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CONTRACTING ORGANIZATION: Duke University

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

A majority of service members who undergo traumatic amputation develop chronic phantom or chronic residual limb pain with 10-15% of these patients developing severe, disabling, long-term pain. 30-40% of traumatic amputees, however, have no clinically significant chronic pain. We believe this dichotomy of outcome is the key to understanding the development of chronic neuropathic pain after nerve injury. Preclinical studies using rodent models have provided some insights into the pathological sequelae of nerve injury, but this knowledge has not resulted in successful translation to the clinic. Recent evidence suggests that interspecies differences are a major barrier to successful translation, since rodent sensory neurons diverge considerably from their human counterparts. Accordingly, in order to better understand the pathological processes that lead to neuropathic pain after nerve injury, it is necessary to comprehensively study injured human nerves. Our colleagues at Walter Reed National Military Medical Center spent three years obtaining sciatic nerve samples from service members undergoing primary amputation revision surgery after suffering traumatic amputation on the battlefield. These unique samples allow, for the first time, study of nerve regeneration and neuroinflammation in humans during the days following traumatic amputation. Utilizing bulk tissue and single nuclei RNA-sequencing and unbiased global proteomics of the distal portion of sciatic nerve collected 1-14 days after initial traumatic amputation, we aim to establish the distinctive transcriptional, protein and glial/immune cell profile of injured sciatic nerve during injury and regeneration.

15. SUBJECT TERMS

Transcriptomics, single nuclei transcriptomics, proeteomics, neuroinflammation, neuropathic pain, nerve regeneration, phantom limb pain, residual limb pain.

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INTRODUCTION:

A majority of service members who undergo traumatic amputation develop chronic phantom or chronic residual limb pain with 10-15% of these patients developing severe, disabling, long-term pain. 30-40% of traumatic amputees, however, have no clinically significant chronic pain. We believe this dichotomy of outcome is the key to understanding the development of chronic neuropathic pain after nerve injury. Preclinical studies using rodent models have provided some insights into the pathological sequelae of nerve injury, but this knowledge has not resulted in successful translation to the clinic. Recent evidence suggests that interspecies differences are a major barrier to successful translation, since rodent sensory neurons diverge considerably from their human counterparts. Accordingly, in order to better understand the pathological processes that lead to neuropathic pain after nerve injury, it is necessary to comprehensively study injured human nerves. Our colleagues at Walter Reed National Military Medical Center spent three years obtaining sciatic nerve samples from service members undergoing primary amputation revision surgery after suffering traumatic amputation on the battlefield. These unique samples allow, for the first time, study of nerve regeneration and neuroinflammation in humans during the days following traumatic amputation. Utilizing bulk tissue and single nuclei RNA-sequencing and unbiased global proteomics of the distal portion of sciatic nerve collected 1-14 days after initial traumatic amputation, we aim to establish the distinctive transcriptional, protein and glial/immune cell profile of injured sciatic nerve during injury and regeneration.

KEYWORDS:

Transcriptomics, single nuclei transcriptomics, proteomics, neuroinflammation, neuropathic pain, nerve regeneration, phantom limb pain, residual limb pain.

1. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

- **Aim 1 - Perform bulk tissue and single nuclei RNA-sequencing and unbiased global proteomics of the proximal and distal portion of sciatic nerve to establish the distinctive transcriptional, protein and glial/immune cell profile of injured sciatic nerve during injury and regeneration.**

- Major Task 1: Amend existing USUHS IRB and obtain approval for transcriptomic work on sciatic nerve samples. **100% complete (Performed at both Duke and USUHS/DVCIPM)**

    - As noted in the last report, IRB approval of the sequencing amendment was obtained. This was a sensitive issue because the IRB at USUHS and Walter Reed are extremely protective of the genetic information of military subjects as is our group. Therefore, we had to carefully describe and educate ourselves and the IRB about the genetic risks.

