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TITLE: Single Nucleus Expression Profiling of Human Sciatic Nerve After Traumatic Amputation: Predicting Pain and Functional Outcomes

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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 unbiased global proteomics, MSD array analysis and immunostaining 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 unbiased global proteomics, MSD array analysis and immunostaining 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.
   - **Summary of Results of Major Task 1: IRB approval of the sequencing amendment was obtained.**

   - Major Task 2: Obtain USAMRMC Office of Research Protections HRPO approval for use of sciatic nerve samples.
   - **Summary of Results of Major Task 2: Approved**

   - Major Task 3: Renew IRB exemption for work on deidentified nerve samples at Duke.
   - **Summary of Results of Major Task 3: Approved**

   - Major Task 4: Process each nerve, collect nuclei and bulk RNA.

- **Summary of Results of Major Task 4: 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). First, we cut 75 um sections for RNA extraction and proteomics and then 10um sections for individual slides. 25 slides were prepared per patient, each with one distal and one proximal section.**

- Major Task 5: Create RNA libraries and complete sequencing.
- **Summary of Results of Major Task 5: 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 that 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. Unfortunately, nucleic and bulk RNA quality was too poor for adequate analysis (Figure 1).**

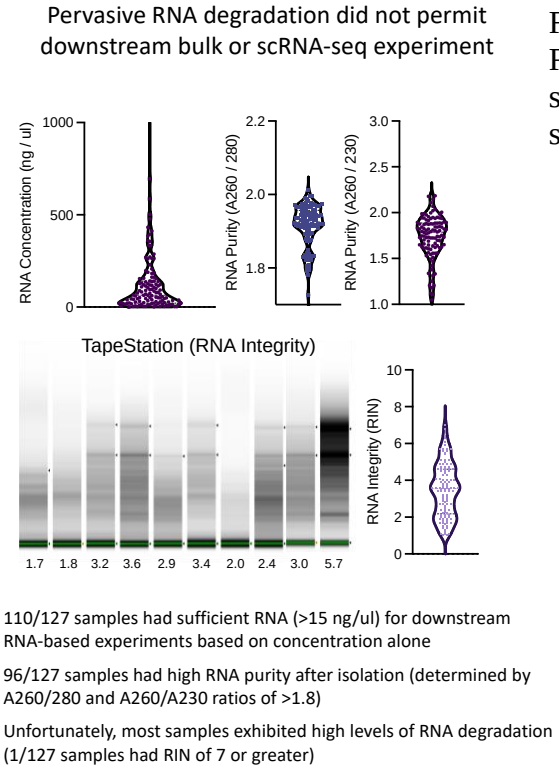
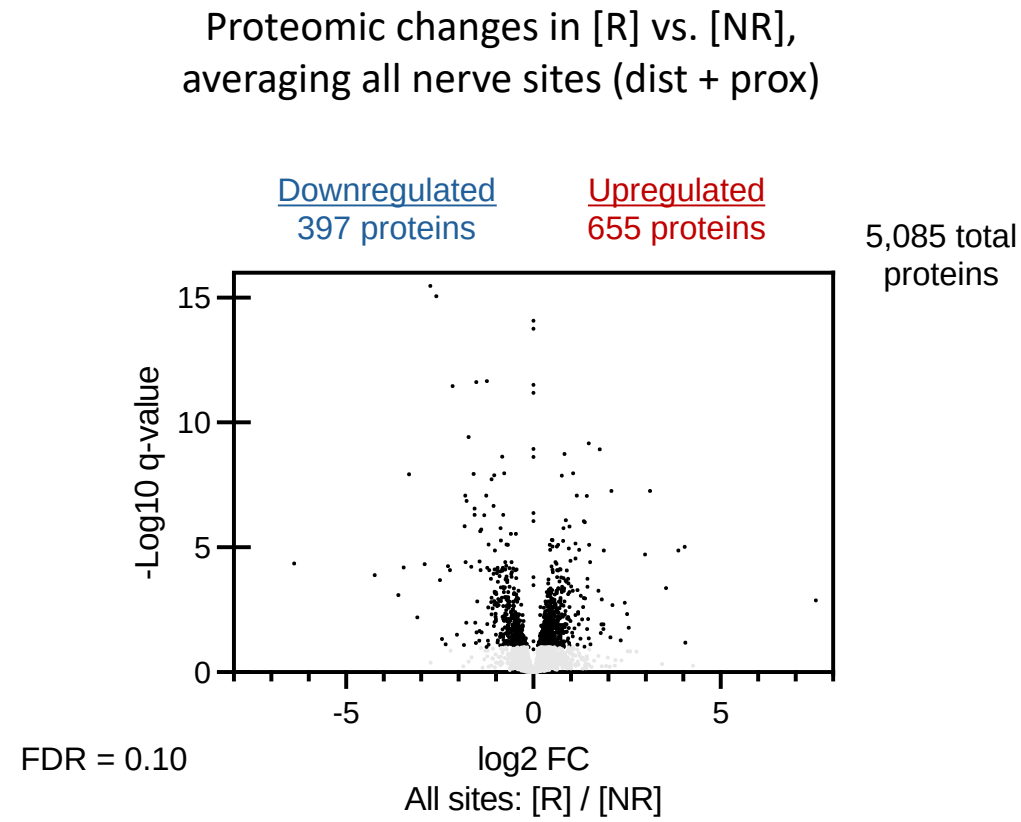


