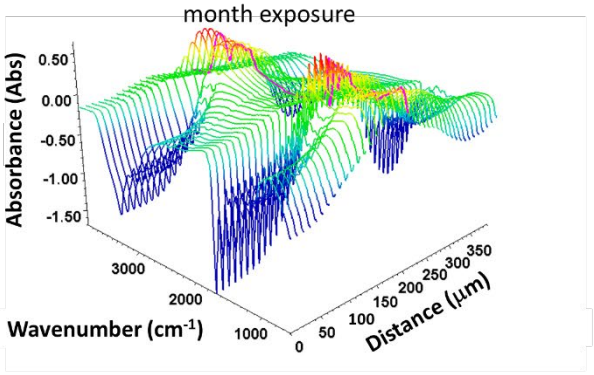
Gastrocnemius muscle containing W pellet, 12 month exposure



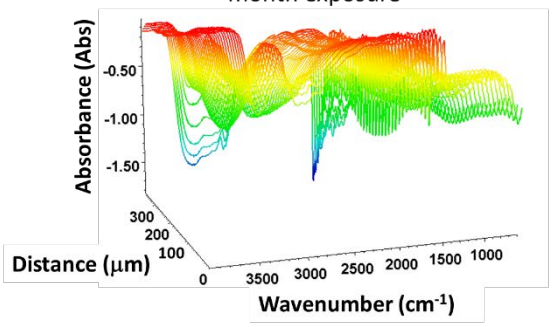


2. FTIR microscopy: Protein secondary structure changes are observed in gastrocnemius muscle surrounding metal pellet implantation site.

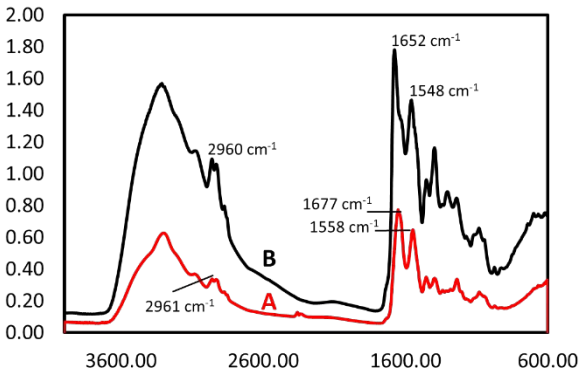
Gastrocnemius muscle exposed to Fe pellet, 1 month exposure



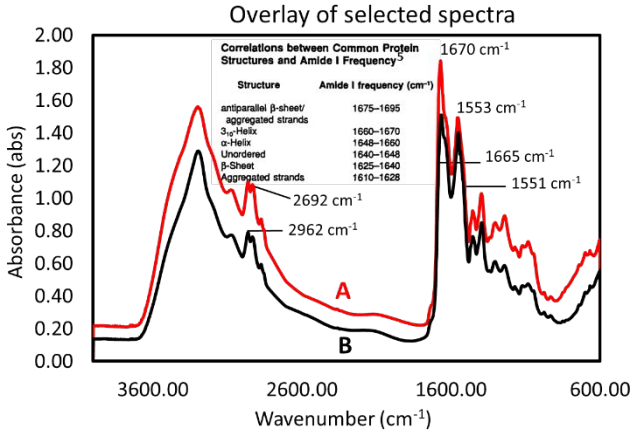
Gastrocnemius muscle exposed to Fe pellet, 12 month exposure

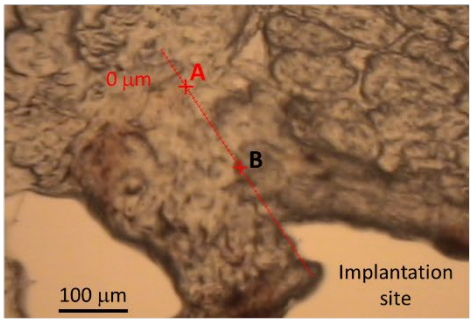


Overlay of selected spectra



Overlay of selected spectra





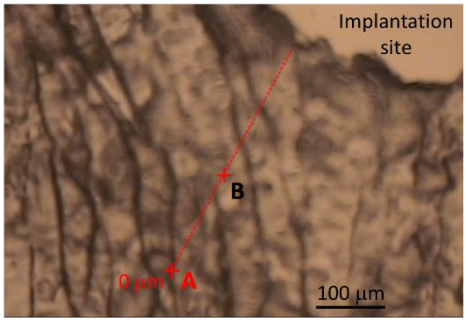
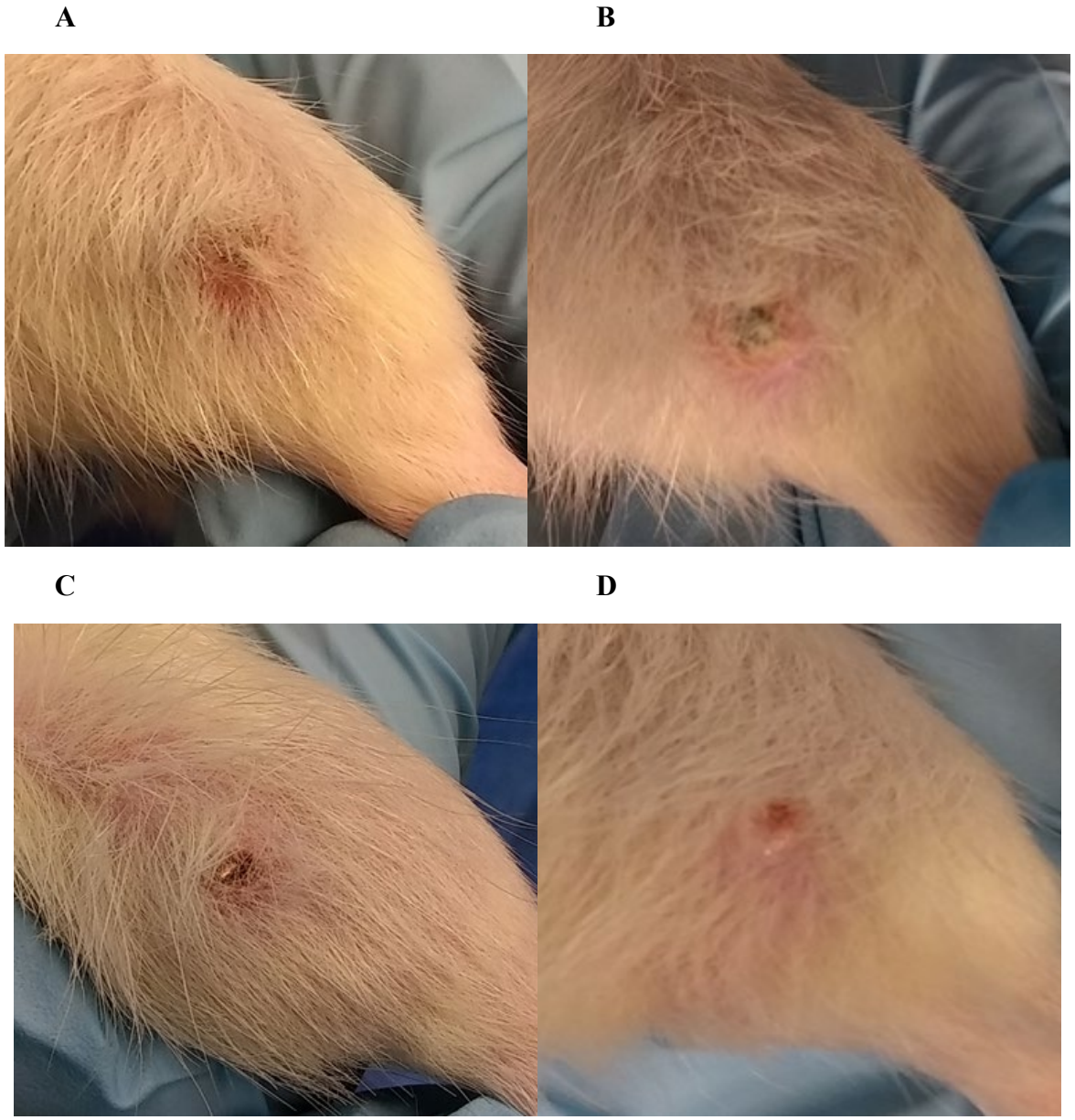


Figure 5: Time Course of Copper Pellet Expulsion from Rat Muscle



Title

Hydrophobic sand is a viable method of urine collection from the rat for extracellular vesicle biomarker analysis

Authors

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Abstract

Previously we have shown in rats a new method of urine collection, hydrophobic sand, to be an acceptable alternate in place of the traditional method using metabolic cages. Hydrophobic sand is non-toxic, induces similar or lower levels of stress in the rat, and does not contaminate clinical urine markers nor metal concentrations in collected samples (Hoffman et al, 2017 and 2018). Urine is often used in humans and many animal models as a readily-attainable biosample which contains proteins and microRNAs (miRNAs) within extracellular vesicles (EVs) that can be isolated to indicate changes in health. In order to ensure hydrophobic sand did not in any way contaminate or disrupt the extraction and analysis of these EVs and miRNAs, we used urine samples from the same 8 rats in the within-subjects crossover experiment comparing hydrophobic sand and metabolic cage collection methods. We isolated EVs and miRNAs from the urine set and examined their quantity and quality between the urine collection methods. We found no significant differences in particle size, particle concentration, total RNA, or the type and abundance of miRNAs contained within the urine EVs due to urine collection method, suggesting hydrophobic sand represents an easy-to-use, non-invasive method to collect rodent urine for EVs and biomarker studies.

Keywords

extracellular vesicles; urine; urine collection; hydrophobic sand; metabolic cage; miRNA

Abbreviations

EVs – extracellular vesicles
LS - LabSand
MC – metabolic cage
miRNAs – microRNAs
PKD – polycystic kidney disease

1. Introduction

Exosomes are a type of small, membrane-bound extracellular vesicles (EVs) that may contain protein, mRNA, and microRNA (miRNA). Exosomes are released by most cells and can be isolated from biofluids such as serum and urine, which has sparked interest in their use as potential non-invasive diagnostic biomarkers for various diseases [1,2,3,4,5]. In humans, exosomes have recently been linked to a number of diseases, including nephronophthisis-related ciliopathies [6], diabetic kidney disease [7], bladder cancer [8,9], liver disease [10], and amyotrophic lateral sclerosis from

cerebrospinal fluid [11]. Rat models of renal disease, such as polycystic kidney disease (PKD), show similar deviations in kidney health biomarkers as humans with PKD. In one study this included increases of urea, guanidinosuccinic acid, creatinine, guanidine, methylguanidine, and N(G)N(G)-dimethylarginine [12], and another study found elevated expression levels of activator of G-protein signaling 3 (AGS3) in urine exosomes for rat and humans with PKD compared to controls [13]. These studies highlight the importance of improving methods of isolating and using extracellular vesicles from humans and animals for the study of disease.

In humans, urine collection is straightforward and standard. In rodents, there are several available methods, but one acceptable standard is the use of a metabolic cage, where rats are isolated in a small, circular plastic cage with a wire mesh bottom that allows urine and feces to pass through into separate collection tubes. Although isolation and collection times can vary, they are usually limited to 24 hours. Habituation of the animal to the metabolic cage is often required and its use must be justified due to its potential for causing stress to the animal [14,15,16,17]. Previously we investigated the use of a new method for rat urine collection, hydrophobic sand (brand name LabSand for scientific use). Originally developed for home urine collection in the cat, LabSand is a biodegradable material with a non-toxic hydrophobic coating that causes urine to pool on its surface, making it easy to collect. We reported no significant differences in stress induction, toxicity to the animals, urine volume collection, or urine quality with several common clinical biomarkers [18], as well as sample integrity when assessing potential contamination during urinary metal concentration analyses [19]. Unlike the metabolic cage, hydrophobic sand is similar to home cage bedding, reducing animal stress and does not require as much time for collection or habituation [discussed in depth in our previous publications, 18 and 19]. Thus we concluded hydrophobic sand has the potential to become a valuable new method for urine collection in the rodent. Given the potential for EVs and miRNAs collected from urine to act as important biomarkers for health conditions in a multitude of research areas, our goal in the work presented here was to take the method comparison a step further and determine if the quantity and quality of EVs collected from rat urine by hydrophobic sand were comparable to that of urine collected by metabolic cage. If we did not find any differences in characterization of EVs and miRNAs collected from urine via these two methods, it would indicate that hydrophobic sand does not contaminate biosamples and thus would be an appropriate alternate method applicable to a broad range of research utilizing urine samples.

2. Materials and Methods

2.1 Animals

All animals in this study are the same as those previously reported in detail [18]. No changes to animal manipulation or urine collection were made for the purposes of this paper. Briefly, 8 male Sprague Dawley rats (Envigo, Frederick, MD, USA) were maintained on a 12:12 light:dark cycle with access to food and water *ad libitum* and pair-housed except during urine collection periods. Rats underwent no treatment or experimental conditions beyond exposure to both urine collection methods. All procedures involving animals were approved by the AFRRI Institutional Animal Care and Use Committee under protocol 2016-05-006.

2.2 Urine collection and experimental design

A crossover within-subjects design was used to compare urine collection from traditional metabolic cages and hydrophobic sand (LabSand, Coastline Global, Palo Alto, CA, USA) [full design and method details can be found in 18]. Briefly, rats were randomly assigned to two groups: (A) metabolic cage followed by LabSand or (B) LabSand followed by the metabolic cage. Both groups were run simultaneously under the same testing conditions. There was a total of 5 collection sessions: at 2 h, at 4 h, and three separate 6 h sessions, each separated by a rest period of at least 48 h. The method crossover occurred after the last session and the entire pattern was repeated. Food and water were not provided to any animal during urine collection sessions, but each animal was provided with a water replacement gel in a plastic cup (HydroGel®, Clear H2O, Westbrook, ME, USA) to avoid dilution from a water bottle drip.

For the metabolic cage method, animals were individually housed in a standard circular metabolic cage (Nalgene Nunc, Rochester, NY, USA) with a wire mesh floor where urine collected into a Nalgene tube at the bottom of a funnel system. Urine could only be collected at the end of the session due to the cage design. For the hydrophobic sand method, animals were individually housed in a rectangular microisolator cage with the sand lining the bottom of the cage in place of regular bedding; urine pools on top of the sand, which is then collected with a pipette. For each rat, we collected urine every half hour which was subsequently pooled at the end of the session. The urine collected in [18] was also analyzed in [19] and an aliquot of the same urine samples were used here for EV isolation and analysis.