- Major Task 2: Obtain USAMRMC Office of Research Protections HRPO approval for use of sciatic nerve samples. **100% complete (Performed at Duke under Dr. Vandeven)**

    - Approved

- Major Task 3: Renew IRB exemption for work on deidentified nerve samples at Duke. **100% complete (Performed at Duke under Dr. Vandeven)**

    - This exemption no longer has an expiration date

- Major Task 4: Process each nerve, collect nuclei and bulk RNA. **75% complete (Performed at Duke under Dr. Vandeven) This task is where we have spent most of our time this year and some of the alterations in research strategy and the progress we've made are detailed below:**
    - Tissue Processing
        - For each nerve, we cut 1 cm off the distal and 1 cm off the proximal ends and embedded them in OCT for precision sectioning on cryostat. We decided against using a manual razor blade cutting strategy for the more precise and reproducible cryostat which allows cutting while sample is frozen and allows the production of thin slices for staining and RNAscope (an additional technique not included in the original research plan). I will discuss RNAscope under Major Task 5 below
            - First, we cut 75 um sections and collected in tube A for RNA extraction
            - Second, we cut 75 um sections and collected in tube A for proteomics
            - Third, we cut 10 um sections (x5), and placed on individual slides.
            - Slides: 25 slides per patient; each slide will have one distal, one proximal section.
- Major Task 5: Create RNA libraries and complete sequencing. **20% complete (Performed at Duke under Dr. Vandeven)**
    - One of the three main tasks in aim 1 of this project was to collect RNA from single nuclei and sequence the RNA to determine what cell populations are present at the injured tip of the sciatic nerve. As we said in the application, we had worries that the RNA in the nuclei in these relatively old samples would not be of sufficient quality to obtain usable sequence data. In the past two years a statistical technique using deconvolution of bulk RNA sequence to determine cell populations has matured. Though this is a change in research method the strategy and output will be unchanged. The advantages of this technique is we bypass the problem of poor-quality nuclei RNA and we also will be able to detect RNA with lower expression levels (likely the more interesting genes) compared to single nuclei sequencing.
    - An exciting and unexpected development over the past year was our discovery that the nerves were still of sufficient quality that we are able to perform RNAscope on thin slices of the distal nerve endings. This means we can use the nerves themselves to validate the findings of RNA sequencing. For example, if we find expression of a certain immune mediator is increased in the bulk RNA sequence of a patient with severe chronic pain we can go back and perform RNAscope to determine directly if and where that RNA is present in the nerve cross section. Example images are attached below in the appendix.
- Major Task 6: Complete proteomics. **20% complete (Performed at Duke under Dr. Vandeven)**
    - We have sent our first two trial nerve samples for proteomics analysis. Each sample was sectioned in the exact same way but either embedded in OCT or not embedded.

Embedding is our plan, but requires an ethanol de-lipidation step and we want to get an idea of how this will affect yields.

- The proteomics facility at Duke is optimistic about running phosphoproteomics to help give us more information about cellular signaling (phosphorylation being a major post-translational modification altering protein activity), so we're going to do normal proteomics and a TiO2 enrichment for subsequent phosphoproteomics so we can get both datasets. This method will catch threonine and serine phospho-sites, which make up the vast majority (95-98%) of phosphorylation events

- Major Task 7: Develop an expression and cell signature of nerve regeneration over the two weeks following traumatic amputation. **0% complete**

  - This aim is dependent on completion of the RNA sequencing and proteomics analysis

- **Aim 2 - Complete a pain and functional outcome database describing each patient enrolled in the SEXI trial and use those outcomes to identify immune cell populations, gene and protein expression changes around the time of amputation that correlate with positive outcomes of good function and minimal residual limb or phantom pain.**

- Major Task 1: Amend existing USUHS IRB to collect physical function and more recent pain and analgesic medication data on the patients who previously donated sciatic nerve samples. **100% complete (performed by the USUHS/DVCIPM team under Dr. Buckenmaier)**

- Major Task 2: Update current clinical outcomes database of SEXI patients for most recent pain scores and medication use (60 total patients) **100% complete (performed by the USUHS/DVCIPM team under Dr. Buckenmaier)**

  - This database of pain scores, medication use, and psychiatric comorbidities is complete.