Figure 1: RNA integrity examples from RNA extractions of sciatic nerve slices. Tapestation results show significant RNA degradation .

- Major Task 6: Complete proteomics.
- **Summary of Results of Major Task 6: We found a number of proteins differentially expressed in the nerves of patients who went on to develop chronic pain (NR or non-resolvers) vs those who didn't (R or resolvers) (Figure 2). Some of the differentially expressed proteins are targets that we have been interested in from separate studies we have performed including the TGF-beta signaling pathway (R > NR), MANF (R > NR) and Gasdermins (GSDMD & GSDME; R > NR) (Figure 3). Network Analysis of the top differentially regulated proteins in resolvers vs nonresolvers shows a surprising result that smooth muscle proteins are upregulated in resolvers (Figure 4). This is supported by MSD**

array findings that show mediators that direct vascular regeneration (angiogenesis) are upregulated in resolvers.



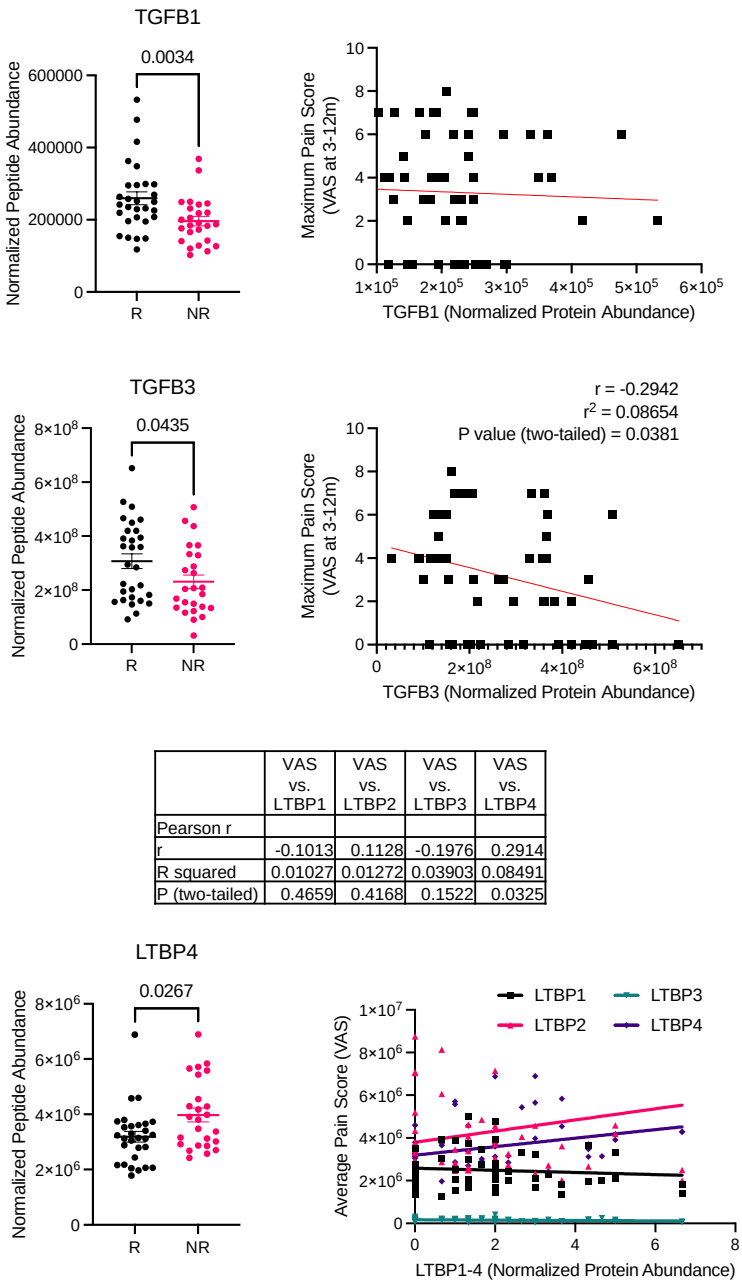
Top 10 downregulated (by q-value)