2.3 Extracellular vesicle isolation and characterization

The experimental design of n=8 rats with a within-subjects crossover design (4 in hydrophobic sand, 4 in metabolic cages for 5 collection sessions, then the groups switched for an additional 5 sessions) provided an n=8 for each session of each collection method. In order to have enough volume to collect extracellular vesicles from each rat, we created a pooled sample (3 mL total) of all 5 sessions within each individual rat's method of urine collection; this gives an n=8 total urine samples collected by each method. Extracellular vesicles were isolated using ExoQuick-TC kit according to the manufacturer's protocol (System Biosciences, Palo Alto, CA, USA). Urine EV size and abundance were measured using nanoparticle tracking analysis (NTA) with ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and corresponding software 8.04.02 sp1. After calibration using 100 nm polystyrene particles, EVs were appropriately diluted using Dulbecco's Phosphate-Buffered Saline (Thermo Fisher, Waltham, MA, USA) to measure the particle size and concentration. NTA measurements were analyzed at 11 positions at a constant temperature of 23°C.

2.4 miRNA microarray analysis and quantification of select miRNA expression by qPCR

A total of 150 ng of RNA isolated from the EVs was pooled from all samples within each urine collection method to conduct a survey of microRNA abundance using microarray analysis. The microarray hybridization and processing were performed at the University of Kentucky Genomic Core Laboratory using Affymetrix miRNA 4.0 array chips (Santa Clara, CA, USA). Raw signal intensity data were normalized with robust multi-array average (RMA) from the Affymetrix data bank and sorted from highest to lowest signal using the LabSand group.

RNA from individual samples was then used to validate the five most abundant miRNAs as determined by microarray. Reverse transcription reactions for let-7b, let-7c, miR-3473, miR-23b, miR-200b and cel-miR-39 were performed with 3 ng of total RNA using Taqman MicroRNA Reverse Transcription Kit (ThermoFisher Scientific) according to the manufacturer's directions. qPCR was carried out with Taqman Gene Expression Master Mix (2x) (ThermoFisher Scientific) and TaqMan gene expression assays (let-7b, #000378; let-7c, #000379; miR-3473, #475642_mat; miR-23b, #000400; miR-200b, #001800; cel-miR-39, #000200) using cDNA in a 10μL reaction volume. The cel-miR-39 was used to normalize miRNA expression; 1nM of a 5'-phosphorylated sequence (#478293_mir) was spiked into each cDNA synthesis reaction.

qPCR reactions were performed in the ABI 7500 qPCR system (Applied Biosystems, Santa Clara, CA, USA). qPCR efficiency was calculated by linear regression from fluorescence increase in the exponential phase in the LinRegPCR software v11.1 [20]. The comparison of urine EV miRNA expression between LabSand and metabolic cage was determined following normalization with cel-miR-39. The relative microRNA expression was measured by using the comparative CT method [21].

2.5 Statistics

All comparisons between metabolic cage and LabSand urine collection methods were performed by paired t-test (EV size, EV concentration, and total RNA) or unpaired t-test (all 5 PCR validations due to lack of enough volume for a few samples to complete the pair) using GraphPad

Prism Software (version 8.01, La Jolla, CA, USA). *P* values less than 0.05 were considered significant.

3. Results

3.1 Characterizing Extracellular Vesicles

Exosomes are typically 50-150 nm in diameter [3,5]. Extracellular vesicles in our samples had a size range between 15-345 nm, with the largest peaks falling within 41-160 nm, as can be seen in the size spectrum analysis in Fig. 1A. There were no significant differences between characteristics of EVs isolated from urine collected with the metabolic cage (MC) method versus the LabSand (LS) method. The size of the particles extracted from the MC urine samples were not significantly different from the size of the particles extracted from the LS urine samples (Fig.1A, entire particle size distribution: MC mean 122 nm, STD 6.46, min 114, max 134; LS mean 125 nm, STD 9.69, min 107, max 136; t_7=0.8685, p=0.4139). Similarly, the particle concentration (number of particles per mL of urine) was not significantly different between the two urine collection methods (Fig. 1B: MC mean $1.27x10^{12}$ particles/mL, STD $0.553x10^{12}$, min $6.0x10^{11}$, max $1.9x10^{12}$; LS mean $1.79x10^{12}$ particles/mL, STD $1.18x10^{12}$, min $4.0x10^{11}$, max $3.9x10^{12}$ t_7=1.871, p=0.1035). Further, total RNA collected from the EVs was also not significantly different between urine from the two methods (Fig. 1C: MC mean 344 ng/µl, STD 80.7, min 257, max 513; LS mean 369 ng/µl, STD 59.9, min 290, max 477; t_7=0.6986, p=0.5073). For all samples, the bioanalyzer electropherogram showed a single peak at the 25 nt location, indicating that the type of RNA in EVs collected from urine samples were primarily miRNAs. It is also important to note that without the presence of rRNA, there is no RIN value to indicate quality of RNA extracted beyond the size peak.

3.2 qPCR validation of top 5 most abundant miRNA

To perform a “survey” analysis of the identification and relative abundance of the miRNAs present within the EVs, all 8 individual rat session-pooled samples were further pooled within each urine collection method and assayed via miRNA microarray (n=1 for each collection method). This data is not shown, since it is just a list of abundant miRNAs, although the list is in the same order for both collection methods. The top 5 most abundant miRNAs as determined by microarray were validated by qPCR, going back to the 8 individual rat session-pooled samples (n=7 for metabolic sand, n=6 for lab sand due to not enough leftover volume for verification). Samples were analyzed by qPCR for expression of miR-3473, let-7c-5p, let 7b-5p, miR-200b-3p, and miR-23b-3p. Expression was normalized to a spike of cel-miR-39. There were no statistically significant differences in the level of expression (arbitrary units, AU) for any of these miRNAs between samples collected from the MC method compared to the LS method (Fig. 1D; see table 1 for mean, STD, and statistical results)

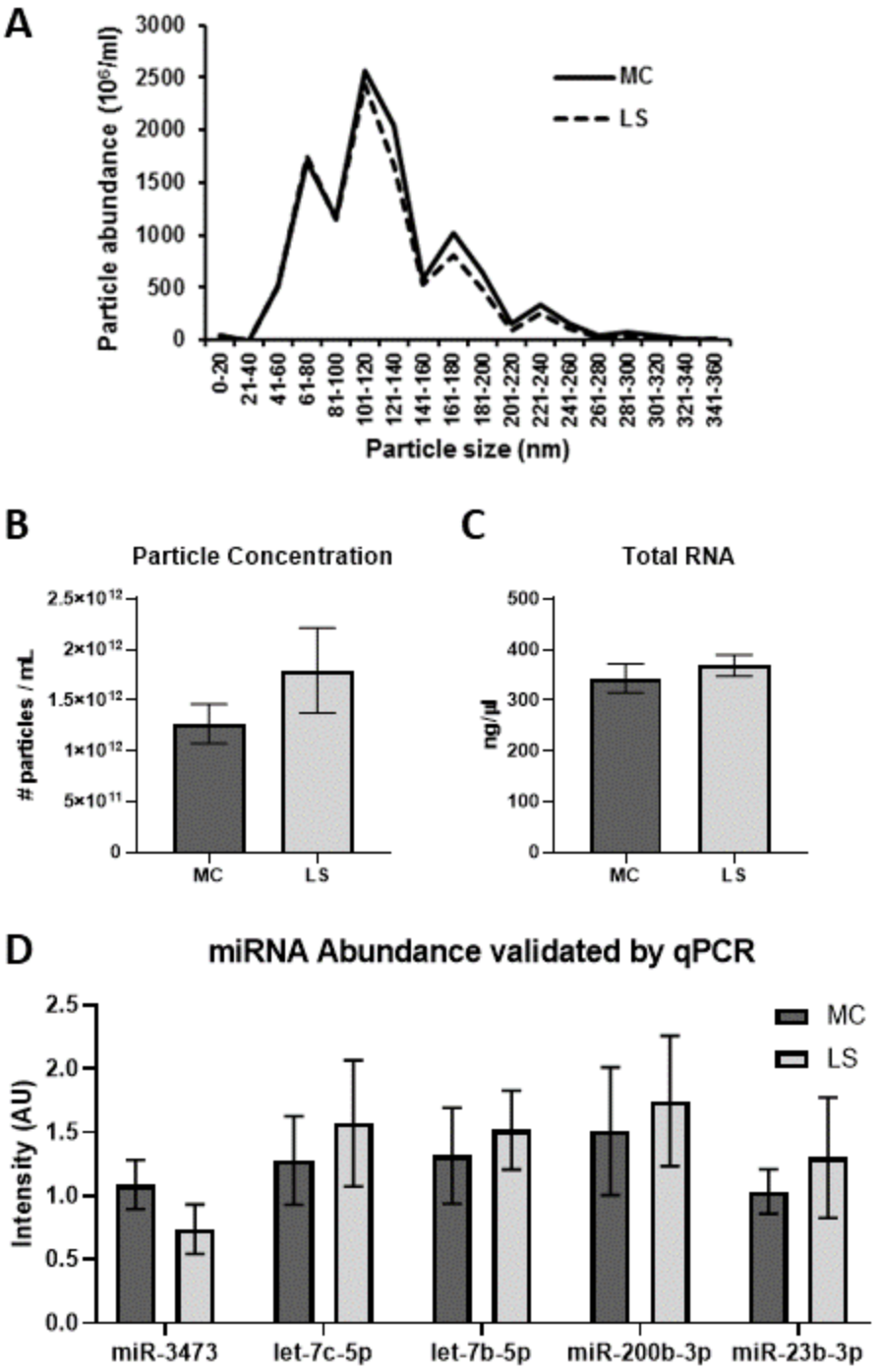

Figure 1: Extracellular vesicles (EVs) isolated from urine samples collected by either metabolic cage (MC) or LabSand (LS) urine collection methods were characterized for their (A) size, (B) particle concentration, (C) total RNA, and type and abundance of miRNAs expressed in the EVs to determine whether the LabSand urine collection method resulted in similar quantity and quality of EV collection as the traditional metabolic cage urine collection method. Relative expression of the top five miRNAs determined by microarray were verified by qPCR (D). Data is presented as the mean +/- SEM.

Table 1 – mean, standard deviation, and statistical results for graphs in Fig. 1D

miRNA	MC		LS		Statistics
	Mean	STD	Mean	STD	
miR-3473	1.09	0.514	0.738	0.476	t_{11}=1.2660, p=0.2316
let-7c-5p	1.28	0.919	1.57	1.21	t_{11}=0.4926, p=0.6320
let-7b-5p	1.32	0.998	1.52	0.760	t_{11}=0.4057, p=0.6927

miR-200b-3p	1.51	1.33	1.75	1.25	t_{11}=0.3299, p=0.7477
miR-23b-3p	1.03	0.461	1.30	1.16	t_{11}=0.5605, p=0.5864

4. Discussion

We have previously shown that a hydrophobic sand material is a suitable alternate method for urine collection in the rat compared to the traditional metabolic cage because it does not increase stress or stress markers in the rats, nor does it contaminate or otherwise alter normal clinical urinary markers or the concentration of various metals. Here we wanted to take this a step further and determine whether the hydrophobic sand compromised the quantity or quality of EVs, and their miRNA cargo, which could be collected from rat urine samples compared to the use of the metabolic cage method. We found no significant differences in particle size or concentration, total RNA collected, or the types and abundance of miRNAs contained within urine EVs due to urine collection method. It is interesting to note that of the most abundant miRNAs detected in EVs isolated from these urine samples, three (let-7c, miR-23b and miR-200c) are associated with kidney function [22-25] . The let-7 family is known to be highly abundant in different bodily fluids with let-7b being a proposed as a potential biomarker for kidney disease [26]. miRNA-3473 was the most abundant urine miRNA detected in the current study and has been reported to be associated with renal tubular injury [27]. We conclude that the use of hydrophobic sand in the collection of rodent urine for studies of changes in EVs as biomarkers of kidney health will not compromise the quality of the miRNAs examined, and is thus an acceptable alternate method of urine collection for an even broader range of studies.

5. Funding and Acknowledgements

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Assessing the Effect of Embedded Metals in Tissues Using Novel Spectroscopic Techniques

Diane Smith[a,b], José A. Centeno[b], John Kalinich[c] and Jessica Hoffman[c]

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b Division of Biology, Chemistry and Materials Science, Office of Science and Engineering Laboratories, Center for Devices and Radiological Health, U.S. Food and Drug Administration, White Oak Federal Research Center, Silver Spring, MD
c Internal Contamination and Metal Toxicity Program, Armed Forces Radiobiology Research Institute, Uniformed Services University, Bethesda, MD

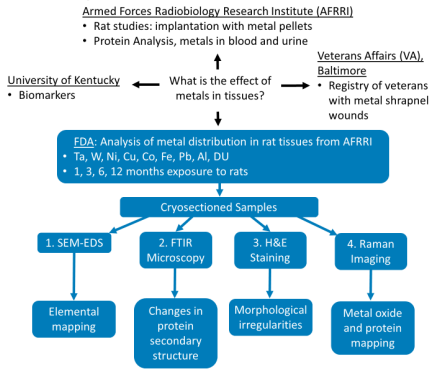
Plain Language Abstract

Metals embedded in tissues are evaluated using highly sensitive chemical analysis. These materials are relevant to medical devices and military wounds, the long-term health ramifications of which are not fully understood.