- Major Task 3: Add functional outcomes data to database. **25% complete**

  - Our USUHS colleagues have attempted to acquire physical function outcomes data using physical therapy notes and by searching for physical function questionnaires in patient records (such as Oswestry and Promis PF short forms). Unfortunately, few of these patients have standardized PF outcomes in their medical records. Our USUHS colleagues are now working with the physical therapy department to determine if there are other ways of extracting this data. Currently only about 10% of the soldiers and veterans who donated nerve tissue have physical function outcomes data in our database.

- Major Task 4: Correlate outcomes with expression signature **0% complete**

  - Dependent on completion of proteomics and RNA sequencing

- **Aim 3 - Correlate perioperative ketamine use with immune cell population and gene and protein expression profile.**

- Major Task 1: Determine whether ketamine treatment produces improved functional outcomes. **0% complete**

- Major Task 2: Identify the protein and RNA expression signature in sciatic nerve unique to patients treated with perioperative ketamine. **0% complete**

- **What opportunities for training and professional development has the project provided?**
  - Nothing to report
- **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?**
  - During the next reporting period all proteomics and RNA sequencing will have been performed. Analysis will also be started but due to some delays during the COVID pandemic we plan to request a no-cost extension at the end of the next reporting period to make sure we have time to fully analyze and publish the proteomics and RNA data.

2. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
  - Nothing to Report yet
- **What was the impact on other disciplines?**
  - Nothing to Report yet
- **What was the impact on technology transfer?**
  - Nothing to report
- **What was the impact on society beyond science and technology?**
  - Nothing to report

3. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
  - The main changes in approach are the decision to determine cell populations within the sciatic nerves using deconvolution of bulk RNA sequencing data rather than single nuclei sequencing. The advantages are the ability to detect low expression genes which are more likely to be useful biomarkers of chronic pain. Also, we have added RNAscope which we didn't initially believe to be possible. After finding RNA of sufficient quality in thin slices of nerve tissue we are going ahead with this method which offers the ability to validate the 'omic analyses.
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - No further delays are expected. We have hired a new highly qualified research technician who is helping to move the project forward quickly. The only problem we anticipate is the inability to get useful physical function data from the medical record without reconsenting enrollees.
- **Changes that had a significant impact on expenditures**
  - None

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
    - We have obtained HRPO approval for the project and also IRB approval at USUHS.
- **Significant changes in use or care of human subjects**
- **Significant changes in use or care of vertebrate animals.**
- **Significant changes in use of biohazards and/or select agents**

4. **PRODUCTS:**

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

Journal publications.

Nothing to Report yet

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

Nothing to report

Other publications, conference papers, and presentations.

Nothing to report

5. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	*Thomas Van de Ven*
Project Role:	*Principal Investigator*
Researcher Identifier (eRA Comm	*THOMAS.VANDEVEN*
Nearest person month worked:	*5*
Contribution to Project:	*Coordinates all aspects of the project and assumes overall responsibility for its success.*
Funding Support:	*No other support*

Name:	*Chester Buckenmaier*

Project Role:	*Site Prinicipal Investigator*
Researcher Identifier (eRA Comm	cbuckenmaier
Nearest person month worked:	*5*
Contribution to Project:	*Coordinates IRB approval of study activities and collection of clinical data on enrolled subjects*
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to Report
- **What other organizations were involved as partners?**

- **Organization Name:** Defense and Veterans Center for Integrative Pain Management (DVCIPM) and Uniform Services University of the Health Sciences (USUHS)
- **Location of Organization:** Bethesda, Maryland
- **Partner's contribution to the projec**t

Collaboration Dr Buckenmaier and the research staff at DVCIPM are responsible for IRB approval of this study and for collection of the clinical data needed to tie molecular changes in the sciatic nerve samples to important clinical functional and pain outcomes

6. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from **BOTH** the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ebrap.org for each unique award.*

- **QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

7. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES.***