Protein	-log10 q-value	Log2FC
TMEM165	15.4766366	-2.7504
ABI2	15.0542065	-2.5877
LRSAM1	11.6631767	-1.2408
TUBG1	11.6232138	-1.5245
GTF3C4	11.4545495	-2.1586
TFRC	9.41493909	-1.7314
ARHGEF7	8.63271779	-0.8270
MPI	7.9647133	-0.7792
SKIV2L	7.94569595	-1.5922
SAA2	7.9258183	-3.3198

Top 10 upregulated (by q-value)

Protein	-log10 q-value	Log2FC
ZNF384	9.16867279	1.4832
HTRA3	8.92683417	1.7698
CSTF2T	8.73758007	0.8347
MRPS6	7.96723704	1.0662
NUP153	7.87271369	0.7595
HLA-DRB1	7.26506178	3.1185
H2AFX	7.26457252	2.0845
GSDME	7.07353277	1.1618
SDF2	7.06217212	1.4228
NDRG3	6.09253624	0.8685

Figure 2: Volcano plot showing proteins upregulated or downregulated in resolvers vs nonresolvers with most significantly regulated proteins listed by significance and fold change.



	VAS vs. LTBP1	VAS vs. LTBP2	VAS vs. LTBP3	VAS vs. LTBP4
Pearson r				
r	-0.1013	0.1128	-0.1976	0.2914
R squared	0.01027	0.01272	0.03903	0.08491
P (two-tailed)	0.4659	0.4168	0.1522	0.0325

Figure 3: Abundance of TGF and LTBP (TGF binding proteins) proteins in patient samples vs VAS. TGFB3 is more abundant in resolvers and LTBP4 is less abundant in resolvers.