Abstract

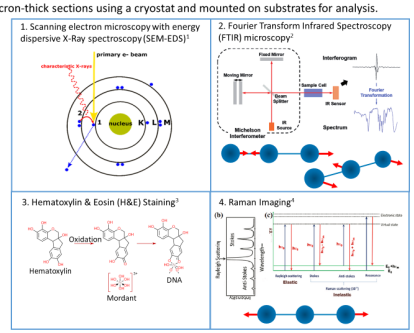
Many medical devices contain metals that may interface with the body. The long-term health consequences of many of these materials are not thoroughly understood. Additionally, embedded metal fragments from military wounds are typically not removed, to avoid the risk of morbidity associated with invasive surgery. The aim of our study is to evaluate the distribution of metals in animal tissues as a model to establish health risk from metal exposure. Metal pellets were implanted in the gastrocnemius muscle of rats for up to 12 months. The muscle with the embedded metal fragment and distant tissues were harvested, flash frozen, and sectioned for analysis. Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDS) and Fourier transform infrared spectroscopy (FTIR) microscopy were used to map the distribution of metals in rat tissues without the use of fixatives or stains, thereby preserving ultrastructural integrity of the tissues. This information will help bridge the gap in our understanding of the potential effects of select metals in the body. In addition, this study will contribute to our assessment of embedded metal fragments in military personnel.

Collaborative Project Overview



Methods

Fresh-frozen rat gastrocnemius (leg) muscles containing metal pellets were sliced in 6 – 12 micron-thick sections using a cryostat and mounted on substrates for analysis.

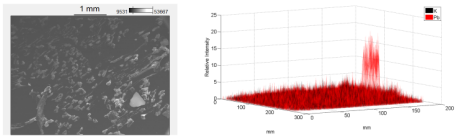


Results

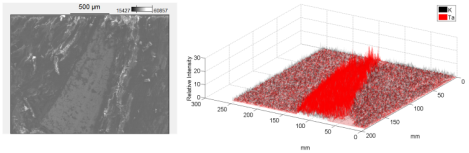
1. SEM-EDS: Elemental mapping of rat gastrocnemius tissue containing exposed metal can be used to verify the foreign material composition.

Acceleration voltage = 20 kV	Spotsize = 72
Working distance = 12 mm	Acquisition time = 500 s
Dead time = 35 ± 5 %	

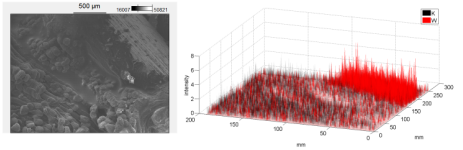
Gastrocnemius muscle containing Pb pellet fragment, 12 month exposure



Gastrocnemius muscle containing Ta pellet, 1 month exposure

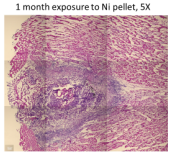


Gastrocnemius muscle containing W pellet, 12 month exposure

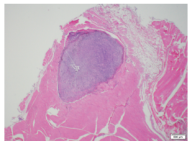


3. H&E Staining: Irregularities in rat gastrocnemius muscle are apparent after 1 month of exposure to Ni pellet. These are not present in gastrocnemius tissue exposed to Ta (control) after 12 months of exposure.

1 month exposure to Ni pellet, 5X



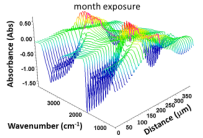
12 month exposure to Ni pellet, 2X



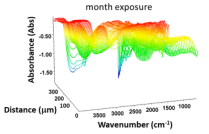
12 month exposure to Ta pellet (control), 5X

2. FTIR microscopy: Protein secondary structure changes are observed in gastrocnemius muscle surrounding metal pellet implantation site.

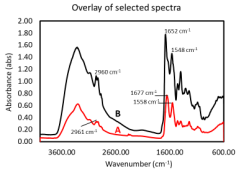
Gastrocnemius muscle exposed to Fe pellet, 1 month exposure



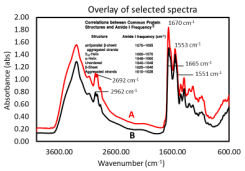
Gastrocnemius muscle exposed to Fe pellet, 12 month exposure

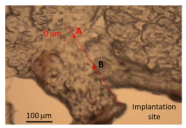


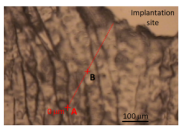
Overlay of selected spectra



Overlay of selected spectra







Conclusions

1. SEM-EDS can identify metal fragments in tissues, but is limited when attempting to identify leached metal in surrounding areas. More sensitive methods including inductively coupled plasma mass spectrometry and X-ray photoelectron spectroscopy can quantitatively determine trace metal presence and should be considered.
2. Changes in protein secondary structure with respect to proximity to the implanted metal pellet can be examined using FTIR microscopy. This analysis can compliment our understanding of the effect of metal exposure on tissue as a function of time.
3. Morphological changes to tissue and inflammation response after metal exposure can be visualized using H&E staining. These images help inform our choices of tissue locations for follow-up analysis.
4. Raman imaging can be used to map metal oxides present in tissues.

Acknowledgments

This work was supported by the grant W81XWH-16-2-0058 from the Congressionally Directed Medical Research Programs (CDMRP) Peer Reviewed Medical Research Program. Much thanks to our colleagues in CDER and CBER for their contributions to these efforts.

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Disclaimer

Figure 1. **Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2**

Validation of isolation of total RNA from urine

- **comparison of total urine microRNA abundance to exosomes (EVs)**

Urine	EVs
rno-miR-3473	rno-miR-3473
rno-let-7c-5p	rno-let-7c-5p
rno-let-7b-5p	rno-let-7b-5p
rno-miR-455-3p	rno-miR-200b-3p
rno-miR-26a-5p	rno-miR-23b-3p
rno-miR-3544	rno-miR-1224
rno-let-7a-5p	rno-miR-328a-5p
rno-miR-3556b	rno-miR-200c-3p
rno-miR-200b-3p	rno-let-7a-5p
rno-miR-182	rno-miR-26a-5p

- **3 most abundant microRNAs the same**
- **8 of top 10 the same**
- **RNA sequencing is completed on urine RNA**

SUBMISSIONS AND APPROVALS TABLES

PROJECTS 3 & 4: Joanna Gaitens, Ph.D., MSN/MPH, RN Stella Hines, M.D., MSPH

Protocol #A-19735.1						
		IRB		DoD HRPO		
Type of Submission	**Number**	**Date Submitted**	**Date Approved**	**Date Submitted**	**Date Approved**	**Main Content**
Modifications	1	8/18/2017	9/1/2017	9/27/2018*	12/14/2018	VA logo replaced UMB logo on recruitment letters
	2	3/12/2018	3/28/2018	3/28/2018	4/3/2018	VA REDCap is replaced by AITC and CSP for development and management of electronic survey; minor revisions to questionnaire
	3	5/11/2018	6/1/2018	6/5/2018	6/13/2018	A 4-digit PIN code is assigned to veterans, along with their unique access code, in order to access the online survey

	4	7/20/2018	7/30/2018	8/3/2018	8/3/2018	CTRIC is replaced by VA Perry Point CSP for production and management of paper-based questionnaires using “Datafax”; New pulm fellow added
	5	11/1/2018	11/25/2018	11/30/2018	12/17/2018	Coordinator’s new extension added to all letters and webpage for online survey
	6	7/3/19	7/12/19	7/16/19	pending	New pulm fellows added; Method of payment changed to ClinCard; Adding “Receive by” date for Batch 7 onward for payment
	7	8/20/19	8/21/19	9/3/19	pending	AITC, the team that manages our electronic survey, required a

						warning issued by the VA to be added to the webpage
Continuing Review	1	7/2/2018	7/12/2018	7/17/2018	7/30/2018	
	2	5/30/19	6/18/19	6/24/19	6/28/19	

*This modification was included in the initial protocol submission to DoD HRPO

Protocol #A-19735.2						
		IRB		**DoD HRPO**		
Type of Submission	**Number**	**Date Submitted**	**Date Approved**	**Date Submitted**	**Date Approved**	**Main Content**
Modifications	1	6/5/2018	6/19/2018	6/25/2018	6/26/2018	Consent language amended to allow veteran to return to complete testing if unable to do so in one visit. Also, a visit reminder letter was added.
	2	8/15/2018	11/5/2018**	11/20/2018	12/17/2018	Consent amended to offer veteran opportunity to be contacted for future studies. HIPAA and Study protocol amended to reflect these changes. Also, the Human Animal Research Protections Officer was updated at the Baltimore site.

	3	9/4/2018	11/5/2018**	11/20/2018	12/17/2018	As a result of recent protocol deviations, HIPAA waiver (form 103) and Alteration of Informed Consent Process (form 112a) were modified to request waivers for scheduling purposes, per IRB recommendations.
	4	3/27/19	4/9/19	4/18/19	6/13/19	Phoenix VA added as 6th recruitment site
	5	5/2/19	5/28/19	5/28/19	7/9/19	San Antonio LSI, Dr. Duch, replaced LSI Dr. Do. Eligibility Criteria updated to specify location and era in which injury occurred resulting in fragment; participant must be able to provide urine specimen without the use of a catheter.
Continuing Review	1	3/22/2018	4/29/2018	5/22/2018	6/12/2018	
	2	3/12/19	4/22/19	5/6/19	5/9/19	

**Notification of approval of amendments 2 and 3 was not received until 11/14/2018

Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments
(Projects 3 & 4)
Investigator Meeting Agenda
Baltimore, MD
September 19-20, 2019
SMC Campus Center, Room 203

Day 1

8:30 Coffee and Welcome

9:00 Introductions – Old and New project members

9:30 Project Timeline Updates –
- Review original plan, compare with current progress

10:00 Recruitment Status
- Current stats
- Updated recruitment lists
- Strategies for improving recruitment

10:45 Break

11:00 Recap of Year 2 (Year 1 of Recruitment) Activities
- Data handling procedures
- Review of troubleshooting
- Anticipated troubleshooting needs

12:00 Lunch Break

1:00 Preliminary Data Review
- Demographics
- Injury Characteristics
- General Health
- Renal History
- Urine Metals
- Renal Injury Markers
- Respiratory Health
- PFT Data
- IOS Data

3:00 Break

3:15 Regulatory Issues
- Review of Modifications & Amendments submitted to C-IRB

- Review of Deviations & how we fixed them
- Review of Feedback from DOD
- Quarterly and Annual Reports
- Trainings due

4:00 Budgetary Information
- Carry-over requests/plans

5:00 Adjourn

6:30 Group Dinner (optional)

Day 2

9:00 Planned Analyses
- What research questions? When?

9:30 Preliminary findings from Questionnaire-Only Study

10:00 Scholarly Works
- Conference abstracts
- Research papers
- Discuss authorship

10:30 Break

10:45 Preliminary findings from Animal Investigators (Kalinich)

11:15 Discussions regarding future studies
- Funding opportunities – CDMRP Extension Award, timing of application
- Consent for re-contact

12:00 Next Steps and Final Q & A

1:00 Adjourn

SLIDE SET FROM ALL-INVESTIGATOR MEETING

SEPTEMBER 19-20, 2019

BALTIMORE, MD

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"

UNIVERSITY of MARYLAND
SCHOOL OF MEDICINE

Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments

CDMRP Grant Meeting
September 18-19, 2019

Introductions

Coordinating Site: **Baltimore VAMC/University of Maryland**

Overall Study PI:
Melissa McDiarmid, MD, MPH

Project Leads and Local Site Investigators:
Joanna Gaitens, PhD, MSN/MPH, RN
Stella Hines, MD, MSPH

Research Coordinator:
Kate Agnetti, BS

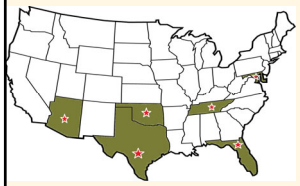
Pulmonary Fellows:
Danielle Glick, MD
Maxwell Reback, MD
Katherine Chin, MD

Statistician:
Clayton Brown, PhD

Database and Share Point Management:
Christopher Crayton

Administrative Staff:
Rachel Coates-Knowles
Sheila Williams

Site Collaborators



- **VA North Florida/South Georgia Veterans Health System (Gainesville)**
  Local PI: Peruvemba Sriram, MD
  Research Coordinators: Nataliya Kirichenko & Katherine Solis
- **South Texas Veterans Health Care System (San Antonio)**
  Local PI: John Duch, MD & Antonio Anzueto, MD
  Research Coordinators: Myra Mireles & Alexander Aguilera
- **Central Tennessee Valley Healthcare System (Nashville)**
  Local PIs: Kerri Cavanaugh, MD, MHS & William Lawson, MD
  Research Coordinator: Audrey Tesi
- **Oklahoma City VA Healthcare System (Oklahoma City)**
  Local PI: Lisa Beck, MD
  Research Coordinator: Vickie Phillips
- **Phoenix VA Health Care Systems (Phoenix)**
  Local PIs: Paska Permana, PhD & Samuel Aguayo, MD
  Research Coordinator: Kelli Bingham

Grant Overview

Background

- The 'signature' wound of current and recent conflicts is from improvised explosive devices (IEDs) or other high kinetic energy weapons.
- These result in traumatic injury and wound contamination with toxic metals potentially posing additional health threats from acute and long-term exposure to embedded fragments.
- Previous management permitted fragments to remain in place, if not accessible, however, this is concerning now due to observations from DU and TEF programs showing
  - ***metal 'mobilization' from fragments permitting systemic absorption and toxicity***
  - ***local, tissue effects including foreign body carcinogenicity.***
- We therefore need additional guidance on fragment management and removal indications.

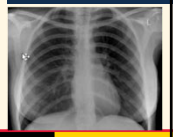
- Research Plan: Leveraging long-standing collaborations between the Armed Forces Radiobiology Research Institute (AFRRI) and the Department of Veterans Affairs (DVA) Depleted Uranium (DU) and Toxic Embedded Fragment (TEF) Surveillance Centers, **we propose a complementary array of both animal investigations and human epidemiology projects to more fully characterize the health threats to** military service members and **Veterans** who are victims **of these injuries**.

- **Project 1:** Health Effects of Embedded Fragments of Military-Relevant Metals will examine the behavior and kinetics of embedded fragments in tissue of animals implanted with metals of toxic concern.

- **Project 2:** Biomarkers for the Early Detection of Adverse Health Effects Resulting from Embedded Metal-Fragment Wounds will identify early biomarkers of tissue injury that may signal developing local toxic effects from the fragment and thus need for removal.

- **Project 3:** Biomarker Assessment of Kidney Injury from Metal Exposure in Embedded Fragment Registry Veterans will assess biomarkers of early kidney damage in Veterans registered in the VA TEF registry and injured with a fragment.