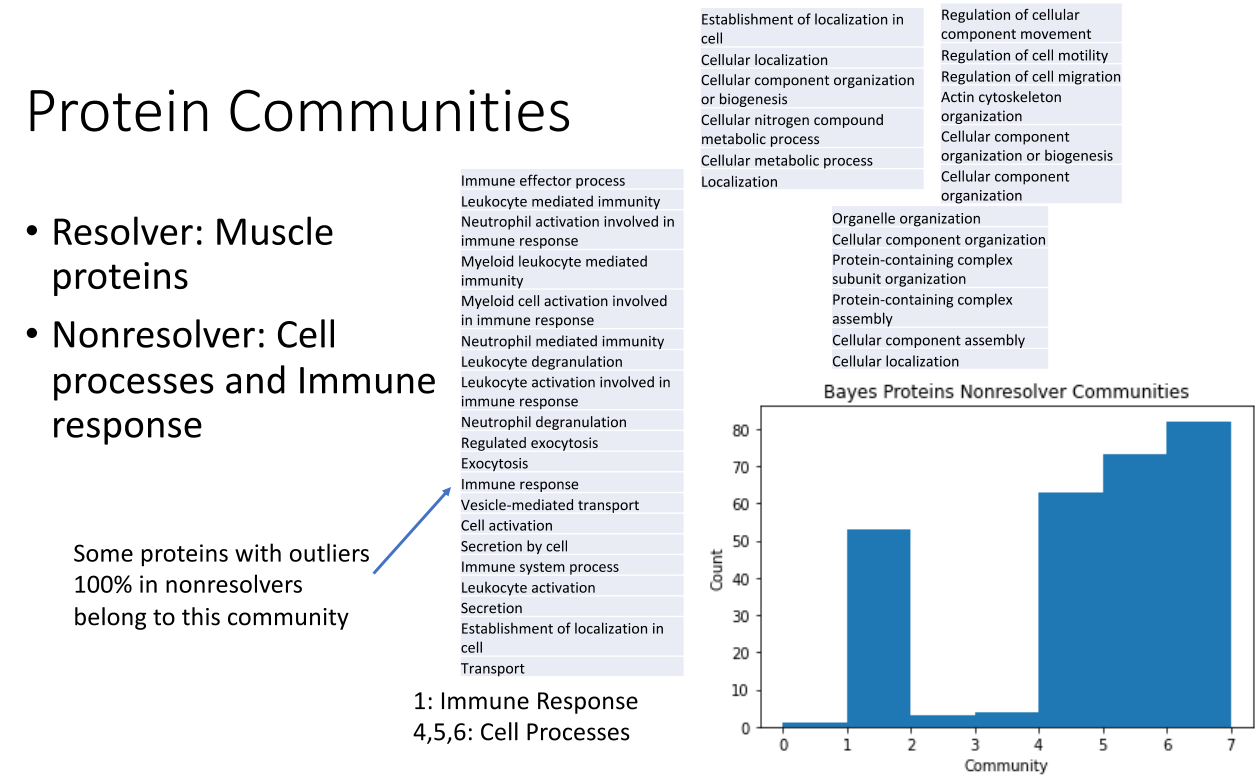


Figure 4: Network Analysis - Protein pairs are assigned a connection if their correlation is above a certain threshold (0.8). Here, the Walktrap algorithm is used to define communities. In brief, the algorithm uses random walks to assign the probability of each node transitioning to each other node. The spectral properties of the transition matrix is then used to establish a distance between nodes, which are then merged according to their heuristic until the communities are established.

The results of the network analysis reveals communities of proteins that have similar functional identity. These communities can show the underlying cellular processes of Resolvers versus Nonresolvers. For example, Resolvers have a unique muscle related community that does not show up for Nonresolvers.

Finally, certain proteins are highly concentrated in only one category of samples. These proteins can be identified and assigned to a community, thus revealing the protein's function. Proteins that are uniquely elevated in Resolvers belong to the muscle-related community; proteins that are uniquely elevated in Nonresolvers belong to an immune-related community and three other

- Major Task 7: Develop an expression and cell signature of nerve regeneration over the two weeks following traumatic amputation.
- **Summary of Results of Major Task 7: Since RNA quality was poor, this major task was completed using two new additional procedures – cytokine and chemokine profiling (to validate the pathways found to be important in major task 6) and immunohistochemistry of various macrophage markers in slices of the distal tip of the nerves. Briefly, resolvers had a robust response to nerve injury characterized by upregulating a number of important inflammatory and angiogenesis markers while nonresolvers did not have a robust inflammatory or angiogenesis response to nerve injury (Figures 5, 6 and Table 1). Immunostaining was completed for the majority of nerve**

samples with successful identification of various inflammatory and neuronal markers. (Figure 7) We found that sciatic nerves from resolvers contained a higher number of M1 Macrophages than nonresolvers again suggesting that resolvers have an important and robust inflammatory response to injury and nonresolvers do not have a robust response (Figure 8).

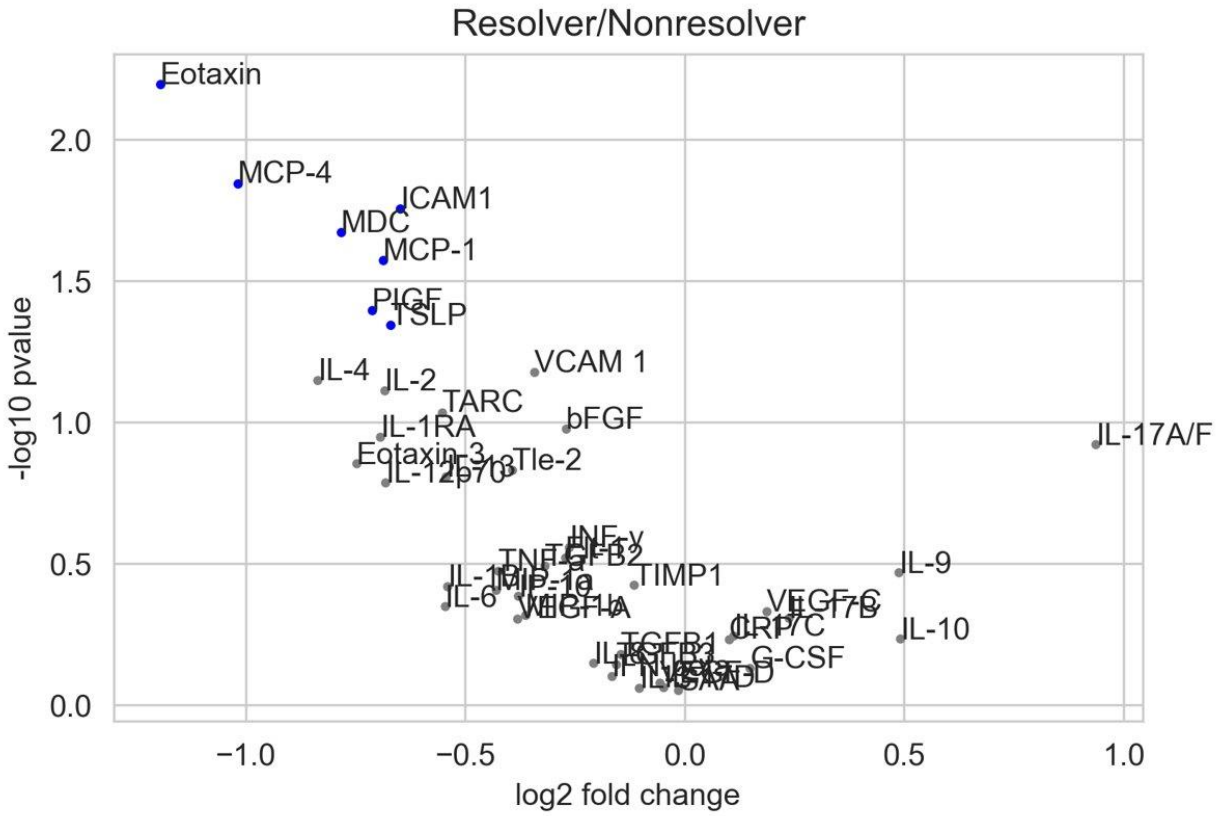


Figure 5: Volcano plot showing differential analyte protein concentrations between resolver and non-resolver patients. Each point on the plot represents an analyte, with the x-axis representing the log2 fold change in analyte protein concentration between the two groups, and the y-axis representing the -log10 of the p-value from a statistical t-test assessing the significance of the difference in analyte concentrations. Proteins exhibiting statistically significant upregulation in resolvers compared to non-resolvers are situated on the right side of the plot, characterized by positive log2 fold change values. Eotaxin (p=0.006), MCP-4 (0.014), ICAM-1(0.018), MDC (0.021), MCP-1 (0.027), PLGF (0.04), and TSLP (0.045) were all significantly higher in resolver group.