- **Project 4:** Respiratory Health in a Cohort of Embedded Fragment Registry Veterans Exposed to Blasts and Metals will examine lung function and insult from both metal inhalation and blast effects from the traumatic injury in this same VA-TEF Registry cohort.



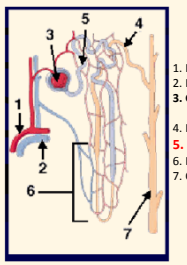
- **Impact:** These projects will address the specific knowledge gaps currently challenging the care of embedded fragment and blast injury patients.
- **Military Relevance:** Results will provide the evidence base to support medical decision-making in the care of the estimated 40,000 injured in recent conflicts.

The kidney is a target organ for heavy metal exposure

- Some metals are nephrotoxic in high-dose acute exposures
- Chronic metal exposure to relatively low metal concentrations over time can adversely affect the kidney (Wallin et al., 2014; Pennemans et al., 2011; Munter et al., 2003).
- Chronic low-level co-exposure to more than one metal can have pronounced effects on the kidney (Cobbina et al., 2015; Hambach et al., 2013; Huang et al., 2009; Wang and Fowler, 2008)

Renal: Hypothesis and Specific Aims

- **Hypothesis 1:** The **prevalence of kidney injury is higher** in Veterans with embedded fragments **with elevated urine metal concentrations** compared to Veterans who have normal metal concentrations.

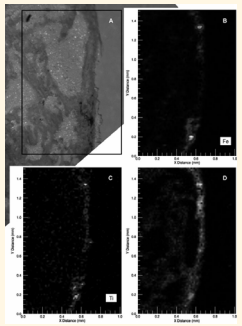


1. Interlobulary artery
2. Interlobulary Vein
3. **Glomerulus**/ Bowman's capsule
4. Distal Tubule
5. **Proximal Tubule**
6. Loop of Henle
7. Collecting duct

Respiratory health in OIF/OEF/OND deployers

Metals and the Lung

- Metals found in lung tissue of deployers
  - Si, Cd, Al, Va, Ti, Fe
    - *Lowers 2015, Zembrzuska 2011, Szema 2012 & 2014*



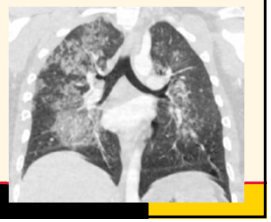
Szema JOEM 2014

Blast and the Lung

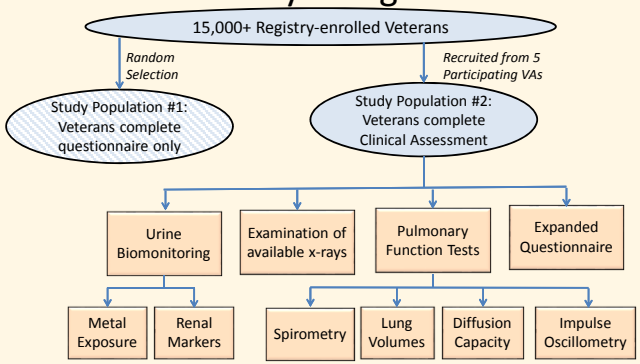
- Lung Injury from 1° Blast Exposure
  - stripping of airway epithelium
- VA's Airborne Hazards Registry
  - Dyspneic: 1.6 x more likely to report exposure to blast *(Jani 2017)*

Pulmonary Hypotheses and Specific Aims

- **Hypothesis 2:** Veterans with **greater metal burdens** will have **more respiratory health abnormalities**.
- **Hypothesis 3:** Veterans **exposed to blasts** will have **more respiratory health abnormalities**.



Study Design



ORIGINAL GOAL

Table 1. Timeline for Major Milestones/Critical Tasks by Project

Major Tasks/Projects	Year 1 (Oct 2016-Sept 2017)	Year 2 (Oct 2017 - Sept 2018)	Year 3 (Oct 2018 - Sept 2019)	Year 4 (Oct 2019 - Sept 2020)	Year 5 (Oct 2020-Sept 2021)
	Quarter 1 2 3 4	Quarter 1 2 3 4	Quarter 1 2 3 4	Quarter 1 2 3 4	Quarter 1 2 3 4
Projects #3 & 4					
1. Questionnaire Development	→				
2. Obtain Regulatory Approvals	→				
3. Recruitment and Questionnaire Administration		→	→	→	
4. Questionnaire Analysis		→	→	→	→
5. Collection and analyses of urine specimens		→	→	→	
6. Collection and analyses of PFT and IOS findings		→	→	→	
7. Summarize metal and renal findings		→	→	→	→
8. Summarize PFT and IOS findings		→	→	→	→
Collaboration Meetings					
1.Clinical Sites All-Investigators Meetings	→		→	→	
2. CDMRP Milestone Meeting			→		

Actual start, April 2018

Based on original timeline…..

- Remaining months to complete recruitment (except Phoenix)
  - **9 MONTHS** – aiming to meet that target
    - Affects some personnel budgets for year 5
    - Allows time to perform analyses; write manuscripts
  - PHX goal: 15 months (or sooner if possible!)
    - 55 participants

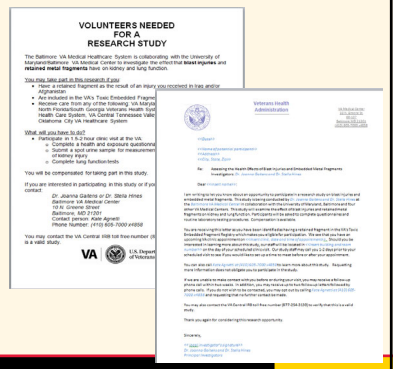
Recruitment

Review of Recruitment Methods

1. Targeted flyers
2. Scheduled Clinic Visits Mailings
3. No Scheduled Clinic Visits Mailings

Baltimore provides each site with a list of eligible Veterans.

Participants receive $100 for completion of protocol



Recruitment Updates

- New Eligibility Checklist approved 5/23/19
  - Clarified eligibility based on when/where injury and fragment were received
  - Must be able to provide urine specimen without use of catheter
- Updated eligibility lists provided to each site in July
- Amended protocol to permit Veterans to come back to complete protocol if a technical issue is encountered
- We have reached 60% of targeted recruitment number!

Enrollment and Recruitment

Site	Total on Eligibility List	Individuals contacted by letter	Initial Contact (in-person or via phone) made	Scheduled for future Study Visit	Completed protocol	Total Targeted Recruitment Number
Baltimore	106	59	25	1	9	35
OKC	304	200	175	7	79	94
San Antonio	636	528	357	15	83	119
Gainesville	354	247	202	5	63	101
Nashville	342	198	139	5	57	72
Phoenix	238					55
Totals	1981	1232	898	34	289	421

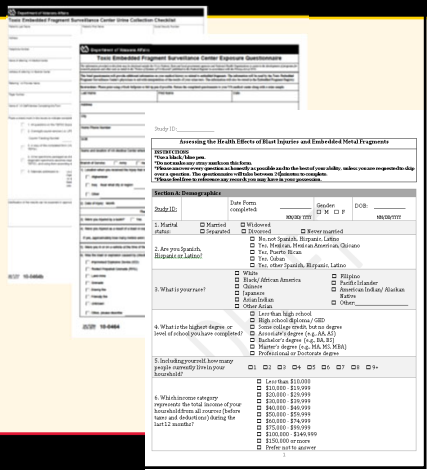
Strategies for Improving Recruitment

- Challenges
  - Some Veterans on eligibility list live several states away
  - Difficulty contacting Veteran
- Strategies for overcoming challenges
  - Addition of Phoenix as a recruitment site
  - Some sites may be able to exceed their targeted recruitment number
  - Recruitment Method 1 – Flyers to PCPs
    - PIs reach out to PCPs?
  - "Local" participants on other sites' list

Clinical Assessment Visits

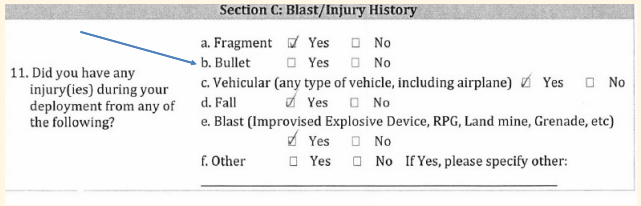
Study Questionnaires

1. Study Questionnaire
2. Clinical Forms
   a. TEF Exposure Questionnaire
   b. TEF Checklist



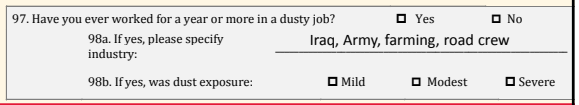
Data Handling Procedures

If multi-response question & some responses checked YES and others left blank, treating the blank ones as "NO."



Coding any reference to military as "military"

Assuming answer is YES if they specified something.



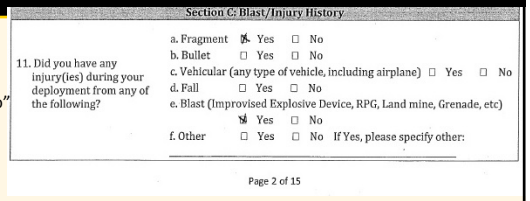
Blast questions-q11, 12, 13, 14

Are the unchecked answers "No" responses?

They should only check "Not applicable" if they did not experience a blast.

If they experienced a blast, they should mark "Yes" to 11e. Blast

Person previously reported "Blast." These answers should be Yes/No, not "NA"

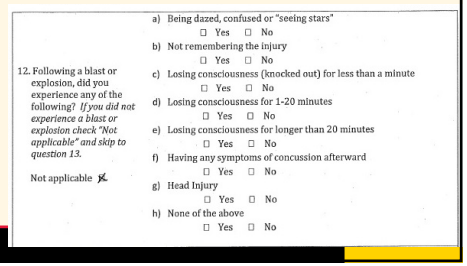


Section C: Blast/Injury History

11. Did you have any injury(ies) during your deployment from any of the following?

a. Fragment ☒ Yes ☐ No
b. Bullet ☐ Yes ☐ No
c. Vehicular (any type of vehicle, including airplane) ☐ Yes ☐ No
d. Fall ☐ Yes ☐ No
e. Blast (Improvised Explosive Device, RPG, Land mine, Grenade, etc) ☒ Yes ☐ No
f. Other ☐ Yes ☐ No If Yes, please specify other:

Page 2 of 15



12. Following a blast or explosion, did you experience any of the following? *If you did not experience a blast or explosion check "Not applicable" and skip to question 13.*

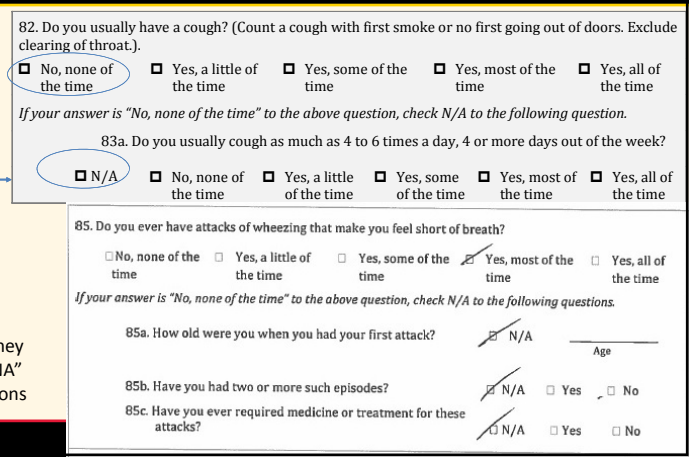
Not applicable ☒

a) Being dazed, confused or "seeing stars" ☐ Yes ☐ No
b) Not remembering the injury ☐ Yes ☐ No
c) Losing consciousness (knocked out) for less than a minute ☐ Yes ☐ No
d) Losing consciousness for 1-20 minutes ☐ Yes ☐ No
e) Losing consciousness for longer than 20 minutes ☐ Yes ☐ No
f) Having any symptoms of concussion afterward ☐ Yes ☐ No
g) Head Injury ☐ Yes ☐ No
h) None of the above ☐ Yes ☐ No

Lung Function section, q82-100

They should only check "Not applicable" if they answered "No," above.

If they check Yes, they should not mark "NA" to follow-up questions



82. Do you usually have a cough? (Count a cough with first smoke or no first going out of doors. Exclude clearing of throat.).

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

If your answer is "No, none of the time" to the above question, check N/A to the following question.

83a. Do you usually cough as much as 4 to 6 times a day, 4 or more days out of the week?

☐ N/A ☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

85. Do you ever have attacks of wheezing that make you feel short of breath?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☒ Yes, most of the time ☐ Yes, all of the time

If your answer is "No, none of the time" to the above question, check N/A to the following questions.