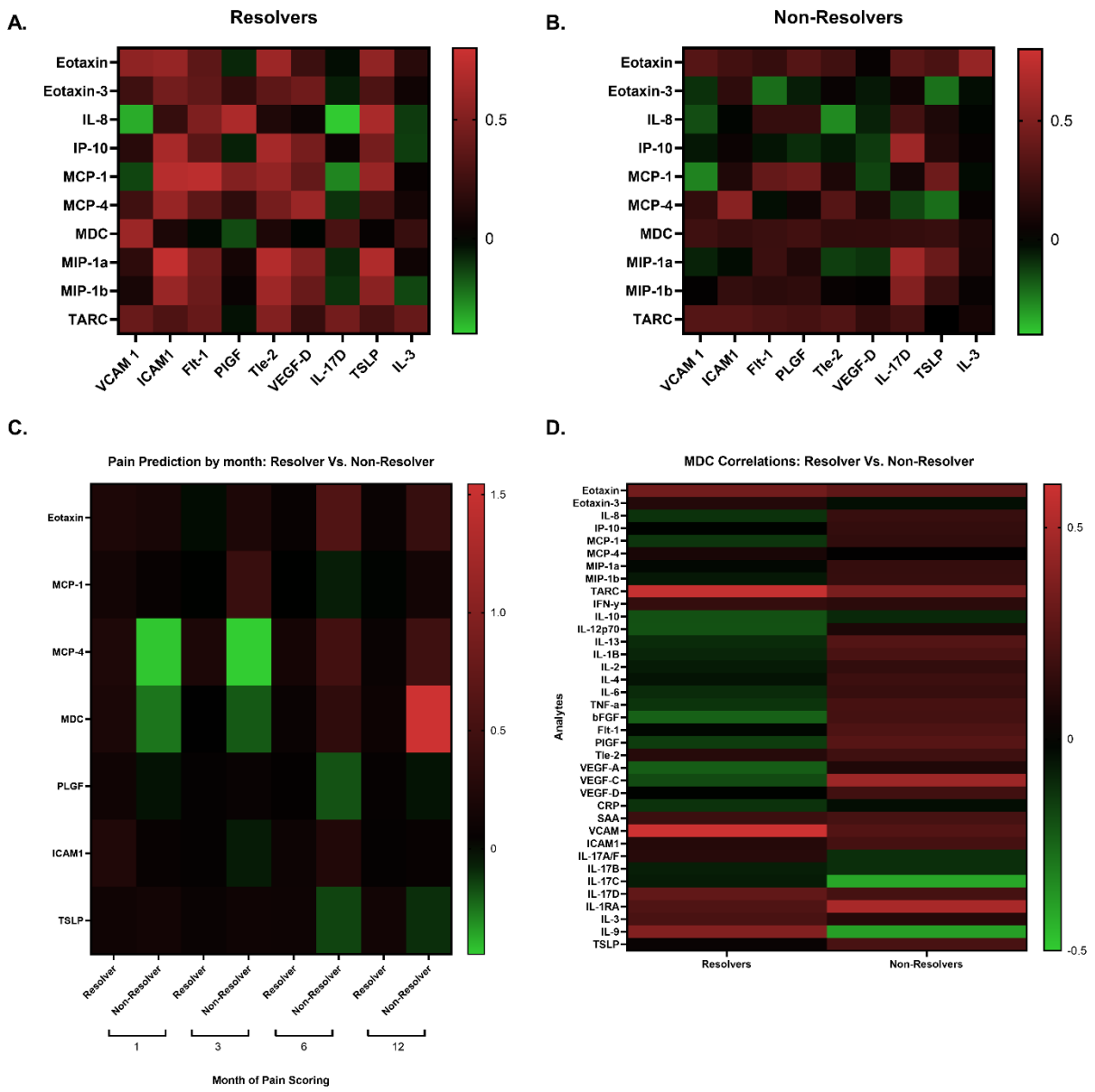


Figure 6: Correlation heat maps showing chemokine analytes on y-axis and their significant strong positive correlation (dark red) or significant strong negative correlation (green) with other analytes on x-axis in both resolvers (**A**) and non-resolvers (**B**). **C.** Heat map illustrating analytes' predictive value of pain in resolvers versus non-resolvers at the month of pain scoring (1,3,6 and 12). MDC is shown to be highly predictive of pain in non-resolvers at 12 months post-surgery. **D.** Heat Map illustrating MDC correlation across all analytes (y-axis) in resolvers versus non-resolvers. A strong positive correlation coefficient is >0.5 and a strong negative correlation coefficient is < -0.5.

Resolver	Non-Resolver
- Involves Th2 response: An important contribution from TSLP and a different path of activation of TSLP which leads to Th2 response, neutrophil activation (leads to IL-6, IL-1B, IL-13, MCP-1) - Involves Th1 Response	No Th2 response: TSLP & IL-1RA Involves Th1 Response
No important contribution from IL-17D	IL-17D & IFNy show a positive correlation. These together inhibit TSLP.
Chemokine response correlated with vascular injury and angiogenesis analytes. More macrophage infiltration pathways involved in resolvers. Important role for chemokines in guiding angiogenesis.	Only ICAM-1 had correlation with chemokine response.
Angiogenesis markers correlated with several cytokines.	N/A
No analytes were predictive of pain after month 1 post-surgery (not surprising since this group has low pain scores at 1,3,6, and 12 months)	MDC Highly predictive of pain at month 12 post-surgery

Table 1: Summary table showing comparison between resolvers and non-resolvers in the analyte response pattern and their correlation pattern.