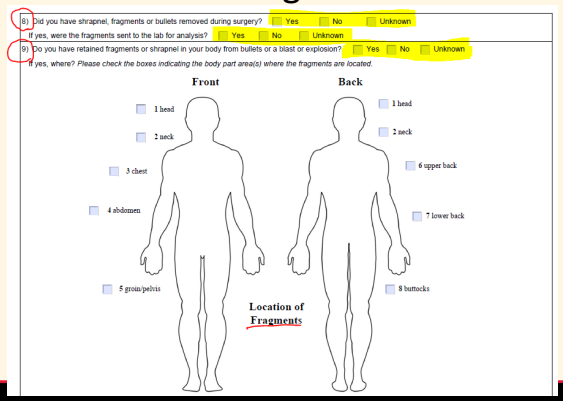
85a. How old were you when you had your first attack? ☒ N/A _____ Age

85b. Have you had two or more such episodes? ☒ N/A ☐ Yes ☐ No

85c. Have you ever required medicine or treatment for these attacks? ☒ N/A ☐ Yes ☐ No

Data Handling Procedures

TEF FORM
Page 3



8) Did you have shrapnel, fragments or bullets removed during surgery? ☐ Yes ☐ No ☐ Unknown
If yes, were the fragments sent to the lab for analysis? ☐ Yes ☐ No ☐ Unknown
9) Do you have retained fragments or shrapnel in your body from bullets or a blast or explosion? ☐ Yes ☐ No ☐ Unknown
If yes, where? *Please check the boxes indicating the body part area(s) where the fragments are located.*

Front

1 head
2 neck
3 chest
4 abdomen
5 groin/pelvis

Back

1 head
2 neck
6 upper back
7 lower back
8 buttocks

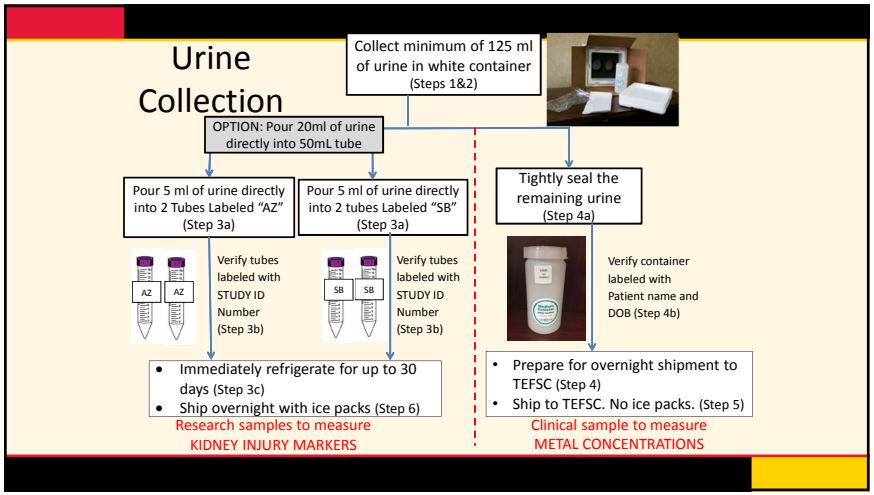
Location of Fragments

Frequently Missed:
Questions 8-9

Questionnaire Review

- While participant is doing PFT, please review questionnaires
  - Flag any unanswered questions and ask veteran after done with PFT if they meant to skip

Urine Collection



Urine Collection Challenges and Updates

- A few samples have been mislabeled
  - System now in place to catch mislabeling errors sooner
- Can be difficult to obtain adequate urine volume
  - Collect urine sample at end of site visit
  - **Collect only one AZ and one SB tube if volume is low**
- A few urine collection tubes have much **more than 5mL** of urine
  - Cases reviewed with our laboratory

Analyses of Urine Specimens

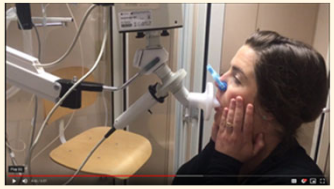
- Urine metals measured by the Joint Pathology Center
- University of Antwerp is measuring kidney injury markers
  - 1st batch of specimens (n=126) sent to Belgium in December 2018
    - Results received in June 2019
  - 2nd batch of specimens to be sent Oct 7

Metals of Interest
Aluminum (Al)
Arsenic (As)
Cadmium (Cd)
Chromium (Cr)
Cobalt (Co)
Copper (Cu)
Iron (Fe)
Lead (Pb)
Manganese (Mn)
Molybdenum (Mo)
Nickel (Ni)
Tungsten (W)
Uranium (U)
Zinc (Zn)

Renal Markers of Interest
Total protein
Retinol binding protein (RBP)
Creatinine
Intestinal alkaline phosphatase (IAP)
Albumin
Alpha-1 microglobulin (α1M)
Beta-2 microglobulin (β2M)
N-acetyl-beta-D-glucosaminidase (NAG)
Kidney injury molecule 1 (KIM-1)
Interleukin 18 (IL-18)
Neutrophil gelatinase-associated lipocalin (NGAL)

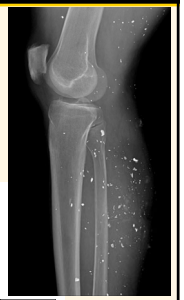
Questions or Comments about Urine Collection Procedures?

PFTs and IOS



Review of Imaging Data

- UMB team will review images to document:
  - Presence of a fragment, reason for imaging, approx. number and size of fragments, location of fragments, presence of a bone fracture



Imaging Data Abstraction Form

STUDY ID: _____ Date of record review: _____ Name of Reviewer: _____

1. **Are images and/or imaging reports available within CPRS?** ___Yes ___No
2. **Complete the following table for each available image where fragments are discussed:**

	Imaging Date (mm/dd/yyyy)	**Type of Image** 1= X-ray 2= Ct scan 3=Other	**Body part imaged***	**Reason for imaging** (check all that apply)						**Was a fragment documented?** (Yes/No)	**Number of fragments***	**Approx Size of largest fragment** (length X width in mm)	**Approx Size of smallest fragment** (length X width in mm)
				Confirm presence of fragment	Routine follow-up of fragment	Pain possibly r/t fragment	Inflammation possibly r/t fragment	Other symptom r/t fragment (specify)	Imaging initially performed for non-fragment related reason				
a													
b													
c													

Preliminary Data Review

Analyses

- All data double-entered
- SAS
- Questionnaire Data
  - Chi square for differences in frequencies
- Renal Markers
  - GLM regressions
- PFT & IOS:
  - T-test with unequal variances for comparison of means
  - Adjusted comparisons, GLM regressions

Data included in the Analyses

- Total N = 242
- Removed data for 13 Veterans who were injured in locations other than Iraq/Afghanistan
  - Examples:
    - Vietnam, 1968
    - Philippines, 1989
    - Saudi Arabia, 1996
    - Stateside, 1995
    - Kuwait, 1990

DEMOGRAPHIC VARIABLE		N (%)
Gender	Male	234 (96.7)
Race	White Black/African American Hispanic Other	145 (59.9) 20 (8.3) 42 (17.4) 35 (14.4)
Marital Status,	Married	151 (62.3)
Education	High School Associates or Bachelors Advanced/or Professional	131 (54) 82 (34) 29 (12)
Income	<$40,000 $40,000-100,000 >$100,000	42 (17.4) 146 (60.3) 39 (16.1)
DEMOGRAPHIC VARIABLE		**MEAN (SD)**
Age		42.1 (7.6)
Height (cm)		177.3 (7.6)
Weight (kg)		97.6 (20.2)
BMI		31.1

Military Demographics

VARIABLE		N (%)	
Service Branch	Army Navy Air Force Marine	186 (76.9) 14 (5.8) 7 (2.9) 43 (17.8)	
Active Duty		225 (93.4)	
Geographic Location	Iraq Afghanistan	190 (78.5) 64 (26.5)	
Injury Mechanism	Blast only Blast + Bullet Bullet Only	196 (81.0) 28 (11.6) 15 (6.2)	} 92.6% exposed to blast
Blast Cause		IED – 131 (54.1) RPG – 58 (24.0) Landmine – 15 (6.2)	Grenade – 22 (9.1) Mortar – 28 (11.6) Other – 55 (22.7)
VARIABLE		**MEAN (SD)**	
Years Since Injury		12.7 (3.2)	

Blast Exposure- 1

Exposure parameter	Source of data	N	% of cohort (n=242)
Injured by blast			
	TEF question 4	224	93.3
	Clinical questionnaire q11e (comes from BTBIS)	219	90.5
No blast			
	Bullet only (constructed from TEF, not BTBIS)	15	6.3

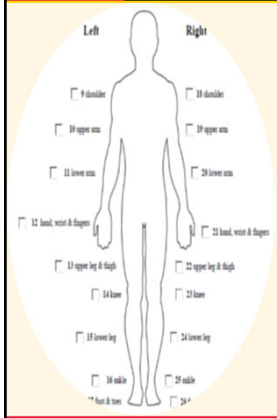
Blast Exposure – 2 (BTBIS)

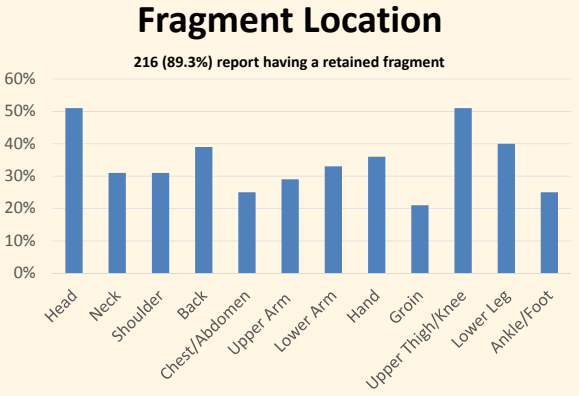
Exposure parameter	Responses included	n	% (n=242)
BTBIS positive	Yes to any:	217	89.7
	Being dazed, confused or "seeing stars"		
	Not remembering the injury		
	Losing consciousness (knocked out) for less than a minute		
	Losing consciousness for 1-20 minutes		
	Losing consciousness for longer than 20 minutes		

Exposure parameter	Responses included	n	% (n=242)
Sxs related to mTBI	Yes to any:	228	94.2
(mTBI possible)	Headaches		
	Dizziness		
	Memory problems		
	Balance problems		
	Ringing in the ears		
	Irritability		
	Sleep problems		
	Other:		

Blast Exposure - 3

Exposure parameter		n	% (of n=242)
TBI Dx by MD		161	66.8
Barotrauma Symptoms positive	Yes to any:	44	18.2
	Pneumothorax		
	Lung Contusion		
	Ruptured ear drum		





Self-Reported Metal Exposure In the Last Year – 84 (34.7%)							
Welding	25 (10.3%)	**Soldering**	12 (5.0%)	**Firing Range**	41 (16.9%)	**Bullets**	5 (2.1%)
Jewelry or Art	1 (0.4%)	**Mining**	1 (0.4%)	**Lead Paint**	1 (0.4%)	**Marine Paints**	1 (0.4%)
Demolition	11 (4.6%)	**Sandblasting**	8 (3.3%)	**Wood Preservatives**	13 (5.4%)	**Fish Weights**	2 (0.8%)
Machining	32 (13.2%)	**Metal Manufacturing**	10 (4.1%)	**Work Dust**	24 (9.9%)	**Hobby Dust**	22 (9.1%)

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Machining	32 (13.2%)	**Metal Manufacturing**	10 (4.1%)	**Work Dust**	24 (9.9%)	**Hobby Dust**	22 (9.1%)
Exposure at Work: Dust or Gas Fumes							
Dusty Job >1 Year		118 (48.8%)		**Gas or Fumes**		119 (49.2%)	

Environmental metal exposures

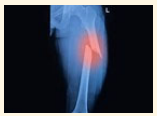
Other sources of metal exposure – 28 (11.6%)	
	N (%)
History of lead poisoning	6 (2.5)
Ever live near lead industry	3 (1.2)
Renovated pre-1960s house	20 (8.3)
Ate seafood in last 24 hours	17 (7.0)
Water supply	
Community water system	204 (84.3)
Private well	35 (14.5)

Presence of Metal Implants

Reported Any Implanted Metal – 96 (39.7%)	
Device	**N (%)**
Pacemaker/Defibrillator	1 (0.4%)
Stents	8 (3.3%)
Surgical Clips/Wires	5 (2.1%)
Plates, Screws, or Rods	62 (25.6%)
Dental Implants	24 (9.9%)
Joint Replacement	7 (2.9%)
Other Metal Medical Devices	18 (7.4%)

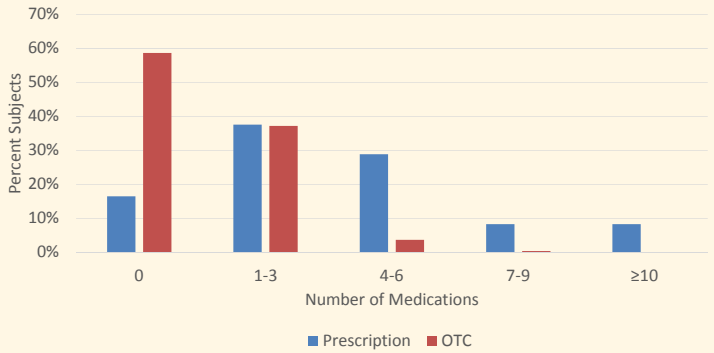
Other Variables of Interest

Diagnosis	N (%)
Ever broken a bone	170 (70.2)
Metal allergy or sensitivity	6 (2.5)

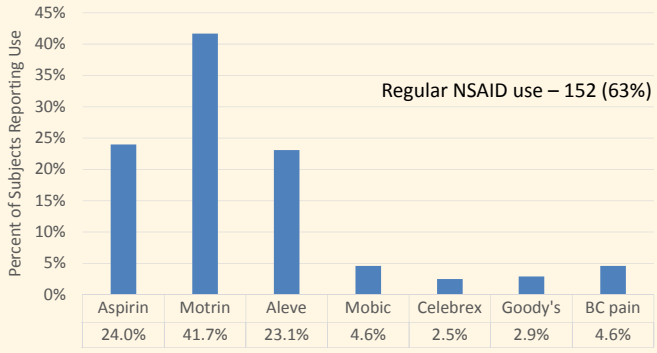


Smoking + tobacco use	
Source	**N (%)**
Ever smoker of cigarettes	129 (53.3)
Current smoker	48 (19.8)
Ever smoker of non-tobacco product	28 (11.6)
Current use of chewing tobacco	35 (14.5)

Medication Use



NSAID Use



Use of products/supplements

Routine use of any supplement - 102 (42.1%)	
Product used	N (%)
Zinc sunblock	7 (2.9)
Supplements	41 (16.9)
Vitamins	86 (35.5)
Ayurvedic medications	0 (0)
Denture cream	3 (1.2)

Urine Metal Results

Distribution of Urine Metal Concentrations-1

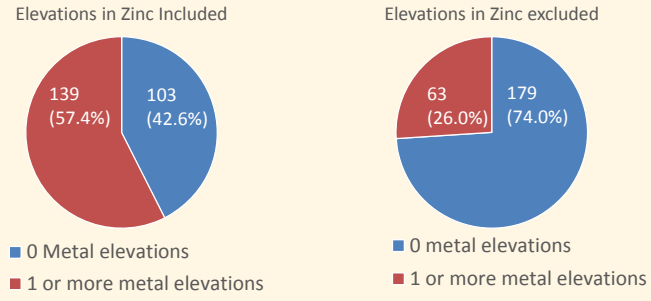
Urine metal concentration (µg/g Cr)				
Metal	**Normal cutoff**	**Mean (SD)**	**Min**	**Max**
Aluminum	30	18.93 (12.53)	4.40	94.16
Arsenic	60	7.05 (12.08)	1.21	131.87
Cadmium	1	0.19 (0.17)	0.05	1.93
Chromium	2	0.63 (0.75)	0.03	6.59
Cobalt	1	0.41 (0.35)	0.095	5.01
Copper	40	5.71 (15.36)	0.88	189.28
Iron	250	18.35 (19.96)	4.40	226.69

Distribution of Urine Metal Concentrations-2

Urine metal concentration (µg/g Cr)				
Metal	**Normal cutoff**	**Mean (SD)**	**Min**	**Max**
Lead	2	0.40 (0.73)	0.006	6.98
Manganese	2	0.46 (0.43)	0.06	3.90
Molybdenum	122	31.30 (22.81)	4.71	207.85
Nickel	8	1.73 (0.81)	0.44	4.71
Tungsten	0.4	0.16 (0.47)	0.01	7.12
Uranium	0.04	0.006 (0.02)	0.0005	0.34
Zinc	1100	1267.37 (889.01)	353.93	10229.50

Percent of subjects with elevated urine metal

Percent of Subjects with 1 or Urine Metal Elevations

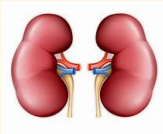


Predictors of Metal Exposure*

*Elevated Zn included in analyses

		N (%)		
Predictor Variable		**0 metal elevations**	**1 or more metal elevation**	**P-value**
Metal implants	No	68 (46.6)	78 (53.4)	0.12
	Yes	35 (36.5)	61 (63.5)	
Exposed to dusty work or gas fumes	No	92 (42.2)	92 (57.5)	0.98
	Yes	35 (42.7)	47 (57.3)	
Metal exposure from work or hobby	No	69 (42.5)	92 (57.1)	0.90
	Yes	34 (42.0)	47 (58.0)	
Routinely take supplements	No	65 (46.4)	75 (53.6)	0.84
	Yes	38 (37.3)	64 (62.8)	
Vitamins	No	75 (48.1)	81 (51.9)	0.02
	Yes	28 (32.6)	58 (67.4)	
Ever broken bone	No	35 (48.6)	37 (51.4)	0.22
	Yes	68 (40.0)	102 (60.0)	

Risk factors for Kidney Disease and Self-reported Outcomes



CKD and comorbidities

Variable	N (%)
Tested for CKD	19 (7.8)
Have CKD	3 (1.2)
Kidney stones	26 (10.7)
Family h/o CKD	6 (2.5)
Hypertension	82 (33.9)
Cardiovascular disease	5 (2.1)
Kidney cancer	3 (1.2)
High cholesterol	10 (4.1)
Kidney infection/inflammation	10 (4.1)

Renal symptoms from MDRD - 1

	Patient N (%)				
Symptom	0 – Not at all	1	2	3	4 – Extreme
Bad taste	62 (25.6)	63 (26.0)	63 (26.0)	44 (18.2)	10 (4.1)
Poor appetite	57 (23.6)	55 (22.7)	73 (30.2)	46 (19.0)	11 (4.6)
Nausea	67 (27.7)	78 (32.3)	48 (19.8)	42 (17.4)	7 (2.9)
Vomiting	175 (72.3)	42 (17.4)	15 (6.2)	9 (3.7)	1 (0.4)
Heartburn	50 (20.7)	48 (19.8)	53 (21.9)	41 (16.9)	50 (20.7)
Bloating	46 (19.0)	51 (21.1)	51 (21.1)	62 (25.6)	32 (13.2)
Diarrhea	52 (21.5)	72 (29.8)	52 (21.5)	40 (16.5)	26 (10.7)
Constipation	111 (45.9)	61 (25.2)	36 (14.9)	23 (9.5)	10 (4.1)

*p-value compares those with metal elevations vs those without

Renal symptoms from MDRD - 2

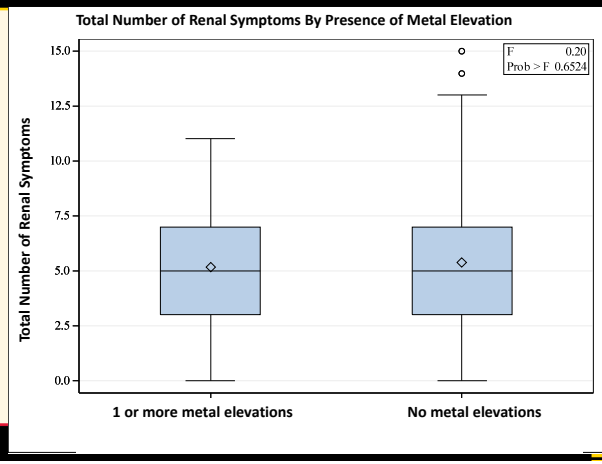
	Patient N (%)				
Symptom	0 – Not at all	1	2	3	4 - Extreme
Hiccups	120 (49.6)	78 (32.2)	29 (12.0)	12 (5.0)	3 (1.2)
Itching	40 (16.5)	77 (31.8)	57 (23.6)	52 (21.5)	16 (6.6)
Hives	138 (57.0)	43 (17.8)	31 (12.8)	21 (8.7)	9 (3.7)
Bruise/bleed	148 (61.2)	48 (19.8)	28 (11.6)	9 (3.7)	9 (3.7)
Low energy	14 (5.8)	39 (16.1)	57 (23.6)	69 (28.5)	63 (26.0)
Weakness	22 (9.1)	55 (22.7)	47 (19.4)	68 (28.1)	50 (20.7)
Muscle cramps	39 (16.1)	67 (27.7)	51 (21.1)	61 (25.2)	24 (9.9)
Lightheaded	80 (33.1)	69 (28.5)	49 (20.3)	36 (14.9)	8 (3.3)

*p-value compares those with metal elevations vs those without

Renal symptoms from MDRD - 3

	Patient N (%)				
Symptom	0 – Not at all	1	2	3	4 - Extreme
Poor sleep	19 (7.9)	20 (8.3)	24 (9.9)	67 (27.7)	112 (46.3)
Drowsy	-	-	-	-	-
Irritable	17 (7.0)	37 (15.3)	50 (20.7)	68 (28.1)	70 (28.9)
Low alertness	96 (39.7)	56 (23.1)	49 (20.3)	30 (12.4)	11 (4.6)
Forgetful	9 (3.7)	37 (15.3)	48 (19.8)	81 (33.5)	67 (27.7)
Blurry vision	51 (21.1)	58 (24.0)	48 (19.8)	62 (25.6)	23 (9.5)
Hematuria	197 (81.4)	28 (11.6)	8 (3.3)	5 (2.1)	3 (1.2)
Puffy eyes	115 (47.5)	62 (25.6)	34 (14.1)	23 (9.5)	8 (3.3)
Frequent urination	76 (31.4)	49 (20.3)	46 (19.0)	40 (16.5)	31 (12.8)

*p-value compares those with metal elevations vs those without



CKD risk: SCORED model

	Variable	N (%)
Age	50-59	36 (14.9)
	60-69	6 (2.5)
	70+	1 (0.4)
Female		8 (3.3)
High blood pressure		74 (30.6)
Anemia		10 (4.1)
Diabetes		27 (11.2)
Heart attack or stroke		6 (2.5)
Congestive heart failure		2 (0.8)
Circulation disease in legs		8 (3.3)
Protein in urine		15 (6.2)

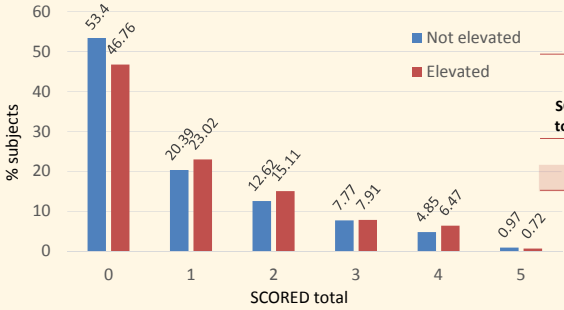
- Age:
  1. I am between 50 and 59 years of age.....Yes 2 _____
  2. I am between 60 and 69 years of age.....Yes 3 _____
  3. I am 70 years old or older.....Yes 4 _____
- I am a woman.....Yes 1 _____
- I had/have anemia.....Yes 1 _____
- I have high blood pressure.....Yes 1 _____
- I am diabetic.....Yes 1 _____
- I have a history of heart attack or stroke.....Yes 1 _____
- I have a history of congestive heart failure or heart failure.....Yes 1 _____
- I have circulation disease in my legs.....Yes 1 _____
- I have protein in my urine.....Yes 1 _____

Total _____

Bang H, et al. Arch Intern Med. 2007;167:374-81.

4 points or more: 18% chance of having CKD (PPV)
99% negative predictive value

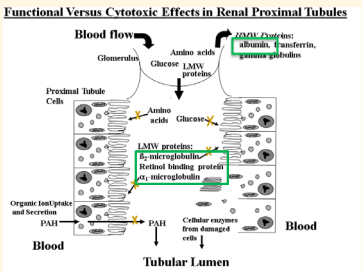
SCORED totals and elevated urinary metals



SCORED total ≥ 4	Elevated urinary metal, N (%) Yes	No	p-value*
Yes	10 (7.2)	6 (5.8)	0.67
No	129 (92.8)	97 (94.2)	

*No difference when zinc is excluded

Kidney Injury Markers



Urinary Markers of Kidney Injury (n = 119)

Marker (unit/g creatinine)	Normal cutoff	Mean (SD)	Min	Max	% above normal
IL-18 (ng)	<20	29.6 (26.8)	0.2	128.0	55.5
KIM-1 (ng)	<560	279.5 (179.6)	31.5	954.0	8.4
IAP (U)	<2	0.6 (0.7)	0.01	3.8	6.7
NGAL (ng)	<1200	1199.3 (1708.0)	14.8	10430.0	25.2
α1M (mg)	<12	4.2 (3.9)	0.6	24.0	5.0
β2M (mg)	<0.2	0.1 (0.2)	0.003	2.1	4.2
NAG (U)	<5	1.7 (1.1)	0.1	7.4	2.5
Total protein* (g)	<0.12	0.03 (0.03)	0.01	0.3	2.5
RBP (μg)	<300	46.7 (60.2)	11.9	500.4	1.7
Albumin* (mg)	<25	18.7 (24.2)	3.4	174.2	17.8

*n=118

Kidney Injury Markers by Metal Status (n = 119)

Marker (unit/g creatinine)	Mean	
	Elevated metal	No elevated metal
IL-18 (ng)	33.9	24.1
KIM-1 (ng)	309.1	241.4
IAP (U)	0.5	0.7
NGAL (ng)	1473.9	845.5
α1M (mg)	5.0	3.3
β2M (mg)	0.1	0.1
NAG (U)	1.9	1.4
Total protein* (g)	0.04	0.03
RBP (µg)	53.6	37.4
Albumin* (mg)	24.3	11.7

*n=118

Effect of elevated metals (0 vs 1 or more) on kidney injury markers adjusted for age and NSAID use (n=119)

Marker	Estimate (SE)	P-Value
IL-18	10.48 (4.88)	0.03
KIM-1	66.01 (33.01)	0.05
IAP	-0.12 (0.13)	0.38
NGAL	609.81 (312.95)	0.05
α1M	1.62 (0.72)	0.03
β2M	0.05 (0.04)	0.21
NAG	0.54 (0.21)	0.01
Total protein*	0.01 (0.01)	0.05
RBP	16.30 (11.12)	0.14
Albumin*	12.80 (4.35)	0.004

*n=118

Effect of elevated metals (0 vs 1 or more) on kidney injury markers adjusted for age and NSAID use (n=119)*

Marker	Estimate (SE)	P-Value
IL-18	8.37 (6.20)	0.18
KIM-1	31.4 (42.0)	0.46
IAP	-1.48 (0.17)	0.38
NGAL	299.4 (398.1)	0.45
α1M	2.63 (0.89)	0.004
β2M	0.10 (0.05)	0.06
NAG	0.55 (0.26)	0.04
Total protein**	0.02 (0.01)	0.003
RBP	17.61 (13.95)	0.21
Albumin**	22.80 (5.24)	<0.0001

*Elevated zinc levels excluded; **n=118

Preliminary Data Conclusions

- There is no association between self-reported renal symptoms and metal elevations.
- There is some evidence that the prevalence of kidney injury is higher in Veterans who have elevated urine metal concentrations compared to those who have normal metal concentrations.