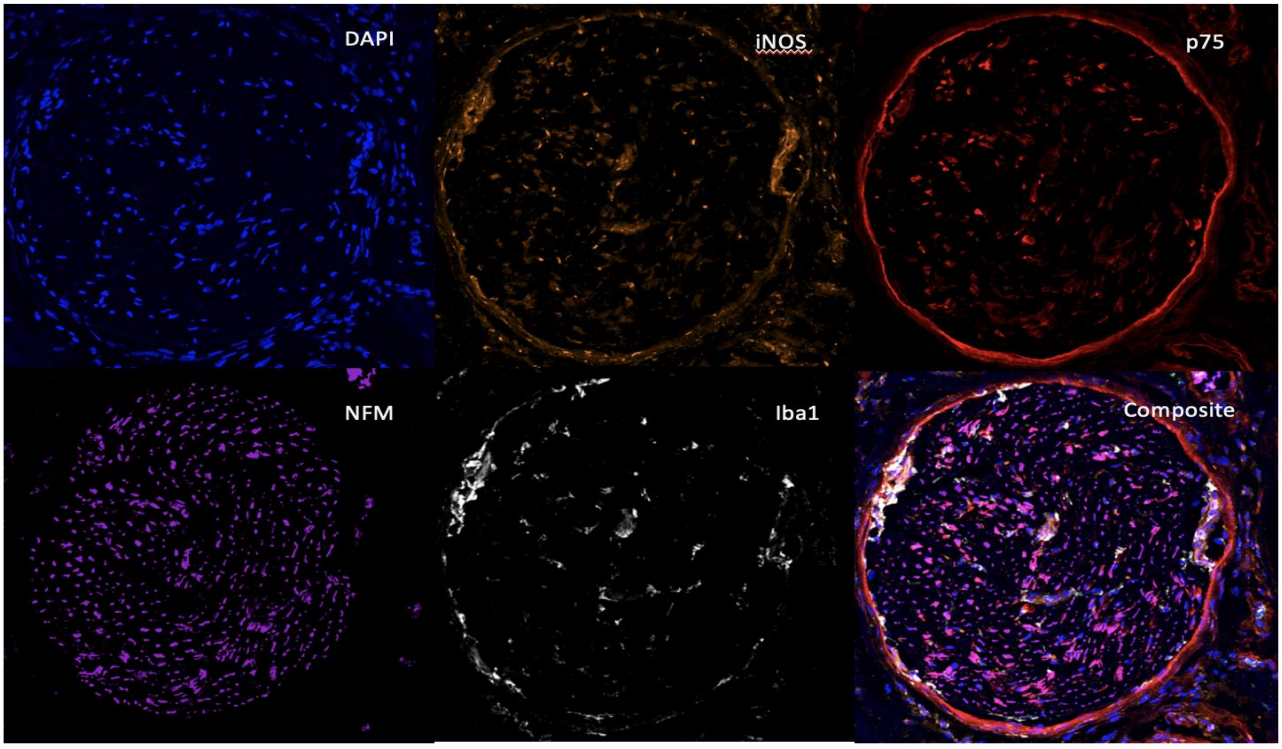


Figure 7: Immunohistochemical staining of sciatic nerve tissue. Markers for nuclei (blue), inflammation (orange), Schwann cells (red), axons (purple), and macrophages (grey) were selected.

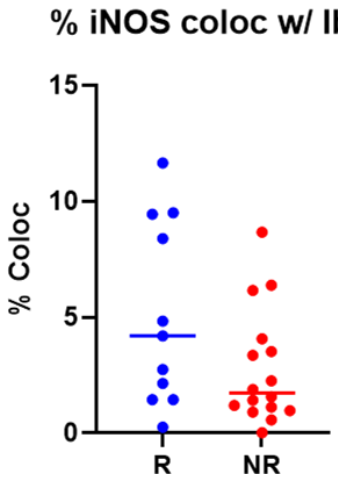


Figure 8: A higher percentage of iNOS (M1 macrophage marker) pixels are colocalized with Iba1 pixels in the resolver group (R) than in the non-resolver group (NR).

- **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.
- **Summary of Results of Major Task 1: Opioid, NSAID, corticosteroid, SNRI and gabapentinoid use at the time of surgery and 1-12 months after surgery was collected for all service members enrolled in this study (performed by the USUHS/DVCIPM team under Dr. Gelfand)**

- Major Task 2: Update current clinical outcomes database of SEXI patients for most recent pain scores and medication use (60 total patients)
- **Summary of Results of Major Task 2: A complete clinical outcomes database including medications, regional anesthesia, ketamine use, psychiatric comorbidities and pain scores was collected for all enrolled service members (performed by the USUHS/DVCIPM team under Dr. Gelfand)**

- Major Task 3: Add functional outcomes data to database.
- **Summary of Results of Major Task 3**: Our USUHS colleagues 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 so this task turned out to be impossible.

- Major Task 4: Correlate outcomes with expression signature
- **Summary of Results of Major Task 4: For correlation of pain to expression signature please see Major Task 7 under Aim 1 above.**

- **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.
- **Summary of Results of Major Task 1: There was no difference in functional/pain outcomes in servicemembers treated with ketamine around the time of amputation revision surgery (Figure 9)**