Self-reported Respiratory Symptoms

Self-report – respiratory symptoms

		Responses N (%)					
Symptom	Elevated metal?*	None	Little of time	Some of time	Most of time	All the time	p#
Cough	No	51 (49.5)	27 (26.2)	19 (18.5)	5 (4.9)	1 (1.0)	0.27
	Yes	63 (45.3)	43 (30.9)	16 (11.5)	14 (10.1)	3 (2.2)	
Phlegm	No	51 (49.5)	28 (27.2)	17 (16.5)	5 (4.9)	2 (1.9)	0.78
	Yes	62 (44.6)	44 (31.7)	19 (13.7)	10 (7.2)	4 (2.9)	
Wheezing	No	48 (46.6)	32 (31.1)	17 (16.5)	5 (4.9)	1 (1.0)	0.79
	Yes	57 (41.0)	47 (33.8)	23 (16.6)	8 (5.8)	4 (2.9)	

*Includes patients with elevated Zn;
unadjusted analysis, chi-square

Self-report – dyspnea

		Responses N (%)					
Symptom	Elevated metal?*	None	Little of time	Some of time	Most of time	All the time	p#
SOB from wheezing	No	78 (75.7)	11 (10.7)	11 (10.7)	3 (2.9)	0 (0)	0.56
	Yes	95 (68.4)	23 (16.6)	14 (10.1)	6 (4.3)	1 (0.7)	
SOB with hurrying or hill	No	28 (27.2)	38 (36.9)	24 (23.3)	6 (5.8)	7 (6.8)	0.58
	Yes	41 (29.5)	55 (39.6)	28 (20.1)	11 (7.9)	4 (2.9)	
SOB with slow walking on level	No	58 (56.3)	21 (20.4)	13 (12.6)	8 (7.8)	3 (2.9)	0.96
	Yes	82 (59.0)	30 (21.6)	14 (10.1)	9 (6.5)	4 (2.9)	
Stop while walking to breathe	No	67 (65.1)	21 (20.4)	10 (9.7)	3 (2.9)	2 (1.9)	0.85
	Yes	87 (62.6)	34 (24.5)	10 (7.2)	6 (4.3)	2 (1.4)	

*Includes patients with elevated Zn; # unadjusted analysis, chi-square

Self-report – respiratory diagnoses

		Responses N (%)		
Diagnosis (Dx)	Elevated metal?*	No Dx	Dx present	p
History of allergies	No	41 (39.8)	62 (60.2)	0.57
	Yes	60 (43.5)	78 (56.5)	
Chronic bronchitis	No	94 (91.3)	9 (8.7)	0.58
	Yes	123 (89.1)	15 (10.9)	
Emphysema	No	103 (100)	0 (0)	0.39
	Yes	138 (99.3)	1 (0.7)	

*Includes patients with elevated Zn;
unadjusted analysis, chi-square

Self-report – respiratory diagnoses

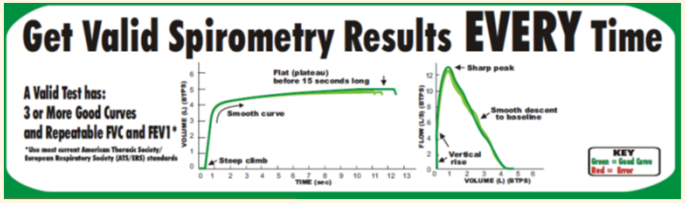
		Patient N (%)		
Diagnosis (Dx)	Elevated metal?*	No Dx	Dx present	p#
Asthma	No	89 (86.4)	14 (13.6)	0.63
	Yes	117 (84.2)	22 (15.8)	
Pneumothorax	No	96 (93.2)	7 (6.8)	0.98
	Yes	125 (93.3)	9 (6.7)	
Lung contusion	No	92 (90.2)	10 (9.8)	0.84
	Yes	118 (89.4)	14 (10.6)	

*Includes patients with elevated Zn;
unadjusted analysis, chi-square

Self-reported respiratory symptoms & diagnoses

- Not yet analyzed according to predictors of blast exposure

Pulmonary Function Tests (PFTs) & Impulse Oscillometry System (IOS) data



- BY METALS
- BY BLAST

Expected PFT Normal Values

This cohort:

Sex: male (97%)
Age: 42
Height: 177.3 cm (69.8 in)

Weight 97.6 kg (215.2 lb)
BMI 31.1

(Weight & BMI not included in prediction equations for lung function normal values)

		Expected Values		
		White	Hispanic	Black
Spirometry (NHANES III) (Airflow)	FEV1 (L)	4.13	4.15	3.52
	FVC (L)	5.22	5.14	4.31
	FEV1/FVC (%)	79.4	80.8	81.6
	FEF (L)	3.85	4.19	3.66
Lung Volumes (Quanjer 1993) (Lung size)	TLC (L)	7.09		
	FRC (L)	3.44		
	RV (L)	2.02		
	RV/TLC (%)	30.3		
Diffusion Capacity (Crapo 1981) (gas exchange)	DLCO (mL/min/mmHg)	37.6		

PFTs– Overall Cohort, n=242

Spirometry	Mean ± SD	Expected values based on GROUP MEANs (age, height, male, white)
Forced Expiratory Volume in 1 sec, FEV1 (L)	3.72 ± 0.80	4.13
Forced Vital Capacity, FVC (L)	4.75 ± 0.95	5.22
FEV1/FVC (%)	78.40 ± 6.01	79.4
Lung Volumes	Mean ± SD	
Total Lung Capacity, TLC (L)	6.40 ± 1.16	7.09
Functional Residual Capacity, FRC (L)	2.81 ± 0.83	3.44
Residual Volume, RV (L)	1.58 ± 0.63	2.02
Diffusion	Mean ± SD	
Diffusion Capacity, DLCO (mL/min/mmHg)	28.14 ± 6.10	37.6

Expected IOS Normal Values

	Airways resistance		Lung Stiffness		
	Total airways Resistance **(R5)**	Proportion of total resistance due to small airways **(R5-20 %)**	Area of Reactance (Ax)	Reactance at 5 Hz (X5)	Resonant Frequency (*Fres*)
"normal"	< 150 % of predicted	<20%	<0.33 kPa	≥-0.1176 kPa/L/s	< 12 Hz

Technical quality:
Coefficient of Variation for R5, R10 R20 – goal is < 10%

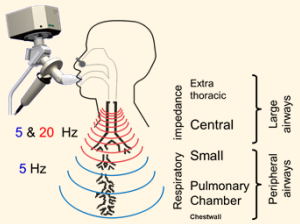
% of predicted values based on Vogel-Smidt 1994

Ax – Capacitative dominates over inertance (higher = abnormal)
X5 – Reflects elastic recoil of peripheral airways (more negative = abnormal)
Fres – Balance point between inertance and capacitance

IOS – Overall (n=242)

Resistance	Mean ± SD (% predicted)
Resistance at **5 Hz**, R5 (kPa/L/sec)	0.42 ± 0.13 **(154)**
Resistance at **20 Hz**, R20 (kPa/L/sec)	0.34 ± 0.09 **(145)**
Frequency Dependence of Resistance, R_{5-20} (%)	**16.5** ± 10.3
Reactance	**Mean ± SD**
Reactance at **5 Hz**, X5 (kPa/L/sec)	**-0.11** ± 0.1
Area of Reactance, **AX** (kPa/L/sec)	**0.62** ± 0.7
Resonant Frequency, **Fres** (Hz)	**14.5** ± 4.8
CV R5, R10, R20	5.9, 5.8, 5.9 (± 5.3)

Impulse frequencies ..5 – 20.. Hz *Multi frequent spectrum*



Elevated airways resistance
- central, not peripheral

Abnormal Ax but normal X5
Slight increase in Fres

PFT & IOS Outcomes by Metal Exposure

PFTs by Urine Metal

		No Metal Elevations N = 103 Mean ± SD	>1 Metal Elevated N = 139 Mean ± SD	p value* (with Zn)	p value* (w/o Zn)
Spirometry	FEV1 (L)	3.78 ± 0.74	3.68 ± 0.84	0.98	0.70
	FVC (L)	4.81 ± 0.91	4.70 ± 0.98	0.93	0.36
	FEV1/FVC (%)	78.6 ± 5.4	78.2 ± 6.4	0.77	0.21
	FEF (L)	3.55 ± 1.05	3.49 ± 1.14	0.82	0.87
Lung Volumes	TLC (L)	6.50 ± 1.12	6.33 ± 1.18	0.74	0.43
	FRC (L)	2.87 ± 0.88	2.76 ± 0.79	0.62	0.30
	RV (L)	1.64 ± 0.61	1.53 ± 0.64	0.32	0.88
	RV/TLC (%)	25.1 ± 8.2	24.2 ± 8.2	0.34	0.92
Diffusion	DLCO (mL/min/mmHg)	28.7 ± 5.7	27.7 ± 6.3	0.62	0.81

*adjusted for age, height, race, and smoking

PFTs by Fragments

		No frags n=26 Mean ± SD	Yes frags n=216 Mean ± SD	p value, adjusted*
Spirometry	FEV1 (L)	3.68 ± 0.94	3.73 ± 0.78	0.88
	FVC (L)	4.73 ± 0.98	4.75 ± 0.95	0.63
	FEV1/FVC (%)	77.5 ± 9.6	78.5 ± 5.5	0.44
	FEF (L)	3.53 ± 1.23	3.51 ± 1.09	0.67
Lung Volumes	TLC (L)	6.62 ± 1.15	6.37 ± 1.16	0.3
	FRC (L)	3.05 ± 0.87	2.78 ± 0.82	0.13
	RV (L)	1.79 ± 0.54	1.55 ± 0.64	0.13
	RV/TLC (%)	26.8 ± 6.8	24.3 ± 8.3	0.23
Diffusion	DLCO (mL/min/mmHg)	27.9 ± 6.0	28.2 ± 6.1	0.72

*adjusted for age, height, race, and smoking

IOS by Urine Metal

		No Metal Elevations N = 103 Mean ± SD	>1 Metal Elevated N = 139 Mean ± SD	p value* (w/ Zn)	p value* (w/o Zn)
Resistance	R5 (kPa/L/sec)	0.40 ± 0.13	0.43 ± 0.14	0.54	0.51
	R5 % predicted	149.1 ± 49.7	157.9 ± 51.4	0.50	0.69
	R20 (kPa/L/sec)	0.33 ± 0.09	0.35 ± 0.10	0.10	0.24
	R20 % predicted	141.1 ± 38.8	147.7 ± 40.6	0.31	0.32
	R_{5-20} (%)	16.7 ± 11.5	16.3 ± 9.3	0.41	0.22
Reactance	X5 (kPa/L/sec)	-0.12 ± 0.07	-0.11 ± 0.08	0.38	0.52
	AX (kPa/L/sec)	0.61 ± 0.66	0.63 ± 0.72	0.75	0.57
	Fres (Hz)	14.4 ± 4.8	14.6 ± 4.8	0.87	0.59

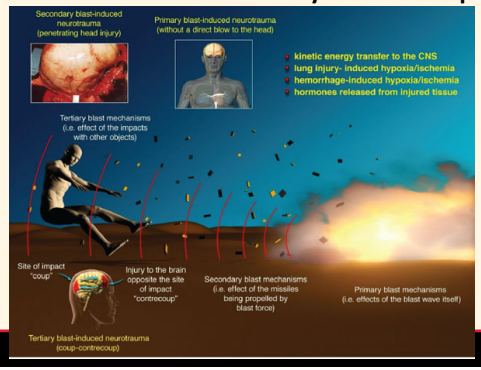
*adjusted for age, height, race, and smoking

IOS by Fragments

		No Frags N = 26 Mean ± SD	Any retained frags N = 216 Mean ± SD	P value, adjusted
Resistance	R5 (kPa/L/sec)	0.41 ± 0.14	0.42 ± 0.13	0.58
	R5 % predicted	149.5 ± 49.3	154.7 ± 51.0	0.6
	R10 (kPa/L/sec)	0.35 ± 0.10	0.37 ± 0.11	0.11
	R20 (kPa/L/sec)	0.31 ± 0.08	0.35 ± 0.10	**0.02**
	R20 % predicted	127.9 ± 30.3	146.8 ± 40.5	**0.03**
	R5-20 (%)	21.0 ± 12.2	15.9 ± 9.9	**0.02**
Reactance	X5 (kPa/L/sec)			0.96
	AX (kPa/L/sec)	0.81 ± 0.80	0.60 ± 0.68	0.23
	Fres (Hz)	15.3 ± 5.0	14.4 ± 4.8	0.45

*adjusted for age, height, race, and smoking

PFT & IOS Outcomes by Blast Exposure



PFTs by No Blast (bullet only) vs Blast

		Bullet Only* N = 15 Mean ± SD	Blast N = 223 Mean ± SD	p value, adjusted*
Spirometry	FEV1 (L)	3.69 ± 0.52	3.72 ± 0.82	0.32
	FVC (L)	4.66 ± 0.62	4.76 ± 0.98	0.14
	FEV1/FVC (%)	79.5 ± 5.0	78.5 ± 6.1	0.46
	FEF (L)	3.48 ± 0.90	3.51 ± 1.12	0.57
Lung Volumes	TLC (L)	6.35 ± 1.21	6.40 ± 1.16**	0.63
	FRC (L)	2.90 ± 1.16	2.80 ± 0.81	0.46
	RV (L)	1.60 ± 0.76	1.57 ± 0.63	0.44
	RV/TLC (%)	24.5 ± 8.3	24.4 ± 6.3	0.44
Diffusion	DLCO (mL/min/mmHg)	28.1 ± 5.0	28.1 ± 6.2	0.43

*adjusted for age, height, race, and smoking

PFTs by BTBIS negative vs positive

		BTBIS negative N = 21 Mean ± SD	BTBIS positive N = 217 Mean ± SD	p value*, adjusted
Spirometry	FEV1 (L)	3.50 ± 0.63	3.75 ± 0.82	0.67
	FVC (L)	4.45 ± 0.81	4.78 ± 0.97	0.56
	FEV1/FVC (%)	78.8 ± 4.9	78.3 ± 6.1	0.78
	FEF (L)	3.31 ± 0.88	3.53 ± 1.13	0.82
Lung Volumes*	TLC (L)	6.19 ± 1.37	6.42 ± 1.16	0.74
	FRC (L)	2.79 ± 0.97	2.81 ± 0.82	0.78
	RV (L)	1.62 ± 0.67	1.57 ± 0.63	0.44
	RV/TLC (%)	25.7 ± 7.2	24.5 ± 8.3	0.48
Diffusion	DLCO (mL/min/mmHg)	28.6 ± 5.5	28.1 ± 6.2	0.15

*adjusted for age, height, race, and smoking

PFTs by MD-Dx TBI negative vs positive

		No MD-Dx TBI n=80 Mean ± SD	Yes MD-Dx TBI n=161 Mean ± SD	p value, adjusted*
Spirometry	FEV1 (L)	3.63 ± 0.72	3.77 ± 0.82	0.87
	FVC (L)	4.62 ± 0.87	4.81 ± 0.99	0.88
	FEV1/FVC (%)	78.6 ± 5.5	78.3 ± 6.3	0.93
	FEF (L)	3.43 ± 1.00	3.56 ± 1.15	0.97
Lung Volumes	TLC (L)	6.38 ± 1.09	6.41 ± 1.20	0.14
	FRC (L)	2.95 ± 0.87	2.73 ± 0.80	**0.003**
	RV (L)	1.68 ± 0.70	1.53 ± 0.59	**0.02**
	RV/TLC (%)	26.0 ± 8.7	23.9 ± 7.8	**0.06**
Diffusion	DLCO (mL/min/mmHg)	27.9 ± 5.6	28.3 ± 6.4	0.35

*adjusted for age, height, race, and smoking

PFTs by Barotrauma Sxs negative vs positive

		Barotrauma Neg N = 198 Mean ± SD	Barotrauma pos N = 44 Mean ± SD	p value, adjusted*
Spirometry	FEV1 (L)	3.73 ± 0.83	3.72 ± 0.63	0.94
	FVC (L)	4.75 ± 0.98	4.76 ± 0.81	0.78
	FEV1/FVC (%)	78.4 ± 6.1	78.3 ± 5.5	0.89
	FEF (L)	3.51 ± 1.13	3.53 ± 0.97	0.84
Lung Volumes	TLC (L)	6.40 ± 1.18	6.39 ± 1.08	0.95
	FRC (L)	2.80 ± 0.83	2.83 ± 0.84	0.94
	RV (L)	1.57 ± 0.64	1.61 ± 0.61	0.99
	RV/TLC (%)	24.5 ± 8.2	24.8 ± 7.9	0.88
Diffusion	DLCO (mL/min/mmHg)	28.4 ± 6.0	27.0 ± 6.3	0.15

*adjusted for age, height, race, and smoking

IOS by No Blast (bullet only) vs Blast

		Bullet Only (No Blast) N = 15 Mean ± SD	Blast N = 223 Mean ± SD	P value, adjusted
Resistance	R5 (kPa/L/sec)	0.38 ± 0.12	0.42 ± 0.13	0.24
	R5 % predicted	139.8 ± 44.9	155.6 ± 51.1	0.15
	R20 (kPa/L/sec)	0.33 ± 0.10	0.34 ± 0.09	0.58
	R20 % predicted	141.9 ± 46.2	145.5 ± 39.6	0.5
	R5-20 (%)	13.3 ± 8.9	16.7 ± 10.4	0.3
Reactance	X5 (kPa/L/sec)	-0.11 ± 0.06	-0.11 ± 0.08	0.91
	AX (kPa/L/sec)	0.47 ± 0.33	0.64 ± 0.71	0.62
	Fres (Hz)	13.0 ± 4.2	14.6 ± 4.8	0.24

*adjusted for age, height, race, and smoking

IOS by BTBIS negative vs. positive

		BTBIS Negative N = 21 Mean ± SD	BTBIS Positive N = 217 Mean ± SD	p value, adjusted*
Resistance	R5 (kPa/L/sec)	0.42 ± 0.14	0.42 ± 0.13	0.63
	R5 % predicted	153.0 ± 55.3	154.4 ± 50.6	0.59
	R20 (kPa/L/sec)	0.34 ± 0.09	0.34 ± 0.10	0.63
	R20 % predicted	140.9 ± 38.0	145.5 ± 40.3	0.47
	R5-20 (%)	18.1 ± 10.9	16.3 ± 10.3	0.64
Reactance	X5 (kPa/L/sec)	-0.13 ± 0.08	-0.11 ± 0.08	0.55
	AX (kPa/L/sec)	0.69 ± 0.60	0.62 ± 0.71	0.98
	Fres (Hz)	14.9 ± 4.4	14.5 ± 4.9	0.94

*adjusted for age, height, race, and smoking

IOS by MD-dx TBI negative vs. positive

		No MD-dx TBI N = 80 Mean ± SD	Yes MD-dx TBI N = 161 Mean ± SD	p value, adjusted*
Resistance	R5 (kPa/L/sec)	0.42 ± 0.14	0.42 ± 0.13	0.68
	R5 % predicted	154.2 ± 54.1	154.2 ± 49.3	0.71
	R20 (kPa/L/sec)	0.35 ± 0.10	0.34 ± 0.10	0.85
	R20 % predicted	146.8 ± 41.4	144.1 ± 39.3	0.87
	R5-20 (%)	15.5 ± 9.7	16.9 ± 10.6	0.22
Reactance	X5 (kPa/L/sec)	-0.12 ± 0.08	-0.11 ± 0.08	0.88
	AX (kPa/L/sec)	0.60 ± 0.74	0.63 ± 0.67	0.44
	Fres (Hz)	14.2 ± 4.9	14.7 ± 4.8	0.22

*adjusted for age, height, race, and smoking

IOS by Barotrauma sxs-negative vs. positive

		Barotrauma Neg N = 198 Mean ± SD	Barotrauma Pos N = 44 Mean ± SD	p value, adjusted
Resistance	R5 (kPa/L/sec)	0.42 ± 0.14	0.39 ± 0.09	0.10
	R5 % predicted	156.6 ± 53.4	143.0 ± 34.8	0.14
	R20 (kPa/L/sec)	0.35 ± 0.10	0.32 ± 0.07	0.14
	R20 % predicted	146.5 ± 41.7	137.4 ± 29.9	0.21
	R5-20 (%)	16.5 ± 10.3	16.1 ± 10.1	0.83
Reactance	X5 (kPa/L/sec)	-0.12 ± 0.08	-0.10 ± 0.06	**0.09**
	AX (kPa/L/sec)	0.65 ± 0.74	0.51 ± 0.41	0.26
	Fres (Hz)	14.6 ± 4.8	14.1 ± 4.9	0.51

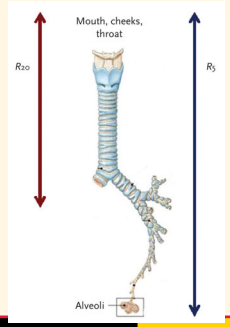
*adjusted for age, height, race, and smoking

Preliminary data conclusions - Pulmonary

- High prevalence of respiratory symptoms
  - 41% have SOB walking on a level surface at own pace
  - 36% have to stop while walking due to SOB
  - 53% have cough
  - 15% have ever been diagnosed with asthma
- Cohort overall may have lower lung function than expected
  - Additional analysis needed to confirm, especially impact of weight

Metal impact on pulmonary outcomes

- No significant differences in symptoms (urinary metal)
  - Have not analyzed by metal surrogates (self-report; presence of frags)
- Lung volumes lower in the + urine metal and + frags groups (NS)
- Higher airways resistance in +urine metal and + frags groups (NS)
  - Appears to be more central, not peripheral



Blast impact on pulmonary outcomes

- High prevalence of blast exposure, symptoms related to TBI
  - May be hard to see differences because of small "unexposed" group
- Have not analyzed symptoms by blast exposure
- PFTs are worse in the no-blast groups (bullet only, BTBIS neg) (NS)
  - FRC, RV, RV/TLC lower in the MD-Dx TBI group (?)
- IOS
  - Higher resistance and Ax in the blast group (NS)
  - No real signal by TBI indices

Day 1
Adjourn

Regulatory Review

Modifications to Date

Modification	IRB Approved	DoD Approved	Main Content
1	6/19/2018	6/26/2018	• Participant allowed to return to complete testing if necessary, and to be compensated an additional $50 • Visit reminder letter added
2	11/5/2018	12/17/2018	• Participant offered option to be contacted for future research studies
3	11/5/2018	12/17/2018	• As a result of recent protocol deviations, HIPAA modified to request waivers for scheduling purposes
4	4/9/19	6/13/19	• Phoenix VA added as 6th recruitment site
5	5/28/19	7/9/19	• New LSI at San Antonio (Dr. John Duch) • Eligibility Criteria updated to specify location and era in which injury occurred • Participant must be able to provide urine specimen without the use of a catheter

Deviations to Date

All PFT-related:
- Orders
- Scheduling
- PFT Lab Staff familiarity

Deviation #	Date of Event	Description
1	6/7/18	PFT with bronchodilator
2	6/26/18	PFT with bronchodilator
3	7/10/18	PFT performed before consent signed
4	7/2/18	PFT with bronchodilator
5	9/7/18	PFT performed before consent signed
6	1/11/19	PFT performed before consent signed

Procedures for reporting unanticipated problems, serious adverse events, protocol deviations

- **Local PIs must inform** their **local VA OR&D** and **Dr. McDiarmid and Baltimore study team** if any unanticipated problems, serious adverse events, or deviations related or probably related to the research or any serious occur.
- **The Baltimore team** must report the event to VA Central IRB within **5 work days of discovering the event**.
  - **In the event of a life threatening problem or death** all parties must be **notified immediately** and **written notification** must be **sent within 48 hours**.

Next Continuing Review

- CR due to Baltimore by 3/14/2020
- Due to VA C-IRB by 3/21/2020
- Study Expiration Date: 5/21/2020

Required Trainings

- CITI (or VA Human Subjects Protection Training)
- TMS
  - Privacy and HIPAA Training
  - VA Privacy and Information Security Awareness and Rules of Behavior
  - Infection Control: Bloodborne Pathogens
- DOT/IATA Shipping Certificates
- Lab Safety

Reporting

Reporting

Next reporting period covers **July 1-Sept 29**

- Technical Reports – **Due October 7th**
  - Due within ***7 days*** from quarter end
  - Submit directly to Dr. McDiarmid and Dr. Gaitens
- Quarterly Invoicing – **Due October 30th**
  - Due **30 days** from quarter end
  - Submit directly to Rachel Coates-Knowles rknowles@som.umaryland.edu

Budgetary Information

- Year 4 budgets included travel for final all-investigators meeting
  - Plan to carry these funds over to Year 5
- Please track if additional participant payments are needed

Special Terms & Conditions

Prior approval from UMB is required for any of the following program or budget revisions:

- Change in Statement of Work
- Disengagement of the approved PI or project director from the project for more than 3 months.
- 25% or more reduction in PI time devoted to project.
- Change in Key Personnel

Reporting Requirements

- Subrecipient must submit a Federal Financial Report (SF425) annually for calendar year end (December 31st).
- Annual financial report due no later than 60 days after the end of the calendar year.

Future Work Products

Planned Analyses - Renal

Aim 1: Characterize the injury and health experiences of Iraq and Afghanistan Veterans who have retained embedded fragment

- Validate self-reports of embedded fragments using available images
- Assess associations between:
  - imaging findings and urine metal concentrations
  - potential predictors of metal exposure and urine metal concentrations
  - self-reported conditions related to kidney disease and kidney outcomes

Aim 2: Evaluate association between kidney injury markers and urine metal concentrations

Aim 3: Evaluate association between self-reported renal outcomes and urine metal concentrations

Planned analyses - Pulmonary

- Aim 1: Evaluate association between urinary metal levels and:
  - Respiratory symptoms
  - PFT abnormality
    - Obstructed/Restricted/Mixed/Hyperinflated/Gas Trapping/Impaired Diffusion
  - IOS abnormality
    - Elevated airways resistance/Small Airways Abnormality/Abnormal Reactance/Abnormal coherence

  Also – evaluate for surrogates of metal exposure (self-reported metal exposure)

Planned Analyses - Pulmonary

- Aim 2: Evaluate association between blast exposure and:
  - Respiratory symptoms
  - PFT abnormality
    - Obstructed/Restricted/Mixed/Hyperinflated/Gas Trapping/Impaired Diffusion
  - IOS abnormality
    - Elevated airways resistance/Small Airways Abnormality/Abnormal Reactance/Abnormal coherence

Additional pulmonary analyses

- Correlate self-reported respiratory symptoms with PFT & IOS parameters
- Correlate PFT parameters with IOS parameters
  - Examples:
    - TLC with X5
    - FEV1/FVC with R5
- Incorporate prediction equations for NHANES III (spirometry)/GLI (spirometry & DLCO-Caucasian), Quanjer 1993 (lung volumes), Crapo 1981 (DLCO)
- Assessment of spirometry quality (variability)

Related Cohorts

Cohort	Source	Size	Age (men); mean or range	Includes women?	Includes OIF/OEF era?	Outcomes
MVP	Veterans	415,694	66	Yes	Yes	Diagnoses, general health status
NewGen	Veterans	20,547	24-60+	Yes	Exclusively	Diagnoses, general health status
MilCo	Active Duty & Veterans	46,077	Mainly born 1960s-70s	Yes	Yes	Diagnoses, general health status
STAMPEDE II	Active Duty (Army)	843	32	Yes	Yes	Respiratory symptoms, diagnoses, PFTs, IOS
AHOBPR	Veterans & Active Duty	>180,000	19-65 (mainly 30s-40s)	Yes	Yes	Diagnoses, general health status

Study Population 1 (Non-clinical population)

- Questionnaires mailed out to 1000 Veterans at a time
- Target Enrollment: 2520
- Goal to have 70% blast exposure; 30% no blast (bullet only injuries)

Batch 5: 186 Blast; 75 Bullet = 71/29%
Batch 4: 159 Blast; 78 Bullet = 67/33%
Batch 3: 204 Blast; 79 Bullet = 72/28%
Batch 2: 169 Blast; 82 Bullet = 67/33%
Batch 1: 209 Blast; 95 Bullet = 69/31%

Questionnaire Mail-Out (Study Population #1)

Mail out	# Questionnaires Sent	Completed Paper (%)	Completed Electronic (%)	Totals
1st	6,013	522 (71%)	209 (29%)	731
2nd	4,982	321 (69%)	147 (31%)	468
3rd	4,413	303 (79%)	79 (21%)	382
4th*	608	35 (78%)	10 (22%)	45
Totals	**16,016**	**1181 (74%)**	**445 (26%)**	**1626**

*4th mailing only done for first round of mailings

Overall Response Rate = 27.0%

Scholarly Works

- Conference Abstracts

Preliminary data | Final data

Conference	2020	2021	2022	2023	2024
SOT					
ATS					
MHSRS (?)					

Potential Papers (clinical population)

- Kidney Injury Markers vs. metal exposure
- Self-report renal outcomes vs. metal exposure
- Self-reported metal exposure vs. urine metal exposure
- Frag characteristics on X-ray vs metal exposure
- SF12 vs Metals
- SF 12 vs Injury Type
- Kidney Injury Markers vs Blast
- Recruitment strategies of OIF/OEF Veterans

Potential papers (clinical populations)

- Blast Exposure vs. PFT
- Blast Exposure vs IOS
- Blast Exposure vs Respiratory Symptoms
- Metal Exposure vs PFT
- Metal Exposure vs IOS
- Metal Exposure vs Respiratory Symptoms
- Respiratory Symptoms vs. Abnormalities in PFT/IOS
- PFT vs IOS
- TBI in TEF Population
- SF12 vs. TBI
- Renal Injury vs. Abnormal PFT/IOS

Thinking towards authorship

- Authors should satisfy 3 conditions *(Uniform Requirements for Manuscripts Submitted to Biomedical Journals, 2007):*
  - Substantially contribute to the conception & design of the study, acquisition of data, or analysis/interpretation of data
  - Participate in drafting or revising the article for intellectual content
  - Review and approve the final, submitted version

http://www.icmje.org/

- Focus on areas of expertise
- May have some limitations based on character counts/# authors on some submissions
  - i.e. SOT Abstract