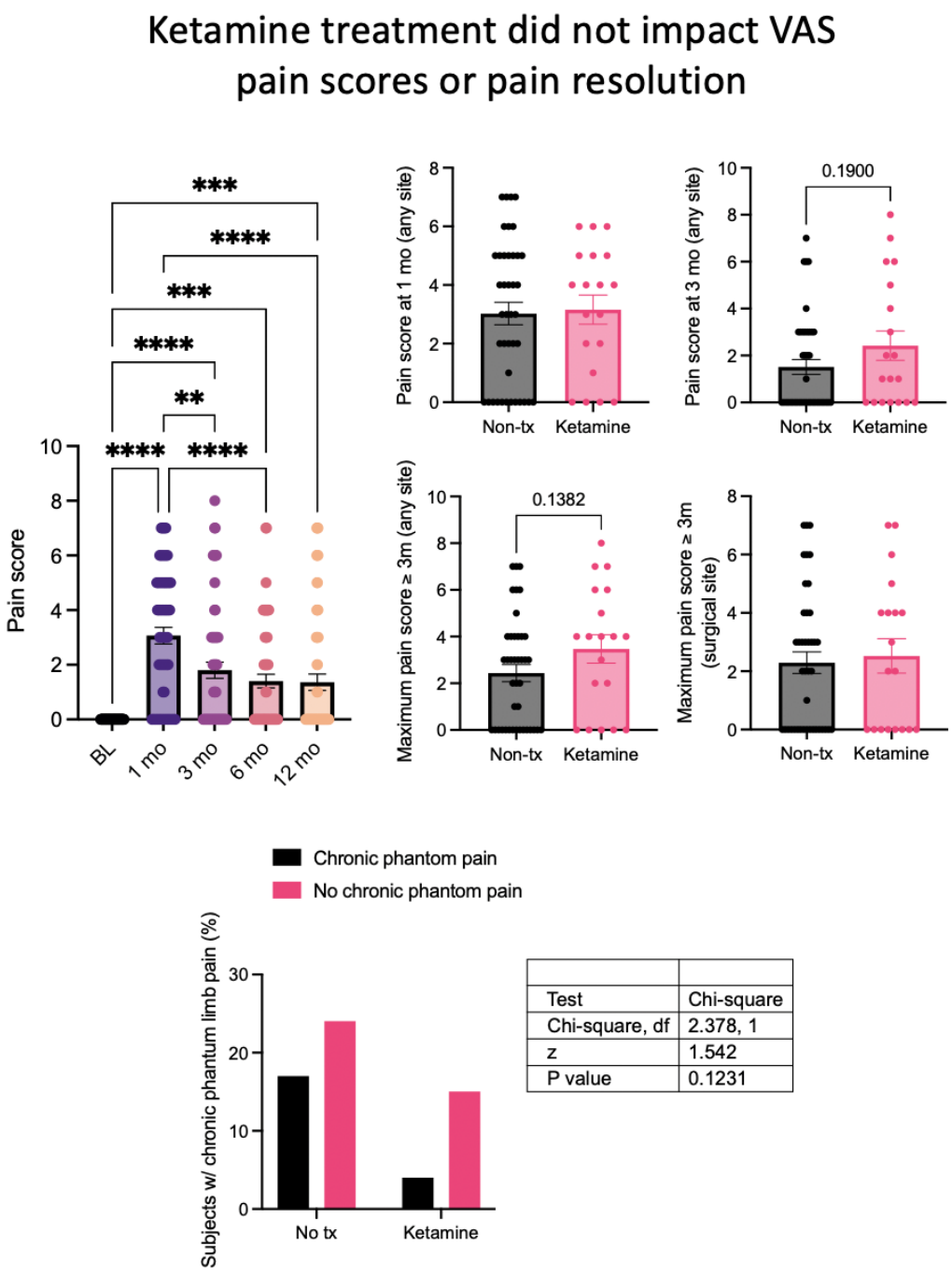


Figure 9: Ketamine vs no ketamine treatment around the time of amputation revision and pain score.

- Major Task 2: Identify the protein and RNA expression signature in sciatic nerve unique to patients treated with perioperative ketamine.
- **Summary of Results of Major Task 2: There was no significant difference in protein expression at the distal nerve tip between patients treated with ketamine and those without ketamine.**

2. **IMPACT:**

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

The conclusions of this study may have a simple but powerful impact on how injured warfighters and veterans are treated - when injured, pain therapies that block the inflammatory response are likely detrimental to their long term recovery. Therefore, reliance on other modalities like nerve blocks, ketamine, and lidocaine should be promoted over steroids, NSAIDS, and opioids.

- **What was the impact on other disciplines?**

The results support a paradigm shift in treatment of acute pain that pertains to many disciplines including orthopedics, rheumatology and physical medicine and rehabilitation – allowing a robust initial inflammatory response may be vital to normal healing and the eventual resolution of pain.

- **What was the impact on technology transfer?**
  - None

- **What was the impact on society beyond science and technology?**
  - Millions of people take anti-inflammatory medications to treat acute injuries: including post-surgical, arthritis associated or fracture related. This may turn out to be deleterious to long term pain outcomes.

3. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

The main changes in approach is an increased reliance on proteomics and shift to inflammatory mediator profiling and immunostaining due to poor quality of bulk and nuclear RNA. These experimental design changes should allow us to identify the molecules and pathways responsible for resolution of pain even though RNA quality was too poor for adequate sequencing.

4. **PRODUCTS:**

Publications, conference papers, and presentations

Poster Presentation - "Proteomic and cellular signatures of pain resolution after traumatic amputation" USASP Conference April 13, 2023

Journal publications.

Two manuscripts in preparation: The first details our global unbiased proteomics findings and the second discusses the MSD array and immunostaining findings.

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 Commons):	*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:	*Harold Gelfand*
Project Role:	*Site Prinicipal Investigator*
Researcher Identifier (eRA Commons):	
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

Collaboration Dr Gelfand 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:** Collaborating site work noted in the summary above
   - **QUAD CHARTS:** *Not Applicable*
7. **APPENDICES:** *None*